Using *Caenorhabditis elegans* as a novel expression system for the generation of recombinant *Teladorsagia circumcincta* vaccine candidates

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Abstract

Teladorsagia circumcincta is a common abomasal parasite of sheep in temperate regions and is one of the major causes of parasitic gastroenteritis (PGE) in growing lambs. Control of infection is achieved using anthelmintic drugs; however, this practice is rapidly becoming unsustainable due to widespread anthelmintic resistance within the *T. circumcincta* population. Sheep can acquire protective immunity against this parasite; immunity involves local and systemic antibodies and immune cells which can impair worm growth and fecundity and lead to expulsion of the parasites from the abomasum. Vaccination against this parasite is therefore a feasible option of control. A recent study showed that a recombinant vaccine cocktail containing 8 *T. circumcincta* antigens significantly reduced the faecal egg count and worm burdens of immunised sheep, compared to an adjuvant-only control group. However, the recombinant antigens induced a suboptimal antibody response to the recombinant antigens. This suggests that differencies between the native antigens and their recombinant versions may exist, possibly due to variations in structure and/or post-translational modifications (PTMs).

The main aim of this work was to use a novel expression system, the free living nematode *Caenorhabditis elegans*, to generate alternative recombinant versions of two of the *T. circumcincta* antigens used in the 8-antigen vaccine, cathepsin F (Tci-CF-1) and monocyte Migration Inhibitory Factor (Tci-MIF-1). This was achieved by micro-injection of *C. elegans* worms with plasmids containing the cDNA sequences of *Tci-cf-1* and *Tci-mif-1* followed by purification of recombinant Tci-CF-1 and Tci-MIF-1 from the transformed worms. Immune recognition, enzyme activity and biological effects on sheep cells of the recombinant antigens were characterised. The results show that immunisation-induced antibodies bind to native Tci-CF-1 purified from *T. circumcincta* L4 ES, whereas infection-induced antibodies were unable to bind the recombinant Tci-CF-1 versions. Further characterisation of recombinant Tci-CF-1 versions expressed in *C. elegans* or *Pichia pastoris* showed that in order to be enzymically active, these proteins require cleavage of the pro-peptide by an exogenous enzyme and that some differences were present in the glycosylation of the recombinant versions and native Tci-CF-1. Characterisation of both recombinant Tci-MIF-1 versions showed that although both are enzymically active, neither showed a significant inhibitory effect on the migration of sheep monocytes or on the activation of sheep macrophages *in vitro* compared to unstimulated controls. It is speculated that Tci-MIF-1 may be involved in *T. circumcincta* larval development rather than host immunosuppression.

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Author’s Declaration

I declare that the work presented in this thesis is my own original work and has not been submitted for any other degree or qualification.

Cassandra W. Longhi-Browne

Date

Abbreviations

°C	degrees Celsius
AMC	7-amino-4-methylcoumarin
APC	antigen presenting cell
ASC	antibody secreting cell
AUC	area under the curve
B cells	B lymphocytes
BCA	bicinchoninic acid
bp	base pair
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
dATP 2’	deoxyadenosine triphosphate
DC	dendritic cell
dH_2O	de-ionised water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	mixture of dATP, dCTP, dGTP and dTTP
DTT	dithiothreitol
EDTA	ethylene-diamine-tetra-acetic acid
ES	excretory/secretory products
EST	expressed sequence tags
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
g	grams
h	hours
hCath L	human cathepsin L (purified from liver)

HCl	hydrochloric acid
HRP	Horse radish peroxidase
Ig	immunoglobulins
IL	interleukin
INFγ	interferon-γ
L	litre
LC-ESI-MS/MS	Liquid chromatography – Electron spray ionisation - Tandem mass spectrometry
M	molar
mAb	monoclonal antibody
MALDI-ToF	matrix-assisted laser desorption/ionisation Time of flight
MHC	major histocompatibility complex
min	minutes
ml	millilitres
ML	macrocyclic lactones
mM	milli-molar
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
MWCO	molecular weight cut-off
NCBI	National Centre for Biotechnology Informations
ng	nanograms
nm	nanometre
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pmol	picomolar
PTM	Post translational modifications
r	recombinant
RBC	red blood cells
RT-PCR	Reverse Transcription - Polymerase Chain Reaction

SDS PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
sec	second
T reg	T regulatory cells
TAE	tris-acetate/EDTA electrophoresis buffer
TCBZ	triclabendazole
TCR	T cell receptors
Th	T helper
Th1	T helper 1
Th2	T helper 2
TNFα	tumor necrosis factor-α
ToF	Time-of-flight
Tris	2-amino-2-2(hydroxymethyl)-1,3-propanediol
V	volts
x g	force of gravity
µg	micrograms
µl	microlitres

"… when you have excluded the impossible, whatever remains,

however improbable, must be the truth."

Sherlock Holmes

(by Sir Arthur Conan Doyle, 1859-1930)

Chapter 1 – Introduction

1.1 *Teladorsagia circumcincta*

Teladorsagia circumcincta (also known as brown stomach worm) is a commonly reported parasite of sheep in temperate areas. Previously known as *Ostertagia circumcincta*, this nematode is the principal causative agent of parasitic gastroenteritis (PGE) in growing lambs of temperate regions (Bartley *et al.* 2003, Taylor M. A. *et al.* 2007). *Teladorsagia circumcincta* is a Strongylid member of the family Trichostrongyloidea and belongs to Clade V of the Phylum Nematoda, based on phylogenetic analysis of small subunit RNA sequences (Blaxter *et al.* 1998, Taylor M. A. *et al.* 2007). As a cause of major production losses and clinical PGE, *T. circumcincta* is a parasite of major importance in the sheep industry (Miller *et al.* 2012, Nieuwhof and Bishop 2005).

1.1.1 Lifecycle

T. circumcincta has a direct lifecycle, with eggs produced by adult female worms present in the faeces of infected sheep (summarised in Figure 1.1). If conditions are favourable, eggs hatch into first-stage larvae (L1) within 24 hours, quickly moulting into the slightly larger second larval stage (L2); both these stages remain within the faecal mass. It can take from 10 to 14 days on pasture for eggs to develop into the infective stage (L3), which retains the external cuticle of the L2 stage and forms the L3 sheath (Keith *et al.* 1990). At the L3 stage larvae move outside the faecal mass and onto the pasture, where they are ingested by the sheep host. The L3 larvae reach the abomasum approximately 24-48 hours after ingestion, where they ex-sheath, burrow into the gastric glands and undergo further moulting into the fourth larval stage (L4). Following development into an immature adult stage (L5) within the abomasal mucosa, the adults then emerge into the abomasal lumen and complete their sexual maturation. The majority of worms complete their maturation and begin releasing eggs approximately 3 weeks after the L3 stages were ingested (Taylor M. A. *et al.* 2007).

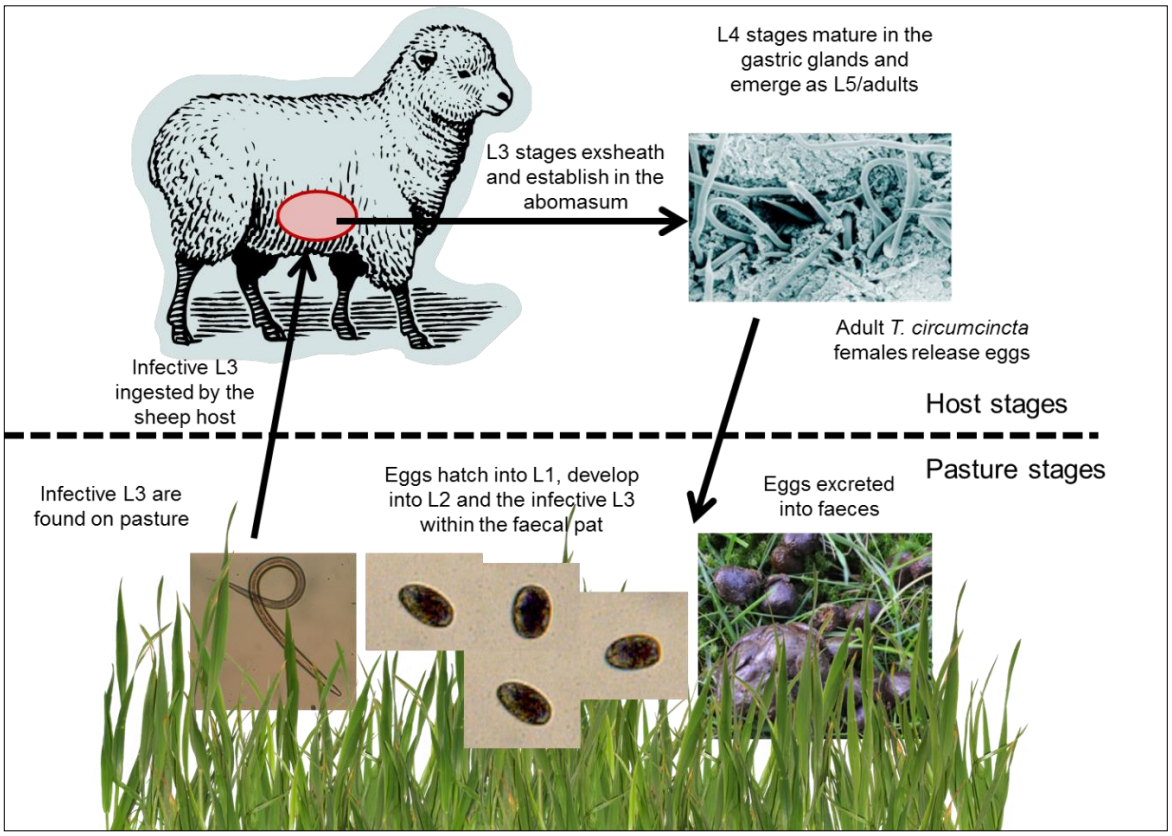

Figure 1.1 The life cycle of *Teladorsagia circumcincta*.
Images of *T. circumcincta* larvae and eggs courtesy of Dave McBean (Moredun Research Institute).

Overwintering on pasture commonly occurs in the case of Trichostrongylid parasites, and *T. circumcincta* is no exception; a study carried out over a period of 3 years showed that mild winter conditions significantly increased the number of established worms within tracer lambs (Makovcova *et al.* 2009). Furthermore, *T. circumcincta* is able to undergo hypobiosis, or larval arrest; this may occur when L3 larvae are ingested by sheep in the autumn/winter seasons and the arrested early L4 can survive for up to 6 months within the host before resuming development. While the processes surrounding larval arrest in parasitic nematodes are still not fully understood, it is hypothesised that there is a role for both environmentally induced hypobiosis and immune-mediated arrest (Gibbs 1986). Furthermore, at lambing time ewes experience a temporary loss of immunity (6-8 weeks peri-partum) which results in increased worm burdens and subsequent increased egg deposition on pasture. These eggs quickly develop into infective L3 stages in the warmer spring temperatures and are abundantly available for recently born, growing lambs to ingest (Barger 1993, Gibbs and Barger 1986, Houdijk *et al.* 2005, Jeffcoate *et al.* 1992), thus optimising spread of infection.

1.1.2 Pathology and disease

Epidemiologically, clinical teladorsagiosis is commonly observed during the summer months (Taylor M. A. *et al.* 2007), although pure teladorsagiosis is less common than PGE caused by mixed parasitic infections. The associated clinical signs of PGE range from suppressed appetite to diarrhoea, quickly followed by dehydration and sometimes death in heavy infections (i.e. a mixed species parasite burden of ~10 000 worms in sheep) (Radostits O.M. *et al.* 2007); however, the major impact of teladorsagiosis is its effect on lamb productivity via a reduction in weight gain (Gibson and Everett 1976). The clinical signs and loss of weight gain arise from the physical damage caused by larvae and adult worms' activity on the mucosal surface of the abomasum. It is known that *T. circumcincta* infection suppresses the appetite and induces significant slowing of muscle tissue deposition compared to uninfected sheep (Greer *et al.* 2008). The same study showed that by suppressing the host immune response with corticosteroids it was possible to reinstate the lost appetite and weight gains, even though the sheep thus treated showed higher FEC and worm burdens. This suggests that part of the economic loss due to *T. circumcincta* infection might be due to misdirected immune responses of the hosts which can put a check on the growth rates (Greer *et al.* 2008).

Parasitic gastrointestinal infection impairs normal abomasal physiology: functional changes in the abomasum occur following *T. circumcincta* infection, which contribute to the clinical signs. For example, production of gastrin (a regulator of gastric secretions produced by gastric antral G cells in the gastric mucosa) increases through loss of negative feedback from gastric acid (Johnson 1987). As *T. circumcincta* infection can induce suppression of gastric acid production, thus increasing the abomasal pH (Simcock *et al.* 1999), parasite infection can lead to hypergastrinemia (increases in serum levels of gastrin) as a result of parasite-induced damage to parietal cells. Hypergastrinemia was shown to occur during nematode infections with *T. circumcincta* and *Haemonchus contortus* in sheep, and *Ostertagia ostertagi* in cattle (Anderson *et al.* 1976, Fox *et al.* 1993, Scott *et al.* 2000, Simpson *et al.* 1997). However, it has also been shown that in some sheep, plasma gastrin levels may return to lower pre-infection levels even though abomasal pH remains elevated (Simcock *et al.* 2006). An increase in the abomasal pH correlated with increased numbers of anaerobic bacteria (Simcock *et al.* 1999). Increased bacterial growth and abomasal pH can both impair protein digestion by the sheep host and contribute to cause diarrhoea (Radostits O.M. *et al.* 2007). Other pathogenic effects of *T. circumcincta* include L3 larvae migrating into the gastric crypts, causing inflammation and immune cell

recruitment due to their antigenicity and tissue damage as they grow. The damaged abomasal mucosa allows increased permeability; in severe cases the mucosal permeability reaches such levels that a protein-losing enteropathy results [reviewed by Stear *et al.* (2003)]. Gross pathological examination shows areas of inflammation of the abomasal mucosa and parasitized glands; these reveal altered cell composition and mucous cell hyperplasia in histological specimens (Simpson 2000). Furthermore, as a sign of immune cell mobilisation following infection, significant increases of eosinophils, $CD4^+$ and γδ T cells were identified within the abomasal mucosa (Balic *et al.* 2003).

1.1.3 Sheep immune response to *T. circumcincta*

A protective immune response can be elicited against *T. circumcincta* following grazing on infected pasture (Taylor M. A. *et al.* 2007), or experimentally by continuous "trickle" infection over a period of several weeks (Seaton *et al.* 1989). Studies have shown that this immunity occurs through several mechanisms, against different stages of the parasite, such as increased worm loss by exclusion/expulsion of L3 stages, impaired larval development at the L4 stage and reduced fecundity in adults (Halliday *et al.* 2007, Smith *et al.* 1985, Stear *et al.* 1995, Stear *et al.* 1999, Strain *et al.* 2002). Locally produced IgA from the gastric environment and lymph nodes (Halliday *et al.* 2007, Smith *et al.* 2009) and circulating IgE (Huntley *et al.* 2001, Pettit *et al.* 2005) were shown to be associated with the protective sheep immune response to *T. circumcincta*. During *T. circumcincta* infection, gastric lymph total IgA and IgA from abomasal mucosae scrapings negatively correlated with the length of worms recovered from naturally or deliberately infected sheep (Smith *et al.* 1985, Stear *et al.* 1995, Stear *et al.* 1999, Strain and Stear 1999). However, the antibody response alone might not be sufficient to elicit a protective immune response to *T. circumcincta* and the recruitment of specialised cells in a T helper 2 (Th2)-biased environment, such as DCs, appears pivotal [reviewed by McNeilly *et al.* (2009)]. Other studies have shown the involvement of cellular immune responses by studying the expression of genes in the sheep abomasal mucosa and local lymph nodes. In these experiments, up-regulation of transcripts related to mucosal and immune cells such as integrins, galectins and various cytokines was shown to occur in sheep that have been previously infected then challenged with *T. circumcincta*, compared to sheep naïve to the parasite (Knight *et al.* 2011, Pemberton *et al.* 2012).

The local immune responses against gastrointestinal nematodes in sheep involve mucosal inflammation, mastocytosis, eosinophilia, hyperplasia of goblet and mucus cells and

reduction in parietal cell numbers (Balic *et al.* 2003, Balic *et al.* 2000, Craig *et al.* 2014, Scott *et al.* 1998, Scott *et al.* 2000, Stevenson *et al.* 1994). A common host mechanism in response to GI inflammation is recruitment of lymphocytes. For example, sheep lymphocytes found in the caecum mucosa were activated by artificially induced inflammation and were found to migrate preferentially from subcutaneous or intestinal locations (Au *et al.* 2001). This is thought to be due to specific homing receptors in the GI tissues, which redirect immune cells to the site where they were first activated (Cahill *et al.* 1977, Chin and Hay 1984, Kimpton *et al.* 1989). For example, in sheep the expression of integrin α4β7 on lymphocytes appears to confer their selective homing to the intestinal tract; furthermore, chemokines and their receptors have also been shown to be involved in sheep lymphocyte homing to the intestinal mucosa [reviewed by McNeilly (2008)]. These studies suggest that lymphocytes activated by *T. circumcincta* infection in the abomasum may be able to recirculate via the local gastric lymph nodes, efferent lymphatic ducts and blood back into the abomasal mucosa. Relevant to this, Smith and colleagues (1983) have shown a significant increase in lymphocytes in gastric lymph of *T. circumcincta*-naïve sheep 8 days after experimental infection with the parasite. In another study, in sheep rendered immune to *T. circumcincta* (by challenge with 50 000 L3 larvae), lymphocytes were collected from gastric lymph for up to 8 days after challenge and transferred intravenously to another genetically identical worm-free sheep (twins). These lymphocytes were able to transfer partial immunity following another *T. circumcincta* challenge, as defined by stunting or loss of worms compared to unchallenged controls (Smith *et al.* 1986). The composition of efferent gastric lymph has since been characterised and showed increased levels of $CD4^+$ and $CD8^+$ T cells, B cells and IgA in sheep experimentally infected with a *T. circumcincta* trickle infection and subsequent bolus challenge, compared to control sheep that received a primary infection by bolus challenge only in an experimental infection setting (Halliday *et al.* 2009, Halliday *et al.* 2007).

With regards to age of the hosts, it appears that immune responses to GI parasites such as *T. circumcincta* take longer to develop in lambs compared to older (> 12 months old) sheep. This is demonstrated by the increased morbidity, faecal egg counts and worm burdens in younger lambs (4.5 months old) following *T. circumcincta* infection compared to older animals (10 months old; Smith *et al.* 1985, Stear *et al.* 1999). These differences may be explained by the time taken for development of the adaptive immune system in young lambs: for example, γδ T cells which have characteristics of both the innate and adaptive immune response, and are not involved in antibody production, constituted 18 % of circulating T lymphocytes in sheep foetuses and 60 % of circulating T cell population in

4 weeks old lambs' (Washington *et al.* 1992). The γδ T cells percentages halved to ~30% when reaching 3-4 months old, and reached values of less than 10% in the adult sheep, suggesting that a more specific adaptive immune response may develop with older age (Hein and Mackay 1991, Watson *et al.* 1994). Moreover, studies carried out using Merino sheep showed that weaner lambs between 4-8 months of age had a lower proportion of blood $CD4^+$ and $CD8^+$ T cells compared to adult sheep (Watson *et al.* 1994). This suggests that the comparatively low numbers of $CD4^+$ cells in younger lambs might be insufficient to generate an effective Th2-type response and that specific adaptive immune responses may be compromised in young lambs. In previous studies, sheep older than 12 months had greater numbers of globule leukocytes and mucosal mast cells in the abomasal and small intestinal mucosae (Douch and Morum 1993), supporting the hypothesis that age plays an important role in the host response against GI parasites.

1.2 Induction of the immune response

It is widely accepted that helminth infections elicit a strong Th2 polarised immune response (Else 2005, Finkelman *et al.* 2004, Maizels *et al.* 2009a). The adaptive immune responses are characterised by the recruitment of naïve T helper cells upon recognition of a specific antigen by Pattern Recognition Receptors (PRRs) found on antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs), though intervention of DCs might not always be required to initiate the process. The bias that a naïve Th cell may take towards Th1 or Th2 is dependent on the dose, type, presentation of the antigen. A soluble antigen (such as those released by helminths) may be sufficient, alone, to initiate a Th2 response but it may not initiate a Th1 response, which instead requires the presence of microbial adjuvants which induce APCs to secrete cytokines such as IFN-γ, IL-2 and IL-12 (Coico *et al.* 2003, Jankovic *et al.* 2001). A number of transcription factors, such as STAT6, GATA3 and the NFAT family to name a few, are involved in the initiation of the Th2 response by T cells; these factors contribute to the production of effector cytokines such as IL-4, IL-5, IL-9, IL-10, IL-13, and IL-21 (Anthony *et al.* 2007, Grencis 1997, Jankovic *et al.* 2001). The effector cytokines recruit other cell types and promote activation and expansion of $CD4^+$ Th2 cells, plasma cells secreting IgE, eosinophils, mast cells and basophils, in a response that is similar to allergic reactions (Anthony *et al.* 2007, de Veer *et al.* 2007). Helminth infections can elicit both Th1 and Th2 responses, though Th2 tend to be more common. It appears that the protective Th2 responses observed during parasite infection can be of two types, either having a direct effect on the parasite (for example, in the model parasite, *Heligmosomoides polygyrus*, the protective Th2 responses mediate

worm expulsion), or protecting the host from pathology as it occurs during *Schistosoma mansoni* infection where the protective Th2 response down-regulates an otherwise pathological Th1-type response [reviewed in detail by Anthony *et al.* (2007)]. Studies using anti-IL-4 antibodies and IL-4 knock-out mice have shown a critical role for IL-4 in driving the host immune response towards a Th2 environment which may confer host protection in murine candidiasis, *B. malayi* and *Nippostrongylus brasiliensis* infections (Kopf *et al.* 1993, Osborne and Devaney 1998, Romani *et al.* 1992). Parasitic helminths also appear to be able to influence the host responses by inducing a regulatory immune response, in order to allow their own survival [reviewed by Maizels *et al.* (2009a, 2009b, 2009c)].

The developing protective immune response against nematode infections is often influenced by the presence of glycosylated antigens. The effect of glycosylation on the host response can be investigated by periodate treatment of parasite antigens using salts of periodic acid such as sodium periodate [$NaIO_4$ (Eberl *et al.* 2001, Fairlie-Clarke *et al.* 2010, Kooyman *et al.* 2007, Smith *et al.* 1994, Tawill *et al.* 2004)]. Sodium periodate salts are able to oxidise compounds that contain hydroxyl groups on two or more adjacent carbon atoms [vicinal diols, Figure 1.2 (O'Neil 2001)]. If the oxidation process takes place in a compound with a ring structure such as saccharide residues, the action of $NaIO_4$ breaks bonds between adjacent carbons carrying a hydroxyl group, and generates aldehyde and ketone groups by forming double bonds with oxygen atoms in the hydroxyl groups. This changes the shape of carbohydrate epitopes (Kristiansen *et al.* 2010), potentially reducing the antibody and cell binding ability.

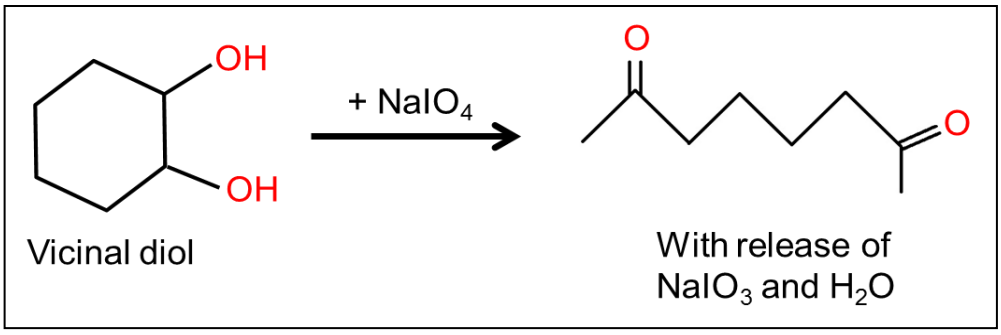

Figure 1.2 The effect of Na periodate ($NaIO_4$) on vicinal diols to form aldehydes and ketones. Adapted from: http://www.masterorganicchemistry.com/2011/10/21/reagent-friday-sodium-periodate/

For example, it was shown that lymph node cells from mice immunised with periodate-treated extracts of *Brugia malayi* or *Caenorhabditis elegans* produced significantly less IL-

4 than mice immunised with the respective mock-treated extracts (Tawill *et al.* 2004). The results suggested that in this model of infection the presence of glycosylated antigens has a pivotal role in inducing a Th2-biased immune response. In cattle, another study showed that deglycosylation of *Dictyocaulus viviparus* antigens using PNGase F resulted in reduced antibody reactivities to Excretory/Secretory (ES) antigens in sera of primary infected calves, suggesting that the carbohydrate moieties represented immunogenic epitopes (Kooyman *et al.* 2007). In the sheep parasite *T. circumcincta*, cathepsin F (Tci-CF-1), a highly immunogenic L4 ES component (Smith *et al.* 2009), was shown to have two putative N-glycosylation sites in the full length Tci-CF-1 sequence (Redmond *et al.* 2006). This could suggest that glycosylation of antigens may have an important role in the immune response against immunogenic antigens of this parasite.

However, while glycan epitopes may stimulate immune responses, numerous studies have also reported immunosuppressive activities associated with helminth glycans. For example, helminths may produce host-like glycosylated antigens to mask their own, or other glycosylated molecules that mimic host cytokines. With these types of mechanisms, the host immune response may be misdirected towards an immunomodulatory response that is non-harmful for the parasite and may allow its survival [reviewed by Maizels and Yazdanbakhsh (2003), van Die and Cummings (2010)]. Many parasitic helminths produce glycosylated molecules that are putative ligands for lectin receptors on the surface of DCs such as Mannose Receptor (MR), CD209 (also known as DC-SIGN) and CD301 (or Macrophage Galactose-type C-type Lectin, MGL) (van Die and Cummings 2010). For example in mice, one of the main glycan antigens of schistosome eggs (lacto-N-neotetraose) has been shown to induce *in vivo* peritoneal macrophages that secrete IL-10 as early as 2 hours after injection with the antigen, and that these cells promote *in vitro* a phenotype suppressive of naïve $CD4^+$ T cell proliferation (Terrazas *et al.* 2001). This ability of parasites and their glycans to stimulate or modulate/suppress the host immune response can represent both an advantage and a disadvantage in the search for novel drug targets and vaccine antigens. Both parasitic and free-living helminths have been shown to possess a surface coat that is mainly composed of molecules that include lipids and glycans (Blaxter *et al.* 1992). The complexity of this surface coat suggests that they may be too complex structures to replicate *in vitro*. Also this additional layer interposed between the worm cuticle and the host immune response can act as decoy mechanism, preventing drugs from having an effect on the parasite, preventing initiation of a protective immune response and complicating immunoscreening methods to identify vaccine targets (Blaxter *et al.* 1992, Maizels 2009c, Tundup *et al.* 2012).

1.2.1 Alternatively activated macrophages (AAMΦ)

A major effector cell type of the Th1 immune response is activated macrophages; these cells are able to, upon activation, express Tumor Necrosis Factor (TNF)-α, inducible Nitric Oxide Synthase (iNOS), IL-1, IL-6 and IL-12 following contact with bacterial lipopolysaccharide (LPS) or Interferon (IFN)-γ (Coico *et al.* 2003). Macrophages of the Th1-type response have been named "classically activated" to distinguish them from a novel subset of macrophages responsive to IL-4 and IL-13, belonging to the Th2 arm of the immune response. These have been identified in recent years and named "alternatively" activated macrophages [AAMΦ; Stein *et al.* (1992) and reviewed by Gordon (2003)]. This type of macrophage has been extensively studied in mice; in this species, stimulation of peritoneal macrophages with IL-4 and IL-13 resulted in alternative activation, with significantly increased products of those genes that are IL-4/IL-13 dependent such as Resistin-like molecules (RELMα), members of the chitinase family (FIZZ), chemotactic factors (Ym-1) and arginase 1 (Arg-1) (Gordon 2003, Loke *et al.* 2002, Raes *et al.* 2002). Properties of both classically and alternatively activated macrophages are shown in Figure 1.3. Activation of the alternative macrophage phenotype is commonly observed during helminth infections (Flynn *et al.* 2007, Loke *et al.* 2000, Loke *et al.* 2002, Nair *et al.* 2003, Nair *et al.* 2005). For example, a study that used mice infected with *Taenia crassiceps* showed that acute infection caused Th1-biased activation of peritoneal macrophages, whereas chronic infections (> 4 weeks) caused a shift towards a Th2 alternatively activated phenotype in these cells (Rodriguez-Sosa *et al.* 2002). *Fizz1* and *Ym1* are genes characteristically upregulated in the Th2 response to helminth infections in mice. Both genes are highly upregulated at the sites of parasite migration and residence during chronic infection with the filarial nematode *Litomosoides sigmodontis* and acute infection with the gastrointestinal nematode *N. brasiliensis* (Nair *et al.* 2005). Alternative activation was demonstrated *in vitro* by stimulation of the ovine macrophage cell line MOCL7 with adult *Fasciola hepatica* homogenates and ES (Flynn *et al.* 2007). Furthermore, there is evidence that expression of a chitinase 3-like molecule (Chit3-L1, a member of the mammalian chitinases family such as Ym1 in mice) is upregulated in the abomasal mucosa and gastric lymph nodes of sheep following trickle infection and challenge with *T. circumcincta*, suggesting this parasite may also induce AAMΦ (Knight *et al.* 2007).

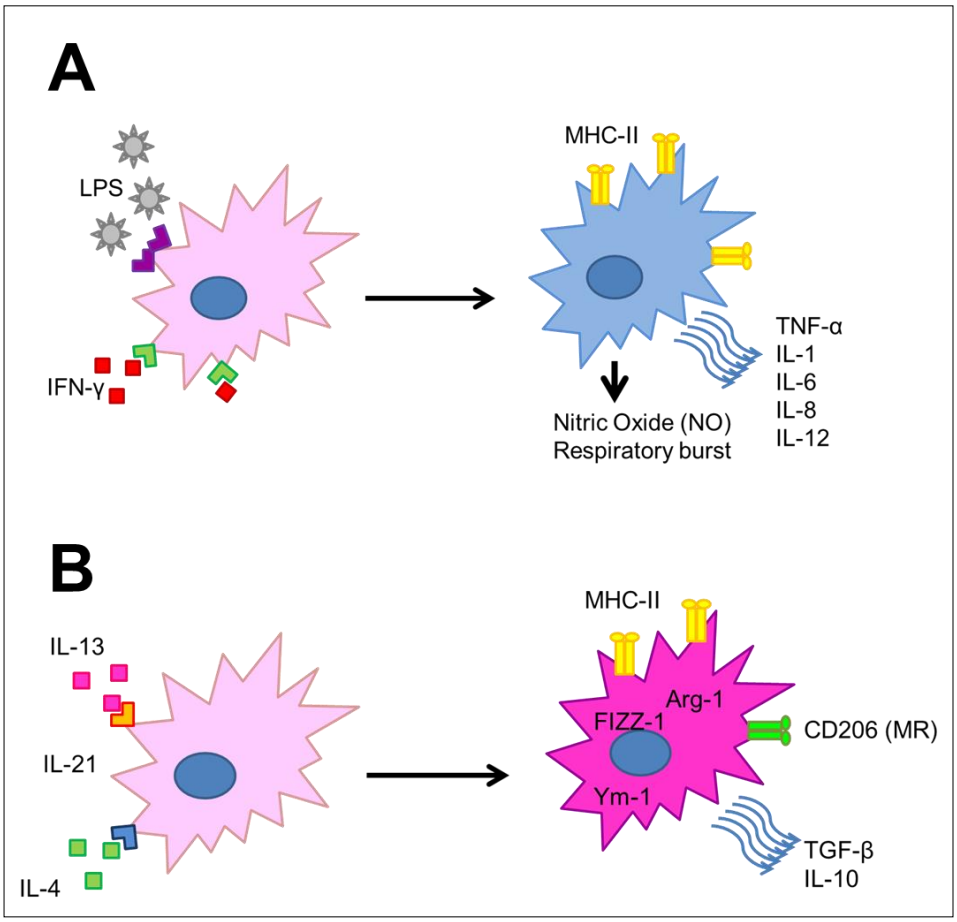

Figure 1.3 Schematic diagram showing the principal differences between classically and alternatively activated macrophages.
Panel A: classical activation; panel B: alternative activation. Legend: LPS, lipopolysaccharide; IFN-γ, interferon-γ; MHC-II, major histocompatibility complex-II; TNF-α, tumour necrosis factor-α; IL, interleukin; MR, mannose receptor; TGF-β, transforming growth factor-β.

A defining characteristic of AAMΦ is also the ability to inhibit cellular proliferation; for example, IL-4-dependent peritoneal macrophages from mice that received a *B. malayi* infection were able to reversibly inhibit the proliferation of human tumour cell lines (Loke *et al.* 2000). Another study showed that mouse macrophages (peritoneal exudate cells, PECs) primed *in vivo* with *S. mansoni* glycoconjugates (LNnT-Dex) were able to suppress the proliferation of $CD4^+$ cells *in vitro*. However this phenomenon did not involve apoptosis, as demonstrated by the significant levels of IL-4 and IL-13 secreted in response to anti-CD3/CD28 stimulation following a period of rest of the $CD4^+$ cells (Terrazas *et al.* 2001). These data indicate that $CD4^+$ T cells remained viable in the presence of suppressive LNnT-Dex-induced PECs and that they were able to resume cytokine production towards a Th2-biased response (Terrazas *et al.* 2001).

IL-10 is a cytokine that has been extensively studied and is produced by a number of cell types including T cells, macrophages and B cells. This cytokine is secreted by cell types of both the Th1 and Th2 response, and its main activity is in inhibiting cytokine synthesis and pro-inflammatory responses in Th1-biased environments (Moore *et al.* 1993). For example, in IL-10 knockout mice, the lack of this cytokine causes chronic enterocolitis and dysregulated inflammatory responses (Kuhn *et al.* 1993, Rennick *et al.* 1995). The inflammatory processes mediated by Th1-driven classically activated macrophages can result in immunopathology, if allowed to persist or escalate in the absence of the immunoregulatory cytokine IL-10 (Li *et al.* 1999).

AAMΦ have been hypothesised to be a Th2 immunoregulatory mechanism that may suppress Th1-derived inflammatory responses, and the tissue damage that can occur (Allen and Loke 2001); both these effects could occur also through production of IL-10. AAMΦ have been shown to play a role during healing processes and able to limit pathology: a study using IL-$4^{-/-}$ knock-out mice showed that IL-4 dependent alternative macrophage activation was necessary to protect the animals from mortality, liver pathology and sepsis due to *S. mansoni*-induced Th1 response (Herbert *et al.* 2004).

1.2.2 Measures of systemic and local antibody responses during parasitic helminth infection

1.2.2.1 Serum samples

The IgG class of immunoglobulins is the predominant class in blood, lymph, cerebrospinal and peritoneal fluid; it is produced in monomers and has the longest half-life of all isotypes (Coico *et al.* 2003), approximately 10 days in the ovine host (Fukumoto and Brandon 1982). The serum IgG response to any antigen is characterized by a primary peak of antibody secretion following the first immunisation with a vaccine or encounter with the pathogen, and by a secondary (anamnestic) response following another exposure to the same antigens. The secondary response peaks earlier, reaches higher titres, and is maintained for longer than the primary IgG response (Coico *et al.* 2003). These features and its long half-life in the serum make IgG the best isotype to measure the systemic immune response to a specific pathogen. On the other hand, the IgA class is the major immunoglobulin component of external secretions (produced not only systemically but by mucosal tissues too) such as saliva, colostrum, milk and mucus. IgA consists of a dimer joined by a secretory component which allows its transportation through the *lamina propria* and surface epithelium, from where it is secreted into the lumen. IgA has a half-

life of 5-6 days (Coico *et al.* 2003). It has been shown that all or part of the IgA found in serum samples may originate from the local tissue/organ of interest (Beh *et al.* 1974, Cripps *et al.* 1974, Curtain and Anderson 1971), and therefore serum IgA may be used as a measure of local immune response. A recently published study investigated the correlations between levels of *T. circumcincta*-specific IgA in serum and abomasal mucus of sheep exposed to natural parasite infection and subsequently twice challenged with 50 000 L3 larvae 12 weeks apart. The results showed that the serum IgA could be correlated to the IgA present in abomasal mucus of the same animals; however, the levels of systemic IgA were also dependent on the worm burdens of the sheep (De Cisneros *et al.* 2014). This suggests that as the sheep immunity responds locally to parasite infections by secreting mucosal IgA, these antibodies bind to the parasite and its ES products and the unbound fraction subsequently equilibrates between the mucosal and the systemic compartment via the lymphatic system (De Cisneros *et al.* 2014). Furthermore, an explanation for low parasite-specific serum IgA levels in sheep that carry a high worm burden could be due to most IgA produced locally binding to nematodes, therefore leaving only a relatively small proportion of nematode-specific IgA that can be transferred into serum (De Cisneros *et al.* 2014). This suggests that although in most sheep serum IgA is directly correlated to the levels of abomasal IgA, in cases of high worm burdens the relationship may be inverted, and this could still pose problems in evaluating the local immune response as the worm burdens are generally not known until post-mortem examination.

1.2.2.2 Antibody Secreting Cell (ASC) probes

When infectious or inflammatory stimuli challenge the immune system, antigens are transported via afferent lymph ducts to the local draining lymph nodes by immune cell trafficking. Thus, lymph nodes become activated and cell proliferation and differentiation takes place (Mirsky *et al.* 2011). For this reason, culturing of cells derived from lymph nodes draining the site of infection can provide a powerful tool to study the local immune response to parasitic pathogens (McWilliam *et al.* 2012). By culturing *in vitro* cells isolated from lymph nodes and collecting the antibody-rich supernatant (ASC probes), it is possible to gain a snapshot view of the local challenge-specific antibody response. The antibodies generated are not only parasite- and stage-specific, they are also specific for the tissue region of the draining lymph nodes collected. This makes them powerful tools for immune studies, as each cell clone is specific for one parasite epitope and analysis of ASC probe reactivity avoids the polyclonality of serum antibodies (reviewed by McWilliam *et al.* (2012). The use of ASC probes in helminth immunology studies is not new. In 1994,

(Meeusen and Brandon 1994a) proved the validity of antigen-specific ASC probes in experiments with the cestode *Taenia hydatigena* in sheep, and in rodent models with the liver fluke *F. hepatica* (Meeusen and Brandon 1994b). Similarly, *H. contortus* antigens were identified, both in the L3 and L4 stages, by using ASC probes generated from the abomasal lymph nodes of infected animals (Bowles *et al.* 1995). One of these antigens, named *Hc*-sL3, was later identified and isolated from the L3 stage (Ashman *et al.* 1995). When injected intradermally with Al-hydrogel as adjuvant, *Hc*-sL3 was able to confer protection in sheep experimentally challenged with 10 000 *H. contortus* L3, as defined by a significant (*p* value 0.027) decrease in FEC compared to the unchallenged group (Jacobs *et al.* 1999). More recent studies using the ASC probe technique have led to the identification of *Ascaris suum* stage-specific antigens, with antibody binding patterns specific to the organ of provenance, of the ASC probes and different from serum antibody responses (Jungersen *et al.* 2001). In sheep infected with *T. circumcincta*, extensive studies on the local immune reactivity of ovine ASC probes have given further insight into the relationship between host and parasites (Balic *et al.* 2003). In this study, 18 months old sheep were sourced from pasture and, following levamisole treatment, housed in a worm-free environment and trickle infected with 3000 *T. circumcincta* larvae each week for 9 weeks. Following a rest period of 12 weeks, a group of sheep was challenged with 50 000 *T. circumcincta* L3 and another group left unchallenged. The results showed a significant ($p < 0.05$) increase in weight of abomasal lymph nodes in the challenged sheep 5 days post challenge (dpc), compared to unchallenged animals. These experiments also showed that ASC probes from sheep trickle infected then challenged with *T. circumcincta* contained antibodies specific for L3, L4 and adult stages antigens (Balic *et al.* 2003). Taken together, their results confirm the ability of cells in the local site of infection (in this case, abomasal lymph nodes) to develop specificity for parasitic antigens in the course of an active infection, and that the antigens recognised are stage-specific; this confirmed the ability of the probes to monitor the immune response at the site of infection which is stage-specific and dependent on an active infection (Balic *et al.* 2003). For these reasons, one of the approaches used in the study here described was to collect abomasal lymph nodes from sheep that had been subjected to a *T. circumcincta* trickle infection and use the ASC probes thus generated as a tool to measure the local immune responses to various *T. circumcincta* native and recombinant antigens (Figure 1.4 shows the location of the abomasal lymph nodes in relation to the body of the abomasum).

1.2.2.3 Gastric lymph

Successful implementation of a protocol to collect and re-infuse gastric lymph in sheep was first described by Smith *et al.* (1981). In this study, the authors showed that surgical cannulation of the common gastric lymph duct is a viable and efficient way to collect lymph samples draining the ovine stomach, which can be used for subsequent experiments (Figure 1.4 shows a diagram of the common gastric lymph duct in sheep). The authors have shown that *T. circumcincta*-specific IgA was significantly higher in lymph from sheep previously infected compared to the controls and that the mean lymph to serum IgA ratio (calculated from the IgA concentrations in paired lymph/blood samples collected on the same day) was suggestive of the abomasum being the source of IgA antibodies during *T. circumcincta* infection (Smith *et al.* 1981). Additionally, the fore-stomachs (rumen, reticulum and omasum) are lined with non-glandular epithelium which contrary to abomasal mucosa, contains very few IgA-staining plasma cells (Smith *et al.* 1981), suggesting that lymph draining the ruminant stomachs has abomasal provenance. In subsequent studies which used the same surgical technique, parasite-specific IgA antibody responses were observed after *T. circumcincta* challenge of animals which had previously been infected with *T. circumcincta* (Smith *et al.* 1983), showing that *T. circumcincta*-specific antibody titres became significantly higher following parasite challenge. *Teladorsagia circumcincta* L4-specific IgA was also detected in gastric lymph of later experiments (Halliday *et al.* 2010, Halliday *et al.* 2007), confirming that gastric lymph is an ideal tool for investigation of local abomasal immunity against the parasite.

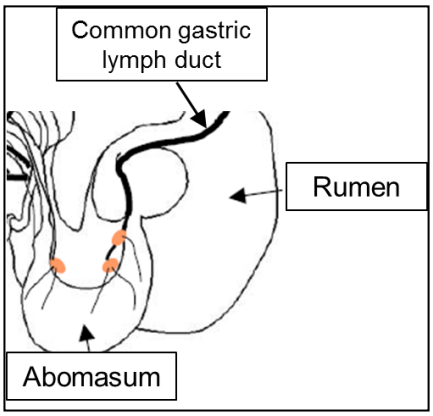

Figure 1.4 Schematic representation of the abomasal lymph node chain and the common gastric lymph duct location in sheep, in relation to the main body of the abomasum. The common gastric lymph duct (indicated by top arrow) drains efferent lymph from the abomasal lymph nodes (pink circles), situated on the smaller curvature of the abomasum. Adapted from Hein *et al.* (2004).

1.3 Control of parasitic infections

1.3.1 Anthelmintic treatments

Teladorsagiosis is a major problem in the sheep industry and control of the parasite relies heavily on the use of anthelminthic drugs. Three major classes of chemicals are used: benzimidazoles (BZs), levamisole and macrocyclic lactones (MLs, of which the prototype is ivermectin). These drugs have different mechanisms of action against the nematodes. Benzimidazole-based drugs bind nematode β-tubulin within the parasite's cells, thus preventing microtubule formation and ultimately leading to cell lysis (Prichard 1990). Levamisole is another type of anthelmintic drug that acts as a cholinergic agonist at neuromuscular junctions (McKellar and Jackson 2004). It is thought that ivermectin and other MLs act by competing with gamma amino butyric acid (GABA) and cause paralysis of the nematode somatic muscles, through stimulation of glutamate-gated chloride channels (Feng *et al.* 2002). Recently, two new classes of anthelmintics have been introduced: the amino-acetonitrile derivatives (AADs, for example monepantel) which act on nematode-specific nicotinic acetylcholine receptors different from those affected by levamisole (Kaminsky *et al.* 2008), and the spiroindoles which are able to block cation channels in nematode muscle cell membranes (Little *et al.* 2011). Despite the number of drugs available to treat sheep against GI nematodes, heavy use of all classes and underdosing of animals has resulted in widespread anthelmintic resistance. Although anthelmintic resistant parasites were already known in the late 1950s (Drudge *et al.* 1957), these had resistance to one class of anthelmintic at any given time and isolates that showed resistance to more than one type of drug had been relatively rare. In recent years however, single *T. circumcincta* isolates showing multiple resistance to the three most commonly used drug classes have been identified (Bartley *et al.* 2003, Bartley *et al.* 2005, Sargison *et al.* 2005, Sargison *et al.* 2010, Sutherland *et al.* 2008). These parasite populations pose a threat to the sheep industry, and it was reported that some farms were forced to stop all sheep operations until clear of the offending parasites (Blake and Coles 2007, Sargison *et al.* 2005). Alternative means of controlling these parasites are urgently needed and an increasing effort has recently focused on studying *T. circumcincta* development and survival, in order to identify other potential drug targets. Studies have focused for example on the moulting mechanism of the parasite (Stepek *et al.* 2010a, 2010b). Other studies have focused on anthelmintic candidate molecules from plants, such as cysteine proteases from papaya latex which have been shown to decrease motility of the mouse parasite *Heligmosomoides bakeri* when co-incubated (Behnke *et al.* 2008, Stepek *et al.* 2005).

1.3.2 Pasture and flock management

Although the current dependency on anthelmintic drugs must change in order to stop the spread of resistant strains, alternative measures of parasite control such as pasture rotation, grazing on tannin-rich plants, manipulation of protein nutrition of the ewes and the use of nematophagous fungi are not sustainable practices in the current intensive farming industry, for reasons of practicality and costs involved (Sargison *et al.* 2005).

Studies have shown that different sheep breeds have different susceptibility to parasitic infection and that individuals respond differently to parasite challenge, depending on their genotype (Bahirathan *et al.* 1996, Golding and Small 2009, Good *et al.* 2006, Miller and Horohov 2006). Flock management practices such as breeding of resistant sheep may be an option; however, tropical breeds which have shown higher resistance to parasitic infection are also less productive under intensive farming techniques than the European-derived breeds (Bisset *et al.* 2001). In addition, although the heritability of genetic traits that appear to correlate with parasite resistance (such as low FEC) is similar to other genes of productive traits (e.g. meat and wool production), these tend to negatively correlate [i.e. sheep resistant to parasitic infection tend to show unfavourable growth rates/fleece weights under parasite challenge (Bisset *et al.* 2001)]. A better option may be to breed parasite resilient sheep (less production losses under parasite challenge), than resistant sheep *per se* (lower FEC under the same infection conditions). Introducing resilience in a flock is a less straightforward process than introducing resistance, as heritability of the latter trait is higher (i.e. a larger proportion of the phenotype observed is due to genetic factors than environmental) [reviewed by Bisset *et al.* (2001)]. The process used to identify the resilient sheep ("drench-on-demand") is similar to the process described as targeted selected treatments [TST (Kenyon and Jackson 2012)], albeit with slightly different aims. Both result in only some of the animals in the flock being treated with anthelmintic drugs (poor-doers under parasite challenge), leaving the others (well-doers) to continue the shedding of parasite eggs. This allows the establishment of a parasite population called *refugia*, which has not been exposed to anthelmintic drugs and therefore less likely to develop resistance (Besier 2012, Kenyon and Jackson 2012). At the same time, sheep that do not require drenching but still perform well (regardless of FEC) are the ones that should be retained for breeding as representative of the resilient flock.

1.3.3 Immunological methods (vaccines)

An alternative approach to control parasitic helminth infection is the use of vaccines. A number of vaccination studies have been carried out with varying results. To date, successful immunization against parasitic nematodes has proved largely elusive, with few exceptions [reviewed by Geldhof *et al.* (2007), Newton and Meeusen (2003)]. Successful vaccines against helminths may constitute different parasite preparations: live attenuated parasite larvae (commercialised against *Dictyocaulus viviparus*); purified native antigens enriched for H11 and the H-gal-GP complex from *Haemonchus contortus* (Knox and Smith 2001) and purified ES-thiol fractions from *Ostertagia ostertagi* adult worms (Meyvis *et al.* 2007). A summary of those tested and the outcome of vaccination are summarised in Table 1.1.

Table 1.1 Examples of immunisation trials against nematodes of livestock using whole parasites or native antigens purified from the parasites.

Parasite species (host)	Antigen(s)	Administration route/adjuvant	Reductions (%)		References
			FEC	Worm burdens	
D. viviparus (cattle)	Irradiated L3 larvae	Oral/no adjuvant	n.c.	n.a.^	Jarrett *et al.* (1958)
O. ostertagi (cattle)	ES-thiol fractions	IM/Quil A	62	0	Meyvis *et al.* (2007)
	Oo-ASP	IM/Quil A	74	47	Meyvis *et al.* (2007)
	Oo-Gal-GP	IM/Quil A	22.9	10.9	Smith *et al.* (2000)
T. circumcincta (sheep)	L4 ES	IM/Freund's	n.c.	n.c.	Rose 1976, (1978)
	Irradiated L3 larvae	Oral/n.a.	n.c.	Stat sig	Smith *et al.* (1982)
H. contortus (sheep)	H-Gal-GP	IM/Quil A	96	71	Smith *et al.* (1994, 2000)
	H11	IM/Quil A	99.9	93.6	Roberts *et al.* (2013)
	*Hcs*L3	SC/AlOH	61	54	Piedrafita *et al.* (2012)
	Irradiated L3 larvae	Oral/n.a.	Signif. delayed but not reduced	0	Smith and Angus (1980), Urquhart *et al.* (1966)

Note: This table is not comprehensive. N.c., data not calculated but a reduction was apparent; Stat sig, statistically significant but % reduction was not provided; n.a.^, data not available as reduction in worm burdens but calculated as statistically significant (*p* value not reported reductions in morbidity and mortality. Adjuvants used: Quil A = Quil A saponin; AlOH = aluminium hydroxide. Administration of vaccination: SC = subcutaneous; IM = intramuscular.

Helminth vaccine production using live attenuated/killed worms or purified native antigens can present difficulties, such as obtaining sufficient quantities of parasite material from which to purify protective antigens, and the need to guarantee batch variability, antigen stability and biosafety (for the potential presence of adventitious agents) in a large industrial production scale (Smith and Zarlenga 2006). For these reasons, parasite vaccine research is currently focused on the development of recombinant antigens which should overcome the issues outlined above. Recombinant antigen vaccines have been demonstrated to be successful against cestodes [reviewed by Lightowlers (2006, 2003)]. Currently, researchers are increasingly focusing on the production and testing with a focus

on antigens identified from parasite Excretory/Secretory (ES) products, gut extracts or somatic extracts (Newton and Meeusen 2003, O'Donnell *et al.* 1989, Redmond *et al.* 2006, Smith *et al.* 2009).

The hypothesis behind the use of recombinant antigens is that immunisation of animals with a synthetic antigen can achieve a protective immune response, by inducing antibody or cellular responses which are able to recognise the native versions of the proteins (Coico *et al.* 2003). This immune response can be similar to that elicited by natural infection against parasite antigens which are presented to the host immune system, but also directed against "hidden" antigens, such as those present in gut tissues of parasites (Knox 2000). There have been a number of helminth recombinant antigens generated with various expression systems, achieving very variable and at times inconclusive results when used in vaccine and challenge experiments. For example, 3 immunisations of dogs with the catalytically active *Pichia pastoris*-expressed Ac-CP-2 antigen (a cysteine protease) from the hookworm *Ancylostoma caninum* achieved significant reductions in the geometric means of faecal egg output [approximately 13 000 eggs/g faeces (epg) and ~ 5000 epg in controls and immunised dogs, respectively] following intradermal challenge with 500 *A. caninum* L3 (Loukas *et al.* 2004). In contrast, a baculovirus-expressed version of the activation-associated secreted protein Oo-ASP-1 did not confer protection against *O. ostertagi* challenge when used to immunise calves (Geldhof *et al.* 2008), although previous immunisation studies showed that native *O. ostertagi* ASP proteins were protective against homologous challenge (Meyvis *et al.* 2007).

Vaccine candidates are selected for recombinant expression on the basis of genomic and transcriptomic information (cDNA and EST data) (Dalton *et al.* 2003, Menon *et al.* 2012, Nagaraj *et al.* 2008), proteomic approaches (Smith *et al.* 2009) and immune recognition of the orthologous/homologous native protein (Nisbet *et al.* 2009). However, if the recombinant version fails to induce a protective immune response it is not known whether this is due to inappropriate adjuvant choice (which would affect antigen presentation), incorrect form/folding of the recombinant, or whether the original native antigen was not an appropriate choice, possibly due to lack of immunological or biological knowledge of the native antigen [reviewed by Geldhof *et al.* (2007), Smith and Zarlenga (2006)]. It has been hypothesised that the recombinant versions of nematode antigens produced using standard expression systems such as bacteria, yeast and baculovirus, may show high degrees of dissimilarity with the native forms. Therefore, antibody or cellular immune responses generated against these versions would be less able to recognise the native forms

when encountered during a challenge infection (Geldhof *et al.* 2002, Meyvis *et al.* 2008, Newton and Meeusen 2003). Examples of immunisation trials using recombinant nematode vaccine antigens are provided in Table 1.2.

Differences in conformation and Post Translational Modifications (PTMs) may occur using traditional expression systems, especially when dealing with organisms taxonomically very distant, leading to the production of enzymes lacking correct folding or with defective PTMs and impaired functions [reviewed by (Geldhof *et al.* 2007). Some nematode proteins are highly glycosylated with unique structures such as core α1→3-fucose residues that may be nematode-specific (Cipollo *et al.* 2002, Dell *et al.* 1999, van Die *et al.* 1999), and appear to be important in stimulating the host immune response. For example, calves vaccinated with irradiated *D. viviparus* larvae show a strong but short-lived serum antibody response directed to N-glycans on the nematode surface (Kooyman *et al.* 2007). It is possible that these glycans may be important for vaccination and could have an important role establishing a protective immune response.

Table 1.2 Examples of immunisation trials using nematode recombinant antigens.

Parasite (host)	Antigen(s)	Expression system	Administration route/adjuvant	Reduction (%)		References
				FEC	Worm burden	
O. ostertagi (cattle)	Oo-ASP-1	Baculovirus	IM/Quil A	0	0	Geldhof *et al.* (2008)
	Oo-HSP-18	Baculovirus	IM/Quil A	-12	-38	Vercauteren *et al.* (2006)
A. caninum (dog)	Ac-CP-2	*P. pastoris*	IM/AS02, AS03, Alum	Stat sig (all 3 adj)	Stat sig (all 3 adj)	Loukas *et al.* (2004)
T. circumcincta, *H. contortus* (sheep)	PP2Ar from *Angiostrongylus costaricensis*	*E. coli*	Intranasal/ Bacterial cell walls	Stat sig †	> 68 †	Mohamed Fawzi *et al.* (2013)
T. circumcincta (sheep)	Tci-CF-1*, Tci-APY-1#, Tci-MEP-1#, Tci-ES20*, Tci-MIF-1#, Tci-TGH-2#, Tci-SAA-1#, Tci-ASP-1#	*E. coli* (#) or *P. pastoris* (*)	IM/Quil A	70-58	55-57	Nisbet *et al.* (2013)
H. contortus (sheep)	H11	*C. elegans*	SC/Vax saponin	0	0	Roberts *et al.* (2013)
	Cathepsin L		IM/Quil A	0	0	Murray *et al.* (2007)
	ES15/ES24	*E. coli*	SC/DDA	0-42	55	Vervelde *et al.* (2002)
	H-Gal-GP	*E. coli*, insect cells	IM/Quil A	2.5	0	Cachat *et al.* (2010)

Note: This table is not comprehensive. Stat sig, statistically significant but % reduction was not provided; †, reduction was significant ($p < 0.05$) in immunised and challenged sheep compared to control animals which were not immunised (and not injected with adjuvant) and received same challenge infection. Adjuvants used: Quil A = Quil A saponin; AS02A = monophosphoryl lipid A and QS21 (saponin)-based adjuvant; AS03 = squalene, DL-α tocopherol and polysorbate 80-based adjuvant; DDA = dimethyl dioctadecyl ammonium bromide. Administration of vaccination: SC = subcutaneous; IM = intramuscular.

1.4 Vaccination trials using *T. circumcincta* recombinant antigens

Sheep can acquire immunity against *T. circumcincta* and therefore vaccination is a possible alternative to anthelminthic control. While a few promising studies have reported progress towards a vaccine using recombinant antigen technology for *T. circumcincta* (Nisbet *et al.* 2013, Redmond *et al.* 2006, Smith *et al.* 2009), no commercial vaccine against this parasite has been developed. Recent studies carried out at MRI identified a series of potential

vaccine candidates for *T. circumcincta*, which have subsequently been tested in immunisation trials (Nisbet *et al.* 2013). This work focused on a panel of 8 immunogenic molecules. These included Tci-CF-1, Tci-MEP-1, Tci-ASP-1 and Tci-ES20, which are present in larval Excretory-Secretory (ES) products. These were identified by proteomics and immunoscreening of *T. circumcincta* L4 ES with abomasal IgA probes generated from experimentally induced *T. circumcincta*-immune sheep (Nisbet *et al.* 2010a, Redmond *et al.* 2006, Smith *et al.* 2009). Another molecule, Tci-SAA-1, was chosen because of its homology with another known surface antigen of hookworm, previously used as a vaccine candidate (Nisbet *et al.* 2009). The final three potential vaccine candidates (Tci-MIF-1 (Nisbet *et al.* 2010b), Tci-APY-1 (Nisbet *et al.* 2011) and Tci-TGH-2 (McSorley *et al.* 2010) were investigated on the basis of their putative immunoregulatory functions at the host:parasite interface. These antigens were cloned, sequenced and expressed in bacterial or yeast expression systems. The cocktail of eight antigens thus developed was used to immunise lambs (by means of 3 injections, each including all 8 antigens), which were subsequently challenged with a *T. circumcincta* trickle infection of 4 weeks duration. Under these experimental conditions, the recombinant vaccine showed, in two immunisation trials, that a significant reduction in egg output (70 % and 58 % reduction in faecal egg count in trial 1 and 2, respectively) and adult worm burdens (75 % and 56 % in trials 1 and 2, respectively) could be achieved (Nisbet *et al.* 2013). These very promising results have shown that indeed it is possible to vaccinate sheep against *T. circumcincta* with recombinant antigens and achieve protection. However, the purified recombinant proteins did not always show optimal expected biological or catalytic activity in *in vitro* assays (e.g. Tci-MIF-1) and it was hypothesised that the recombinant proteins could have various degrees of dissimilarity from the native forms, and may therefore induce sub-optimal immune responses against the native forms of the antigens. It is also still unclear whether all the components in this recombinant vaccine had a critical role in inducing the protective immune response and further work is required to characterise the recombinant vaccine components.

1.5 Parasitic nematode Excretory/Secretory (ES) products

A number of parasitic nematodes have been shown to excrete and secrete a variety of molecules during *in vitro* culture, and it is thought that this also happens during their persistency within the host. These molecules have been named Excretory/Secretory (ES) products; ES products were classified as molecules released from specialized secretory-

excretory organs or as antigens associated with the parasite's gut or derived from the external tegument of the parasite. An extensive description of helminth ES products was published more than 20 years ago (Lightowlers and Rickard 1988). This complex mixture includes molecules released by the parasite during *in vitro* culture, which are known or presumed to be released *in vivo*; many of these molecules are released in a stage-specific manner (Knox 2000). Studies have focused on the investigation of ES components and functions, hypothesising various functions carried out by ES products, including aid during host penetration, parasite feeding and evasion of host anti-parasite immune responses, including enzymes such as proteases, acetylcholinesterases and superoxide dismutases [reviewed by Hewitson *et al.* (2009), Knox (1994, 2000)]. For example, *S. mansoni* egg antigen (SEA) stimulation induced *in vitro* suppression of human T cell populations by an IL-10-mediated mechanism (King *et al.* 1996). In another *in vitro* experiment with the filarial parasite *B. malayi*, PBMC from infected patients stimulated with live L3 or microfilariae showed significantly lower cytokine production compared to PBMC from uninfected people (Babu *et al.* 2006). In mice, ES products from the parasite *N. brasiliensis* can inhibit LPS-dependent neutrophil recruitment to the lungs following experimental intranasal administration; in addition, *H. polygyrus* ES and somatic extracts could inhibit T cell proliferation and macrophage nitric oxide production in *in vitro* studies (Marsland *et al.* 2005, Rzepecka *et al.* 2006).

In a number of parasite ES products, including *T. circumcincta*, venom allergen-like (VAL) molecules (part of the Activation-associated Secreted Protein (ASP)-like family of antigens) have been identified and a role in immunomodulation was suggested (Hewitson *et al.* 2009). Indeed immunosuppressive effects of ES products appear to be conserved in the interaction of *T. circumcincta* with the sheep host. A recent study by Grainger *et al.* (2010) showed the ability of *T. circumcincta* L4 ES to induce Foxp3 expression (a transcription factor associated with T regulatory cell functions) on the surface of murine activated $CD4^+$ T cells *in vitro*, suggesting that the parasite may be able to induce this type of cell during infection in the definitive host. Furthermore, McNeilly and colleagues (2013) were able to confirm that $Foxp3^+$ cells were present in the abomasum of infected sheep during the early stages of infection, when the L4 stages are predominant. Even more interestingly, a major effect of *T. circumcincta* L4 ES on ovine PBMC was the profound suppression of mitogen-induced proliferation; this effect was observed to be heat labile, and associated with lack of IL-2Rα up-regulation, suggesting that *T. circumcincta* ES may be able to influence the early activation of T cells.

It has been previously shown that ES products from the liver fluke *F. hepatica* can suppress *in vitro* proliferation of ovine lymphocytes (Jefferies *et al.* 1996). In particular, one *F. hepatica* ES component, Cathepsin L, was shown to cleave CD4 receptors from ovine T cells and that by preventing activation of T *helper* cells this trematode may be able to evade the host immune system (Prowse *et al.* 2002). In mice, the same *F. hepatica* Cathepsin L protease provoked suppression of *Bordetella pertussis*-induced IFN-γ production, suggesting that this molecule may have an important role in suppressing the host Th1 immune response to infection (O'Neill *et al.* 2001). A cysteine protease, named cathepsin F (Tci-CF-1), had been identified as the most abundant L4 ES antigen in *T. circumcincta* (Redmond *et al.* 2006), and it was hypothesised that this enzyme may be responsible or contribute to the suppression of PBMC proliferation observed in sheep. Whole *T. circumcincta* L4 ES was shown to suppress the *in vitro* proliferation of sheep lymphocytes, and the addition of the cysteine protease inhibitor E-64 did not appear to affect the inhibitory effect observed (McNeilly 2013). However, direct *in vitro* experiments using purified Tci-CF-1 have not yet been published and at the time of writing it was not possible to completely rule out the involvement of Tci-CF-1 in inhibiting sheep cell proliferation.

1.6 Selection of a panel of antigens for this study

In order to immunise sheep with *T. circumcincta* antigens in the search for a successful vaccine against this parasite, *E. coli* and *P. pastoris* expression systems have previously been used (Nisbet *et al.* 2013). However, traditional expression systems such as bacterial and yeast cells have potential issues for the generation of vaccine candidates against nematodes, in terms of incorrect protein folding, or inappropriate or missing PTMs. In addition, not all recombinant vaccine candidates are able to induce a protective antibody response able to recognise the native homologues [reviewed by Geldhof *et al.* (2007), Smith and Zarlenga (2006)]. For these reasons, the choice of expression system in this study was directed towards a novel type, the nematode *C. elegans*, which has been shown to generate enzymatically active recombinant enzymes and is taxonomically closer to *T. circumcincta* than *E. coli* or yeast cells (Blaxter *et al.* 1998, Murray *et al.* 2007, Roberts *et al.* 2013). The choice for the panel of *T. circumcincta* antigens selected for expression in *C. elegans* was based on the following criteria i) recombinant versions have already been generated and are available for comparative studies, ii) where the *in vitro* activity of these existing recombinants has been characterized it appears that they may not exhibit full

functional activity, prompting further studies, and iii) their biological properties are yet to be fully characterised.

1.6.1 Cysteine protease, Tci-CF-1

The large group of Cysteine (C) Peptidases is subdivided into families (e.g. C1, C2, etc) and sub-families (e.g. C1A and C1B); Cathepsin F enzymes belong to the sub-family C1A. The C1 family contains enzymes with very diverse enzymatic activity ranges. This includes endopeptidases with broad specificity (as in the prototype of this family, papain from *Carica papaya*) and others with narrower endopeptidase specificity (such as glycyl endopeptidase), aminopeptidases, and peptidases with both endo- and exopeptidase activity (such as Cathepsins B and H) while some family members do not have catalytic activity (Rawlings and Barrett 1994). The catalytic residues shared by members of the C1 family are cysteine (Cys) and histidine (His). Two other active site residues are found, a glutamic acid (Gln) residue preceding the catalytic Cys and an asparagine (Asn) residue following the catalytic His. The Gln helps in the formation of the 'oxyanion hole' and the Asn in orientating the imidazolium ring of the catalytic His. In those non-peptidase homologues, the catalytic residues have been replaced by other residues [see http://merops.sanger.ac.uk/index.shtml and (Rawlings *et al.* 2010)]. Peptidases of the C1 family are synthesized with a signal peptide to allow their secretion or transportation to the lysosome; they also possess a pro-peptide region at the N terminus, which is cleaved for activation of the enzyme (Rawlings and Barrett 1994) and they may be modified at N-glycosylation sites within their amino acidic sequence (Redmond *et al.* 2006, Santamaria *et al.* 1999). Cathepsin-like cysteine proteases have been identified in many species, both prokaryotes and eukaryotes (Berti and Storer 1995, Rawlings and Barrett 1994). In helminths, various types of cathepsin enzymes have been identified in the phylum *Trematoda* (O'Neill *et al.* 2001, Pinlaor *et al.* 2009, Prowse *et al.* 2002, Robinson *et al.* 2008, Sripa *et al.* 2010), *Cestoda* (Li *et al.* 2006, Sako *et al.* 2007) and *Nematoda* (Britton and Murray 2002, Murray *et al.* 2007, Redmond and Knox 2004, Redmond *et al.* 2006).

Cathepsin F is one member of the C1A sub-family that has been characterised in many species including humans, mice and helminths (Deussing *et al.* 2000, Pinlaor *et al.* 2009, Santamaria *et al.* 1999, Wang *et al.* 1998). Cathepsin F protein was identified by immunoblot in *T. circumcincta* in ES material from L4 larvae (Redmond *et al.* 2006). The protein, Tci-CF-1, has an amino acid sequence of 364 residues (mature enzyme: 214 residues) and the mature enzyme has an estimated molecular mass of ~24 kDa (Redmond

et al. 2006). Two N-glycosylation sites were predicted, one in the pro-region and one in the mature enzyme; glycosylation was later confirmed in lectin-binding affinity experiments (Redmond *et al.* 2006). The relationship between Tci-CF-1 and homologous sequences from *O. ostertagi*, *H. contortus*, *C. elegans* and *Brugia malayi* was explored by phylogenetic analysis of EST data, which showed the presence of close homologues in these other species (Redmond *et al.* 2006). The transcript is only found in L4 and adult stages of *T. circumcincta*, and not in the pre-parasitic stages, suggesting a particular role in the host (Redmond *et al.* 2006). More recent studies have identified Tci-CF-1 as the predominant transcript expressed in the L4-specific nucleotide dataset, and the most abundant molecule in L4 ES (Nisbet *et al.* 2008, Redmond *et al.* 2006). This enzyme has been expressed in recombinant form using the yeast *P. pastoris* and was included in a protective vaccine against *T. circumcincta* (Nisbet *et al.* 2013). In sheep immunised with this recombinant protein then trickle infected with *T. circumcincta* L3 there was an increase in Tci-CF-1-specific serum IgA and IgG; however in the serum of control sheep (trickle infected but not immunised) neither IgA nor IgG binding occurred to the recombinant Tci-CF-1 version (Nisbet *et al.* 2013). These results suggest that Tci-CF-1 expressed in *P. pastoris* may not have an optimal structure or correct PTMs in order to stimulate the optimal protective immune response required against natural infection.

1.6.2 Monocyte Migration Inhibitory Factor (MIF), Tci-MIF-1

A cytokine identified in the mid-1960s and later named Migration Inhibitory Factor (MIF) had been shown to play an important role during delayed hypersensitivity reactions in guinea pigs (Bloom and Bennett 1966, David 1966). The first type of biological activity identified was the inhibition of cell migration. Studies showed that this effect can span species differences, when Thor *et al.* (1968) used human MIF to inhibit guinea pig peritoneal macrophage migration. Since then, human and mouse MIF proteins have been cloned and characterised (Bernhagen *et al.* 1994, Weiser *et al.* 1989), and other functions of this cytokine have been described. The first report of the tautomerase activity of MIF was provided by Rosengren *et al.* (1996). Tautomerase activity was defined as the ability of an enzyme to catalyse the reaction from dopachrome to 5,6-dihydroxyindole-2-carboxylic acid [DHICA (Rosengren *et al.* 1996)]. The same research group showed that MIF is also a keto-enol isomerase (Rosengren *et al.* 1997). Furthermore, mammalian MIF has been shown to also possess oxidoreductase activity, dependent on a specific amino acid motif (the CALC motif; Kleemann *et al.* 1998). Biological effects of MIF include a marked pro-inflammatory effect, as shown by the increase in lethality of LPS-induced endotoxic

shock in mice when recombinant murine MIF was injected (Bernhagen *et al.* 1993, Calandra *et al.* 1995, Roger *et al.* 2001).

MIF protein can be produced not only by activated T cells but also by macrophages (Calandra *et al.* 1995). More recently, a further role in the complex activity of MIF has been shown: parasite-derived MIF was associated with the *in vivo* induction of the alternatively activated phenotype (AAMΦ) in peritoneal exudate cells (PEC; a type of macrophages) of mice which received intraperitoneal injection of *B. malayi* L3 stages or purified recombinant Bm-MIF-1 (Falcone *et al.* 2001, Loke *et al.* 2002). In another study, recombinant Bm-MIF-1 was able to induce *in vitro* the alternatively activated phenotype in bone marrow-derived mouse macrophages (Prieto-Lafuente *et al.* 2009). For example, it is thought that the continuous release of Bm-MIF by adult *Brugia malayi* worms in the host may activate an anti-inflammatory pathway in accord with the anti-inflammatory phenotype of helminth infection (Kleemann *et al.* 2000, Maizels and Yazdanbakhsh 2003). The debate on the complete roles of MIF is still open, and research on this cytokine continues; as described above, the apparent contrasting pro- and anti-inflammatory qualities make MIF an interesting subject in the study of the more general biology of the immune response to pathogens. A number of MIF homologues have been identified in numerous species, including sheep, cattle, ticks, fish and a number of parasitic nematode species (Galat *et al.* 1993, Jaworski *et al.* 2001, Lopes *et al.* 2011, Sato *et al.* 2003, Vermeire *et al.* 2008); such widespread conservation suggests important biological functions among organisms [reviewed by Calandra and Roger (2003)]. In studies aimed at identification of *T. circumcincta* vaccine candidates, a MIF-like protein was characterised in *T. circumcincta* L3 stage (Nisbet *et al.* 2010b). It has been hypothesised that *T. circumcincta* might excrete Tci-MIF-1 from its gut into the host, from where it could influence monocyte movement and activation in the abomasum. In that study, preliminary data showing Tci-MIF-1 acting as host cytokine mimic was presented; however, its ability to inhibit sheep monocyte migration was unconvincing and the presence of Tci-MIF-1 in *T. circumcincta* ES material has not been demonstrated (Nisbet *et al.* 2010b, Smith *et al.* 2009). This suggests that the current existing recombinant version of Tci-MIF-1 may not have the correct structure and/or PTMs required to carry out the biological function of inhibitor of monocyte migration. However, the potential biological effects and immunoregulatory activity on the sheep host make Tci-MIF-1 a promising vaccine candidate worth further characterisation in its recombinant forms.

1.7 *Caenorhabditis elegans* as a novel expression system

1.7.1 Model organism

Caenorhabditis elegans is a free-living nematode which normally resides in soil. It is a multicellular organism with a 3.5-day lifecycle and exists in two sexes, hermaphrodite and male organisms. The gonad occupies most of the body cavity and is bilobed [see http://www.wormbook.org/]. *C. elegans* belongs to Clade V of the Phylum Nematoda (Blaxter *et al.* 1998) and is therefore related phylogenetically to *T. circumcincta* and other important veterinary trichostrongylid nematodes.

C. elegans is an ideal model system in which to study nematode biology due to its simplicity, short life-cycle, availability of annotated genome sequence and ease of genetic manipulation (Brenner 1974, *C. elegans* Sequencing Consortium 1998). In this study, both Tci-CF-1 and Tci-MIF-1 will be expressed in recombinant form using *C. elegans* as an alternative expression system. Expressing proteins using *C. elegans* involves the cloning of the desired gene into an appropriate plasmid vector, which is then micro-injected into the gonad of young adult *C. elegans* hermaphrodites. The selected gene is injected together with another plasmid encoding a marker gene which generates an identifiable marker phenotype (Mello *et al.* 1991). The resulting progeny are screened for expression of the marker phenotype as an indication of successful transformation (summarised In Figure 1.5). Individual worms are picked, separated and maintained in culture to assess their ability to generate a stable clone expressing the injected genes. After at least two generations, the line can be considered stable and the worms screened by PCR for inclusion of the gene of interest, or directly for protein expression (Murray *et al.* 2007, Roberts *et al.* 2013). Previous studies have shown that *C. elegans* can be used as an alternative to the traditional bacterial and yeast systems to express nematode proteins and to study conservation of function across species (Britton and Murray 2002, Murray *et al.* 2007, Pillai *et al.* 2005, Redmond *et al.* 2001). Indeed *C. elegans* has been the expression system of choice in characterisation of the activity and immunogenicity of *H. contortus* cathepsin L cysteine protease (Murray *et al.* 2007) and more recently for the aminopeptidase H11 (Roberts *et al.* 2013). Both were shown to be active and glycosylated in *C. elegans* with similar glycans to those of the native parasite proteins.

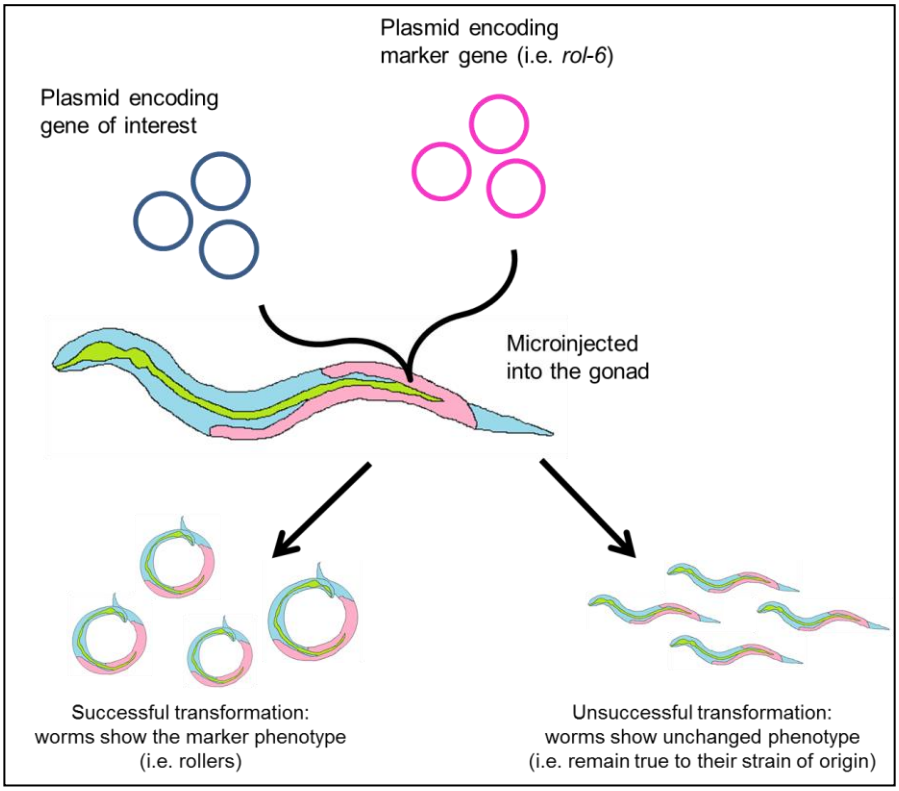

Figure 1.5 Diagram showing the theory of the *C. elegans* transformation protocol by microinjection.
Plasmids encoding the gene of interest and a marker gene are microinjected into the gonad of each *C. elegans* hermaphrodite (in pink). The progeny of each injected worm is screened for the presence/absence of marker phenotype; transformation is considered successful if worms maintain the transformed phenotype for subsequent generations, which can be isolated as a stable line.

1.8 Aims of this study

The overall aim of this work was to contribute to the further development of the prototype recombinant subunit *T. circumcincta* vaccine (Nisbet *et al.* 2013), by understanding the differential host immune response to native and recombinant versions of putative protective antigens and engineering the recombinant subunits to overcome such differences. Existing recombinant versions of *T. circumcincta* vaccine candidates may be inducing a sub-optimal immune response, most likely due to differing structure and/or PTMs compared to the native versions. In this respect, the overall aim of this work is to evaluate a nematode-based expression system, in an attempt to improve the existing recombinant versions in regards of their immune recognition and biological functions.

The specific aims of this work were:

i) To investigate the sheep antibody response against native preparations of native Tci-CF-1 (purified from *T. circumcincta* L4 ES) and compare these with immunisation-induced antibody responses to recombinant *P. pastoris*-expressed Tci-CF-1. This will be achieved using a combination of ELISA and Western blotting techniques with ovine biological samples of different provenance (serum, lymph node cell supernatants and gastric lymph), to highlight the differences between systemic and local immune responses to naive and recombinant Tci-CF-1.

ii) To generate *C. elegans*-expressed recombinant *T. circumcincta* vaccine candidates.

iii) To investigate the properties of recombinant versions of Tci-CF-1 and Tci-MIF-1; this will be achieved by characterisation of the specific enzyme activity, immunogenicity and/or effects on cell migration, cytokine release and gene expression as appropriate for each molecule.

Chapter 2 - Characterisation of Ovine Antibody Binding to Native and Recombinant *Teladorsagia circumcincta* cathepsin F (Tci-CF-1)

2.1 Introduction

Both cellular and humoral responses are involved in the acquired immunity to *T. circumcincta* parasites in sheep (Halliday *et al.* 2009, Halliday *et al.* 2007, Smith *et al.* 1986, Smith *et al.* 1983). In this context, IgA antibodies have been associated with reductions in adult worm length by inhibiting worm growth and delaying worm development (Halliday *et al.* 2007, Stear *et al.* 1995, Stear *et al.* 1999, Strain *et al.* 2002), whereas IgE responses were correlated with reduced faecal egg counts (Huntley *et al.* 2001, Pettit *et al.* 2005). Significantly higher levels of IgE against a "High Molecular Weight Allergen", an L3 stage-specific *T. circumcincta* antigen, were observed in 6 month old lambs termed "responders" (with low cumulative FEC scores; mean values not available) when compared to sheep termed "non-responders" (those with high FECs; mean values not available; Huntley *et al.* 2001). Previous studies have shown that, in sheep that received a *T. circumcincta* L3 trickle infection followed by an anthelmintic treatment and a 50 000 L3 bolus challenge seven days later, gastric lymph IgA reactivity to L4 Excretory/Secretory (ES) products increased significantly from pre-challenge levels, between 6-10 days post challenge (dpc) (Halliday *et al.* 2007). During the course of a similar experiment, a negative correlation was found between worm burdens and L4 ES-specific lymph IgA levels at 10 dpc (Routledge 2008). *Teladorsagia circumcincta* L4 ES-specific serum IgG levels were shown to be significantly higher between 2 and 10 dpc in sheep that received an L3 trickle infection followed by a 50 000 L3 bolus challenge (previously infected) compared to the control group (primary infected *T. circumcincta* naïve sheep which only received a bolus challenge; Routledge 2008). Although there is strong evidence to suggest that immunity against this parasite depends on the production of specific antibodies, the involvement of cellular immune responses cannot be excluded. Indeed, as discussed in a review by McNeilly *et al.* (2009), the recruitment of immune cells in a Th2-biased environment, such as DCs, mast cells, eosinophils and lymphocytes, appears pivotal in building a complete immune response to gastric parasites such as *T. circumcincta* (Balic *et al.* 2003, Balic *et al.* 2000, Craig *et al.* 2014, Scott *et al.* 1998, Scott *et al.* 2000, Stevenson *et al.* 1994).

Although serum IgA levels to *T. circumcincta* L4 somatic extracts were shown to be positively correlated with mucus IgA against the same antigens (Stear *et al.* 1995), serum IgA binding to these antigens showed heterogeneous antigen recognition patterns in individual sheep (Strain and Stear 1999). Variability in the individual sheep serum IgA response to L4 ES antigens was also documented (Routledge 2008). In an experiment carried out by Stear *et al.* (1995), 6-month old lambs exposed to natural mixed parasite infection were included in an experimental model where, after housing in a helminth-free environment for three months, they received two *T. circumcincta* bolus challenges (50 000 L3, 12 weeks apart). In this study it was shown that as the worm burdens in the sheep hosts increased, there was a decrease in the proportion of *T. circumcincta* L4 somatic extract-specific IgA transferred from mucosa to serum (Stear *et al.* 1995). These results were further confirmed by De Cisneros and colleagues (2014). Taken together, these examples suggest that, although easily accessible for sampling, serum might not represent the optimal sample type to measure local immune responses and the immunogenicity of *T. circumcincta* antigens in the sheep host. The possibility of surgically accessing the efferent lymph ducts draining the cranial digestive tract (including the abomasum) allows the collection of lymph draining the site of *T. circumcincta* infection (Smith *et al.* 1981). This technique has proved useful in understanding the kinetics of the local mucosal responses to *T. circumcincta*, providing an alternative to post-mortem derived abomasal mucus (Halliday *et al.* 2010, Halliday *et al.* 2009, Halliday *et al.* 2007, Smith *et al.* 1983, 1983). Additionally, Antibody Secreting Cell (ASC) probes, generated from lymph nodes anatomically close to the site of infection, have been shown to be a useful tool to investigate the parasite antigen-specific immune responses, providing a stage- and tissue-specific snapshot of the antibody reactivity elicited by the parasites *T. circumcincta*, *Ascaris suum*, *Haemonchus contortus*, *Ostertagia ostertagi*, *Taenia hydatigena* and *Fasciola hepatica* (Balic *et al.* 2003, Bowles *et al.* 1995, De Maere *et al.* 2002, Jungersen *et al.* 2001, Meeusen and Brandon 1994b, Meeusen and Brandon 1994a).

ES products of helminths can provide antigenic stimulus to the host immune system, providing a valuable source of antigens and making ES products promising vaccine candidates against ruminant parasites (Knox 2000, Lightowlers and Rickard 1988, Nisbet *et al.* 2013, O'Donnell *et al.* 1989, Schallig *et al.* 1997, Vercauteren *et al.* 2004). For example, immunising calves with *O. ostertagi* thiol-enriched ES products resulted in a protective immune response against the parasite, with a significant reduction of 56–62 % in cumulative faecal egg counts (FECs) and a significant reduction in worm length following 25 days (1000 L3/day, 5 days/week) of trickle infection (Geldhof *et al.* 2002, Geldhof *et*

al. 2004, Meyvis *et al.* 2007). The choice of adjuvant was also found to influence the outcome of the protection trials: calves vaccinated with *O. ostertagi* ES-thiol fractions and Quil A as adjuvant showed a 56 % reduction in FEC and significantly less eggs/female worms compared to animals vaccinated with *O. ostertagi* ES-thiol/aluminium hydroxide and an adjuvant control group (Geldhof *et al.* 2004). *Ostertagia ostertagi* thiol-bound ES was fractionated using a Q-Sepharose anion exchange column to separate components in activation-associated secreted proteins (ASPs), cysteine proteases and a further remaining fraction. Each was used to immunise calves and a significant reduction in FEC (70-80 %) and worm length was induced by each fraction compared to the control group (Meyvis *et al.* 2007). In another study, preliminary data on the immunisation of lambs using concentrated ES products from mixed stage *T. circumcincta* in Freund's complete adjuvant resulted in lower worm burdens, worm lengths and FECs compared to the control lambs (Rose 1976, 1978). In these studies, although no statistical analysis was carried out, the data collected showed protective potential of the concentrated mixed-stage ES products used. Based on success of *O. ostertagia* ES-fractionation studies, further immunisation trials were carried out using a detergent-soluble, ConA-binding fraction of *T. circumcincta* L4 whole worms (Halliday and Smith 2011). This preparation however, contrary to the encouraging results shown by Geldhof *et al.* (2002, 2004) and Meyvis *et al.* (2007), showed that no protection could be achieved against *T. circumcincta* L3 bolus or trickle infection challenge with the L4 fraction (Halliday and Smith 2011).

It has been shown that native ES products exhibit stage-specificity and are low in protein levels when produced in vitro; for example, *Trichostrongylus colubriformis* produced ~1 mg ES protein per 25 000 adult worms per day (Emery 1996). *Teladorsagia circumcincta* L4 harvested from five donor sheep seven days after challenge with 150 000 L3 each produced ~2 mg protein over two days of *in vitro* culture (David Frew, personal communication). These factors would make native antigen production from ES an expensive way to manufacture vaccines, with the added problems of batch variability and difficulties in performing quality controls (Emery *et al.* 1993, Smith and Zarlenga 2006). The low protein yield has led to further analysis of the composition of parasite ES, for identification and expression of protective antigens as recombinant proteins (Schallig *et al.* 1997, Smith *et al.* 2009). The advantages in a vaccine development approach would be that recombinant antigens can be engineered and manipulated to be as closely structured as possible to the desired native protein if this was not available or easily accessible, or to specifically add different features [e.g. modifying signal sequences to improve purification steps, targeting a specific promoter to induce expression, modifying glycosylation sites or

engineering fusion proteins and viral vectors by adding epitopes from other antigens (chimeras); Hansson *et al.* 1994, Keyt *et al.* 1994, Koths 1995, Liljeqvist and Stahl 1999, Makrides *et al.* 1996, Suarez *et al.* 1997, Walmsley and Arntzen 2000, Yusibov *et al.* 1997)]. In addition, recombinant proteins can be produced on larger scales, with less cost and reductions in the use of donor animals compared to using native ES preparations, although the knowledge on the native antigens on which they are based on must be sound (Geldhof *et al.* 2007).

Parasite ES products contain, among other proteins, a wide range of proteases [(Yatsuda *et al.* 2003), reviewed by Knox (1994)]; for example, the activity of four classes of proteases has been shown in *Trichinella spiralis* L1 ES (Lun *et al.* 2003). *Teladorsagia circumcincta* ES products are also a complex mix of molecules (Craig *et al.* 2006). Proteomic data were obtained from *T. circumcincta* L3- and L4-derived ES from larvae cultured *in vitro* following collection at 1, 3 and 5 days post infection (Smith *et al.* 2009). BLAST analysis of the data against *T. circumcincta* EST databases revealed 15 significant matches, ten of which were found to be L4-specific [an aspartyl protease, several ASP-like proteins, an apyrase, an astacin-like metalloprotease, a cysteine protease and several proteins of unknown function (Smith *et al.* 2009)]. One of the matches, a cysteine protease named Tci-CF-1, had been previously identified as the most abundant protein in *T. circumcincta* L4 ES (Redmond *et al.* 2006), and as the predominant transcript present in the L4-specific nucleotide dataset (Nisbet *et al.* 2008). Tci-CF-1 has an estimated molecular mass of 24 kDa and it was shown to have close homologues in other helminth species such as *O. ostertagi, H. contortus, Caenorhabditis elegans* and *Brugia malayi.* Two N-glycosylation sites were predicted and subsequently confirmed in lectin-binding affinity experiments (Redmond *et al.* 2006). Tci-CF-1 was shown to be an immunogenic component of L4 ES in immunoblot experiments in which lymph IgA derived from sheep previously infected by trickle infection with *T. circumcincta* L3 (2000 L3 three times a week for 9 weeks) and mucus IgA from sheep grazed on naturally infected pasture, then trickle infected with *T. circumcincta* L3 (2000 L3 three times a week for 10 weeks), bound to this and other antigens in *T. circumcincta* L4 ES (Redmond *et al.* 2006, Smith *et al.* 2009). Serum IgG and IgA from sheep immunised with a recombinant version of Tci-CF-1 and trickle infected with *T. circumcincta* also showed reactivity to native Tci-CF-1 in immunoblots of L4 ES. These characteristics were pivotal in choosing Tci-CF-1 as a vaccine candidate (Nisbet *et al.* 2013).

Recombinant Tci-CF-1 expression was first carried out in *E. coli* but the protein was expressed in an insoluble form. During an experimental infection regime, serum and gastric lymph antibody binding (IgA, IgG1 and IgG2) to *E. coli*-expressed Tci-CF-1 showed no significant difference between sheep which received a primary infection and those previously infected with *T. circumcincta* (in the form of a trickle infection followed by a 50 000 L3 bolus challenge; Routledge 2008). This showed that the recombinant *E. coli*-expressed protein was not recognised by infection-induced antibodies. These results also suggests that *E. coli*-expressed Tci-CF-1 may not have undergone the correct post-translational modifications required for solubility and/or antibody binding observed previously in gastric lymph of trickle infected and challenged animals against the native form of Cathepsin F (nTci-CF-1) (Redmond *et al.* 2006). *E. coli* expression systems do not always complete the post-translational modifications required to generate a recombinant protein similar to the native form. Notable is the lack of glycosylation and the difficulty in expressing proteins containing disulphide bonds, due to the reducing environment of the prokaryotic cytoplasm (Macauley-Patrick *et al.* 2005). To overcome these limitations, an alternative approach, using yeast *Pichia pastoris* as an expression system has several advantages, among which are very high levels of secretion into an almost protein-free medium, ease of fermentation, high cell density and the possibility to maintain genetic stability and scale-up expression without loss of yield (Darby *et al.* 2012, De Schutter *et al.* 2009, Romanos 1995). Importantly, *P. pastoris* is also capable of performing post-translational modifications such as folding and glycosylation that cannot be performed in prokaryotic systems (Bretthauer and Castellino 1999, Macauley-Patrick *et al.* 2005). This may be an advantage when expressing a glycosylated protein such as nTci-CF-1 (Redmond *et al.* 2006).

A recent study (Nisbet *et al.* 2013) showed the protective effect of a *T. circumcincta* vaccine containing 8 different recombinant antigens; one of the antigens included in the panel was a Tci-CF-1 version expressed in the yeast *Pichia pastoris*. In these experiments, the development of serum IgG and IgA responses which recognised the recombinant Tci-CF-1 versions was shown, following immunisation with the recombinant protein cocktail. These antibodies in a serum pool from immunised sheep were shown to bind to native Tci-CF-1 in immunoblots of *T. circumcincta* L4 ES. In the control group animals (which only received trickle infection following immunisation with adjuvant only), the IgG and IgA binding to native Tci-CF-1 in immunoblots of *T. circumcincta* L4 ES was markedly less compared to the immunised animals. However, in the control trickle-infected only sheep there was also lack of either IgG or IgA reactivity against the recombinant *P. pastoris*-

expressed Tci-CF-1, suggesting that infection-induced antibodies were able to a certain extent to bind native Tci-CF-1 but unable to recognise the recombinant version. Moreover, the *T. circumcincta* trickle infection did not induce a serum antibody booster in the immunised animals (anamnestic response), also suggesting that vaccination-induced B cells and antibodies were not able to respond to the native antigens (Nisbet *et al.* 2013). Therefore, this study proposed to generate a recombinant version of Tci-CF-1 expressed in *P. pastoris* and to compare its immune recognition with that of the native form (nTci-CF-1) purified from *T. circumcincta* L4 ES.

The overall aims of this chapter are:

i) to purify native Tci-CF-1 from L4 ES material;

ii) to generate a full length nucleotide sequence of Tci-CF-1, to be used for downstream cloning and recombinant protein expression in the yeast *Pichia pastoris*;

iii) to assess the differential immune recognition of nTci-CF-1 and *P. pastoris*-expressed Tci-CF-1 (PiTci-CF-1) using serum, antibody secreting cell (ASC) probes and gastric lymph samples from a number of animals with different infection status in order to investigate the recognition of these antigens by the local and systemic antibody response to *T. circumcincta*.

2.2 Materials and methods

2.2.1 Purification of native Tci-CF-1 (nTci-CF-1)

2.2.1.1 Purification of nTci-CF-1 from L4 ES samples

Teladorsagia circumcincta L4 ES was fractionated by Stuart Smith, George Newlands and Neil Inglis at MRI. The ES samples were obtained by a stock previously generated at MRI and following application to a Superose 12 HR 10/30 size exclusion column (GE Healthcare, Little Chalfont, UK) using an Äkta Protein Purifier HPLC system (GE Heathcare), were eluted in PBS. Fractions thus obtained by gel filtration were collected and stored at -80°C until use. SDS-PAGE analysis was carried out as described in Appendix B to determine which fractions contained nTci-CF-1.

2.2.1.2 Enrichment of nTci-CF-1 using a thiol-sepharose column

A protocol adapted from (Geldhof *et al.* 2002) was used to further purify nTci-CF-1 from one of the L4 ES fractions previously obtained. An empty column (GE Healthcare) was pre-packed with 1 g of thiol-sepharose matrix (Activated Thiol-Sepharose® 4B, GE Healthcare) that had been re-hydrated overnight in 10ml of PBS to give a ~5 ml gel bed column volume. The packed column was equilibrated in PBS using a chromatography pump (Pharmacia Biotech, GE Healthcare) at 4°C for 2.5 h, at a flow rate of 10 ml/h. Native Tci-CF-1 samples were incubated with 2.5 mM Dithiothreitol (DTT, Sigma Aldrich, Gillingham, UK) at 37°C for 30 min, then washed in PBS using Amicon Ultra-15 centrifugal filter with a 10 kDa MWCO (Merck-Millipore, Billerica, MA, USA), to reduce the concentration of DTT to < 0.02 mM. The samples were applied to the column and re-circulated (1 ml/h) overnight at 4°C. The unbound fraction was collected until the UV absorbance levelled to base line; the column was washed with 5 column volumes of PBS. The bound fraction was eluted in 10 ml of PBS/50 mM DTT. The eluted fraction was buffer exchanged and concentrated in PBS using an Amicon Ultra-15 centrifugal filter with a 10 kDa MWCO (Merck-Millipore). Concentration of thiol-enriched nTci-CF-1 was estimated by band densitometry on a Coomassie-stained gel using the ImageQuant 4000 (GE Healthcare) instrument and accompanying software. The protein purity before and after thiol-enrichment was estimated using the software ImageJ by densitometry analysis of the mean grey value for each entire lane and bands within, after background subtraction; the purity was calculated as % purity = [value(band)/value(lane)] × 100. The concentrated fractions were aliquoted and stored at -80°C until use. The identity of the major band

present in pre- and post-thiol enrichment fraction was confirmed by Liquid chromatography – Electron spray ionisation - Tandem mass spectrometry (LC-ESI-MS/MS) performed at MRI by the Proteomics Department.

2.2.2 Generation of full length cDNA *Tci-cf-1* sequence

2.2.2.1 PCR amplification of Tci-cf-1 from L4 cDNA

Fourth stage larval (L4) *T. circumcincta* cDNA and primers for PCR amplification of *Tci-cf-1* full length sequence were provided by Dr Alasdair Nisbet (MRI) (see primers set no.1, Table 7.1, Appendix A). Each reaction was made up with of 5 µl 10x Advantage® 2 PCR Buffer (Clontech, Saint-Germain-en-Laye, France), 1 µl each of 5' and 3' primers set 1 (10 pmol working solution), 1 µl 50x dNTP Mix (Clontech, Saint-Germain-en-Laye, France), 2 µl of L4 cDNA, 1 µl of 50 X Advantage® 2 Polymerase Mix (Clontech), made up to 50 µl with dH_2O. Cycling conditions were: 1 min at 95°C, 30 cycles of (15 sec at 95°C, 3 min at 55°C, 3 min at 68°C). Negative controls included dH_2O instead of cDNA template.

2.2.2.2 Cloning procedures

The full length *Tci-cf-1* PCR products were column purified (QIAquick PCR Purification Kit, Qiagen, Manchester, UK), cloned into pGEM-T Easy and transformed into 50 µl of JM109 competent cells by heat shock according to the manufacturer's instructions (Promega, Southampton, UK). The transformed cells were incubated in 950 µl SOC medium (Appendix D) at 37°C for 2.5 h, with shaking. Luria-Bertani (LB) agar plates were prepared as described in Appendix D; to 50 ml of molten LB agar, 200 µl of 25 mg/ml ampicillin stock (final concentration 100 µg/ml) were added. Once set, 20 µl of 50 mg/ml X-Gal stock (Promega) and 10 µl of 1M IPTG were spread over each plate. One hundred microliters of transformed cells in SOC medium were spread over each plate. The plates were left overnight at 37°C, white colonies picked the following day with a sterile toothpick and placed in 40 µl of dH_2O. Colony PCR was carried out as follows: 2.5 µl 10 X PCR reaction buffer with $MgCl_2$ (Roche, Welwyn Garden City, UK), 0.5 µl each of 5' and 3' primer set 1 (10 pmol working solution, Table 7.1), 1 µl dNTPs (Roche), 2.5 µl of a dH2O solution in which individual colony stabs have been suspended, 1.25 U of Taq DNA Polymerase (Roche), made up to 25 µl with dH_2O. Cycling conditions were: 5 min at 94°C, 30 cycles of (30 sec at 94°C, 30 sec at 55°C, 1 min 30 sec at 72°C) and a final extension period of 7 min at 72°C. Colonies that resulted positive for PCR product ligation into plasmid were amplified overnight in 10 ml LB broth (Appendix D) with the addition of

100 µg/ml ampicillin. Plasmid DNA was purified from overnight cultures using the Wizard® Plus SV Minipreps DNA Purification System (Promega) following the manufacturer's instructions.

2.2.2.3 Sequencing and sequence analysis

Plasmid insert DNA was sequenced by Eurofins MWG Operon (Germany) using the commercially available primers SP6 and T7. Further sequencing primers were used as indicated in each relevant section of this chapter. The sequence data were analysed with the software packages DNASTAR Lasergene 8 (DNASTAR Inc., Madison, WI, USA) and the freeware Chromas Lite (Technelysium Pty Ltd) to visualise chromatograms. The free online alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for sequence alignment studies.

2.2.3 Generation of recombinant Tci-CF-1 in the yeast *Pichia pastoris*

The full length *Tci-cf-1* generated in Section 2.2.2 was modified by PCR to omit the signal sequence and insert restriction sites as follows: primer set no. 2 (Table 7.1) was used in reactions containing 5 µl of 10 X PCR Buffer for KOD Hot Start DNA Polymerase (Toyobo Novagen, supplied by Merck4Biosciences UK), 2 µl of $MgSO_4$ (final concentration 1 mM), 5 µl of dNTPs (final concentration 0.2 mM), 1.5 µl (15 pmol) each of the 5' and 3' primers, 0.5 µg of a plasmid template from one of the clones containing full length *Tci-cf-1*, 1 unit of KOD Hot Start DNA Polymerase (Toyobo Novagen) made up with dH_2O to a final volume of 50 µl. Cycling conditions were: 2 min at 94°C, 30 cycles of (15 sec at 94°C, 30 sec at 58°C, 1 min 30 sec at 72°C) and a final extension period of 5 min at 72°C. The *Tci-cf-1* product (with restriction sites and without signal sequence) was double digested with *Xba*I and *Pml*I (New England Biolabs, Hitchin, UK). The plasmid pPICZα C (Invitrogen, Paisley, UK) was linearised using *Xba*I and *Pml*I (New England Biolabs), gel-purified and then used to ligate the *Tci-cf-1* sequence as follows: 5 µl of 2 X Ligation Buffer (Promega), 1 µl (210 ng) digested *Tci-cf-1*, 3 µl (11 ng) of gel-purified, linearised pPICZα C and 1 µl of T4 Ligase enzyme (Promega). The ligation reaction was used to transform *E. coli* JM109 competent cells (Promega) by heat shock method and cultured on Low Salt Luria-Bertani (LB) agar plates (Appendix D) with 25 µg/ml of Zeocin®. Colony PCR was carried out using primers sets no. 2 or the α-factor (F) and 3'AOX (R) oligonucleotide primers (all in Table 7.1, Appendix A) in separate reactions. Colony PCR was carried out using primer pair 5'AOX (F) and 3'AOX (R) and

set no. 3 [*Tci-cf-1* internal primers, ForIN (F) and RevIN (R); Table 7.1]. Reactions were made up of 12.5 µl of SapphireAmp® Fast PCR Master Mix (TaKaRa BioInc, Shiga, Japan), 0.5 µl of each forward and reverse primer (working solution 10 pmol) and 9.5 µl of dH_2O. The following cycling conditions were used: 5 min at 94°C, 30 cycles of (30 sec at 94°C, 30 sec at X°C, 1 min 30 sec at 72°C) and a final extension period of 5 min at 72°C, where X was Tm - 2°C.

Sequences of isolated plasmids were analysed as described in section 2.2.2.3. A clone of the pPICZα C plasmid containing *Tci-cf-1* was purified then linearised using *Pme*I (New England Biolabs). The transformation of *Pichia pastoris* cells (strain X33 Mut^+) was carried out following the manufacturer's instructions (EasySelect™ Pichia Expression Kit, Invitrogen, Paisley, UK). *Pichia pastoris* cells were made competent and transformed by electroporation using the following conditions: 1.5 kV, 25 µF and 200 Ω using a Gene Pulser® II (Bio-Rad, Hemel Hempstead, UK) apparatus. The transformed cells were plated on Yeast Peptone Dextrose Sorbitol (YPDS) agar plates (Appendix D) with 100 µg/ml Zeocin®, and incubated at 29°C for 2 days until colonies developed. Colony PCR was carried out as described above using a primer combination consisting of forward: α factor (primer set 4), reverse: RevIN (primer set 3) (see Table 7.1). Colonies were grown in BMGY (Appendix D) and subsequently maintained in BMMY (Appendix D) for 96 h, when the expression of Tci-CF-1 was induced with 0.5 % methanol following the manufacturer's instructions. SDS-PAGE analysis was used to identify a colony expressing Tci-CF-1.

2.2.4 Recombinant protein purification using Ni^{2+} columns

The cell culture supernatants of *P. pastoris* cultures were applied to a Ni^{2+} column (HisTrap™ HP Column, GE Healthcare) to purify the expressed, histidine-tagged proteins. Briefly, the column was attached to a bench-top chromatography pump system (Pharmacia Biotech) and washed with dH_2O for 5 min at a flow rate of 1 ml/min. The column was then equilibrated with 5 column volumes of Binding Buffer A (Appendix D). The *P. pastoris* supernatant was applied neat to the column and left to re-circulate through the column for 1-2 h at a flow rate of 1 ml/min. The column was then washed with 5 column volumes of Binding Buffer A before applying Elution Buffers 1-4 (Appendix D) containing an imidazole step gradient ranging between 165-500 mM. The purified recombinant Tci-CF-1 was dialysed against 0.1 M Sodium Phosphate Buffer, 0.5 M NaCl pH 7.4 using a Mini Dialysis Kit (GE Healthcare) with a molecular weight cut-off (MWCO) of 8 kDa. Protein

expression and identity as Tci-CF-1 was confirmed by SDS-PAGE analysis and by Matrix Assisted Laser Desorption/Ionisation - Time of Flight (MALDI-ToF) spectrometric analysis carried out at MRI Proteomics Unit.

2.2.5 Experimental animals

2.2.5.1 Serum and lymph nodes provenance

As described previously by Nisbet *et al.* (2013), fourteen Texel crossbred lambs, 204-206 days old at the start of a prototype recombinant vaccine experiment, were reared under conditions to exclude helminth infection and separated in groups of 7 (Group A: immunised, B: control). Subsequently, twenty-eight Texel crossbred lambs, 172-178 days old at the start of the experiment, were also separated in groups of 7 animals each (Groups C: immunised, killed at d 84 post immunisation (p.i.), D: control, killed at d 84 p.i., E: immunised, killed at d 112 p.i., F: control, killed at d 112 p.i.). Each animal in groups A, C and E received three immunising doses 3 weeks apart, each containing 50 μg of each of 8 recombinant antigens, one of which was PiTci-CF-1. The injections contained also PBS/urea and Quil A as adjuvant (immunised groups). Each animal in groups B, D and F only received PBS/urea/Quil A injections (control groups). The injections were administered for each animal subcutaneously, in the cervical area, following this pattern: left side of the neck for the first injection, right for the second injection, and left side for the last injection. Following the final antigen immunisation or mock immunisation, every animal in each group received a trickle infection of 3 000 *T. circumcincta* L3, three times a week for 4 weeks. Blood samples were taken weekly and at post mortem, which took place 84 days (groups A-D) or 112 days (groups E-F) after the start of the experiment. All animals from groups A and B and two randomly chosen animals from each group C and D were used in the work described herein for the collection of serum and lymph node samples. The experimental plan for groups A-D is outlined in Fig. 2.1.

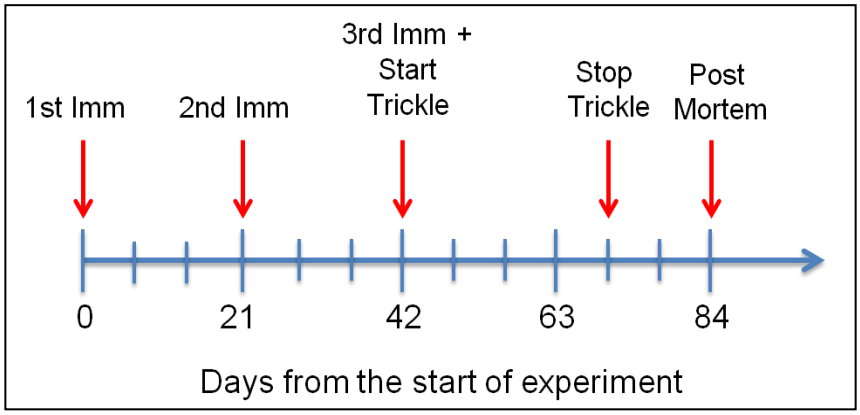

Figure 2.1 Diagram outlining the experimental protocol for animals in groups A-D. Serum samples were collected weekly, but only the dates on which samples relevant to this project were collected are shown by annotation with red arrows. "Imm" represents dates on which sheep were immunised; "Trickle" represents the trickle infection (3 times weekly between day 42 and 70). At post mortem, the last serum and lymph node samples were collected.

Antibody Secreting Cell (ASC) probe samples were collected, as described below, from 18 animals included in the study outlined in Fig. 2.1, listed in Table 2.1. To reflect the local immune responses to *T. circumcincta* following trickle infection, two to four lymph nodes from the abomasal lymph node chain were collected from each of the 14 animals (7 immunised, 7 non-immunised controls) in groups A and B, and from 4 randomly chosen animals in groups C (2 immunised) and D (2 non-immunised controls). From the same animals both left and right pre-scapular lymph nodes were also collected, as the immunisation protocol was administered on rotation between the left and right side of the neck, as described above. The collection method is described in Section 2.2.5.4. Within the same animal, cells from the same lymph node location (i.e. abomasal chain or left/right pre-scapular) were pooled prior to in vitro culture. This yielded two supernatant samples from each animal, one pool of abomasal lymph node cells secreted antibodies (Abo), and one pool of pre-scapular cells secreted antibodies (PreSc). Table 2.1 (below) summarizes the animals used for serum and lymph node collection.

2.2.5.1.1 Additional serum samples

Two serum samples, representing pre- and post-immunisation with a previously generated PiTci-CF-1 version, were made available by Dr Alasdair Nisbet and Dr Tom McNeilly (MRI). These samples originated from a pilot immunisation trial of sheep immunised with 50 µg each of three *T. circumcincta* recombinant proteins, one of which was PiTci-CF-1, for each immunisation (2 in total, administered intramuscularly at day 0 and 28 of the

experiment). Quil A was used as adjuvant. Serum samples were collected weekly for 6 weeks throughout the experiment, though for the purposes of this study only the pre-immunisation (day 0) and the final (day 42, at post-mortem examination) samples were used. The sheep did not receive *T. circumcincta* trickle infections (see Table 2.1).

Table 2.1 List of all the experimental animals from which serum and lymph node samples were collected for this project.

Animal number	Code given	Breed/Age at start of experiment	Infection/Immunisation status	Time points used in this project	Reference
6450H	Imm.1	Texel X, 204-206d	Immunised, trickle infected for 4 weeks	From start of trial: Serum: 0, 21, 42, 63, 84 d ASC: 84 d (PM)	Nisbet *et al.* (2013)
6451H	Imm.2				
6455H	Imm.3				
6474H	Imm.4				
6496H	Imm.5				
6507H	Imm.6				
6508H	Imm.7				
679J	Imm.8	Texel X, 172-178d			
683J	Imm.9				
6453H	Ctrl.1	Texel X, 204-206d	Adjuvant-only, trickle infected for 4 weeks		
6462H	Ctrl.2				
6468H	Ctrl.3				
6482H	Ctrl.4				
6484H	Ctrl.5				
6492H	Ctrl.6				
6494H	Ctrl.7				
639J	Ctrl.8	Texel X, 172-178d			
661J	Ctrl.9				
Sheep 1		Unknown	Immunised, not trickle infected	Serum: 0, 42 d (PM)	Tom McNeilly and Alasdair Nisbet, unpublished data
Sheep 2					
Sheep 3					

2.2.5.2 Gastric lymph provenance

A number of gastric lymph samples had been collected during previous experiments in collaboration between MRI and R(D)SVS, described by Halliday *et al.* (2007, 2010). Animals had been treated as follows: sheep were trickle infected with 2 000 *T.*

circumcincta L3, three times a week, for 8 weeks, then 2 days later cleared of infection with an anthelminthic treatment (benzimidazole class, at the dose of 5 mg/kg) and 7 days later challenged with a bolus of 50 000 *T. circumcincta* L3 larvae (termed "previously infected" sheep from here onwards). Lymph samples from 6 of those animals between +4 and +10 days post-challenge infection (dpc) (representing the anamnestic response to *T. circumcincta*) were retrieved from the collection stored at MRI. Multiple lymph samples from each individual animal at different time points between +4 and +10 dpc were pooled to give a representative anamnestic lymph sample for each animal. Controls were animals which only received a primary infection as a bolus of 50 000 L3 *T. circumcincta* at the same time as the previously infected animals (termed "primary infection" sheep from here onwards). The samples were collected between -2 and +2 dpc from 6 animals, before a primary immune response to *T. circumcincta* antigens was developed [as shown by Halliday *et al.* (2007), the peak lymph IgA following *T. circumcincta* infection occurs after +6 dpc, before decreasing around +10 dpc]. Multiple lymph samples from each individual animal at different time points between -2 and +2 dpc were pooled to give a representative anamnestic lymph sample for each animal.

In addition, a smaller set of 3 gastric lymph samples was obtained courtesy of Dr John Huntley and Dr David Smith (Moredun Research Institute). The animals in this group had been part of a previous experiment (David Smith, 1987 unpublished data). Included in this group were gastric lymph samples from 3 animals which received an 8-week *T. circumcincta* trickle infection and subsequently treated with fenbendazole, then received a 50 000 infective *T. circumcincta* L3. From one of the animals, lymph was collected -5dpc to represent the antibody levels after trickle infection, but before the bolus challenge. From the remaining 2 animals, the lymph was collected +5 dpc to reflect the anamnestic response to *T. circumcincta* following bolus challenge.

In certain experiments indicated in the results section of this chapter, lymph samples from each animal in the previously infected or primary infection group were pooled and named +ve lymph pool and -ve lymph pool respectively. Table 2.2 (below) summarizes the animals used for serum and lymph node collection.

Table 2.2 List of all the experimental animals from which lymph samples were retrieved for this project.

Animal number	Breed/Age at start of experiment	Infection/Immunisation status	Time points used in this study	Reference
B067	Dorset x Suffolk, 12 months	"Primary Infection" Single challenge with L3 bolus	2 dpc	Halliday *et al.* (2009)
B072			-1, 0 dpc	
B103			2 dpc	
1820	Dorset x Suffolk, 5 months		0 dpc	n/a
1858			-2, -1 dpc	
1872			-3 dpc	
A295	Blackface x BF Leicester, 11 months	"Previously-infected" Trickle infected for 8 weeks, cleared then challenged with L3 bolus	8, 10 dpc	Halliday *et al.* (2007)
A611			4, 8, 9 dpc	
A1008			6 dpc	
No Tag			7, 8 dpc	
1831	Dorset x Suffolk, 5 months		8, 9 dpc	n/a
1880			5, 6, 7, 8 dpc	
614, 17/2/87	Suffolk X, 9 months	"Previously infected" Trickle infected for 8 weeks, cleared then challenged with L3 bolus	-5 dpc	Courtesy of Dr D. Smith and Dr J. Huntley (MRI)
614, 27/2/87			5 dpc	
1138K, 27/2/87				

L3 bolus = 50 000 *T. circumcincta* infective stage larvae (L3); dpc = Days post L3 bolus challenge.

2.2.5.3 Collection of serum samples

Blood samples were collected by jugular venepuncture using plain Vacutainers (BD Biosciences, Oxford, UK). Samples were left at room temperature overnight to facilitate clot formation and retraction. The following morning, sera was collected from each tube, centrifuged at 1200 *x g* for 10 min, then aliquoted and stored at -20°C until use.

2.2.5.4 Collection of lymph nodes for the generation of Antibody Secreting Cell probes (ASC)

Lymph nodes were collected aseptically at post-mortem using sterile scalpel blades and autoclaved surgical scissors and forceps, sprayed thoroughly in 70 % ethanol between samples. Each lymph node was placed in 35 ml of ice cold Sheep Wash Medium (SWM,

Appendix D) and kept on ice until processed. In sterility in a tissue culture hood the lymph nodes were placed in a Petri dish and residual adipose tissue was removed, five ml of SWM were added and then using a sterile scalpel/scissors/forceps kit the tissue was cut into ~1-2 mm^3 pieces. The tissue was placed in sterile Stomacher® 80 bags and stomicated for 1 min using a Colworth Stomacher® 80 paddle blender (Seward Ltd. Worthing, UK), after which the cell suspension was filtered through sterile microscope lens cleaning tissue which had been folded three times. The filtered cells were washed twice in 25 ml SWM at 100 *x g* for 10 min then re-suspended in 10 ml of ASC culture medium (ASC-CM, Appendix D). Cells were counted by Trypan Blue exclusion in a haemocytometer and incubated for 5 days at 37°C, 5 % CO_2 in 24- or 48-well plates at a concentration of 5 x 10^6 viable cells/ml in ASC-CM. After 5 days the cell cultures were harvested, centrifuged at 100 *x g* for 10min and the supernatant containing antibodies (ASC probes) aliquoted and stored at -20°C.

2.2.5.5 Collection of gastric lymph

This process was described in detail by Smith *et al.* (1981) and was followed with minor changes in the studies from which the samples used in this project were retrieved (Halliday *et al.* 2010, Halliday *et al.* 2007). Briefly, the common gastric lymph duct, which contains efferent lymph draining all four stomachs, was cannulated during abdominal surgery of selected sheep. An intravenous indwelling catheter was also placed, to allow re-infusion of lymph. Cannulated sheep were housed in individual pens and lymph was collected into sterile, heparinized drainage bags supported by a harness. Every day the lymph flow rate was estimated, aliquots collected into heparinised tubes and the remaining lymph re-infused under gravity.

2.2.6 Indirect ELISA for detection of *T. circumcincta* antigen-specific antibody responses

Unless otherwise indicated, all ELISA experiments were conducted as follows: flat bottom high binding plates (Bio-one, Greiner, Stonehouse, UK) were coated with 50 µl antigen/well (Table 2.3) in carbonate coating buffer pH 9 (Appendix D) and incubated at 4^oC overnight. The following day the plates were washed 4 times in wash buffer (PBST, Appendix D) and 100 µl/well of blocking buffer (TBST/5 % soya, Appendix D) were added. Plates were incubated for 1 h at room temperature (RT), then washed 4 times in PBST. Samples were distributed at 50 µl/well final volume of the appropriate serum/lymph/ASC probe dilution in TBST (Table 2.4) and incubated for 1 hr at RT. After

another 4 washes, the plates received 50 µl/well of secondary antibody (as detailed in Table 2.4), diluted in TBST. Following 4 further washes in PBST, the plates were incubated for 1 hr at RT with 50 µl/well of a tertiary antibody (HRP-conjugated, as detailed in Table 2.4) diluted in TBST. Plates were washed 4 times in PBST then developed with *o*-Phenylenediamine dihydrochloride substrate (Sigmafast OPD, Sigma-Aldrich, Gillingham, UK), 50 µl/well, at RT for 5-15 min. The reactions were stopped by the addition of 30 µl/well of 2.5 M H_2SO_4 then read at 490 nm using a Sunrise™ plate reader (Tecan, Männedorf, Switzerland). Table 2.4 reports the antibody combinations used.

Table 2.3 Concentration of antigens used in ELISA assays.

Antigen	µg/ml
L4 ES*	5
nTci-CF-1^	1
PiTci-CF-1†	1

Description of antigens: * *T. circumcincta* L4 ES products; ^ native Tci-CF-1 purified from *T. circumcincta* L4 ES; † recombinant Tci-CF-1 purified from *P. pastoris* cell culture supernatant.

Table 2.4 Concentration of antibody used in ELISA assays.

Indirect IgG detection					
Sample (primary antibody)		**Secondary antibody**		**Tertiary antibody**	
Type	Dilution	Type	Dilution	Type	Dilution
Serum	1:1000	Anti-goat/sheep IgG:HRP (clone GT-34, A9452, Sigma-Aldrich)	1:1000	n/a	
ASC probes	neat	As above		As above	
Lymph	1:10	As above		As above	
Indirect IgA detection					
Sample (primary antibody)		**Secondary antibody**		**Tertiary antibody**	
Type	Dilution	Type	Dilution	Type	Dilution
Serum	1:10	Mouse anti bovine-ovine IgA (clone K84 2F9, MCA628, Serotec)	1:250	Rabbit anti mouse IgG:HRP (clone n/a, P0260, Dako)	1:1000
ASC probes	neat	As above		As above	
Lymph	1:10	As above		As above	

All data were normalized for plate-plate variations, allowing comparison using the following method: all ODs were blank-corrected and a normalization coefficient was calculated for each plate using the formula below:

$$NC\ [plate\ A] = \frac{\text{Average OD of positive sample [plate A]}}{\text{Average [positive sample OD of all plates]}}$$

Subsequently, all ODs in each plate were divided by the correspondent normalization coefficient for the same plate.

2.2.7 Immunoassay for sodium periodate sensitive/insensitive epitopes

For these ELISA experiments, standard steps as detailed in Section 2.2.6 were used and antigen periodate treatment was carried using a protocol adapted from Fairlie-Clarke *et al.* (2010): plates were coated overnight with native or recombinant *T. circumcincta* antigens, then blocked for 1h with TBST/5 % soy milk (Appendix D) and washed. After the wash steps, wells were incubated with 50 µl/well of 20 mM $NaIO_4$ solution (Appendix D) for 1 h

at 37°C, in the dark. Wells were washed with 50 mM Sodium Acetate Buffer (Appendix D) for three times and subsequently incubated with 50 μl/well of 50 mM $NaBH_4$/TNTT for 30 min at 37°C. Control wells were incubated in 50 mM Sodium Acetate Buffer, instead of 20 mM $NaIO_4$ solution. Wells were then washed with PBST for 4 times, resuming the standard ELISA protocol as described above.

2.2.8 SDS PAGE and Western blotting

The techniques used in this Chapter to carry out protein electrophoresis using polyacrylamide gels (SDS-PAGE) are described in detail in Appendix B (Section 8.1). Unless otherwise stated, 130 ng of protein preparation was added to 5 μl of NuPAGE® 4 X LDS Sample Loading Buffer with the addition of 2 μl of NuPAGE® Reducing agent (all from Invitrogen), for a total volume of 20 μl. These samples were heated at 70°C for 10 min and loaded into individual wells of the gel. Gel and electrophoresis chambers assembly were carried out following the manufacturer's instructions. Running conditions were 200 Volts for 35 min. Gels were routinely stained with Coomassie-based dye (SimplyBlue™ SafeStain, Invitrogen) or silver staining (SilverQuest™ Silver Staining Kit, Invitrogen). If gels were used for Western blotting, the transfer onto nitrocellulose membrane was carried out using iBlot System (Invitrogen). The membranes were stained with Ponceau S (Sigma-Aldrich) to identify bands and cut into strips. Blocking was routinely carried out overnight using 20 ml TNTT (Appendix D). The protein preparations and the serum or lymph samples used to probe each strip are detailed in the relevant Results sections. Secondary and tertiary antibodies and their working dilutions are detailed in Table 2.5. Detection of bound antibodies was carried out using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) following the manufacturer's instructions.

Table 2.5 Working dilutions of the antibodies used in Western blot experiments.

Sample (primary antibody)		Secondary antibody		Tertiary antibody	
Type	Dilution	Type	Dilution	Type	Dilution
Serum	1:1000 (IgG)	Anti-goat/sheep IgG:HRP (clone GT-34, A9452, Sigma)	1:1000	n/a	n/a
	1:100 (IgA)	Mouse anti bovine-ovine IgA (clone K84 2F9, MCA628, Serotec)	1:500	Rabbit anti mouse IgG:HRP (clone n/a, P0260, Dako)	1:1000
Gastric lymph	1:100	Anti-goat/sheep IgG:HRP (clone GT-34, A9452, Sigma)	1:1000	n/a	n/a
		Mouse anti bovine-ovine IgA (clone K84 2F9, MCA628, Serotec)	1:500	Rabbit anti mouse IgG:HRP (clone n/a, P0260, Dako)	1:1000

2.2.9 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.01 and GenStat 12th Edition. Arithmetic means with standard errors and replicate number (n) are shown throughout this chapter. Data were checked for normal distribution by analysing the histogram of frequencies and residual plots. If data were not normally distributed, a Box-Cox transformation (Box 1964, Peltier *et al.* 1998) was used to choose the appropriate transformation method; the details of each transformation are provided for the data sets in each figure throughout this chapter. For data normally distributed (with or without transformation), a one-way Analysis of Variance (ANOVA) test was used, followed by the Tukey post hoc test for pair wise comparison of means; when only one pair of means was compared, an unpaired two-tailed *t*-test was used. The Area Under the Curve (AUC) data were calculated for each group of animals and each antibody isotype over the 84 days of the experiment and used to compare statistically the antibody responses between groups using an unpaired, two-tailed *t*-test. This test was also used to determine whether there was statistical significance in the increase of antigen-specific antibody levels detected within each sample group between day 0 of the experiment and day 84. If data were not normally distributed and Box-Cox analysis failed to suggest an appropriate transformation method,

the non-parametric Mann-Whitney test and Kruskal-Wallis test were used (details are provided for the data sets in each figure). *P* values of < 0.05 were considered significant.

2.3 Results

2.3.1 Purification of native Tci-CF-1 (nTci-CF-1)

A typical profile of *T. circumcincta* L4 ES fractionation by gel filtration chromatography, monitoring absorbance at 280 nm, is shown in Figure 2.2. The presence and relative abundance of nTci-CF-1 was confirmed by SDS PAGE analysis of ES fractions; by using silver staining, it was possible to identify the eluted fractions containing enriched nTci-CF-1 (Figure 2.2). The identity of the prominent band observed in lanes C5 to D12 of Figure 2.2 was confirmed as Tci-CF-1 by LC-ESI-MS/MS analysis carried out by the Proteomics Facility at MRI.

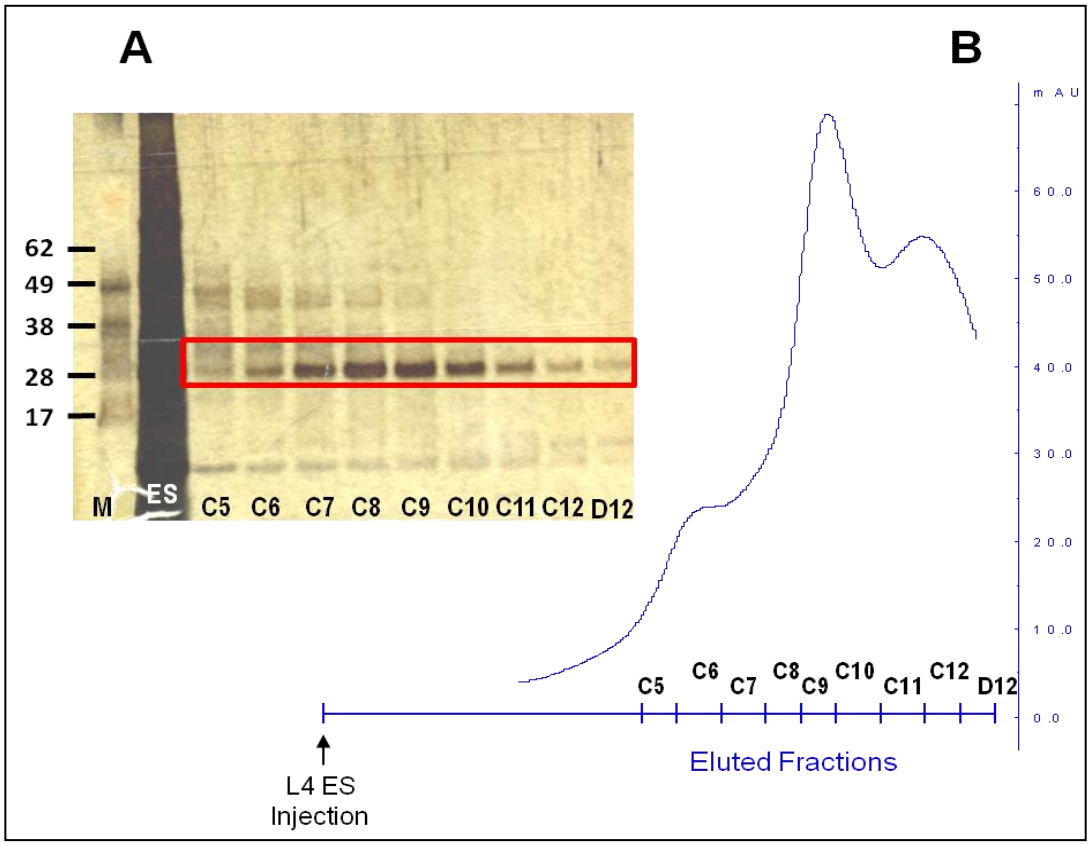

Figure 2.2 Size Exclusion Chromatographic Fractionation of *Teladorsagia circumcincta* L4 ES Products.
Panel (A): an equal amount (13 µl) of each eluted fraction was loaded per lane under reducing conditions. Protein bands were visualized with silver staining. Red box highlights the presence of nTci-CF-1 bands. M: molecular weight marker (kDa); ES: L4 ES prior to fractionation. Panel (B): representative chromatograph showing typical absorbance of elution fractions of *T. circumcincta* L4 ES during size exclusion chromatography using a Superose 12 HR 10/30 size exclusion column. X axis: progressive number of eluted fractions; y axis: UV absorbance in mAU.

Only one of the 250-500 µl fractions eluted contained what visually appeared to be the most abundant and pure nTci-CF-1 (lane C9). The fractions preceding and following C9

also contained nTci-CF-1 but, in these fractions, it was still mixed with other parasite products of similar, but not identical, mass. These more complex fractions were treated with a protocol adapted from Geldhof *et al.* (2002). By pooling and treating the fractions containing nTci-CF-1 with DTT and loading the sample onto a thiol-sepharose pre-packed column, it was possible to further purify nTci-CF-1 from the more complex fractions. Silver stained SDS PAGE gels showed that a large amount of nTci-CF-1 did not bind to the thiol column and was therefore lost in the unbound fraction (Figure 2.3). The relative purity of nTci-CF-1 was estimated by band densitometry to be 85.13 % before thiol enrichment, and 84.9 % afterwards. The concentration of nTci-CF-1 was also estimated by band densitometry against a BSA standard curve to be ~10 μg/ml before thiol enrichment, and ~5 μg/ml after. The low yield of thiol-purified nTci-CF-1 (1-2 μg from each 200 μg L4 ES aliquot) was not sufficient for downstream applications. Taking into account the relative purity of the nTci-CF-1 fractions before and after thiol enrichment (equivalent to C9 in Figure 2.3), the nTci-CF-1 fraction as eluted from the size exclusion chromatography was considered of acceptable quality to be used for ELISA assays without further purification steps.

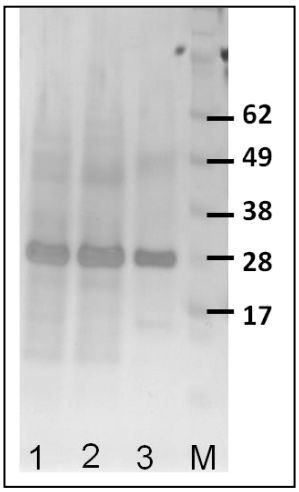

Figure 2.3 SDS-PAGE analysis of thiol-eluted nTci-CF-1.
Silver stained gel. Lane 1: gel filtration fraction enriched with nTci-CF-1 (collected from a subsequent enrichment and equivalent to C9 in Figure 2.2); lane 2: flow-through (unbound) from thiol column; lane 3: thiol-eluted nTci-CF-1; M: molecular weight marker (kDa).

2.3.2 Generation of *P. pastoris*-expressed Tci-CF-1 (PiTci-CF-1)

Full length Tci-CF-1 was amplified omitting the signal peptide from *T. circumcincta* L4 cDNA by PCR, subcloned into pPICZα C and transformed into yeast cells. Colony PCR and DNA gel electrophoresis, from randomly selected yeast colonies, confirmed successful

transformation. Protein expression was induced and recombinant Tci-CF-1 (PiTci-CF-1) was purified. Protein identity was confirmed by SDS-PAGE analysis (Figure 2.4 A, lane 2), MALDI-ToF and finally by Western blot using a PiTci-CF-1 anti-serum previously raised in sheep at MRI (Figure 2.4, B). Mean purified protein yield was calculated as 109.56 µg/ml of packed cells volume (n = 2). SDS-PAGE analysis revealed that purified PiTci-CF-1 migrates as two bands at around 48 kDa and 34.7 kDa, suggesting a degree of post-translational processing occurring within the yeast cultures. The identity of both bands had been confirmed as PiTci-CF-1 by MALDI-ToF. The process of cloning and generating another recombinant version of PiTci-CF-1, instead of using the previously purified form (Nisbet *et al.* 2013), was necessary in view of the subsequent generation of Tci-CF-1 in *C. elegans*. Thus, the two recombinant versions would have identical amino acid sequences and therefore also allow later comparisons of antibody recognition and enzyme activity between the two.

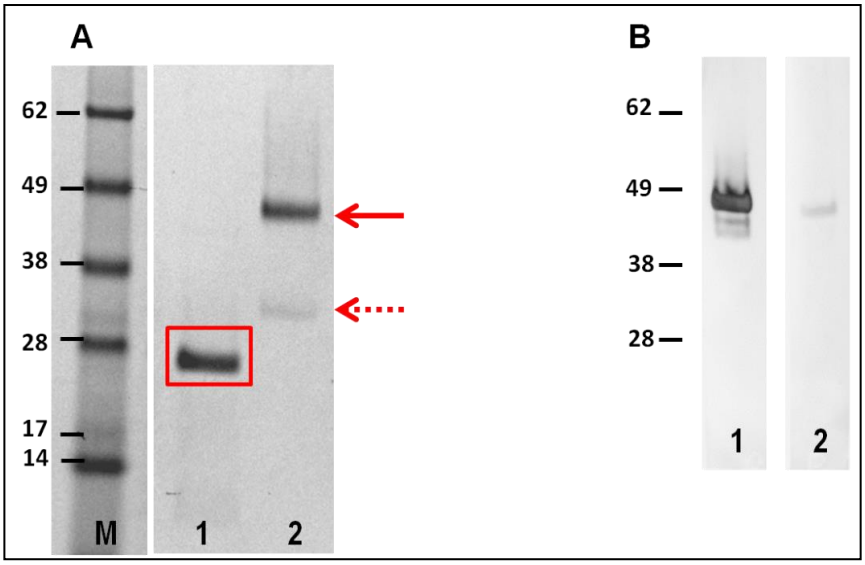

Figure 2.4 SDS-PAGE analysis of enriched nTci-CF-1 and purified PiTci-CF-1 and immunoblot of PiTci-CF-1.
(A) Lane M: molecular weight marker (kDa); lane 1, red box: nTci-CF-1; lane 2: PiTci-CF-1, both solid and dotted red arrow (as determined by MALDI-ToF). (B) Western blot of PiTci-CF-1 probed with, lane 1: sheep anti-PiTci-CF-1 serum, lane 2: control serum (pre-immunisation). Bound antibody was detected by chemiluminescence using ECL substrate.

2.3.3 Differential recognition of nTci-CF-1 and PiTci-CF-1 by antibodies from serum

To investigate the systemic and local immune responses against native *T. circumcincta* antigens and PiTci-CF-1 in the serum of sheep which had been immunised with PiTci-CF-1 [as part of an 8-recombinant antigen cocktail in adjuvant (Nisbet *et al.* 2013)] or

adjuvant-only, and subsequently trickle challenged with *T. circumcincta*, an indirect ELISA was carried out. This evaluated the serum IgA and IgG reactivity to L4 ES products [(of which native Tci-CF-1 is the most abundant antigen (Redmond *et al.* 2006)] and purified PiTci-CF-1.

Serum IgG and IgA reactivity against L4 ES in the control group, which received a *T. circumcincta* trickle infection and mock immunisation with adjuvant only, were detectable after day 42, when the trickle infection started (Figure 2.5), showing the effect of exposure to the parasite. Although serum IgA levels in the group immunised with the cocktail vaccine replicated the same pattern as the control group and no difference was observed (AUC $p = 0.2685$; Figure 2.5, Panel A), the serum IgG response against L4 ES antigens in the immunised group was significantly higher (AUC $p < 0.0001$) than the control animals. IgG levels in immunised sheep started rising at an earlier time point than in the control sheep during the experiment (day 21), after two immunisations and three weeks before the initiation of the trickle infection [Nisbet *et al.* (2013) and Figure 2.5, Panel B]. However, this antibody response started to decline from day 63 onwards.

In sheep immunised with the cocktail vaccine, containing a version of PiTci-CF-1, a significantly higher serum IgA and IgG response was observed to this recombinant protein in the immunised group compared to the controls, starting after the first immunisation (AUC $p = 0.0001$ and $p < 0.0001$ for IgA and IgG respectively; Figure 2.5, panels C and D). However it is shown that the IgG response started to decline at day 63 [Figure 2.5, Panel D and Nisbet *et al.* (2013)]. The lack of IgA and IgG reactivity against PiTci-CF-1 in the control animals (Figure 2.5, panels C and D) suggested that the antibodies elicited by *T. circumcincta* trickle infection, which bound to L4 ES (Figure 2.5, panels A-B), did not cross-react with the recombinant yeast-expressed PiTci-CF-1.

An unpaired, two-tailed *t*-test showed that in each group considered (i.e. immunised or control sheep) the antibody levels at day 84 of the experiment (both IgA and IgG) were significantly higher ($p < 0.005$) than at day 0 of the experiment, with the exception of PiTci-CF-1-specific IgA in the control group, where the increase was not significant.

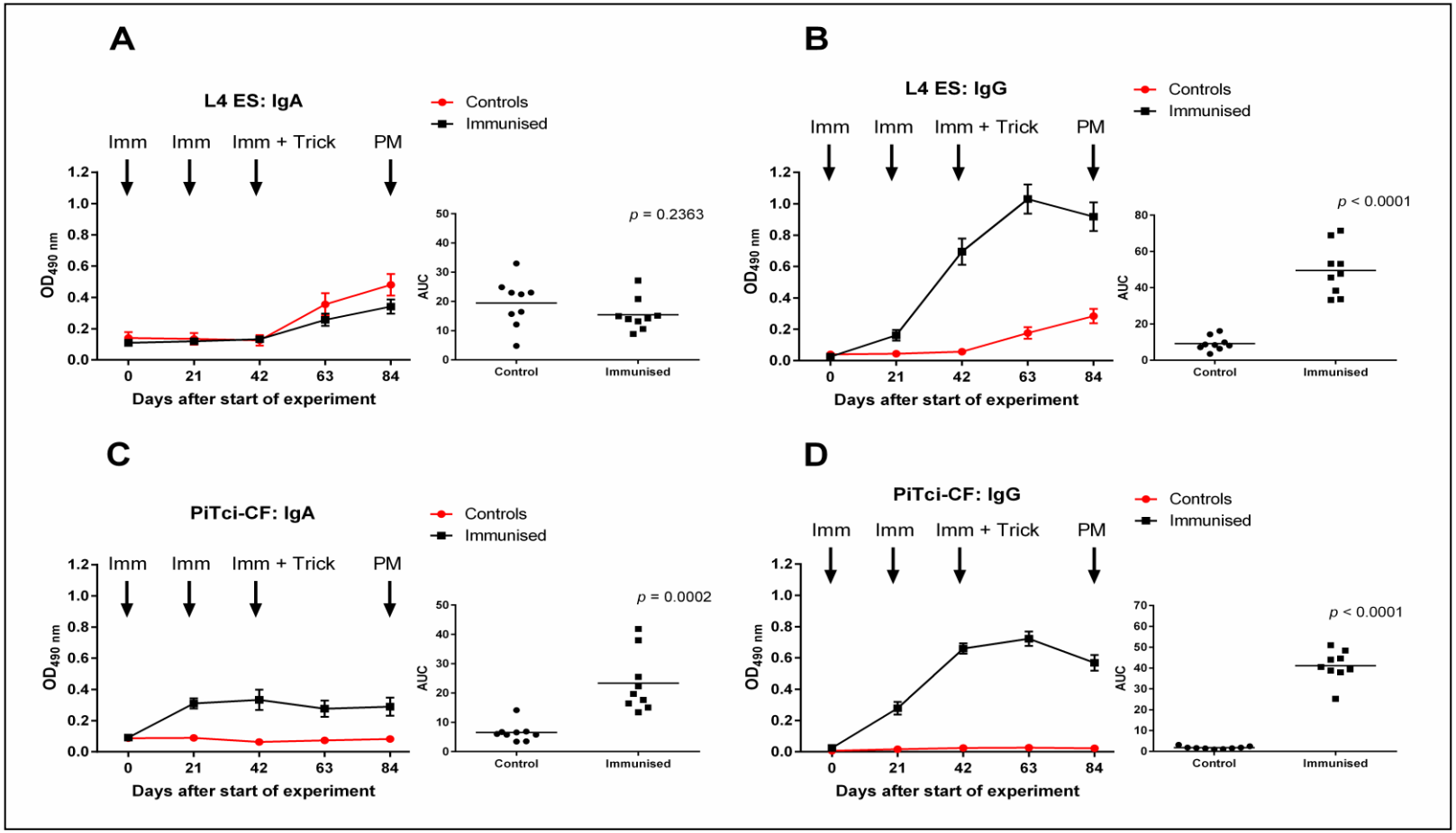

Figure 2.5 The serum IgA and IgG responses in immunised and control sheep to *T. circumcincta* L4 ES antigens and recombinant PiTci-CF-1. Panels A-B: serum IgA and IgG responses to L4 ES during an experimental infection regime. Panels C-D: serum IgA and IgG responses to PiTci-CF-1 during an experimental infection regime. Red circles: control animals; black squares, immunised animals. Data represent mean±SEM, n=9 per group. Plots on the right of each graph show the statistical analysis of the calculated AUC for each group, with respective *p* values shown on the top right corner.

The serum IgG response in immunised animals against PiTci-CF-1 was further investigated in ELISA experiments to examine the specificity for glycan residues that may have been present in PiTci-CF-1. This was achieved by treating the coating antigen (PiTci-CF-1) with a solution of sodium periodate ($NaIO_4$). "Untreated" wells were incubated with the same buffers as described in the materials and methods section 2.2.7 with the exception of periodate solution. The serum samples used were from day 63 of the experiment when IgG levels had peaked against PiTci-CF-1 (see Figure 2.5), and were significantly higher in the immunised animals compared to the controls (Figure 2.6, panel A). Following periodate treatment, a statistically significant loss of PiTci-CF-1-specific IgG binding was observed in the immunised animals, whereas no appreciable change was seen in the control animals. Serum IgG binding to PiTci-CF-1 in immunised animals was still significantly higher following periodate treatment, relative to reactivity of control animals (Figure 2.6, panel A).

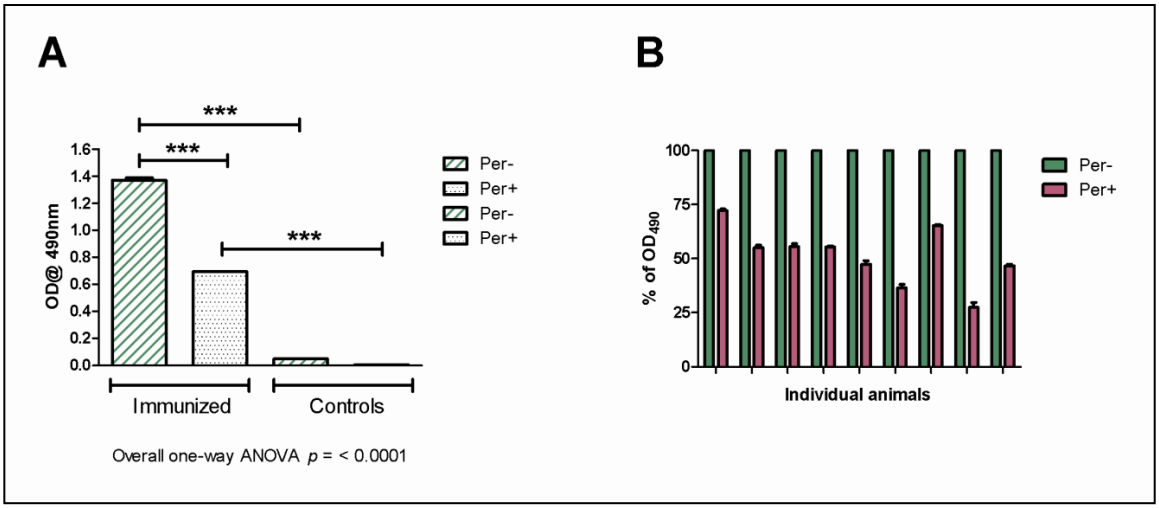

Figure 2.6 The effect of sodium periodate treatment of PiTci-CF-1 on binding of serum IgG from PiTci-CF-1 immunised sheep.
(A): green striped bars: periodate untreated (Per-) samples; black dotted bars: periodate treated (Per+) samples. Data represent mean ±SEM, n=9 per group with all samples analysed in duplicate. Overall one-way ANOVA *p* value displayed below the graph. Post-ANOVA analysis: Tukey's multiple comparison test. *** = p <0.001. Panel (B): percentage of IgG binding loss in serum from immunised animals following periodate treatment of PiTci-CF-1. Mean OD loss 48.72 %, range 27.72-72.57 %. Green bars: $NaIO_4$ untreated (Per-) samples. Purple bars: periodate treated (Per+) samples. Error bars represent SEM.

The loss of IgG binding to PiTci-CF-1 was observed in each of the individual serum samples of immunised animals, and it was shown to have a wide range of variability between animals (mean OD loss 48.72 %, range 27.72-72.57 %, Figure 2.6, panel B).

2.3.4 Differential recognition of nTci-CF-1 and PiTci-CF-1 by antibodies from Antibody Secreting Cell (ASC) probes

A snapshot of the local immune response against a specific stage/antigen of a parasite can be obtained by investigating the antibody responses of ASC probes generated from local lymph node cells (McWilliam *et al.* 2012). Abomasal and pre-scapular lymph nodes were collected from 7 animals in groups A and B, and from 2 randomly chosen animals in groups C and D (see Section 2.2.5.1 for a summary of experimental plan), to match the serum samples for which antibody reactivity was investigated in Section 2.3.3. Abomasal lymph nodes were collected to reflect the local antibody response to *T. circumcincta* infection. The pre-scapular lymph nodes were chosen as they drained the site of immunisation, and therefore immune responses at this lymphatic site would reflect immunisation-induced immune responses. From these lymph nodes, ASC probes were generated and used in ELISA experiments to test the IgA and IgG binding to L4 ES antigens, enriched nTci-CF-1 fractions and purified recombinant PiTci-CF-1. Figure 2.7 summarizes the findings.

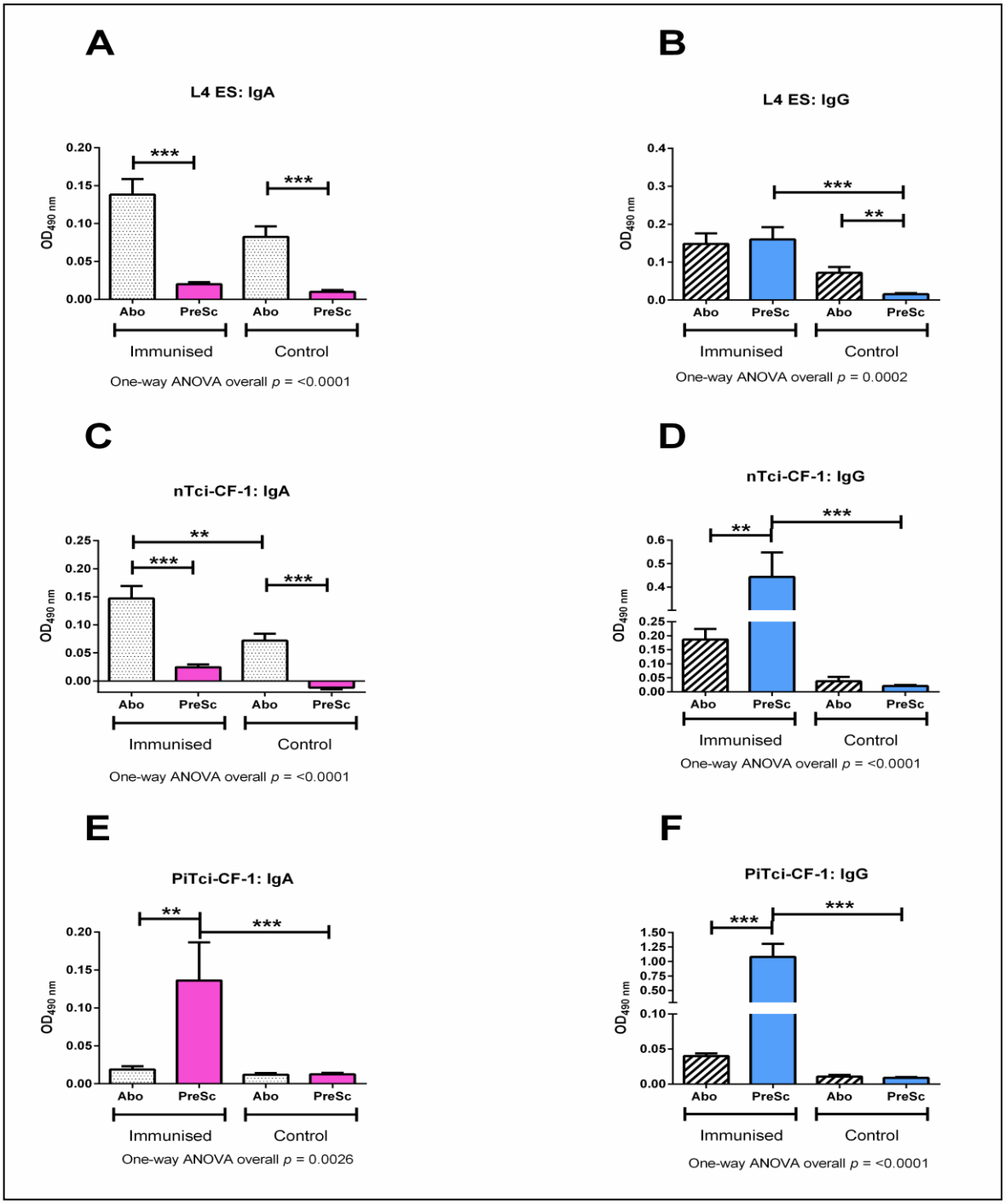

Figure 2.7 The ASC probes IgA and IgG responses from lymph nodes of immunised and control sheep to *T. circumcincta* L4 ES antigens, nTci-CF-1 and recombinant PiTci-CF-1, during an experimental infection regime.
Sheep were immunised with a *T. circumcincta* recombinant antigen cocktail containing PiTci-CF-1. All animals received a trickle infection (3 000 L3, 3 times a week for 4 weeks) and ASC probes generated at 6 weeks post-infection. Rows share the antigen used to coat the plates, columns share the antibody isotype (IgA or IgG). Abo: abomasal ASC probes; PreSc: pre-scapular ASC lymph node probes. Data represent mean ±SEM, n=9 per group with all samples analysed in duplicate. Overall one-way ANOVA *p* value displayed below each graph. Post-ANOVA analysis: Tukey's multiple comparison test. *** = p <0.001; ** = p < 0.01; * p <0.05. Statistical analysis was carried out on untransformed (panels C, D and F) or transformed (panels A, C and E) data. Untransformed data represented in all panels. Data in panels A, B and E was transformed prior to statistical analysis using the following: $OD^{0.5}$, $1/OD^{0.25}$ and $OD^{0.25}$ respectively.

IgA from Abomasal lymph node ASC probes showed significantly higher binding to L4 ES and nTci-CF-1, but not to PiTci-CF-1, compared to the correspondent pre-scapular probes in both groups of animals (immunised and controls; Figure 2.7, panels A, C and E). In control animals, IgA from pre-scapular ASC probes were not able to bind any of the three antigen preparations tested. IgA binding to PiTci-CF-1 was significantly higher in pre-scapular ASC probes from immunised animals compared to both the abomasal probes from the same (immunised) animals, and pre-scapular probes from the control animals (Figure 2.7, panel E). These results show that trickle infection-induced abomasal IgA was able to bind native parasite antigens such as nTci-CF-1 and those found in L4 ES; however, these did not bind to recombinant PiTci-CF-1.

ASC abomasal and pre-scapular IgG reactivity was also investigated. In immunised sheep, IgG binding from pre-scapular ASC probes to nTci-CF-1, L4 ES and PiTci-CF-1 was significantly higher than in the control group (Figure 2.7, panels B, D and F). Pre-scapular ASC probe IgG from immunised animals showed significantly higher binding to PiTci-CF-1 than either L4 ES ($p < 0.001$) and nTci-CF-1 ($0.001 < p < 0.01$). In the control sheep, IgG from pre-scapular ASC probes did not bind to any antigen tested, and IgG from the abomasal probes did not bind either nTci-CF-1 or PiTci-CF-1.

IgG reactivity to periodate treated PiTci-CF-1in the pre-scapular ASC probes from the immunised animals was evaluated. The immune response elicited by the vaccine antigen PiTci-CF-1 was periodate sensitive (Figure 2.8, panel A) and individual serum samples showed a wide range of OD loss following periodate treatment (mean OD loss 56.39 %, range 86.82-36.17 %; Figure 2.8, panel B).

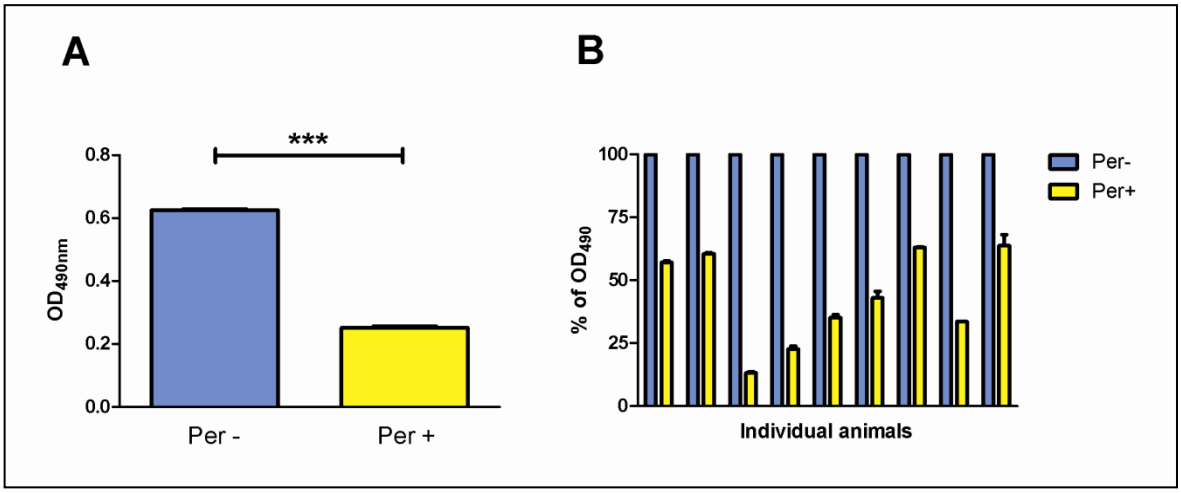

Figure 2.8 The effect of sodium periodate treatment of PiTci-CF-1 on binding of pre-scapular ASC IgG from PiTci-CF-1 immunised sheep.
(A): blue bar: periodate untreated (Per-) samples; yellow bar: periodate treated (Per+) samples. Two-tailed, unpaired t-test *p* = 0.0002; Data represent mean ±SEM, n=9 per group with all samples analysed in duplicate. Panel (B): percentage of IgG binding loss in immunised animals to purified PiTci-CF-1 following periodate treatment. Mean OD loss 56.39 %, range 86.82-36.17 %. Blue bars: periodate untreated (Per-) samples. Yellow bars: periodate treated (Per+) samples. Data represent mean ±SEM.

2.3.5 Differential recognition of nTci-CF-1 and PiTci-CF-1 by antibodies from gastric lymph

IgA antibodies in the gastric lymph of sheep previously infected with a *T. circumcincta* trickle infection then bolus challenged showed significantly higher binding to L4 ES and nTci-CF-1 than those in the lymph of *T. circumcincta*-primary infection animals (collected from helminth-free animals, at time points between -2 and +2 days of primary bolus challenge). There was no significant difference between previously infected and primary infection animals for lymph IgA binding to PiTci-CF-1. Lymph samples from sheep that had been previously infected showed significantly higher binding to L4 ES and nTci-CF-1 than to PiTci-CF-1 (Figure 2.9).

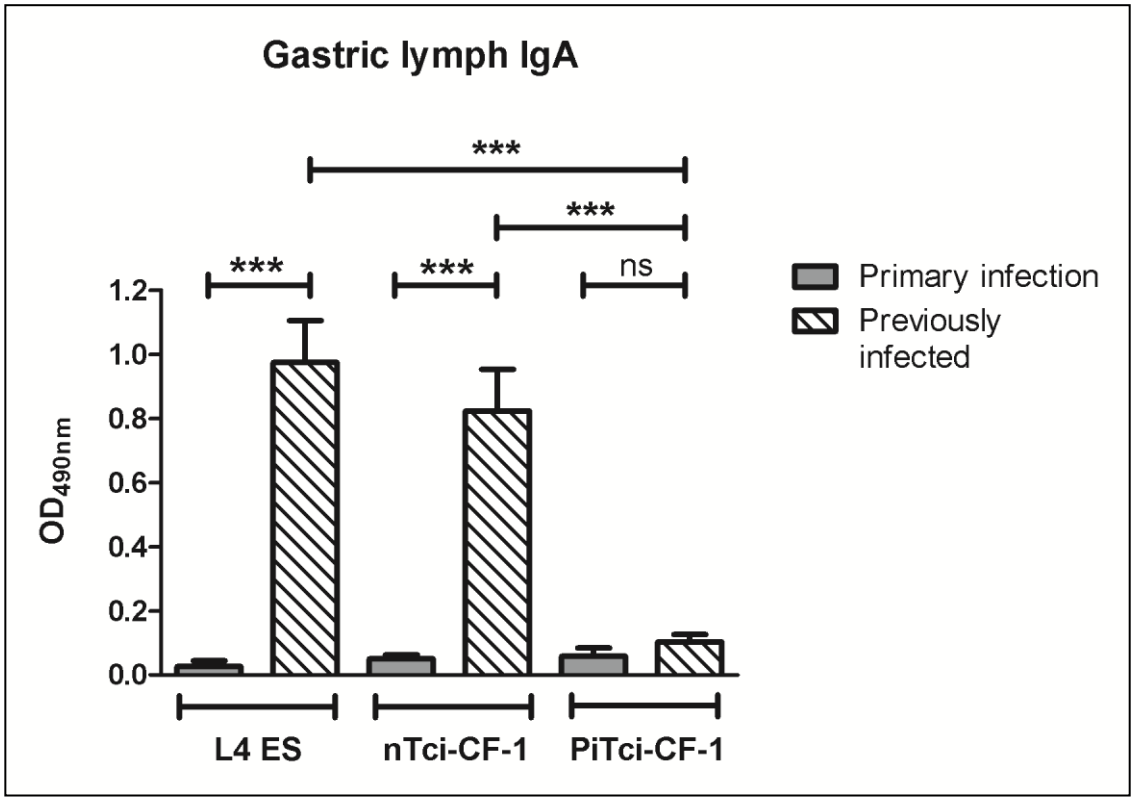

Figure 2.9 The gastric lymph IgA responses from previously infected and primary infection sheep to *T. circumcincta* L4 ES antigens, nTci-CF-1 and recombinant PiTci-CF-1, during an experimental infection regime.
Data represent mean ±SEM, n=5 (primary infection) and 8 (previously infected); all samples analysed in duplicate. Overall one-way ANOVA *p* <0.0001. Post-ANOVA analysis: Tukey's multiple comparison test. * = *p* <0.001; ns = *p* not significant.**

Periodate treatment of nTci-CF-1 showed loss of IgA reactivity in gastric lymph (Figure 2.10, panel A), with a wide range of variability between animals (mean OD loss 60.05 %, range 40.86-81.62 %; Figure 2.10, panel B). When PiTci-CF-1 was treated with sodium periodate (Figure 2.11), the binding of lymph IgA to the periodate-untreated antigen was not significantly different between the previously infected and the primary infection groups (as seen in Figure 2.9). However, samples from previously infected animals showed significantly higher binding to periodate treated PiTci-CF-1 compared to lymph from primary infection animals.

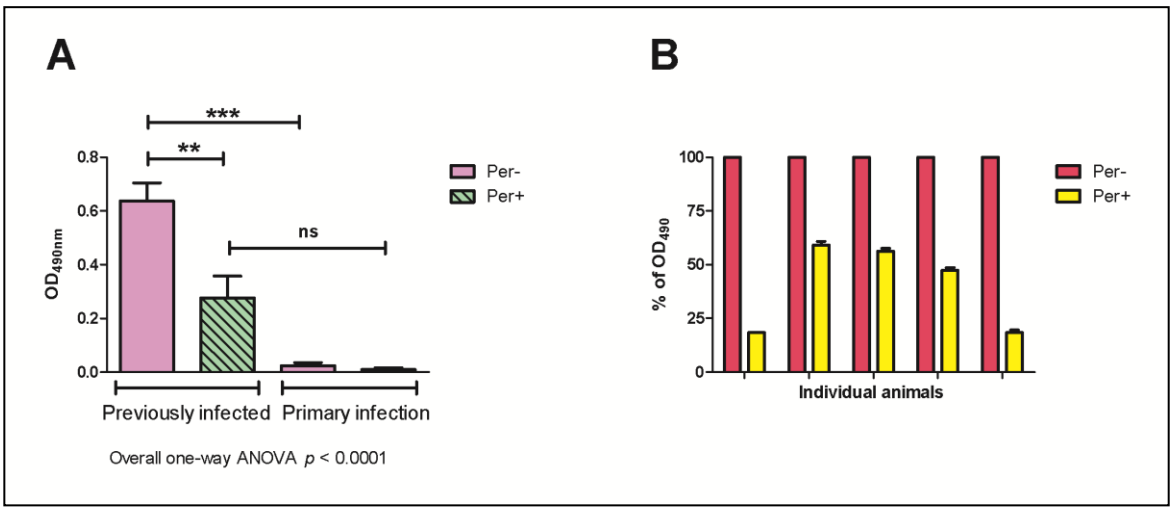

Figure 2.10 The effect of sodium periodate treatment of nTci-CF-1 on binding of gastric lymph IgA from previously infected and primary infection sheep during an experimental infection regime.
Data represent mean ±SEM, n=3 (primary infection) and 5 (previously infected); all samples analysed in duplicate. Panel (A): pink bars: periodate untreated (Per-) samples; green striped bars: periodate treated (Per+) samples. Overall one-way ANOVA *p* value displayed below the graph. Post-ANOVA analysis: Tukey's multiple comparison test. * = *p* <0.001; ** = *p* < 0.01; ns = not significant. Panel (B): the percentage of IgA binding loss to nTci-CF-1 following periodate treatment in previously infected animals. Mean OD loss 60.05 %, range 40.86-81.62 %. Red bars: periodate untreated (Per-) samples. Yellow bars: periodate treated (Per+) samples. Data represent mean ±SEM.**

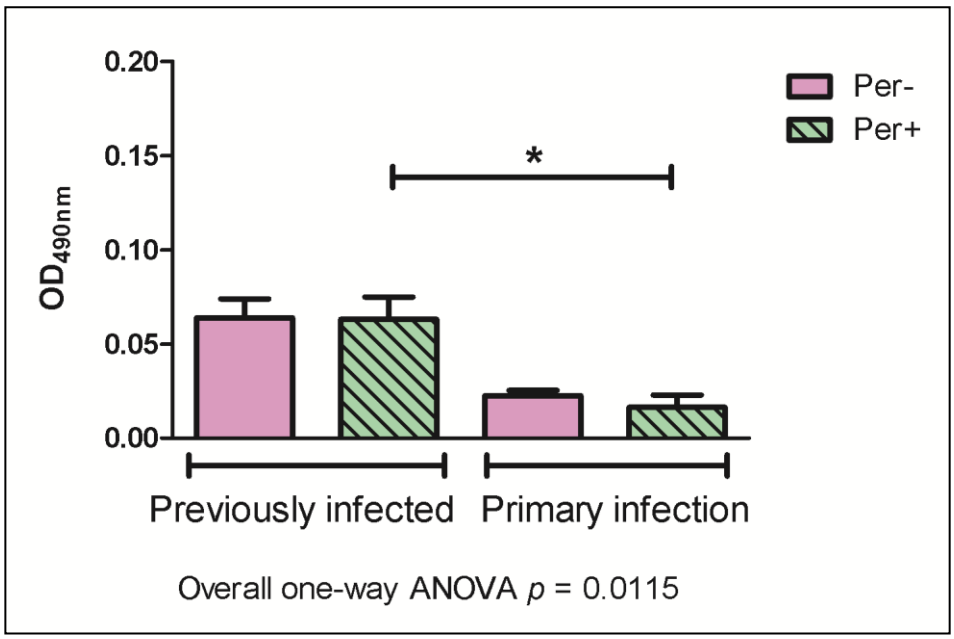

Figure 2.11 The effect of sodium periodate treatment of PiTci-CF-1 on binding of gastric lymph IgA from previously infected and primary infection sheep during an experimental infection regime.
Data represent mean ±SEM, n=3 (primary infection) and 5 (previously infected); all samples analysed in duplicate. Pink bars: periodate untreated (Per-) samples. Green striped bars: periodate treated (Per+) samples. Overall one-way ANOVA p value displayed below the graph. Post-ANOVA analysis: Tukey's multiple comparison test. * = *p* < 0.05.

Lymph IgG also experienced loss of reactivity to nTci-CF-1 following periodate treatment of the antigen (Figure 2.12). In this experiment primary infection animals showed pre-periodate treatment IgG reactivity comparable to previously infected animals; this may have been due to non specific cross-reactivity of the sheep gastric lymph IgG to nTci-CF-1. Both decreases in OD observed following periodate treatment were statistically significant.

Tables 2.6 to 2.8 summarise the findings of this chapter in regards to IgG and IgA reactivity.

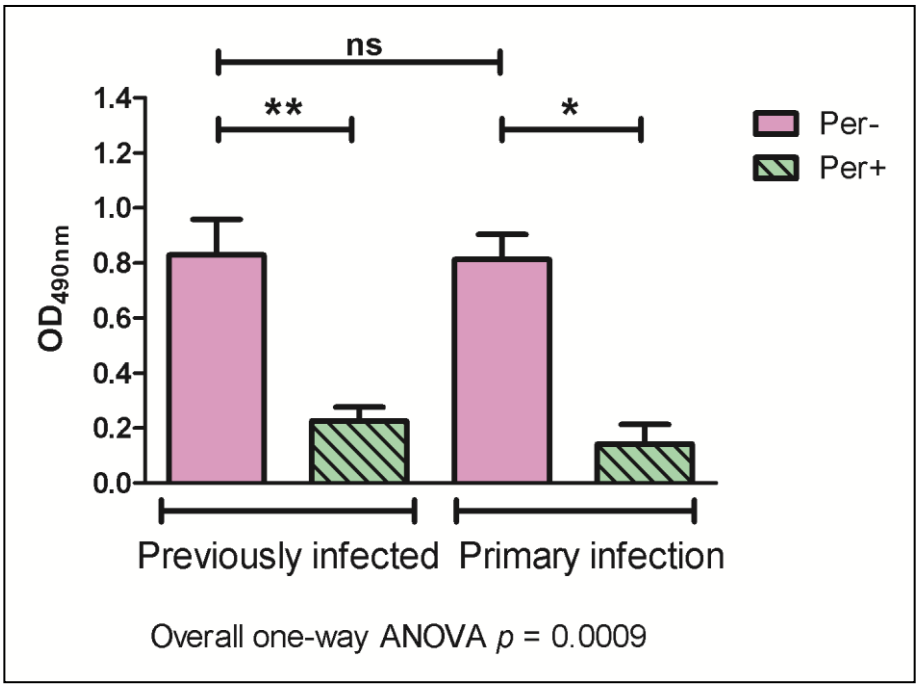

Figure 2.12 The effect of sodium periodate treatment of nTci-CF-1 on binding of gastric lymph IgG from previously infected and primary infection sheep during an experimental infection regime.
Data represent mean ±SEM, n=3 (primary infection) and 7 (previously infected); all samples analysed in duplicate. Pink bars: periodate untreated (Per-) nTci-CF-1. Green striped bars: periodate treated (Per+) nTci-CF-1. Overall one-way ANOVA p value displayed below the graph. Post-ANOVA analysis: Tukey's multiple comparison test. ** = 0.001 < p < 0.01; * = 0.01 < p < 0.05; ns = *p* > 0.05.

Table 2.6 Tables summarising the IgA and IgG reactivity of serum to different *T. circumcincta* antigens in immunised (then trickle infected) and in control (trickle infected only) sheep.

Antigen	Serum IgA	
	Immunised	Adjuvant-only controls
L4 ES	- (^)	- (^)
nTci-CF-1	n/d	n/d
PiTci-CF-1	+	-

Antigen	Serum IgG	
	Immunised	Adjuvant-only controls
L4 ES	+	-
nTci-CF-1	n/d	n/d
PiTci-CF-1	+	-

Legend: + signs: antibody detection significantly higher than the other sample group considered in the same experiment; - signs: antibody detection not significantly different from the other sample group; n/d: not determined; (^): antibody binding was detected in both groups but there was no significant difference. Immunised: sheep were immunised with a PiTci-CF-1 preparation and trickle infected with *T. circumcincta* L3 for 4 weeks; adjuvant-only controls: sheep received a mock immunisation and were trickle infected for 4 weeks.

Table 2.7 Tables summarising the IgA and IgG reactivity of serum to different T. circumcincta antigens in immunised (then trickle infected) and in control (trickle infected only) sheep.

Antigen	ASC probes (IgA)			
	Immunised		Adjuvant-only controls	
	Abo	Pre-Scap	Abo	Pre-Scap
L4 ES	+	-	+	-
nTci-CF-1	+	-	+	-
PiTci-CF-1	-	+	-	-

Antigen	ASC probes (IgG)			
	Immunised		Adjuvant-only controls	
	Abo	Pre-Scap	Abo	Pre-Scap
L4 ES	-	+	-	-
nTci-CF-1	-	+	-	-
PiTci-CF-1	-	+	-	-

Legend: + signs: antibody detection significantly higher than the other sample group considered in the same experiment; - signs: antibody detection not significantly different from the other sample group. Immunised: sheep were immunised with a PiTci-CF-1 preparation and trickle infected with *T. circumcincta* L3 for 4 weeks; adjuvant-only controls: sheep received a mock immunisation and were trickle infected for 4 weeks. Abo: abomasal ASC probe; Pre-Scap: pre-scapular ASC probe.

Table 2.8 Table summarising the IgA reactivity of gastric lymph to different *T. circumcincta* antigens in previously infected (trickle + bolus challenge) and primary infection (bolus challenge only) sheep.

Antigen	Gastric Lymph	
	Previously infected	Primary infection
L4 ES	n/d	-
nTci-CF-1	+	-
PiTci-CF-1	-	-

Legend: + signs: antigen-specific IgA detection significantly higher than the other sample group considered in the same experiment; - signs: antibody detection not significantly different from the other sample group. Prev. Inf.: lymph collected 6-10 days *T. circumcincta* 50 000 L3 bolus challenge from animals that received previous trickle infection (8 weeks); Primary infection: lymph collected -2 to +2 days post *T. circumcincta* 50 000 L3 bolus challenge.

2.4 Discussion

In this chapter it was demonstrated that native Tci-CF-1 (nTci-CF-1), a major component of *T. circumcincta* L4 ES, can be purified from the complex ES mix by size exclusion chromatography. However, treatment with DTT and application of the ES-purified nTci-CF-1 fraction to a thiol-sepharose column did not yield further purification of this antigen, and protein loss occurred in the process. Thiol columns have been previously used to enrich cysteine proteases in *Ostertagia ostertagi* ES material (Geldhof *et al.* 2002, Geldhof *et al.* 2004). In these studies, DTT was used prior to application of the ES material and in the elution buffer, and the process was replicated for this project using *T. circumcincta* ES. DTT is a protective agent for disulphide groups (Cleland 1964, O'Neil 2001) and it is capable of maintaining them in the reduced state of thiols. This ability is favourable in the context of cysteine protease purification, as they possess a cysteine residue in the active site, and cysteine, containing a sulphide group (-SH), is able to form disulphide bonds with other –SH residues in the amino acid chain in order to achieve correct folding. Therefore DTT may help during the purification process, as the disulphide bonds in the amino acid chain are reduced to thiols that are then able to bind to the thiol-sepharose matrix of the HPLC column. However, this process may lead to protein unfolding (due to reduction of disulphide bonds to -SH between amino acid residues) and precipitation, potential disruption of the active site and unexpected interactions between amino acid and carbohydrate residues (Alliegro 2000, Cho *et al.* 2008, Cleland 1964); these could lead to loss of antibody binding and enzymatic activity. For the above reasons, it was decided not to pursue further purification of native Tci-CF-1 by using thiol columns, as it is shown here that this process would not lead to a purer fraction, with in addition loss of protein quantity and potential loss of activity and correct folding.

In this chapter, differential antibody recognition of L4 ES antigens, purified nTci-CF-1 and recombinant PiTci-CF-1 was observed in three different experiment types, using a range of ovine biological samples to reflect the differences in local and systemic immune response. Antibody responses appear to have an important role in the control of parasitic infections in ruminants, and it has been shown that excretory-secretory (ES) products and purified native antigens were the target of antibodies found in systemic and local environment in several ruminant host-parasite systems (Britton *et al.* 1993, De Maere *et al.* 2002, Geldhof *et al.* 2002, Halliday *et al.* 2007, Smith *et al.* 1986, Smith *et al.* 1994, Stear *et al.* 1995, Stear *et al.* 1999 b). For example, in previous studies on the ovine immune response to *H. contortus* experimental infection, significantly higher ($p < 0.05$) serum and abomasal

mucus IgG and IgA responses against ES products of adult worms were observed in previously infected sheep compared to uninfected animals (Lacroux *et al.* 2006). In cattle infected with the lungworm *Dictyocaulus viviparus* it was shown that in both groups of previously infected (by trickle infection with 100 L3/day for 5 days, then challenged with 1500 L3) and control animals (only challenged), all serum immunoglobulin isotypes tested (IgA, IgG1 and IgG2) were reactive with adult ES antigens (Kooyman *et al.* 2007). In *T. circumcincta* trickle infection and challenge experiments, L4 ES-specific gastric lymph IgA levels were significantly higher in previously infected sheep compared to sheep subjected to primary challenge (Halliday *et al.* 2007). In an immunisation experiment of cattle with native purified Oo-ASP proteins (obtained by ES fractionation) plus Quil A adjuvant, followed by trickle infection with *O. ostertagi*, native ASP-specific IgG1 and IgG2 levels were significantly higher in both the serum and the abomasal mucus of the immunised animals than in the adjuvant only control group animals (Van Meulder *et al.* 2013). In this experiment, the Oo-ASP/QuilA immunised cattle also showed significantly lower FEC compared to the adjuvant only animals; although correlations between ASP-specific serum antibodies and FEC were not investigated (Van Meulder *et al.* 2013), the findings discussed here have underpinned the use of ES proteins as prototype vaccines.

In the work presented in this chapter, serum IgA and IgG responses to *T. circumcincta* L4 ES, native Tci-CF-1 and *P. pastoris*-expressed Tci-CF-1 were analysed during the course of a *T. circumcincta* experimental immunisation trial in sheep: groups of animals were immunised with 8 recombinant antigens or received a mock immunisation of adjuvant only, three times each; immunisations were three weeks apart (d 0, d 21 and d 42). All the animals received a *T. circumcincta* L3 trickle infection for 4 weeks, starting on the day of the last immunisation (d 42), and were euthanized 84 days after the start of the experiment (experimental plan Fig. 2.1). *Teladorsagia circumcincta* L4 ES-specific IgA and IgG were detected in all groups except one (serum IgG in immunised sheep) from day 42 onwards. In immunised sheep, the serum IgG response against L4 ES was evident three weeks before the start of the trickle infection, from day 21 [Figure 2.5 and Nisbet *et al.* (2013)]. This suggests that the IgG response induced by immunisation and directed against the 8 components of the recombinant was to a certain degree reactive to the native ES antigens used to coat the plates.

In addition, the serum IgG response to L4 ES in immunised animals was significantly higher than in control animals, demonstrating that immunisation-induced IgG antibodies were able to bind native antigens present in the L4 ES preparation used in ELISA

experiments. In this chapter, serum IgA was only detectable after the start of the trickle infection at day 42 in each group of animals, suggesting a mucosal origin for parasite-specific serum IgA, probably triggered by the trickle infection.

In the immunised animals, serum IgA and IgG responses against recombinant PiTci-CF-1 started to increase shortly after the start of the immunisation protocol (between day 0 and 21), as these animals had received parenteral administration of PiTci-CF-1. However IgA and IgG levels peaked shortly afterwards, and both were shown to decline by the end of the trickle infection at day 70 (see experimental plan in Figure 2.1 and Figure 2.5), suggesting that, although *T. circumcincta* infection induced parasite-specific antibodies, these did not recognise recombinant PiTci-CF-1 and did not contribute to boost the PiTci-CF-1-specific antibody responses. This is an interesting finding in the light of the differential IgG and IgA binding observed in the control animals against PiTci-CF-1. In this case, none of the animals showed any serum antibody reactivity against the recombinant antigen, supporting the hypothesis that parasite-induced responses may not optimally recognise recombinant antigens and that the recombinant PiTci-CF-1 version may have differed subtly from the native nTci-CF-1 form. Indeed previous work using a *T. circumcincta* recombinant antigen showed no significant difference between primary infection or previously infected animals in regards to serum IgA or IgG binding to recombinant Tci-CF-1 expressed in *E. coli*, which was not recognised by either antibody isotype (Routledge 2008).

Culturing of cells derived from lymph nodes draining the site of infection can provide a powerful tool to study the local immune response to parasitic pathogens [reviewed by McWilliam *et al.* (2012)]. Antigen-specific ASC probes have been validated to study the local immune response to parasitic antigens in various experiments with different helminths: cestodes such as *Taenia hydatigena* in the sheep host (Meeusen and Brandon 1994a), in rodent models with the liver fluke, *Fasciola hepatica* (Meeusen and Brandon 1994b) and nematodes such as *Haemonchus contortus* (Bowles *et al.* 1995). In ovine experimental *T. circumcincta* infection models, ASC probes have been successfully used in previous experiments, where probes from sheep challenged with *T. circumcincta* showed differential antibody binding to antigens from L3, L4 and adult stages (Balic *et al.* 2003) when harvested at different time points post-infection.

The results of this chapter show that in abomasal ASC probes generated from sheep trickle infected with *T. circumcincta*, the IgA binding to native antigens such as L4 ES and nTci-CF-1 was significantly higher than the control probes from pre-scapular lymph nodes in

the same animals. This suggests that an immune response against *T. circumcincta* was present locally in the abomasal lymph nodes and was significantly higher than at the pre-scapular site which is anatomically distant. This showed how the physical distance and absence of lymph mixing from the abomasal infection site maintains a relatively naïve state of the pre-scapular lymph node cell populations to *T. circumcincta* antigens, making these a suitable negative control.

In this chapter, cross-recognition of immunisation-induced antibodies to nTci-CF-1 was shown. The binding of pre-scapular probe IgG to this antigen in the immunised sheep was statistically higher than in the abomasal probes from the same animals. In contrast, those sheep that only received a mock immunisation and trickle infection did not show any difference between abomasal and pre-scapular probe IgG binding to L4 ES and nTci-CF-1. A very similar picture was observed when comparing the antibody responses to recombinant PiTci-CF-1: the control, trickle infected only sheep did not show reactivity to this antigen, whereas the immunised sheep showed a significantly higher IgA and IgG response in the pre-scapular probes compared to the abomasal probes. This phenomenon reflected the close proximity of the pre-scapular lymph nodes with the injection sites in the neck; because these lymph nodes drain the injection site, the recombinant antigens present in the vaccine appeared to stimulate a B cell response in the local lymph nodes, observed as IgA and IgG binding to PiTci-CF-1 which was a component of the vaccine. However, there was no binding to PiTci-CF-1 by any of the abomasal probes, in any of the groups, suggesting that the trickle infection-induced antibody response observed against the native antigens in L4 ES and purified nTci-CF-1 was not cross-reactive with the recombinant, yeast-expressed Tci-CF-1.

A technical limitation of ASC probes is that collection of the lymph nodes of interest can only be carried out at post mortem, or subjecting the experimental animals to numerous surgical operations, which could be considered unpractical and unethical. With these considerations in mind, the availability of efferent lymph from the common gastric duct still gives the opportunity to study the local immune response when investigating gastro-intestinal parasites such as *T. circumcincta*, without having to kill the experimental animals. The antibodies secreted in the ASC probes supernatant could be considered a purer representative of the efferent lymph from a few very specific lymph nodes of the abomasal chain. However, the antibodies in lymph found in the common gastric lymph duct would ultimately have been produced in the same lymph nodes, by the same infection-induced local B cell populations. To these antibodies, in gastric lymph there is

also the addition of antibodies from other plasma cells in the abomasal *lamina propria*. This effectively enhances the potential parasite-induced specific reactivity of the lymph sample compared to the ASC probes. Furthermore, the samples retrieved for the work described in this chapter had been generated (for the previously infected animals) by trickle infecting sheep for 8 weeks, followed by a subsequent 50 000 *T. circumcincta* L3 bolus challenge. Because of the prolonged exposure to the parasite, this protocol was considered appropriate to have generated lymph samples with higher levels of specific anti-*T. circumcincta* IgA and IgG than those found in the ASC probes used in previous ELISA experiments. The description of gastric lymph collection from sheep infected with *T. circumcincta* was first published in 1981 by Smith *et al*., and it has proven to be an useful tool in investigating the local immune response against this parasite (Halliday *et al.* 2010, Halliday *et al.* 2009, Halliday *et al.* 2007, Smith *et al.* 1981, Smith *et al.* 1983, 1983). In this chapter it is shown that *T. circumcincta* trickle infection-induced serum and ASC probe antibodies were not able to bind to PiTci-CF-1, whereas recombinant antigen immunisation-induced systemic antibodies and antibodies generated by pre-scapular ASC probes were able to bind native antigens in L4 ES and nTci-CF-1. These results were further confirmed by gastric lymph ELISA experiments: here it is reported that the binding of lymph IgA from *T. circumcincta* previously infected animals (which received a trickle infection and a bolus L3 challenge) to L4 ES was significantly higher than in control primary infection animals (effectively naïve to *T. circumcincta*, as samples were collected before the insurgence of a primary immune response), as observed previously (Halliday *et al.* 2007). Moreover, binding of lymph IgA from the previously infected animals to purified nTci-CF-1 also was significantly higher compared to that of primary infection animals. Neither group of sheep lymph samples showed significant IgA binding to the recombinant PiTci-CF-1, except in one case. In periodate-treated PiTci-CF-1 wells, lymph IgA from previously infected sheep showed significantly higher binding than lymph from primary infection sheep (Figure 2.11). There was not a significant difference in the periodate-untreated wells, as previously shown (Figure 2.9). This suggests that the use of periodate on the yeast-expressed recombinant Tci-CF-1 might have uncovered epitopes previously hidden by carbohydrate structures, for example within the peptide backbone, and that the Tci-CF-1-specific IgA present in lymph from previously infected sheep could then bind.

Taken together, the results of this chapter showed lack of binding by *T. circumcincta* infection-induced antibodies to the recombinant protein PiTci-CF-1, compared to native L4 ES antigens and native Tci-CF-1. However, a certain degree of cross-reactivity was

observed by immunisation-induced antibodies with the native L4 ES and purified nTci-CF-1. This suggests that the immunisation-induced immune response may be able to recognise epitopes shared between the native and yeast-expressed Tci-CF-1, but also that other factors are involved for optimal immune recognition.

Salts of periodic acid such as the sodium periodate used in this work oxidise hydroxyl groups of carbohydrate residues on two or more adjacent carbon atoms (vicinal diols) to aldehyde and ketone groups (O'Neil 2001), changing the shape of such epitopes (Kristiansen *et al.* 2010). Periodate-induced disruption of the shape of the epitopes in these carbohydrate residues (forming part of the N-linked glycan residues) has the potential of reducing the antibody binding ability (Fairlie-Clarke *et al.* 2010). Previous work conducted in a mouse system of malaria-nematode co-infection showed that periodate treatment of a *P. pastoris*-expressed merozoite antigen of the malarial parasite *Plasmodium chabaudi chabaudi* (MSP-1_{19}) was able to significantly reduce serum IgG3 binding in mice infected with *P. chabaudi*. Also significantly reduced was serum IgG1 and IgG2a binding to periodate-treated *Nippostrongylus braziliensis* somatic extract (NbA) in mice co-infected with *P. chabaudi* and the nematode *N. braziliensis* (Fairlie-Clarke *et al.* 2010). In another study on the lungworm parasite *D. viviparus*, results showed that several adult ES antigens carry N-linked glycans, and that de-glycosylation by PNGase F resulted in reduction of serum IgA, IgG1 and IgG2 binding from cattle that received a primary infection (Kooyman *et al.* 2007).

In this chapter, a marked loss of antibody binding in each of the sample types investigated was observed following periodate treatment of *T. circumcincta* native and recombinant PiTci-CF-1. This finding supports the hypothesis that periodate-sensitive epitopes may be a prominent feature of Tci-CF-1 proteins and potently affect the ability of serum, ASC probes and lymph antibodies to bind to their target antigens. However, in the *D. viviparus* study discussed above, serum IgG1 of previously infected animals binding to de-glycosylated ES antigens was increased compared to the glycosylated native version, suggesting that the primary immune response was directed against glycan antigens and that subsequent parasite challenge elicited an IgG1 response against the protein backbone (Kooyman *et al.* 2007). Fairlie-Clarke *et al.* (2010) also observed that periodate treatment of *Pichia pastoris*-expressed recombinant MSP-1_{19} (not glycosylated in the native form) significantly increased serum IgG2a binding compared to the untreated MSP-1_{19} in mice co-infected with *P. chabaudi* and *N. braziliensis*. In this case it was suggested that the periodate treatment exposed protein epitopes previously masked by the inappropriate

glycosylation by *P. pastoris*. Although work carried out in this chapter showed that both native and recombinant Tci-CF-1 contained periodate-sensitive epitopes and gave indications on the presence of glycan structures, the serum and ASC probes IgG recognition to PiTci-CF-1 in control animals was not enhanced by periodate treatment of the antigen. This suggests that the differences in antibody recognition observed between the two Tci-CF-1 versions may not only be due to differing glycan structures. Here it is also demonstrated that gastric lymph IgA binding from previously infected animals to periodate-treated PiTci-CF-1 was significantly higher than in lymph from *T. circumcincta* primary infection animals. A significantly higher serum IgG binding to periodate-treated PiTci-CF-1 was also observed in immunised animals compared to controls. This result indicates a degree of antibody recognition in protein epitopes of periodate-treated PiTci-CF-1.

Following the periodate treatment experiments, the conclusions that can be drawn are i) antibody responses elicited during *T. circumcincta* trickle infection were not able to cross-react with recombinant PiTci-CF-1, suggesting that there were conformational or post-translational differences between native and recombinant forms of the protein; ii) vicinal diol epitopes, which are periodate-sensitive and commonly found on glycan structures, are a prominent feature of each Tci-CF-1 version and variably but constantly affect the ability of serum, ASC probes and lymph antibodies to bind to nTci-CF-1. Taken together, these results suggest that although PiTci-CF-1 was not recognised by serum, ASC probe, or lymph antibodies from *T. circumcincta* infected sheep, a few shared protein epitopes conferred by the shared amino acid chain may become available if the recombinant version was disrupted by means of chemicals. For this reason, in a following chapter the antibody reactivity to nTci-CF-1 and PiTci-CF-1 was investigated following the denaturing effects of protein blotting.

The findings discussed above have shown once again that engineering a recombinant vaccine candidate against nematode infections can be more complex than anticipated (Geldhof *et al.* 2007). To begin with, different types of native antigens have been used as vaccine candidates against helminth parasites. These include a commercialized live attenuated parasite vaccine (Bovilis™ Huskvac against *Dictyocaulus viviparus*), purified native antigen vaccines such as H11 and H-gal-GP complex in *Haemonchus contortus* (Knox and Smith 2001) and *Ostertagia ostertagi* ES fractions (Meyvis *et al.* 2007). The major issue with vaccines using live attenuated/killed worms or purified native antigens is the difficulty in obtaining sufficient quantities of worms to produce the vaccine, with the

added problems of production on a larger scale, such as batch variability and antigen stability, being the most difficult to overcome (Smith and Zarlenga 2006). Currently, research is increasingly focused on the production of recombinant antigens, following the identification of candidates in the parasites' ES products or somatic extracts (Meyvis *et al.* 2007, O'Donnell *et al.* 1989, Smith *et al.* 2009). There have been a number of helminth recombinant antigens generated with various expression systems, achieving variable and mostly inconclusive results [reviewed by Geldhof *et al.* (2007), Lightowlers *et al.* (2003), Smith and Zarlenga (2006)]. For example, vaccination with the yeast-expressed Ac-CP-2 antigen (a cysteine protease) from the hookworm *Ancylostoma caninum* achieved a significant reduction in the egg output and stunted worm growth compared to the controls (Loukas *et al.* 2004). However a *C. elegans*-expressed cysteine proteinase from *H. contortus*, Hc-CPL-1, did not elicit protection against homologous challenge (Murray *et al.* 2007), although protection afforded by native Hc-CPL-1 is not known due to its low abundance. In many cases, if the recombinant version fails to induce a protective immune response it is not known whether the original native antigen was an inappropriate choice, possibly due to lack of knowledge about the native antigen, or whether the unsuccessful efficacy was due to incorrect formation of the recombinant [reviewed by Geldhof *et al.* (2007), Smith and Zarlenga (2006)]. Differences in conformation and Post Translational Modifications (PTMs) can occur using traditional expression systems, especially when dealing with organisms taxonomically very distant, leading to the production of enzymes lacking correct folding or with defective PTMs and impaired functions (Geldhof *et al.* 2007). Nematode proteins are highly glycosylated with unique structures, such as core α1→3-fucose residues that may be nematode-specific (Cipollo *et al.* 2002, Dell *et al.* 1999, van Die *et al.* 1999), and appear to be important in the host immune response. Sera from animals vaccinated with irradiated *D. viviparus* larvae have shown that there is a strong but short-lived immune response directed against N-glycans on the surface of antigens (Kooyman *et al.* 2007) and that glycans of nematodes are able to induce a strong Th2 immune response (Tawill *et al.* 2004, Thomas and Harn 2004). Therefore, correct glycosylation may be important for effective vaccination.

The hypothesis emerging from the results discussed in this chapter is that a likely cause of the differential antibody binding observed was due to altered folding and/or post-translational modifications (PTMs), such as glycosylation of the recombinant PiTci-CF-1 compared to the native version. For this reason, we aimed to express the same antigen in a different, novel expression system, the free-living nematode *Caenorhabditis elegans*, to

further investigate the enzymatic activity as an indication of correct folding, and glycosylation characteristics of each Tci-CF-1 version.

Chapter 3 - Generation of *Teladorsagia circumcincta* Proteins Using *Caenorhabditis elegans*

3.1 Introduction

Local and systemic antibodies elicited by *Teladorsagia circumcincta* trickle infections of sheep bind native, but not *Pichia pastoris*-expressed Tci-CF-1 (see Chapter 2). The discrepancy observed may be attributed to differences between the recombinant and native protein in regards to protein folding and post-translational modifications (PTMs), as these can differ depending on the expression system chosen (Darby *et al.* 2012, Ferrer-Miralles *et al.* 2009, Macauley-Patrick *et al.* 2005). A system which expresses parasitic nematode proteins in a form resembling the native protein, including correct folding and appropriate PTMs, would therefore be advantageous to vaccine development. For this reason, it was proposed to use an alternative expression system, the free-living nematode *Caenorhabditis elegans* to generate a recombinant version of Tci-CF-1 more similar to the native version. This model organism belongs to Clade V of the phylum Nematoda (Blaxter *et al.* 1998). Its phylogenetic relationship to Clade V parasitic nematodes, such as *Haemonchus contortus* and *Trichostrongylus colubriformis*, and its amenability to genetic manipulation suggested *C. elegans* may be an appropriate system for the study and expression of parasite genes (Grant 1992, Murray *et al.* 2007). For example, the insertion of a β-tubulin gene, *tub-1*, from benzimidazole susceptible *H. contortus* isolates conferred thiabendazole (TBZ) sensitivity when expressed in a *C. elegans* TBZ-resistant mutant (Kwa *et al.* 1995). Others reported that *C. elegans* was able to express a cystatin gene from the filarial nematode parasite *Acanthocheilonema viteae*, as observed in immunoblots of the recombinant protein probed with a monoclonal anti-his tag antibody or cystatin-specific antibodies (Pillai *et al.* 2005). Another study into the functions of nematode proteases showed, in RNAi experiments, that a *C. elegans* cathepsin L protease (*Ce-cpl-1*) was pivotal in controlling embryogenesis of this worm (Hashmi *et al.* 2002). As this gene was found to be highly conserved among nematodes, it was hypothesised that cathepsin L may be essential for parasitic nematode development (Britton and Murray 2002). To prove this hypothesis, a *C. elegans* genetic mutant of *cpl-1* (strain ok360) was rescued by micro-injection with the orthologous cathepsin L gene from *H. contortus* (*Hc-cpl-1*), resulting in almost complete rescue of the embryonic lethal phenotype: 4-5 % arrested embryos compared to the

untransformed ok360 worms (100 % embryo mortality; Britton and Murray 2002). More recently, it was shown that *H. contortus* Hc-CPL-1 could be successfully expressed and purified in active form using *C. elegans* as an expression system (Murray *et al.* 2007).

An effective way to genetically modify *C. elegans* is to micro-inject a DNA construct encoding the desired protein into the gonad of hermaphrodite worms; this may be incorporated into germline cells in the gonad and form an extrachromosomal array in the following generations. Selection of transformed progeny can be achieved by co-injecting a marker gene (Han and Sternberg 1990, Mello *et al.* 1991). The use of dominant marker genes such as *dpy-7:gfp* or *rol-6* is a useful tool to identify transformed worms when generating *C. elegans* transgenic lines (Britton and Murray 2002, Murray *et al.* 2007). In *C. elegans*, *rol-6* encodes a collagen protein (Kramer *et al.* 1990), and mutations of the *rol-6* gene generate worms with a specific locomotion defect – a right-handed twisted phenotype when moving (Cox *et al.* 1980). Worms expressing a gene of interest together with *rol-6* can therefore be identified and selected based on their roller phenotype. An alternative approach is to use a wild-type gene as a marker to rescue a specific mutant phenotype. Gene rescue (or functional complementation) can be used to distinguish untransfomed/mutant from transformed/wild type organisms. The technique was originally devised in the fruit fly *Drosophila melanogaster* (Lewis 1945) and the original aim of the technique was to ascertain whether a wild-type gene can restore the function of a defective mutant, to demonstrate that the mutation is occurring in the same gene as the wild-type. This can be achieved in *C. elegans* by using genes from the same species (Gomez *et al.* 2012), but also from different species, to identify the functions of orthologous genes [for example, rescue using human and rat *NLGN1* genes (Calahorro and Ruiz-Rubio 2012), human *GRB2* and *Drosophila drk* genes (Stern *et al.* 1993) or *T. circumcincta cat-4* gene (Baker *et al.* 2012)]. In this chapter, two approaches for transforming *C. elegans* with two different *T. circumcincta* protein expression constructs were used; first, the micro-injection of *rol-6* as dominant phenotypic marker gene in wild type *C. elegans*, alongside a plasmid encoding *Tci-cf-1*. Second, the rescue of the *C. elegans* mutant strain DR96 (*unc-76* defective) was carried out by micro-injecting *Ce-unc-76* gene to rescue the wild type phenotype, along with a plasmid encoding *T. circumcincta* monocyte Migration Inhibitory Factor (*Tci-mif-1*) to distinguish successfully transformed worms from untransformed mutants.

Macrophage Migration Inhibitory Factor (MIF) was first identified during studies of delayed-type hypersensitivity reaction in guinea pigs (Bloom and Bennett 1966), however

its functions had been unclear until the cloning of human MIF cDNA (Bernhagen *et al.* 1994). In more recent years, it was demonstrated that MIF molecules have pivotal functions as regulators of innate immunity in infection and inflammation models [reviewed by Calandra and Roger (2003)]. A MIF-like molecule (Tci-MIF-1) has been identified in *T. circumcincta* extracts and Tci-MIF-1 mRNA was shown to be more abundantly present in eggs, L3, and adult stages and, in lower levels, in L4 larval stages. Tci-MIF-1 protein abundance was higher in L3 somatic extract than extracts from L4 and adult worms, and immunohistochemical staining of *T. circumcincta* of L3, L4 and adult worms showed localization of the protein in the worm gut (Nisbet *et al.* 2010b). By virtue of its postulated immunoregulatory functions (Bernhagen *et al.* 1993, Calandra *et al.* 1995, Calandra and Roger 2003, Falcone *et al.* 2001), and its abundance in the infective L3 stages, a recombinant version of Tci-MIF-1 was expressed in *E. coli* (EcTci-MIF-1) and used in immunisation trials of sheep which were then challenged with a *T. circumcincta* trickle infection (Nisbet *et al.* 2010b, Nisbet *et al.* 2013). The recombinant Tci-MIF-1 showed the conserved dopachrome tautomerase enzyme activity associated with MIF; however monocyte migration inhibition, which is a known function of MIF proteins from other nematodes (Pastrana *et al.* 1998, Tan *et al.* 2001), was not demonstrated (Nisbet *et al.* 2010b). This may have been due to partial failure of the expression system to deliver correct Post-Translational Modifications (PTMs), affecting the cell recognition of the recombinant protein expressed in *E. coli*. Therefore it was proposed to express Tci-MIF-1 in *C. elegans* to try to deliver a vaccine candidate with structure and PTMs more similar to the native protein found in *T. circumcincta* L3 larvae.

The proposed method of purification for the *C. elegans*-expressed recombinant proteins involved the insertion of a poly-histidine tag at the protein C-terminus and the use of metal ion affinity chromatography. This was based on the fact that histidine is the main amino acid in proteins that interacts with immobilized metal ions [reviewed by Kuo and Chase (2011)]. Metal ions have been used to purify proteins for decades, thanks to the affinity of histidine-rich proteins for heavy metals such as copper (Cu), zinc (Zn), nickel (Ni) and cobalt (Co) (Porath *et al.* 1975). The metal ions can be immobilized in an agarose or silica gel matrix and then used to bind amino acid residues in chromatographic systems [Immobilized Metal Affinity chromatography (IMAC)]. Nickel ions are highly specific for binding peptides and proteins containing neighbouring histidine residues and the use of poly-histidine fusion tags ranging between 2-10 residues fused with recombinant proteins has become the most widely used type of affinity fusion tag since its first application (Hochuli *et al.* 1987, Hochuli *et al.* 1988, Terpe 2003). As this method was used in

previous studies to purify recombinant *T. circumcincta* recombinant antigens such as *Pichia pastoris*-expressed Tci-CF-1 (PiTci-CF-1) and *E. coli*-expressed Tci-MIF-1 (EcTci-MIF-1; Nisbet *et al.* 2010b, Nisbet *et al.* 2013), the same system was considered appropriate for purification of *C. elegans*-expressed proteins in this project.

The overall aims of this chapter are therefore:

i) to express a recombinant version of Tci-CF-1 (that shares the same amino acid sequence as PiTci-CF-1) in the free-living nematode *C. elegans*;

ii) to express a recombinant version of Tci-MIF-1 (that shares the same amino acid sequence as EcTci-MIF-1) in the free-living nematode *C. elegans*;

iii) to efficiently purify both proteins.

3.2 Materials and methods

3.2.1 Generation of vector encoding *Tci-cf-1* for *C. elegans* microinjection

A PCR product containing the full length *Tci-cf-1* gene sequence generated in Chapter 2 was used at a 1:100 dilution as template for PCR reactions aimed at introducing restriction enzyme digestion sites and a C-terminal poly-histidine (10x) tag by two subsequent PCR rounds. The primer sets used for PCR amplifications, colony PCR and sequencing are listed in Table 7.2 (Appendix A). Standard PCR, cloning into pGEM-T (Promega) and sequencing (Eurofins MWG Operon, Germany) conditions are detailed elsewhere [Chapter 2, Sections 2.2.2 (cloning and sequencing) and 2.2.3 (PCR methods)]. Plasmid insert DNA from one colony containing the correct *Tci-cf-1* sequence in frame with a 10x histidine tag and with *Not*I and *Sal*I restriction sites inserted was PCR amplified and purified as described in Chapter 2. This product was used in downstream cloning into a *C. elegans*-expression plasmid vector. This vector was originally generated by Dr Brett Roberts (University of Glasgow), and its structure is similar to that of the plasmid vector described by Britton and Murray (2002); the areas flanking the restriction sites upstream included 1.7 kb of *C. elegans* cathepsin L gene promoter (*Ce-cpl-1*) and 500 bp of *Ce-cpl-1* 3' untranslated region (3' UTR) downstream (Figure 3.1). The plasmid was named ABR-09, and this name is maintained throughout this work. The vector ABR-09 was engineered to contain a *Xho*I restriction sequence, which creates an overhang (TCGA) identical and therefore compatible with the one generated by *Sal*I, which was used for the cloning of the *Tci-cf-1* insert.

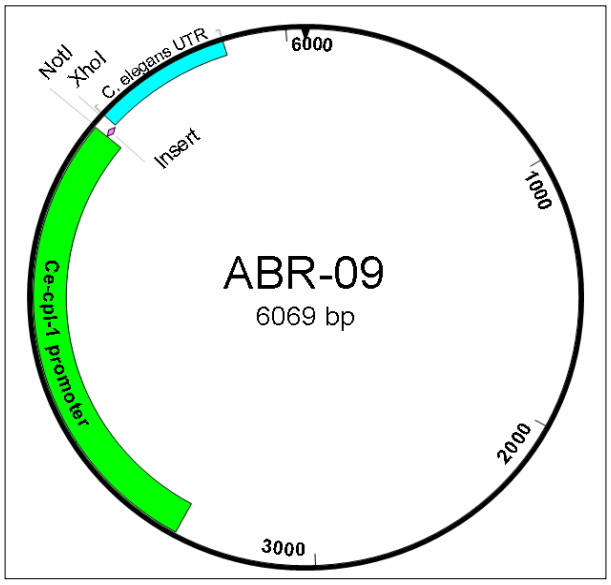

Figure 3.1 Map of the vector ABR-09 used for cloning and expression of *Tci-cf-1* and *Tci-mif-1* genes in *C. elegans*.
The map shows the restriction sites relevant to this study (*Not*I and *Xho*I), Ce-cpl-1 promoter (blue arrow) and *C. elegans* cpl-1 3' UTR (green bar). The free software SnapGene Viewer was used to generate this map.

Restriction enzyme digests were carried out to release the *Tci-cf-1* sequence from pGEM-T and to linearize ABR-09. The restriction digests included 5 µl Reaction Buffer D (Promega, Southampton, UK), 10 µg BSA, 7 µl of *Tci-cf-1* plasmid and 10 U of enzyme, made up to 50 µl with dH_2O. The *Tci-cf-1* insert was released from pGEM-T using 10 U *Sal*I in a first reaction incubated at 37°C for 1 h, after which 10 U *Not*I (all from Promega) was added and the reaction incubated for another 2 h. The released *Tci-cf-1* insert was gel purified (QIAquick Gel Extraction Kit, Qiagen, Manchester, UK) following the manufacturer's instructions. In addition, after the last wash with the buffer provided, one extra isopropanol wash was carried out before eluting the DNA with pre-warmed (50°C) dH_2O. The ABR-09 vector was linearized following a similar procedure with the restriction enzymes *Xho*I and *Not*I (all from Promega) to create compatible ends. The ligation reaction between linearized ABR-09 and released *Tci-cf-1* was: 7 µl of gel-purified *Tci-cf-1*, 1 µl of double digested ABR-09, 1 µl 10 X T4 Ligase buffer and 1 µl of T4 Ligase enzyme (Promega). Cloning into JM109 cells and colony PCR were carried out as previously detailed in Chapter 2.2.2 using primer sets 2, 3 and 4 as listed in Appendix A, Table 7.2. Colonies containing *Tci-cf-1* were amplified and plasmid DNA column purified using a Plasmid Mini Kit (Qiagen). A final restriction analysis was carried out in single reactions using *Asu*II and *Bam*HI (Roche) and *Eco*RI and *Hind*III (Promega) to confirm the insertion of *Tci-cf-1* sequence into ABR-09 in the correct position. The final construct

encoding Tci-CF-1 (Figure 3.2) was sent to Dr Collette Britton at the University of Glasgow for micro-injection into *C. elegans* wild type, strain Bristol N2.

Figure 3.2 Diagram of the Tci gene constructs inserted into plasmid ABR-09 for micro-injection into *C. elegans*.

3.2.2 Generation of vector encoding *Tci-mif-1* for *C. elegans* microinjection

A plasmid containing *Tci-mif-1* insert in pET-22b(+) (Novagen, Darmstadt, Germany) was provided by Dr Alasdair Nisbet at MRI as a scraping of cell culture glycerol stock. The scraping was suspended in 40 µl of dH_2O and used as template for PCR reactions aimed at introducing *Not*I and *Xho*I restriction sites and a C-terminal poly-histidine tag at the 3' end of the amplicon by two PCR rounds, using primer set number 5 listed in Table 7.2 (Appendix A). Standard cloning into pGEM-T (Promega) and sequencing (Eurofins MWG Operon, Germany) conditions are detailed elsewhere (Chapter 2, Sections 2.2.2.2 and 2.2.2.3). One colony containing the correct *Tci-mif-1* sequence in frame with 10x poly-histidine tag, *Not*I and *Xho*I restriction sites was digested to release the *Tci-mif-1* insert from pGEM-T following the procedure detailed in Section 3.2.1; ABR-09 was also linearized. The digested *Tci-mif-1* insert DNA was column purified (QIAquick PCR Purification Kit, Qiagen) and ligated into linearized ABR-09 (Figure 3.1). The ligation reaction was as detailed above in Section 3.2.1. The transformation of ligated products, colony PCR (using primer sets 4 and 5, Table 7.2), and sequencing methods (using primer sets 3 and 6, Table 7.2) have been detailed in Chapter 2. One colony including the correct *Tci-mif-1* sequence in frame with 10x poly-histidine tag was amplified overnight in LB broth and column purified (Plasmid Mini Kit, Qiagen), before being sent to Dr Collette Britton at the University of Glasgow for micro-injection into *C. elegans unc-76* mutant strain DR96.

3.2.3 Routine maintenance of *C. elegans*

Unless otherwise stated, all protocols and media recipes were adapted from the publicly available Wormbook (http://www.wormbook.org/). Briefly, *C. elegans* worms of mixed stages were routinely maintained on Nematode Growth Media (NGM, Appendix D) poured

in 90mm Petri dishes and left to solidify. After 1-2 days of plates resting upside down to let all condensation evaporate, a thin lawn of *E. coli* OP50 strain was seeded onto each plate, making sure not to reach the edge of the agar. Within 2 days the plates were ready to use for worm growth. Fresh plates were left at 4°C until use. Individual worms showing the transformed phenotype were picked from plates in which they had been left to proliferate for at least 3 days, using a worm pick made of a 0.2 mm platinum wire fused to the end of a glass Pasteur pipette. The worms were deposited gently onto the agar of a new NGM plate, not in contact with the *E. coli* lawn, and allowed to crawl off the pick. The plates were maintained in incubators at a constant temperature of 20°C (± 1°C).

If worm stocks became contaminated with yeast or bacteria other than *E. coli* OP50, the plates were treated as follows to remove the contamination (adapted from Wormbook, http://www.wormbook.org/chapters/www_strainmaintain/strainmaintain.pdf). Briefly, plates containing contaminated worms were selected for presence of gravid hermaphrodites and washed with sterile H_2O to free the worms. A 1:1 solution of 1 N NaOH and household bleach (5 % NaClO) was prepared and two 20 µl drops were positioned at diagonal edges of a clean NGM plate. Five gravid hermaphrodite *C. elegans* were put in each drop and left to die. The solution killed the contaminant and worms leaving viable eggs. The following day, hatched larvae had crawled free from contaminant. If the contaminants were not eliminated but only reduced, the procedure was repeated.

3.2.3.1 Storage of worms

For short term storage of worms, 10-15 worms were moved onto a fresh NGM plate in which 25-30 ml of liquid NGM had been allowed to set and an *E. coli* OP50 lawn allowed to grow. The plates were wrapped in Parafilm and left in the incubator at 15°C for up to three weeks. Worms were then picked individually or chunks of agar (carrying worms) from the old plate transferred onto a fresh NGM plate to allow feeding and recovery.

For long term storage of worms, two methods were used: i) starved L1-L2 worms were collected from NGM plates by washing the plates with ~1 ml of S Buffer (Appendix D) for each vial and worms collected in sterile Eppendorfs. An equal volume of S Buffer/ 30 % glycerol was added and mixed. One millilitre of worm suspension was aliquoted into several labelled 1.8 ml vials for long term freezer storage (Nunc® CryoTubes®); the vials were packed in a Styrofoam box appositely prepared with slots to hold cryovials. The box was kept at -80°C until further use. To recover worms it was sufficient to thaw one vial

and pour the contents onto a fresh NGM plate. Thawed vials could not be re-frozen. Freezing method ii) the Soft Agar Freezing Solution was melted in a microwave and left to settle in a water bath at 55°C for 15-20 min. Worms were collected at the L1-L2 stage from starved plates using ~1 ml S Buffer for each vial to freeze and put on ice in an Eppendorf. The same volume of Soft Agar Freezing Solution was added and mixed well. One millilitre of this suspension was aliquoted into several 1.8 ml cryovials (Nunc) and put at -80°C using the Styrofoam box. To recover worms, a small scoop of frozen suspension was collected and deposited to thaw onto a fresh NGM plate. The thawing recovery procedure was repeated if an insufficient number of worms colonized the plate.

3.2.4 *C. elegans* microinjection

This procedure was carried out by Dr Collette Britton and Dr Brett Roberts at the University of Glasgow. To allow positioning for microinjections, young adult *C. elegans* were picked and placed in a drop of mineral oil on a glass cover slide onto which a drop of molten 1 % agarose had been previously poured and left to cool to create a "sticky" pad for the worms to adhere to. The cover slide was transferred to the micro-injecting microscope and a glass needle used to inject DNA into the gonads of the worms. The injected *C. elegans* hermaphrodites were recovered from the agar pad with a drop of M9 Buffer (Appendix D) and deposited in a fresh NGM plate to recover. The plates were kept at 20°C and screened every day for progeny expressing the marker phenotype. Each generation was maintained in a separate plate. Generated *C. elegans* transgenic lines were also screened by Western blot using anti-His antibody for the presence of the desired protein [*C. elegans* expressed Tci-CF-1 (CeTci-CF-1) or *C. elegans* expressed Tci-MIF-1 (CeTci-MIF-1)] in the separate lines.

3.2.5 Generation of *C. elegans* biomass

Stable worm lines showing recombinant protein expression by Western blot, were harvested from NGM plates with 2 ml M9 buffer and 100 µl of the worm suspension were transferred onto Peptone Rich agar plates (Appendix D), pre-seeded with a lawn of *E. coli* BL22 strain. The plates were incubated at 20°C for 3.5-5 days depending on the growth rate of the worms and subsequently harvested before the plates reached complete depletion of the *E. coli* lawn. The worms were harvested by washing the plates with ice cold 0.1 M NaCl, washing at least twice with the ice cold solution. The worm suspension was placed in centrifuge tubes and centrifuged at 7000 *x g* for 5 min. The pellet was resuspended in 20

ml 0.1 M NaCl, then the same volume of 60 % sucrose was added before centrifugation at 3500 *x g* for 5 min. Sucrose is toxic to *C. elegans* at such concentrations, therefore the worms were quickly harvested with a broken glass Pasteur pipette and transferred to a fresh centrifuge tube pre-filled with ice cold 0.1 M NaCl. The worm solution was washed 3 times in 0.1 M NaCl at 7000 *x g* for 5 min. After the final wash, the pellet was resuspended in the same volume of Binding Buffer B (Appendix D) before freezing at -80°C for at least 30 min or until ready to process. The worm suspension was thawed on ice before sonication (10 cycles of 10 sec on/20 sec off) on ice. One millilitre aliquots of the sonicated suspension were applied to a glass homogeniser and worms disrupted manually into a homogenate. To complete protein release from the worm lysate, Triton X-100 to a final concentration of 0.1 % was added to the worm homogenate and left on a rocking device for 30 min at 4°C. Subsequently the homogenate was aliquoted in 1.5 ml Eppendorfs and centrifuged at 13 000 *x g* for 5 min. The cleared lysate (CL) was collected and centrifuged again at 13 000 *x g* for 3 min, the supernatant collected and the pellet resuspended in 2-3 ml Binding Buffer B before repeating the process from the sonication onwards. The pool of cleared lysate was filtered using a 20 ml sterile syringe (BD Bioscience) and single-use Minisart filters (Sartorius, Goettingen, Germany); first the lysate was applied to a 0.45 μm filter followed by a 0.2 μm filter to remove all residual debris. The samples thus obtained were immediately used for protein purification.

3.2.6 Protein purification using metal ion columns (IMAC)

3.2.6.1 Cobalt (Co^{2+}) ion columns

The cleared lysate generated from *C. elegans* transformed worms in section 3.2.5 was applied to the Co^{2+} Immobilized Metal ion Affinity Chromatography [IMAC; HiTrap Talon® crude, GE Healthcare UK Ltd (Little Chalfont, UK)] purification system, following a protocol adapted from (Murray *et al.* 2007). Briefly, the column was attached to a bench-top chromatography pump system (Pharmacia Biotech, now part of GE Healthcare) previously washed with dH_2O for at least 5 min at a flow rate of 1 ml/min; the column was washed with 5 column volumes of dH_2O and equilibrated with 5 column volumes of Binding Buffer B (Appendix D).

The *C. elegans* cleared lysates containing Tci-CF-1 or Tci-MIF-1 were applied in their entirety (~20-40 ml) to the column and left to re-circulate through the column for 1-2 h at a flow rate of 1 ml/min. This allowed the whole sample volume to circulate at least three

times through the column, which was then washed with 5 column volumes of Wash Buffer (Appendix D) before applying Elution Buffers 1-5 (all in Appendix D) containing an imidazole step gradient ranging between 165-500 mM imidazole. The recombinant proteins were dialysed against Dialysis Buffer 2 (Appendix D); Mini Dialysis Kit (GE Healthcare) with a molecular weight cut-off (MWCO) of 8 kDa (Tci-CF-1) or 1 kDa (Tci-MIF-1) were used to dialyse the purified proteins in two washes of 500 ml buffer, overnight and 3 hours in length respectively. On one occasion explained in the relevant results section, Slyde-a-lyzer dialysis cassettes, with a MWCO of 20 kDa (Pierce), were used for dialysis in Dialysis Buffer 2 for recombinant Tci-CF-1.

Protein identity was confirmed for both Tci-CF-1 and Tci-MIF-1 by Matrix Assisted Laser Desorption/Ionisation - Time of Flight (MALDI-ToF) spectrometric analysis performed at MRI Proteomics Unit.

3.2.6.2 Nickel (Ni^{2+})

Purification of *C. elegans*-expressed Tci-CF-1 protein was carried out using IMAC systems with Ni^{2+} ions as described previously in Section 2.2.4 of this thesis.

3.2.7 Whole worm SDS-PAGE and Western blot protocols

Western blot analyses of *C. elegans* transformed adult worms were carried out adapting the general Western blot procedure described in Appendix B. Equal numbers (between 100-200) of *C. elegans rol-6* phenotype (rollers), rescued DR96 or N2 (Bristol strain, wild type) worms were picked into 65 µl of ice cold PBS, to which 25 µl of NuPAGE® sample loading buffer and 10 µl of NuPAGE® reducing agent were added (Invitrogen, Paisley, UK), for a total volume of 100 µl. The samples were heated at 70°C for 10 min and 13 µl were loaded into wells of NuPAGE® Bis-Tris 4–12 % gels; electrophoresis and standard Western blotting were carried out as described in Appendix B. Wash buffer was PBST (Appendix D) and blocking and diluting buffer was PBST-Milk (Appendix D). The primary antibody was a mouse Anti-His (C-term) antibody (clone #3D5, 1:1500 dilution, Invitrogen) and secondary antibody was a rabbit anti-mouse IgG:HRP diluted 1:1000 (clone n/a, P0161, Dako) in PBST-Milk. Blots containing *rol-6/Tci-cf-1* transformed worm extract were probed with a 1:1000 dilution of anti-PiTci-CF-1 sheep serum [previously generated at MRI by Dr Tom McNeilly and Dr Alasdair Nisbet, unpublished data)] and an anti-goat/sheep IgG:HRP (clone GT-34, A9452, Sigma-Aldrich) diluted 1:1000 in TNTT as secondary antibody. Pre-immune serum from the same sheep was used as a control.

Western blots of DR96 *unc-76* rescued/*Tci-mif-1* worms were routinely blocked and washed in TNTT, probed with a 1:50 dilution of anti-EcTci-MIF-1 rabbit serum (Nisbet *et al.* 2010b) and goat anti-rabbit IgG:HRP conjugate (clone n/a, P0488, Dako) diluted 1:1000 in TNTT as secondary antibody. All blots were developed with ECL or DAB as previously detailed in Appendix B. Western blots of IMAC-purified protein were carried out as described in Appendix B, with the antibodies detailed above.

3.2.8 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.01 and GenStat 12th Edition. Arithmetic means with standard errors and replicate number (n) are shown. Data were checked for normal distribution by analysing the histogram of frequencies and residual plots. For comparison of one pair of means, an unpaired two-tailed *t*-test was used. *P* values of < 0.05 were considered significant.

3.3 Results

3.3.1 Generation and purification of *C. elegans*- expressed Tci-CF-1 (CeTci-CF-1)

The transgenic *C. elegans* injected with *Tci-cf-1* and *Ce-rol-6* expressed the roller phenotype. A high proportion of the worms had incorporated the marker plasmid (at least 50 % of the worms displayed the transformed phenotype). A protein of the predicted size of Tci-CF-1 (~45 kDa including 10x His tag) was detected in whole roller worm Western blots using an anti-His tag antibody. However, a band of similar size was also detected in blots of wild type worms (Figure 3.3). The lane containing the transformed roller worm extract showed a more intense band compared to the lane containing N2 worms extract. Considering that the same number of N2 and roller worms had been loaded onto the gel, this suggested that His-tagged recombinant Tci-CF-1 and other *C. elegans* histidine rich protein(s), each similar in size, could be detected by the anti-his tag monoclonal antibody.

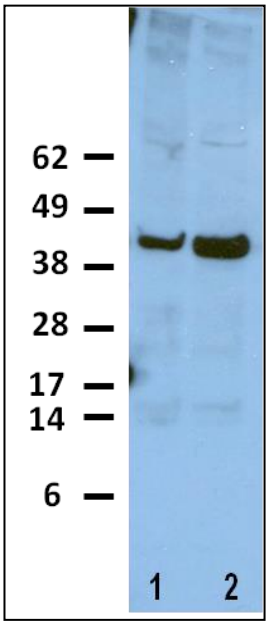

Figure 3.3 Immunoblot of *C. elegans* N2 and *Tci-cf-1/rol-6* roller worms, showing the expression of CeTci-CF-1 in the transformed worms.
Lane 1: 100 whole N2 worms; lane 2: 100 whole roller worms. One hundred worms were incubated in SDS loading buffer and reducing agent at 70°C for 10 min. The proteins were transferred onto nitrocellulose membrane. Immunoblot was probed with anti-His tag:HRP antibody (1:1500, Invitrogen) and developed using the ECL system.

To address this, another immunoblot was probed with an anti-PiTci-CF-1 ovine serum previously generated at MRI. A band of the expected size (between 38 and 49 kDa markers) was present only in the lane loaded with extract of roller worms, when probed with anti-PiTci-CF-1 anti-serum, but not with pre-immune serum from the same animal

(Figure 3.4). This indicated that *C. elegans* transformed with *rol-6/Tci-cf-1* expressed a recombinant version of Tci-CF-1. Additional bands are observed due to cross-reactivity of the antiserum with *C. elegans* extract proteins. This may have been due to epitope similarities between *C. elegans* antigens and other immunogenic antigens commonly encountered by sheep immune system, resulting in a complex polyclonal serum antibody population. The serum used for Fig. 3.4 was collected from sheep that had not been, to the writer's knowledge, previously infected with *T. circumcincta*.

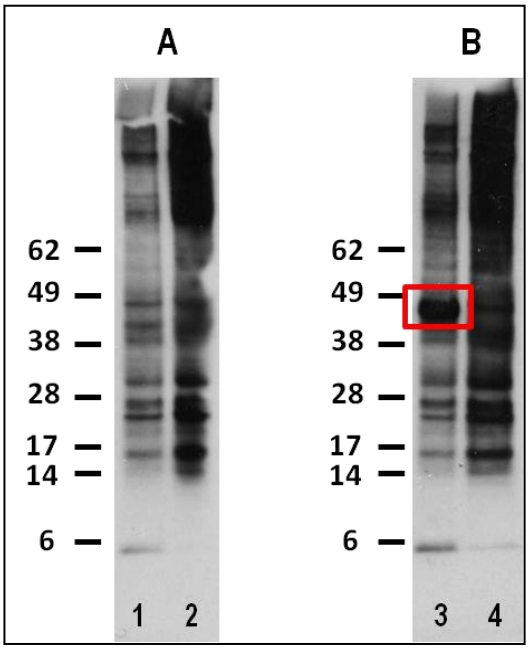

Figure 3.4 Immunoblot of *C. elegans* roller and N2 worms, showing the expression of CeTci-CF-1 (red box).
Lanes 1 and 3: 100 whole roller worms; lanes 2 and 4: 100 whole N2 worms. Worms were incubated in SDS loading buffer and reducing agent at 70°C for 10 min. The proteins were transferred onto nitrocellulose membrane. Immunoblot A probed with sheep serum before immunisation with PiTci-CF-1. Blot B was probed with serum post immunisation. The blots were developed with ECL.

A protein of the expected size of Tci-CF-1 was purified from the cleared lysate of roller worms using a Ni^{2+} chelating column. However, the eluted fractions contained many contaminating proteins (Figure 3.5).

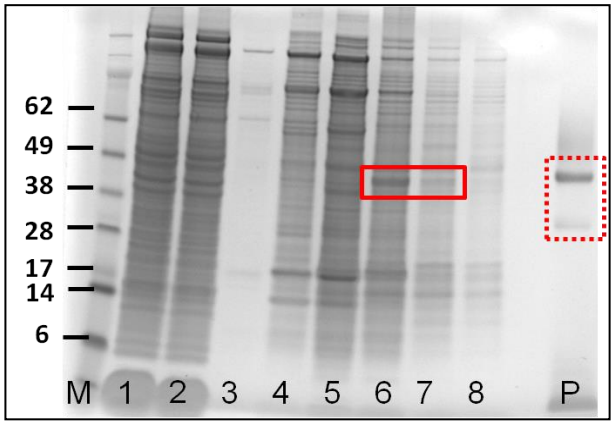

Figure 3.5 SDS-PAGE analysis of *C. elegans* fractions from roller worms expressing CeTci-CF-1, eluted from a Ni^{2+} IMAC system.
Coomassie stained gel. Lane M: molecular weight marker (kDa); lanes 1-2: flow-through (unbound fractions); lane 3: fraction eluted with 50 mM imidazole; lane 4: 200 mM imidazole; lane 5: 350 mM imidazole; lanes 6-8: 500 mM imidazole (putative CeTci-CF-1, solid line box). Lane P: control of PiTci-CF-1 (dotted line box).

A more sensitive method was developed using a Co^{2+} chelating column, in an attempt to purify CeTci-CF-1 alone. The result was partially successful, as all contaminating proteins except one were eliminated from the eluted fraction containing CeTci-CF-1 (eluted using 165 mM imidazole; Figure 3.6). The identity of CeTci-CF-1 was confirmed by the MRI Proteomics Facility by MALDI-ToF analysis.

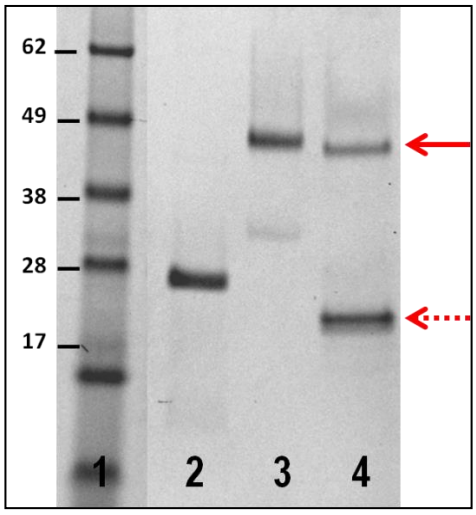

Figure 3.6 SDS-PAGE analysis of *T. circumcincta* L4 ES-purified native Tci-CF-1 (nTci-CF-1), purified recombinant PiTci-CF-1 and CeTci-CF-1.
Coomassie stained gel. Lane 1: molecular weight marker; Lane 2: nTci-CF-1; lane 3: PiTci-CF-1; lane 4: CeTci-CF-1 (solid arrow) and contaminant *C. elegans* protein (dotted arrow).

3.3.2 Identification of a *C. elegans* galectin in the CeTci-CF-1 eluted fractions

Upon investigation and analysis by mass spectrometry (Lc-ESI MS/MS), the contaminant protein in each CeTci-CF-1 eluted fraction was identified as Ce-LEC-8, a galectin (NCBI Protein accession number NP509649, estimated molecular weight: 20.4 kDa). This protein possesses a high number of histidine residues (25 of 180 total residues, 13.8 %, Table 3.1), giving it potential affinity for the chelating ions in the columns used for purification. Protein structure was predicted using the free software Jalview v2.6.1, integrating Jmol (Cole C. 2008, Waterhouse 2009) and the position of histidine residues highlighted to show the presence of surface clusters of this amino acid (Figure 3.7).

Table 3.1 The amino acid composition of Ce-LEC-8, highlighting histidine as the most abundant residue.

Amino Acid	Frequency
His	25
Val	14
Gly	14
Ala	13
Ser	13
Phe	12
Glu	12
Ile	11
Asn	10
Leu	8
Asp	8
Thr	7
Tyr	7
Pro	7
Arg	6
Lys	4
Gln	4
Met	2
Trp	2
Cys	1

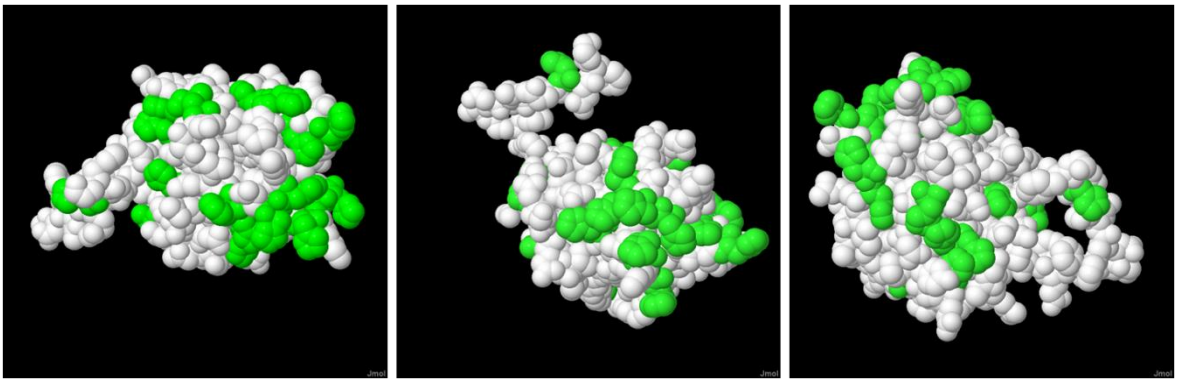

Figure 3.7 Predicted tertiary structure of Ce-LEC-8, viewed from different angles to show the histidine clusters.
Histidine residues highlighted in green. The predicted 3D structure was generated importing the Ce-LEC-8 amino acid sequence into Jalview software v2.6.1; settings were Style: perspective depth, and Scheme: CPK spacefill.

The contaminant protein could be purified from N2 wild type *C. elegans* worms following the same protocols used for CeTci-CF-1 and its identity was confirmed by SDS-PAGE analysis and Lc-ESI MS/MS as Ce-LEC-8 (Figure 3.8).

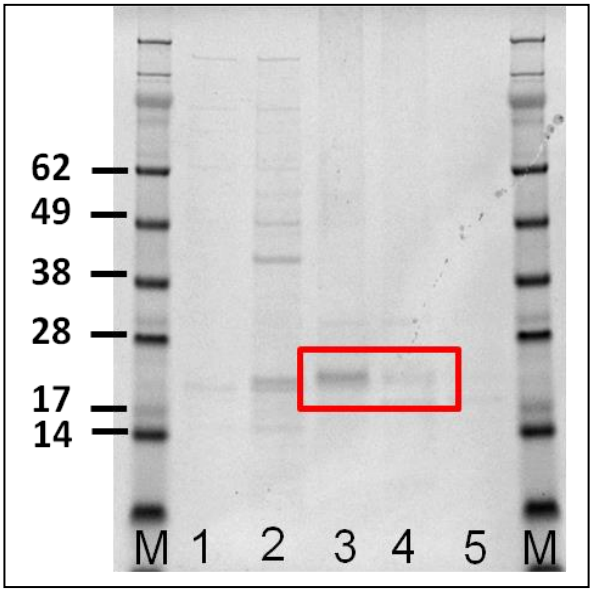

Figure 3.8 SDS-PAGE analysis of *C. elegans* fractions from N2 worms showing Ce-LEC-8, eluted from a Co^{2+} IMAC system.
Coomassie stained gel. Lane M: molecular weight marker (kDa); lane 1: flow-through (unbound fractions); lane 2: wash fraction (40mM imidazole); lanes 3-4: 165 mM imidazole; lane 5: 350 mM imidazole. Ce-LEC-8 bands are shown in the solid line box.

3.3.3 Generation and purification of *C. elegans*-expressed Tci-MIF-1 (CeTci-MIF-1)

The *C. elegans* unc mutant strain DR96 transformed with *unc-76* wild type gene and *Tci-mif-1* showed a wild type phenotype, in contrast to the un-transformed worms that still displayed the *unc-76* deficient DR96 phenotype. A very high proportion of the worms had incorporated the marker plasmid (at least 75 % of the worms displayed the transformed phenotype). A protein of the predicted size of Tci-MIF-1 was detected in Western blots of transformed worms displaying the wild-type phenotype, using an anti-His tag antibody (data not shown). No other protein except CeTci-MIF-1 was bound by the anti-His tag antibody in the extract from DR96 worms, though another unidentified *C. elegans* protein previously observed in N2 worm extracts (~49 kDa, Fig. 3.4 lane 1) was again bound by the anti-His tag antibody in control strips of N2 worms extracts. Following Co^{2+} chelation chromatography and elution using 500 mM imidazole, a protein of the expected size of recombinant Tci-MIF-1 (~13.7 kDa including 10x His tag) was purified from the cleared lysate of rescued DR96 worms (Figure 3.9). The identity of CeTci-MIF-1 was confirmed by Western blot using anti-Tci-MIF-1 rabbit serum [previously generated by immunising rabbits with a recombinant *E. coli* version of Tci-MIF-1 (Nisbet *et al.* 2010b)]; further analysis was carried out by the MRI Proteomics Facility by MALDI-ToF and the identity of CeTci-MIF-1 was confirmed.

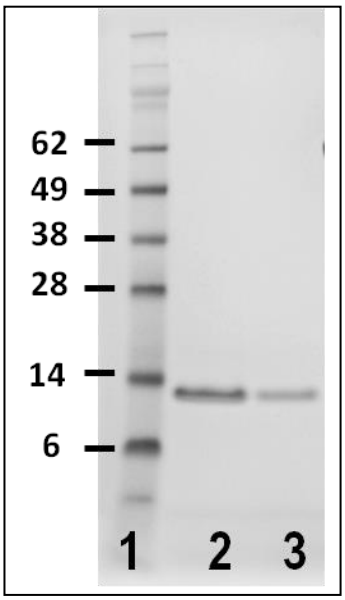

Figure 3.9 SDS-PAGE analysis of CeTci-MIF-1 purified from DR96 *C. elegans* worms rescued with *unc-76* and *Tci-mif-1* genes.
Coomassie stained gel. The fractions shown were eluted from a Co^{2+} IMAC system. Lane 1: molecular weight marker; Lane 2: CeTci-MIF-1 (pre dialysis); lane 3: CeTci-MIF-1 (pooled fractions post dialysis).

A protein of the same estimated molecular weight as Ce-LEC-8 was also eluted at the same imidazole concentration as before (~165 mM) from the cleared lysate of rescued *C. elegans* DR96 worms (Figure 3.10). The protein identity was not further investigated as Tci-MIF-1 eluted at higher imidazole concentration (> 350 mM) and therefore, in contrast to CeTci-CF-1, no further purification of CeTci-MIF-1 was required.

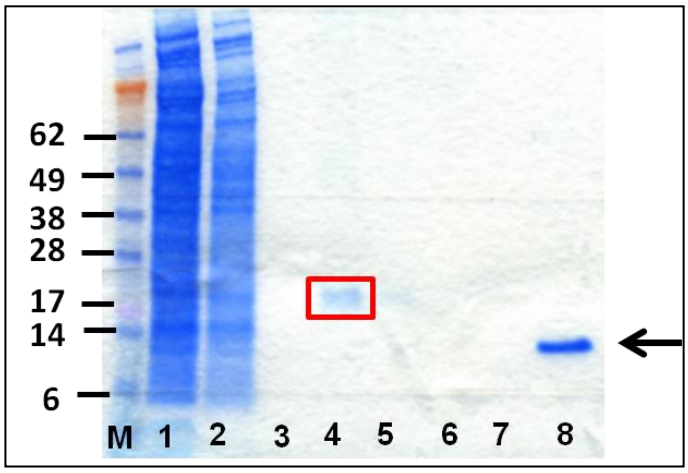

Figure 3.10 SDS-PAGE analysis of CeTci-MIF-1 and hypothesised Ce-LEC-8 purified from DR96 *C. elegans* worms rescued with *unc-76* and *Tci-mif-1* genes.
Coomassie stained gel. The fractions shown were eluted from a Co^{2+} IMAC system. Lane M: molecular weight marker; Lane 1: Flow through; lane 2: wash buffer (40mM imidazole); lanes 3-5: elution buffer (imidazole 165 mM), hypothesised Ce-LEC-8 inside box; lane 6: eluted fraction (imidazole 200 mM); lane 7: eluted fraction (imidazole 350 mM); lane 8: eluted fraction containing CeTci-MIF-1 indicated by the arrow (imidazole > 350 mM).

3.3.4 Characteristics of *C. elegans* transformed strains

Transgenic *C. elegans* populations appeared to differ in regards to the efficiency of transformation in the stable transformed progeny, the time needed to reach a similar number of worms per NGM agar plate following transfer onto fresh substrate, their movement abilities, and the total yield of purified recombinant protein. Following micro-injections and screening of subsequent generations, each transformed *C. elegans* population showed a mixed phenotype, with a high proportion of worms displaying the marker phenotype in any one generation. From previous experience and PCR experiments using transformed *C. elegans* worms, it was shown that worm lines carrying the marker plasmid also carried the protein expression plasmid (Dr Collette Britton, personal communication). Therefore, the presence of transformed phenotype in the *rol-6/Tci-cf-1* injected *C. elegans* population suggested that not only the marker gene had been incorporated as extrachromosomal arrays, but also the plasmids encoding *T. circumcincta*

proteins. However, roller populations constantly displayed a lower transformation efficiency (50-60 % transformed worms/plate) compared to rescued DR96 strain (75-90 %). The discrepancy was maintained when equal numbers of transformed worms were transferred onto fresh NGM agar plates.

Following transfer of ~15-20 roller worms, rescued DR96 or N2 worms onto fresh NGM, daily microscopic observations showed that roller worms completed consumption of all bacterial lawn (reaching near starvation point) on average 2-2.5 days later than either N2 or rescued DR96 worms. This phenomenon was maintained during the process of generating biomass for protein purification, as roller worms required between 4 and 5.5 days to reach the levels of worm biomass considered appropriate for protein purification, whereas either rescued DR96 and N2 generally required 3-4 days (see Table 3.2).

Table 3.2 Observations on some transformed *C. elegans* strain characteristics.

Worm phenotype	Estimate transformation efficiency (% of transformed worms/plate)	Approximate number of days needed to reach near starvation (on NGM)	Movement ability
Roller	60 %	5-6	Limited to circular areas; sinusoidal movements at times
Rescued DR96	75-90 %	3.5-4	Sinusoidal
N2	n/a	3.5-4	Sinusoidal

Roller worms have limited freedom of movement compared to rescued DR96 or N2, with their movements only limited to circular areas as defined by their rotation axis, with rare linear movements. This might have had limiting effects on their ability to forage on fresh bacterial lawn, and move away from exhausted areas. On the contrary, N2 and rescued DR96 worms displayed the wild type movement phenotype, mostly in linear direction.

Data on the total yield of recombinant protein per harvest was collected following protein purification. A harvest was defined as ~35 peptone-rich agar plates onto which *C. elegans* biomass was generated. Following extraction of the protein-rich cleared lysates and their application to Co^{2+} columns, multiple eluted fractions of CeTci-CF-1 and CeTci-MIF-1 were collected (at least two eluted fractions/harvest); the protein concentration in each eluted fraction was estimated either by band densitometry or BCA assay, and the total μg protein/ml of packed worms volume data extrapolated. The mean recombinant protein

yield/ml of packed worm volume was 5.18 µg (CeTci-CF-1; SD = 3.76 and n = 4) and 11.17 µg (CeTci-MIF-1; SD = 7.64 and n = 4); the range of yields was broad: 2.83-10.78 µg/ml of packed worms for CeTci-CF-1 and 4.13-20.67 µg/ml of packed worms for CeTci-MIF-1. The difference observed between *C. elegans*-expressed recombinant proteins total yields was not significant ($p = 0.1926$, n = 4), as shown in Figure 3.11. However, care must be taken interpreting the results comparing the yield of two recombinant *C. elegans* populations, as further data on the effect that different *T. circumcincta* genes might have on *C. elegans* is currently not available.

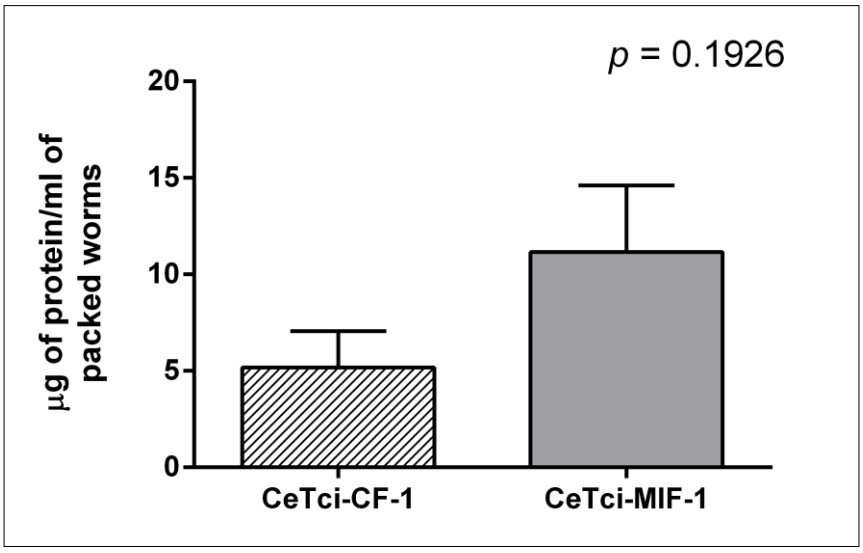

Figure 3.11 The average recombinant protein yield (CeTci-CF-1 and CeTci-MIF-1)/ml of packed worms (*C. elegans* rollers and rescued DR96 worms, respectively).
The ranges of protein yields/ml of packed worms was broad: 2.83-10.78 µg/ml of packed worms for CeTci-CF-1 and 4.13-20.67 µg/ml of packed worms for CeTci-MIF-1; data shown were the mean±SEM of yield from 4 different experiments. Unpaired, two-tailed *t*-test *p* = 0.1926, not significant.

3.3.5 Attempts to further purify CeTci-CF-1

Denaturing SDS-PAGE analysis consistently showed that CeTci-CF-1 was co-eluted in tandem with another protein identified as Ce-LEC-8 (Figure 3.6). The contaminating protein was estimated to be twice as abundant as CeTci-CF-1 in the eluted fractions (~2:1 ratio). Attempts to separate CeTci-CF-1 from Ce-LEC-8 were made as follows:

1) size exclusion chromatography (using the same conditions described in Chapter 2 to fractionate *T. circumcincta* L4 ES), as the two proteins differed substantially in size (~43

kDa and ~20 kDa, see Fig. 3.6); however this approach was not successful as the recovery of CeTci-CF-1 in the 250 µl eluted fraction was negligible and estimated below 2 µg/ml.

2) Slyde-a-lyzer dialysis cassettes, molecular weight cut-off (MWCO) 20 kDa (Pierce), as opposed to the Mini Dialysis Kit (GE Healthcare) described in Chapter 2, were also unsuccessful in separating the two proteins as some of the contaminant and most of CeTci-CF-1 were undetectable post-dialysis (Figure 3.12).

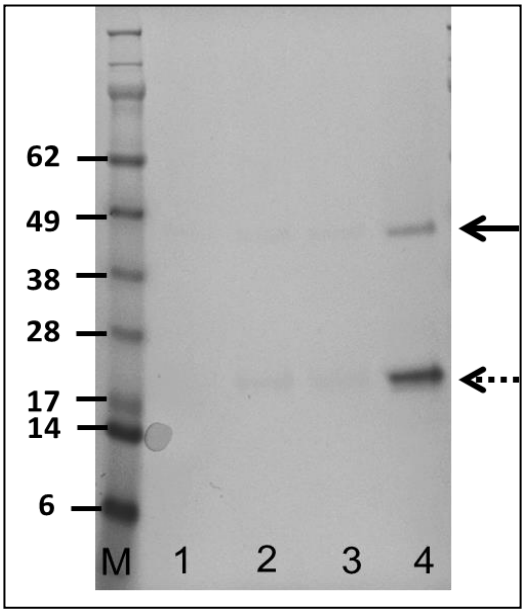

Figure 3.12 SDS-PAGE analysis of Co^{2+} eluted CeTci-CF-1 before and following dialysis with a Slide-A-Lyzer device.
Coomassie stained gel. Lane M: molecular weight marker (kDa); lanes 1-3: representative of each 1 ml aliquots of CeTci-CF-1 fraction following dialysis; lane 4: CeTci-CF-1 before dialysis (solid arrow), and contaminant protein (dotted arrow).

3) Shallower imidazole gradient in the Co^{2+} column elution buffers, as opposed to one single step from 40 mM imidazole wash to 165 mM imidazole elution buffer, to try and separate the two proteins. This approach was also unsuccessful, as the contaminant protein and CeTci-CF-1 shared overlapping elution conditions (Figure 3.13); in addition, this process contributed to further diluting CeTci-CF-1.

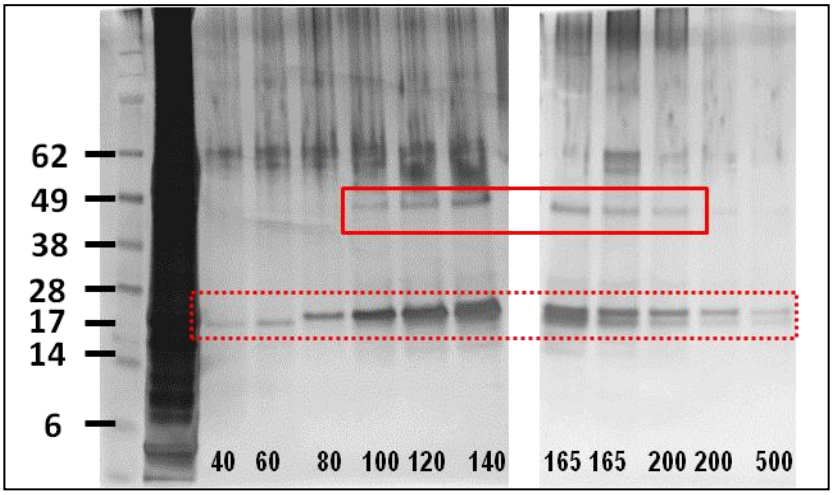

Figure 3.13 SDS-PAGE analysis of CeTci-CF-1 elution from roller *C. elegans* worms lysate over a shallow imidazole gradient.
Silver stained gels. Two gels were needed to analyse all fractions. At the bottom of each lane the imidazole concentration (mM) used to elute each fraction is indicated. From the first lane on the far left: molecular weight marker (kDa); second lane: roller worms cleared lysate before application to the Co^{2+} column; all other lanes: the fractions eluted from the Co^{2+} column. CeTci-CF-1 eluted at a narrower imidazole gradient (100-200 mM, solid line box) than the contaminant *C. elegans* proteins (40-500 mM, dotted line box).

4) As the contaminant protein was identified as *C. elegans* galectin Ce-LEC-8, and it was hypothesised that it may bind to galactose residues, a further attempt was made to separate the two proteins using D-Galactose agarose beads to bind Ce-LEC-8 leaving CeTci-CF-1 in solution. The method was partially successful, as the beads were able to remove most of the Ce-LEC-8; however, at the same time, much of the CeTci-CF-1 was also lost (Figure 3.14).

With these results in mind, it was decided not to pursue further purification of CeTci-CF-1, and all its subsequent uses were adjusted for the presence of *C. elegans* LEC-8.

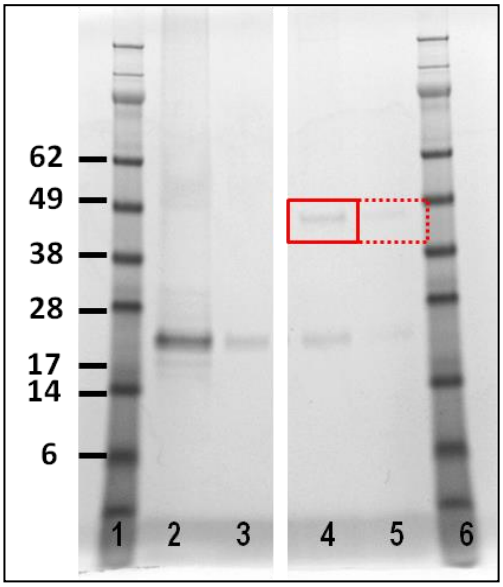

Figure 3.14 SDS-PAGE analysis of proteins purified from *C. elegans* N2 and roller worms following D-galactose agarose chromatography.
Coomassie stained gel. Lanes 1 and 6: molecular weight markers (kDa); lane 2: Ce-LEC-8 as purified from N2 (wild type) worms, before galactose agarose beads chromatography. Lane 3: the same Ce-LEC-8 from N2 worms, after galactose chromatography. Lane 4: CeTci-CF-1 (solid line box), containing similar amounts of Ce-LEC-8 (lower band). Lane 5: the same CeTci-CF-1 fraction (dotted line box) and Ce-LEC-8 (lower band), after galactose agarose chromatography.

3.4 Discussion

The work described in this chapter represents the first successful attempt, to date, of generating and expressing *T. circumcincta* vaccine candidates using *C. elegans*. This chapter reports the generation and purification using *C. elegans* of two distinct *T. circumcincta* vaccine candidates, Tci-CF-1 and Tci-MIF-1, previously generated in other expression systems (Nisbet *et al.* 2010b, Nisbet *et al.* 2013). It was demonstrated that both micro-injection approaches taken (transformation of wild type N2 *C. elegans* worms with *rol-6/Tci-cf-1* genes and rescue of wild type phenotype of DR96 using *unc-76/Tci-mif-1* genes) are viable methods for the expression of *T. circumcincta* genes using *C. elegans*. Both proteins were purified in amounts sufficient for their subsequent characterisation. The experiments carried out have shown that *C. elegans*-expressed recombinant proteins were better purified using IMAC systems based on Co^{2+} ions, rather than Ni^{2+}.

Initial immunoblots of transformed roller worms using an anti-His tag antibody showed the presence of a histidine-rich protein of similar mass (~45 kDa) to CeTci-CF-1. However, the anti-His tag antibody also bound to this protein in extracts of N2 worms, indicating that it was a *C. elegans* contaminating protein and not related to Tci-CF-1. As variable reliability in the binding of anti-his tag antibodies to poly-histidine tags situated at the C-terminus of recombinant proteins was previously demonstrated by Debeljak *et al.* (2006), further experiments were carried out to identify the presence of CeTci-CF-1. To confirm that CeTci-CF-1 was expressed in the roller worms, extracts were probed on western blot with an anti-PiTci-CF-1 sheep serum which contains PiTci-CF-1-specific IgG. As the two proteins shared the same amino acid sequences, serum raised against the *Pichia pastoris*-expressed version of Tci-CF-1 was likely to contain antibodies that recognised epitopes of another recombinant version expressed in *C. elegans*. This identified a protein of the correct size only in transformed roller worms and allowed identification of CeTci-CF-1. In addition, as previously shown in Chapter 2 of this thesis, antibodies elicited by immunisation with PiTci-CF-1 were able to bind native Tci-CF-1, therefore it was very likely that these antibodies would be cross-reactive with a *C. elegans*-expressed Tci-CF-1 version.

Numerous recombinant nematode proteins have been generated in bacterial, yeast, baculovirus and more recently, in *C. elegans* expression systems [reviewed by Geldhof *et al.* (2007)]. Recombinant protein purification by using matrix-immobilized Ni^{2+} ions has been commonplace since its first application (Hochuli *et al.* 1988), and applied

successfully to previous purifications of *T. circumcincta* recombinant proteins [Tci-ASP-1; Tci-MIF-1; Tci-TGH-2; Tci-APY-1; Tci-SAA-1; Tci-CF-1; Tci-ES20; Tci-MEP-1 (Nisbet *et al.* 2010b, Nisbet *et al.* 2013, Nisbet *et al.* 2011)]. In this chapter, it is demonstrated that Ni^{2+} ions were insufficiently specific in their affinity for recombinant CeTci-CF-1, with multiple *C. elegans* proteins eluting from the chromatography column in each fraction. A similar problem has been noted before in the *E. coli* expression system, where most of the contaminants were found to be proteins that tightly bind to metal ions through surface clusters of histidine residues or other metal-binding residues [reviewed by Bolanos-Garcia and Davies (2006)]. For example, the peptidyl-prolyl *cis–trans* isomerase SlyD (also known as WHP) is a major host cell contaminant protein in *E. coli* which binds nickel ions and often co-purifies with heterologous recombinant histidine tagged proteins when using nickel-based IMAC (Hottenrott *et al.* 1997, Wülfing *et al.* 1994). SlyD (WHP) was, however, found to have reduced affinity for Co^{2+} than Ni^{2+} ions, showing an alternative solution to the problem of co-purification (Wülfing *et al.* 1994). In this chapter it was shown that most *C. elegans* contaminant proteins that co-eluted with CeTci-CF-1 also had lower affinity for cobalt IMAC system and that, with the exception of one contaminant, no other *C. elegans* protein bound to the Co^{2+} columns used, thus yielding an almost pure CeTci-CF-1. Indeed in the last remaining contaminant *C. elegans* protein, identified as the *C. elegans* galectin LEC-8, histidine was found to be the most abundant residue in its amino acid sequence (13.8 % histidine residues), as also described by (Nemoto-Sasaki *et al.* 2008). In addition, the histidine residues appeared to form surface clusters in the predicted tertiary protein structure, suggesting that they could have mimicked the presence of a his-tag and therefore increasing Ce-LEC-8 affinity for the Co^{2+} ions used for purification, as shown by [Hayama (2000), meeting abstract available at http://www.wormbase.org/resources/paper/WBPaper00011877#0--10]. A protein of the same estimated molecular weight as Ce-LEC-8 could also be identified in fractions eluted at low imidazole concentration (165 mM) from DR96 rescued *C. elegans* lysate. However, in this case further identification and purification steps were not required, as CeTci-MIF-1 eluted at higher imidazole concentrations (> 350 mM) without other contaminant proteins being identified, at least by Coomassie staining.

In this chapter, *rol-6* was incorporated as a marker gene for transformation in extra chromosomal arrays of wild type N2 *C. elegans*. The marker gene *rol-6* has been used in micro-injections of *C. elegans* worms since the early 1990s (Han and Sternberg 1990, Mello *et al.* 1991). Worms carrying this semi-dominant roller allele are characterised by a right-hand twisted helix in the muscle and cuticle layers of the body. Normal motion

observed in wild type *C. elegans* propels the worms forward or backward in a sinusoidal manner, whereas *rol-6* instead causes the worms to roll along its longitudinal axis and to move in circles (Higgins and Hirsh 1977, Mello *et al.* 1991). Variability of the transformation efficiency of worms transformed with *rol-6* has been shown (Link 1995, Mello *et al.* 1991). Following gonadal injection in *C. elegans*, transgenes form large extrachromosomal arrays which, once established, are transmitted to between 10 and 90 % of the progeny. Therefore, some offspring of transgenic animals will lose the transgenes and will not show the marker phenotype (Mello *et al.* 1991). For example, in *C. elegans* worms transformed to express human β-amyloid peptide, inheritance of the *rol-6* marker gene and β-amyloid injected gene was variable between lines, ranging between 20-70 % (Link 1995). This was supported by the findings of this chapter, where roller worm populations constantly displayed lower estimated transformation efficiency (50-60 % transformed worms/plate) compared to *unc-76* rescued DR96 worms (75-90 %). The most likely explanation for this is that greater expression of *rol-6* is required to distinguish this phenotype from untransformed worms, compared to the *unc-76* rescued phenotype; this could mean that worms expressing low levels of *rol-6* could be missed during screening. In an RNAi study using feeding of bacterial clones, the *rol-6* RNAi worms were associated with a dumpy and egg laying variant phenotype [defined as abnormal oviposition by WormBase vWS238; http://www.wormbase.org/species/all/phenotype/WBPhenotype:0000640#0--10] (Kamath *et al.* 2003). Inefficient egg laying of roller worms may explain the upward trend observed in this chapter in the number of days required for roller worms to completely consume all the bacterial lawn, compared to either rescued DR96 or N2 worms.

Using the gene rescue transformation approach, the marker gene *unc-76* was used in this chapter to rescue the mutated strain DR96, restoring the wild type phenotype. Both the techniques here described have been previously used to express *H. contortus* vaccine candidate genes: roller worms were used to express Hc-CPL-1 and DR96 worms rescued with *unc-76* expressed *H. contortus* pepsinogen (Murray *et al.* 2007, Redmond *et al.* 2001). Worms from the DR96 strain have a slower growing rate than wild type and co-injection of the rescue plasmid containing *unc-76* resulted in phenotypic rescue to the wt phenotype, which grows more quickly than the DR96 strain (Redmond *et al.* 2001).

Recombinant protein expression in bacterial and yeast systems can yield considerable amounts of protein [up to 25 g/L for recombinant nitrilase from the *Pasteurella fluorescens* system (Chen 2012)]. In addition, each recombinant protein-expressing cell population

would consist of a very large number of cells and would have descended from a single clone. In the case of *C. elegans*, micro-injection in the gonad results in the construct encoding the desired protein being incorporated into extrachromosomal arrays. As these are not stably inherited, not all individuals in the population derived from the injected worms will express the recombinant protein (Evans, 2006). Using *C. elegans* as an expression system, recombinant protein yield has been shown to be comparatively low for some proteins: for example, recombinant *H. contortus* pepsinogen was occasionally detected in immunoblots as a weak band in extracts of some batches of transgenic worms, indicating that expression levels were low (Redmond *et al.* 2001). However, sufficient amounts of other proteins, such as Hc-CPL-1 and Hc-H11, have been expressed for use in immunisation studies (Murray *et al.* 2007, Roberts *et al.* 2013). Integration of extrachromosomal arrays into the genome to allow stable inheritance in all progeny has been tried but did not increase protein yield, possibly due to the lower copy number of plasmid following integration compared to extrachromosomal maintenance (Dr Collette Britton, personal communication).

In this chapter, a wide range of recombinant protein yield/harvest was shown between CeTci-CF-1 (mean recombinant protein/harvest 5.18 μg/ml packed worms volume, SD = 3.76 and n = 4) and CeTci-MIF-1 mean recombinant protein/harvest 11.17 μg/ml packed worms volume, SD = 7.64 and n = 4), but never reached the amounts of protein purified from either *P. pastoris* (PiTci-CF-1; mean recombinant protein/harvest 109.56 μg/ml packed cells volume, n = 2) or *E. coli* (EcTci-MIF-1; mean recombinant protein/harvest 155.50 μg/ml packed cells volume, SD = 145.74 and n = 3). The data above only refers to an estimate of the packed worm or cell volume as appeared immediately before sonication [also described by Roberts *et al.* (2013)]; a calculation of the % of recombinant protein as fraction of the total protein content was not possible in this study as total protein was not always determined prior to recombinant protein purification.

During these experiments, the number of purification attempts differed throughout and therefore comparisons between yields must be interpreted carefully. Indeed if the density of two worm populations (rollers and rescued DR96) appeared roughly equal between plates at the time of harvest, and a roughly equal volume of packed worms was collected, the higher efficiency of transformation observed in DR96 rescued *C. elegans* most likely explains the higher yield, with greater numbers of worms expressing the recombinant Tci-MIF-1, compared to rollers expressing Tci-CF-1. However, the aim of this study was to generate and purify *T. circumcincta* vaccine candidates using *C. elegans* transformed

worms, which was successfully achieved. The variable yields may be attributable to a dilution factor of the *T. circumcincta* proteins on preparation of worm protein extracts. It is also possible that expression of recombinant proteins, especially of active enzymes such as Tci-CF-1 and Tci-MIF-1, might be harmful to *C. elegans*, and therefore the worm organism would regulate its expression. However, there was no evidence of any toxicity in the transgenic worms generated in this study. Comparison between the two methods used in this chapter to generate *T. circumcincta* recombinant proteins should be carefully interpreted and would require further studies. At the time of writing, no information was available regarding the possible effect that the two different genes (*Tci-cf-1* and *Tci-mif-1*) might have had on the *C. elegans* transformed populations or indeed on the efficiency of transformation.

The *C. elegans* specific promoter used (*Ce-cpl-1* promoter) allows constitutive protein expression only in those cell populations where the promoter is activated [gut and hypodermic cells (Hashmi *et al.* 2002)]. Tissue/cell-specific promoters might be used to direct transgene expression in certain parts of the worm's body, however gene regulation is a complex matter even in a small organism like *C. elegans* (Okkema and Krause, 2005). If gene activation and protein expression only happened in certain cell types, as might be the case for the *C. elegans* modified strains used in this project, this would lead to a reduced protein production compared to bacterial or yeast populations of millions of unicellular organisms where each cell is over-expressing the desired product. Previous studies using a heat shock promoter for inducible expression of *H. contortus* genes in *C. elegans* did not enhance expression (Dr Collette Britton, personal communication) and this approach is limited by the short time that worms can tolerate heat shock treatment. Other approaches using the Q repressible binary system [by inducing expression of the transcriptional activator QF (Wei *et al.* 2012)] and ligand binding to engineered protein destabilising domains [small domains that bind to a protein of interest and will degrade the complex in absence of a specific ligand (Cho *et al.* 2013)] can result in inducible gene and protein expression in *C. elegans*, but these have not been widely tested for high-throughput protein purification purposes in mind.

Two different approaches have been used in this thesis to transform *C. elegans* worms, employing two marker genes to screen the progeny for successful transformation; both approaches were validated for this purpose. However, improvements may be necessary to increase the efficiency of transformation and protein expression, especially when using *rol-6*: for example, micro-injecting the plasmid encoding for *Tci-cf-1* into DR69 worms

together with *unc-76* (restoring the wild type phenotype) might be an alternative to try and increase the amount of recombinant protein purified. Another possibility could be to engineer a different vector for *C. elegans* micro-injection that included a less specific promoter than the one used (*Ce-cpl-1* promoter), so that most *C. elegans* tissues would be expressing the recombinant protein.

During the purification process of CeTci-CF-1, a contaminant protein was consistently co-purified. This was later identified as a *C. elegans* protein rich in histidine residues, Ce-LEC-8, as also shown by Nemoto-Sasaki *et al.* (2008). Various methods were used to try and isolate CeTci-CF-1 from the contaminant protein; however, all attempts failed to deliver a purer CeTci-CF-1 fraction, and the recovery rate of CeTci-CF-1 was very poor. Therefore, subsequent experiments involving CeTci-CF-1 took into account the presence of Ce-LEC-8. Co-purification of host proteins in recombinant expression systems has been described previously (Bolanos-Garcia and Davies 2006, Wülfing *et al.* 1994). To try and resolve this problem, further experiments of CeTci-CF-1 expression could be carried out using a *C. elegans lec-8* mutant strain (available from the National BioResource Project, Japan) in an attempt to obtain a purer preparation.

Overall, the results of this chapter showed that *C. elegans* was a viable method to express recombinant *T. circumcincta* vaccine candidates; however, further work is required to improve the yield of recombinant proteins from the transformed *C. elegans* populations. This could be achieved by investigating larger scale *C. elegans* biomass production, for example by culturing transformed *C. elegans* in liquid medium as previously shown (Murray *et al.* 2007); or by engineering the worms so that they could be induced to over-express the recombinant protein, as it is the standard procedure in *E. coli* and yeast cell systems.

Chapter 4 – Characterisation of Antibody Reactivity, Enzyme Activity and Glycosylation of Native and Recombinant Tci-CF-1 from *Teladorsagia circumcincta*

4.1 Introduction

A highly immunogenic cysteine protease enzyme, termed cathepsin F (Tci-CF-1), has been shown to be the most abundant protein in *T. circumcincta* L4 Excretory/Secretory (ES) products. This molecule is bound by IgA antibodies from the gastric lymph and mucus of sheep rendered immune to *T. circumcincta* by experimental infection (Redmond *et al.* 2006, Smith *et al.* 2009). By virtue of its immunogenicity and abundance in L4 ES, a recombinant Tci-CF-1 version expressed in the yeast *Pichia pastoris* was included in a panel of 8 vaccine candidates in recent immunisation studies (Nisbet *et al.* 2013). Findings of Chapter 2 of this thesis have shown that Tci-CF-1 purified from *T. circumcincta* L4 ES (nTci-CF-1) was recognized by IgA and IgG antibodies from serum and by IgA from gastric lymph of sheep which had been rendered immune by trickle infection with *T. circumcincta*. However, these antibodies were not cross-reactive with a *Pichia pastoris*-expressed version of Tci-CF-1 (PiTci-CF-1). In contrast, IgG generated from pre-scapular antibody secretory cell (ASC) probes of sheep immunised subcutaneously (in the neck) with PiTci-CF-1 was able to bind PiTci-CF-1 and, to a lesser degree, nTci-CF-1. The differences observed may be caused by differences in the protein folding or Post-Translational Modifications (PTMs) between the two Tci-CF-1 versions. In this respect, Western blotting techniques may allow investigation of the immune response to an antigen (or antigen mix) without the influence of tertiary protein structures, as the standard protein electrophoresis techniques involve denaturing and reducing conditions which facilitate linearization of peptides (Collins *et al.* 2004, Geldhof *et al.* 2003, Harper *et al.* 1990, Jungersen *et al.* 2001, Kurien and Scofield 2006, Vercauteren *et al.* 2004, Wedrychowicz *et al.* 1994).

PTMs occur at distinct amino acid side chains or peptide termini; common PTMs are polypeptide cleavage (e.g. cleavage of pro-peptides from pro-enzymes or "zymogens" in enzyme maturation), acetylation, phosphorylation and glycosylation of amino acid residues. PTMs which result in the addition of extra moieties are covalent chemical

modifications of proteins mediated by enzymes (Farley and Link 2009). One post-translational modification that may be carried out by lysosomal enzymes is cleavage of the pro-peptide, also known as maturation of the enzyme [reviewed by Turk *et al.* (2012)]. The secondary and tertiary structure of enzymes is such that, once correctly folded and maturation is completed, the enzyme is able to carry out its catalytic functions by using the active site thus formed (Rawlings and Barrett 1994, Santucci *et al.* 2008). Proteolytic enzymes such as cysteine proteases undergo maturation by cleaving of the pro-peptide (and signal peptide if initially present); this can occur by *self*-activation, in which a small subfraction of the enzyme becomes catalytically active by a change in environment (e.g. drop in pH, within lysosomal granules or *in vitro*), which in turn is able to activate the remaining enzyme molecules (McQueney *et al.* 1997, Rozanov and Strongin 2003). Enzyme maturation can also occur by *trans*-activation, in which a simple environment change is not sufficient to induce initial pro-peptide cleavage and the involvement of another, different, protease is necessary to activate the cysteine protease pro-enzyme (Menard *et al.* 1998, Sajid *et al.* 2003).

Members of the C1 family of cysteine proteases, such as Tci-CF-1, are characterised by a "catalytic diad" of cysteine (Cys) and histidine (His). The crystal structure of this type of cysteine protease shows an amino acid chain folded to form two domains, with a cleft (the active site) lying between them. The active site residues cysteine and histidine are situated across from each other on either side of the cleft, to aid in the positioning of the substrate [reviewed by Rawlings and Barrett (1994) and Storer and Menard (1994)]. The correct folding of the peptide chain is therefore strongly associated with the protease's ability to degrade its substrate: if the folding is incorrect it is likely to affect not only the overall tertiary structure, but more specifically the active site. If the active site is missing or misshapen due to inappropriate on incomplete folding, the protease would not be able to degrade the substrate and therefore is considered enzymically inactive (Hosfield *et al.* 1999, Santucci *et al.* 2008). Recombinant protein expression systems may generate correctly folded zymogens which do not require activation; or they may require further steps to induce their maturation, such as lower buffer pH; or they may generate insoluble, incorrectly folded proteins (often in inclusion bodies) that may not be activated because of defects of their conformation before or after the processes used to purify them. For example, a recombinant version of the *H. contortus* antigens H11 and Hc-CPL-1 were generated using the free-living nematode *Caenorhabditis elegans* and both showed conserved aminopeptidase or cathepsin L activity (Murray *et al.* 2007, Roberts *et al.* 2013). The cysteine protease Cathepsin L1 of the trematode parasite *Fasciola hepatica* was

expressed in the yeast *P. pastoris* and the recombinant protein could auto-activate at low pH, although the cleavage site of the pro-enzyme was different to that observed in the presence of exogenous activating enzymes (Collins *et al.* 2004). In another study, the *Haemonchus contortus* cysteine proteases hmcp-1, -4, and -6 were expressed as insoluble proteins in *Escherichia coli* and, although their enzyme activity was not reported, the authors implied an associated lack of catalytic activity by suggesting a need to express the enzymes in a eukaryotic system for catalytically active hmcp-1, -4 and -6 (Redmond and Knox 2004). More recently, a *P. pastoris*-expressed Tci-CF-1 generated at MRI (Nisbet *et al.* 2013) had been demonstrated to be enzymically inactive (Dr Tom McNeilly and Dr Alasdair Nisbet, personal communication). Another *P. pastoris*-expressed Tci-CF-1 version generated during this study (PiTci-CF-1, see Chapter 2), sharing 96 % amino acid sequence identity with the previously generated *P. pastoris* recombinant version, showed lack of reactivity with antibodies from serum and gastric lymph of sheep which had been rendered immune by trickle infection with *T. circumcincta*. These combined results using *P. pastoris*-expressed Tci-CF-1 versions suggested that the recombinant system might have produced incorrectly folded enzyme, resulting in an enzymically inactive protein which was not optimally bound by antibodies raised against the native version.

Cysteine proteases of the C1 family are synthesized as zymogens (or pro-enzymes) with a pro-peptide region at the N terminus, which must be cleaved for activation of the enzyme (Rawlings and Barrett 1994). The PiTci-CF-1 version considered here had been generated as a zymogen and, although two bands of the protein were visualized in some polyacrylamide gels of the recombinant protein, suggesting a degree of cleavage may occur during expression in *P. pastoris*, there had been no prior evidence that this cleavage enzymically activated PiTci-CF-1 by removing all or part of the pro-peptide. Thus, the presence of the pro-peptide (or part of it) in the PiTci-CF-1 version used to immunise sheep might have influenced the differences in antibody recognition shown in Chapter 2 of this work, where the immunised animals mounted an immune response to the recombinant and the native Tci-CF-1 versions, but the antibodies from *T. circumcincta* trickle infected animals were not able to bind PiTci-CF-1 perhaps because of "epitope masking" by the pro-peptide on the recombinant version.

Another post-translational modification that can affect the antibody recognition of an antigen is glycosylation: The presence of glycans, their number, size and structure confers a specific 3-dimensional shape to the glycosylated protein, determining epitope structure and availability for cell and antibody binding in the immune response, along with potential

immunomodulatory effects (Kooyman *et al.* 2007, Paschinger *et al.* 2012, van Die *et al.* 1999). Glycosylation, or lack of it, can also affect enzyme activity, as shown by Fan *et al.* (1997); in this study mutagenesis of naturally occurring glycan groups (i.e. deletions of one of 5 glycans) of rat dipeptidyl peptidase IV (DPPIV, CD26) was carried out. The results showed that upon expression of the mutated DPPIV versions in Chinese Hamster Ovary (CHO) cells, the proteolytic activity of one of the mutants was reduced by 90 % compared to the wild type enzyme (Fan *et al.* 1997). In the trematode parasite *Schistosoma mansoni,* studies have identified immunogenic glycoproteins (such as the schistosome soluble egg antigen, SEA) which provoke peritoneal B cell expansion and cytokine production, and that this activity is lost upon deglycosylation [reviewed by Thomas and Harn (2004)]. Nematode glycans and glycoproteins have also been shown to be highly immunogenic, with bias towards a Th2 immune response [reviewed by Dell *et al.* (1999) and Harn *et al.* (2009)]. This was shown by experiments using BALB/c mice immunised with PBS-soluble extracts of mixed adult *Brugia malayi* antigen (BmA) or mixed-stage *C. elegans* antigen (CeA; Tawill *et al.* 2004). The results showed that lymph node cells collected from mice in either immunisation protocol, stimulated with sodium periodate-treated soluble extracts of both nematodes (therefore with disrupted glycan structures) induced significantly less IL-4 production than the respective untreated extracts (Tawill *et al.* 2004). Many of the potential vaccine candidates identified from parasitic nematodes of sheep such as *Haemonchus contortus* [H11 and the H-gal-GP complex (Knox and Smith 2001, Smith *et al.* 1997)] and *T. circumcincta* (Tci-CF-1; Redmond *et al.* 2006) are glycosylated proteins or protein complexes. In cattle, a study showed that there is a strong but short-lived antibody response (IgG1) directed against N-glycans on the surface of *Dictyocaulus viviparus* L3 larvae, as shown by serum from calves vaccinated with irradiated L3 larvae (Kooyman *et al.* 2007). Studies have shown that vaccination of lambs with adult *H. contortus* ES antigens resulted in multiple anti-glycan antibodies, the specificity of which varied depending on the adjuvant used (van Stijn *et al.* 2010). In these animals, IgG antibodies from serum that had in a previous study correlated with protection against *H. contortus* challenge [by FEC and worm burden reductions, (Vervelde *et al.* 2003)] showed binding to the glycan moiety Galα1–3GalNAc, suggesting its contribution to protection against parasite infection (van Stijn *et al.* 2010).

Five distinct types of carbohydrate-peptide bonds have been identified, and the linkage of N-acetyl-D-glucosamine (GlcNAc) to asparagine (“Asn” or “N”) residues (N-linked glycosylation) represents the most widely distributed peptide-carbohydrate bond. This is the site of attachment of a large variety of complex and polymannose oligosaccharides

[reviewed by Spiro (2002)]. Cathepsin F enzymes from both mammals and helminths possess predicted N-glycosylation sites within their amino acid sequences (Deussing *et al.* 2000, Pinlaor *et al.* 2009, Redmond *et al.* 2006, Santamaria *et al.* 1999). Analysis of the inferred amino acid sequence from the mRNA representing *T. circumcincta* Tci-CF-1 demonstrated the presence of two potential N-linked glycosylation sites, one on the pro-peptide region and another on the mature enzyme. Previous lectin binding experiments confirmed the presence of glycans on Tci-CF-1 (Redmond *et al.* 2006); however no more detail about the glycosylation pattern of native Tci-CF-1 has been published.

From the examples described above, it appears that PTMs may be playing a role in influencing the enzyme activity and the specific antibody binding of recombinant *P. pastoris*-expressed Tci-CF-1 (PiTci-CF-1). In particular, the presence of the pro-peptide and/or inappropriate glycosylation by yeast cells may be a hindrance to substrate interactions and antibody binding to this antigen. To examine this further, another recombinant version of Tci-CF-1 expressed using the nematode *C. elegans* was generated (see Chapter 3), in an attempt to deliver a vaccine candidate with structure and PTMs more similar to native Tci-CF-1 than to PiTci-CF-1.

The overall aims of this chapter therefore are:

i) To investigate the hypothesised differences in PTMs between Tci-CF-1 versions by inferred amino acid sequence alignment and annotations, to reveal the overall amino acid residue identity and whether active site and glycosylation-linked amino acid residues were conserved between Tci-CF-1 versions.

ii) To further investigate the ovine gastric lymph antibody recognition of native and recombinant *P. pastoris*-expressed (generated in Chapter 2) and *C. elegans*-expressed Tci-CF-1 (generated in Chapter 3) versions by (a) ELISA and (b) Western blotting of denatured proteins to expose shared epitopes.

iii) To investigate the cysteine protease activity of each Tci-CF-1 version by using substrates specific for papain-like enzymes such as cathepsin F (Fonovic *et al.* 2004, Pinlaor *et al.* 2009, Wang *et al.* 1998) and a cysteine protease-specific inhibitor and to investigate the activation of both recombinant PiTci-CF-1 (generated in Chapter 2) and CeTci-CF-1 (generated in Chapter 3) by inducing cleavage of the pro-peptide.

iv) To investigate the glycosylation of each purified Tci-CF-1 version using glycan-specific stains, enzymatic deglycosylation and lectin binding experiments.

4.2 Materials and methods

4.2.1 Sequence alignment, annotations and BLAST analysis

4.2.1.1 Tci-CF-1 amino acid sequence alignment and annotations

Alignment of PiTci-CF-1 and CeTci-CF-1 amino acid sequences with the inferred protein sequence from the published mRNA sequence for Tci-CF-1 [NCBI accession number ABA01328 (Redmond *et al.* 2006) was carried out using the free online software ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Annotation of predicted signal peptide cleavage site and N-glycosylation sites was carried out by comparison with the previously published sequence (Redmond *et al.* 2006).

4.2.1.2 Ce-LEC-8 nucleotide and amino acid sequence BLAST analysis

The Ce-LEC-8 amino acid sequence (NCBI protein accession number NP509649) identified by LC-ESI MS/MS (see Chapter 3, section 3.3.2) was used in BLAST analyses to search for *T. circumcincta* homologues. The databases used were: i) NCBI EST database (nBLAST), in which a *T. circumcincta* adult cDNA library clone was identified; ii) Nembase sequence database (tBLASTn); iii) in-house (data collected by Dr Alasdair Nisbet) *T. circumcincta* fourth larval stage (L4) 454 sequence database (tBLASTx). The software ClustalW was used for amino acid and nucleotide sequence analysis.

4.2.2 Purification of native Tci-CF-1

Native Tci-CF-1 (nTci-CF-1) was purified from *T. circumcincta* fourth larval stages (L4) Excretory/Secretory (ES) products as described in Chapter 2 of this thesis.

4.2.3 Generation and purification of recombinant Tci-CF-1 versions

4.2.3.1 *Pichia pastoris*-expressed Tci-CF-1

PiTci-CF-1 was expressed using the yeast *P. pastoris* and purified as described in Chapter 2 of this thesis.

4.2.3.2 *Caenorhabditis elegans*-expressed Tci-CF-1

CeTci-CF-1 was expressed using the free living nematode *C. elegans* and purified as described in Chapter 3 of this thesis.

4.2.4 Provenance of sheep gastric lymph and serum samples

4.2.4.1 Gastric lymph

The provenance and collection of sheep gastric lymph samples used in ELISA experiments and Western blots are described in Chapter 2 of this thesis (Sections 2.2.5.2 and 2.2.5.5).

4.2.4.2 Anti-*Pichia pastoris*-expressed Tci-CF-1 serum

Ovine serum samples containing anti-PiTci-CF-1 specific IgG were provided by Dr Alasdair Nisbet and Dr Tom McNeilly (MRI). Details of the animals and the immunisation protocol are detailed in Chapter 2 (Section 2.2.5.1.1) of this thesis.

4.2.5 Purification of Ce-LEC-8 from N2 *C. elegans* worms

This procedure was carried out as described in Chapter 3 of this thesis. Biomass of N2 *C. elegans* worms was generated using Peptone-rich agar plates (Appendix D) and the harvested worms used to extract a cleared lysate as previously described (Chapter 3). The cleared lysate was applied to a Co^{2+} IMAC system and the identity of the eluted protein confirmed by SDS-PAGE analysis and MS/MS-LC/ESI carried out at MRI Proteomics Facility.

4.2.6 Indirect ELISA for detection of *T. circumcincta* antigen-specific antibody responses

These experiments were carried out using purified CeTci-CF-1 at a dilution of 1 μg/ml as coating antigen (Ce-LEC-8 was also present in these preparations at approximately twice the concentration of Tci-CF-1, resulting in ~ 2 μg/ml after dilution). Control wells were coated with Ce-LEC-8 purified from N2 *C. elegans* worms, as described below (section 4.2.6.1), at 2 μg/ml. The protocol used and the antibody combinations were as previously detailed in Section 2.2.6 of this thesis.

4.2.6.1 Immunoassay for sodium periodate sensitive/insensitive epitopes

The immunoassay experiments using sodium periodate were carried out without further modifications from the protocol detailed in Section 2.2.7 of this thesis. Briefly, antigens at the concentrations detailed above were used to coat high binding microtitre plates at 4°C overnight. The following day, the plates were washed 4 times in PBST (Appendix D) and blocking of the wells occurred for 1h with TBST/5 % soy milk (Appendix D). After further 4 washes, wells were incubated with 50 µl/well of 20 mM $NaIO_4$ solution in 50 mM Sodium Acetate Buffer (Appendix D) for 1 h at 37°C, in the dark. Wells were washed three times with 50 mM Sodium Acetate Buffer (Appendix D) and subsequently incubated with 50 µl/well of 50 mM $NaBH_4$/TNTT for 30 min at 37°C. Control wells were incubated in 50 mM Sodium Acetate Buffer, instead of 20 mM $NaIO_4$ solution. Wells were then washed four times with PBST, resuming the standard ELISA protocol as described in Section 2.2.6 of this thesis.

4.2.7 SDS-PAGE and Western blots

Native and recombinant proteins were electrophoresed in denaturing conditions in polyacrylamide gels (NuPAGE® Bis-Tris 4–12 %, Invitrogen, Paisley, UK) as detailed in Appendix B. Transfer of proteins onto nitrocellulose membrane and general immunoblotting techniques are also detailed in Appendix B. Following overnight blocking of membranes in TNTT, strips were incubated with sheep serum (1:1000 for detection of IgG or 1:100 for detection of IgA) or gastric lymph (1:100) samples. Following three 20 min washes in TNTT, some blots were treated with periodate to disrupt glycan structures as follows: blots were washed twice in 50 mM sodium acetate buffer (Appendix D) for 20 min. Subsequently, periodate-treated blots were incubated for 1 h at RT in sodium periodate buffer (Appendix D), whereas untreated strips were incubated in acetate buffer. All blots were washed twice in 50 mM sodium acetate buffer (Appendix D) for 10 min, then washed twice in TNTT for 10 min. All blots were incubated in freshly prepared 50 mM sodium borohydride solution (Appendix D) for 30 min, then washed three times in TNTT for 10 min.

Periodate-treated and untreated blots developed for IgA-binding were incubated for 1 h at RT with a monoclonal mouse anti-bovine/sheep IgA antibody (clone K84 2F9, MCA628, Serotec) at 1:500 dilution. Blots were washed three times for 20 min in TNTT then incubated for 1 h at RT with a rabbit anti-mouse IgG:HRP antibody (clone n/a, P0260, Dako) or a rat anti-mouse IgG1 heavy chain:HRP antibody (clone LO-MG1-2, MCA336P,

Serotec), both at a dilution of 1:1000. Subsequent washes were carried out twice for 20 min then overnight in TNTT. Strips developed for IgG detection were incubated for 1 h at RT with a monoclonal mouse anti-goat/sheep IgG:HRP antibody (clone GT-34, A9452, Sigma-Aldrich, Gillingham, UK) at 1:1000 dilution, washed twice for 20 min in TNTT then one last wash overnight. Bound antibodies were revealed with the chemiluminescent substrate ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) following the manufacturer's instructions. Some strips were also developed with 3,3′-Diaminobenzidine (DAB, SIGMA*FAST*™ DAB with Metal Enhancer, Sigma Aldrich).

4.2.8 Cysteine protease activity assays

4.2.8.1 Microplate methods for *self*- and *trans*-activation assays of Tci-CF-1

The procedure used in these assays was adapted from methods described by Nisbet and Billingsley (2000) and Pinlaor *et al.* (2009). Each "working buffer" was prepared fresh at RT by adding 100 X cysteine and 100 X DTT solutions to the stock buffers (Appendix D) to give final concentrations of 5 mM cysteine and 8 mM DTT. An aliquot of each buffer thus prepared was set aside for the addition of the inhibitor E-64 (stock prepared as 100 X, Appendix D) to a final concentration of 10 µM. Ten stock solutions of the reaction product 7-Amino-4-methylcoumarin (AMC, Sigma Aldrich) were prepared in DMSO. These were used as individual components of a 10-point standard curve in each assay, in which 6 µl of each stock was added to the reaction buffer. The range of the final concentration of substrate for the AMC standard curve was 476.19-0.05 µM. A serial dilution of the substrate Z-Phe-Arg-AMC (Z-FR-AMC, Enzo Life Sciences, Exeter, UK) was also prepared in DMSO; seven dilutions were made in 100 % DMSO and the range of the final concentrations used was 476.19-0.48 µM. A black flat bottom 96-well plate was used for this assay; reaction buffers were distributed at 115 µl/well (120 µl/well for the standard curves), then 5 µl of PBS were added (for the "substrate control" wells). To investigate the *trans*-activation of Tci-CF-1 versions, 5 µl (13.7 ng) of liver-purified human Cathepsin L (hCath L; diluted from stock solution 0.274 mg/ml in buffer pH 5.5; Enzo Life Sciences) were added to the positive control wells. In the test wells, 50 ng of each recombinant Tci-CF-1 version (diluted from stock solution in buffer pH 4.5) were added to 13.7 ng of hCath L in a final volume of 5µl. When *T. circumcincta* L4 ES was used, 100 ng were added to the reaction buffer in a final volume of 5 µl. Buffer + PBS only wells were used as blank. The plate was incubated at 37°C for 20 min to allow enzyme activation in the wells containing recombinant Tci-CF-1 and hCath L, then 6 µl/well of a serial dilution of substrate Z-FR-AMC were added. The reactions were incubated at 37°C for the whole

duration of the assay (120 min) and the fluorescence emitted was measured with a Synergy™ HT fluorescence plate reader and Gen5 Data Analysis Software (both from BioTek, Winooski, USA) every 60 sec, using an excitation wavelength of 370 nm and measuring emission at 450 nm. Each assay was carried out in triplicate and repeated three times. A plot of time *vs* fluorescence was generated and the velocity of each reaction in the linear phase (during the first 20 min) was calculated from the raw data (Relative Fluorescence Units, RFU) using the following method:

i) Blank-adjusted RFU value was obtained with this equation:

$[RFU_{21\ min}\text{-Blank } RFU_{21\ min}]\text{- } [RFU_{1\ min}\text{-Blank } RFU_{1\ min}]$

ii) The blank-adjusted RFU value obtained was then used to calculate the relative concentration of product [P] based on the AMC standard curve generated for each experiment.

iii) The [P] value thus obtained was the amount of product generated by the enzyme during a 20 min period.

iv) To calculate the reaction rate per minute (the amount of product /min or initial velocity, v_i), each value obtained in iii) was divided by 20; this value was the reaction rate in each reaction, which contained between 13.7-63.7 ng of enzyme.

v) The values obtained in iv) were adjusted for each mg of enzyme present in the reactions; adjusted reaction rates were plotted for each enzyme on histograms representing *vi*/min/mg during the linear phase of the reactions.

For the initial pilot experiments and the investigation of *self*-activation of Tci-CF-1 versions at different pH, the chromogenic substrate Z-FR-pNA (Enzo Life Sciences) was used instead of the fluorogenic substrate. In these experiments a 10 mM stock solution of the chromogenic substrate Z-Phe-Arg-pNA (Z-FR-pNA) was prepared in DMSO. The assays were carried out without modifications from the method described above, except that no pNA standard curve was prepared and the reactions were incubated at RT and absorbance readings ($OD_{405\ nm}$) were taken every 60 sec for 120 min.

4.2.8.2 Investigation of the activation of recombinant PiTci-CF-1 using SDS-PAGE analysis, Western blot and ELISA

For the *self*-activation of PiTci-CF-1, working buffer was prepared fresh by adding 100 X cysteine and 100 X DTT solutions to 100 µl of stock buffer pH 4.5 (Appendix D) to give final concentrations of 5 mM cysteine and 8 mM DTT. Five microliters of PiTci-CF-1 (1.15 µg) were added and mixed by pipetting. An identical reaction was prepared alongside, with the addition of 100 X E-64 to a final concentration of 10 µM. A 13 µl aliquot (0 min) was collected from each reaction and prepared for gel electrophoresis by mixing with 5 µl of NuPAGE® 4 X LDS Sample Loading Buffer with the addition of 2 µl of NuPAGE® Reducing agent (all from Invitrogen) then heated at 70°C for 10 min. The reactions were incubated at 37°C and further aliquots were collected at 30, 60, 90 and 120 min and prepared as above for gel electrophoresis and Coomassie staining, which were carried out under the same conditions described in Appendix B.

For the *trans*-activation of PiTci-CF-1, reactions were prepared as follows: to 1 ml of stock buffer pH 3.5 (Appendix D) were added 100 X cysteine and 100 X DTT solutions to give final concentrations of 5 mM cysteine and 8 mM DTT. To 345 µl of this buffer, E-64 was added to give a final concentration of 10 µM. To 115 µl aliquots of each working buffer thus prepared (with or without E-64), one of the following were added: i) 5 µl (1.15 µg) of PiTci-CF-1; ii) 5 µl (13.7 ng) of hCath L diluted in buffer pH 5.5; iii) 1.15 µg of PiTci-CF-1 were added to 13.7 ng of hCath L (stock concentration 0.274 mg/ml) in a final volume of 5 µl. One 115 µl aliquot did not receive enzyme to serve as negative control. A 13 µl aliquot (0 min) was collected from each reaction and prepared for gel electrophoresis as described above. The reactions were incubated at 37°C for 20 min and further aliquots were collected and prepared as above for gel electrophoresis. To each reaction, 6 µl of Z-FR-AMC (final concentration 476 µM) was added and all were incubated at 37°C. At 80 and 140 min further aliquots were collected and prepared for gel electrophoresis and silver staining, which were carried out under the same conditions described in Appendix B. Aliquots of each reaction were also collected at 80 min to use in Western blot analysis. The samples were loaded in a polyacrylamide gel and blotted as described in Appendix B. Immunoblotting was carried out as described previously in Section 4.2.7.

For the ELISA assay of the recognition of *trans*-activated PiTci-CF-1, reactions were prepared as follows: to 100 µl of stock buffer pH 4.5 (Appendix C) were added 100 X cysteine and 100 X DTT solutions to give final concentrations of 5 mM cysteine and 8 mM DTT. To each reaction 4.35 µl of PiTci-CF-1 (final concentration 10 µg/ml) were added.

In the reactions where hCath L was added, this was diluted 1:100 in the final reaction volume (i.e. 1 µl of stock 0.274 mg/ml hCath L). Some reactions were prepared and immediately diluted 1:10 in carbonate buffer (Appendix D; PiTci-CF-1 final concentration 1 µg/ml) to coat wells of a high binding microtitre plates. The remaining reactions were incubated at 37°C for 20 min, before being also diluted 1:10 in carbonate buffer to coat wells of a high binding microtitre plates. Three reactions received the addition of E-64 to give a final concentration of 10 µM. The following reactions were prepared: i) PiTci-CF-1 alone, immediately diluted; ii) PiTci-CF-1 alone, incubated at 37°C for 20 min; iii) hCath L alone, incubated at 37°C for 20 min; iv) PiTci-CF-1 and hCath L, immediately diluted; v) PiTci-CF-1 and hCath L, incubated at 37°C for 20 min; vi) PiTci-CF-1, hCath L and E-64, incubated at 37°C for 20 min; vii) hCath L and E-64, incubated at 37°C for 30 min, then PiTci-CF-1 added, then further 20 min incubation; and viii) hCath L and E-64, incubated at 37°C for 20 min. The plates thus coated were incubated overnight at 4°C, subsequently blocked and ELISA protocol carried out with gastric lymph samples as described in Section 2.2.6 of this thesis.

4.2.9 Staining of protein-linked glycans

Protein-linked glycans were visualized by staining SDS-PAGE gels of native and recombinant Tci-CF-1 with a glycan-specific stain supplied with the Pro-Q® Emerald 300 Gel and Blot Stain Kit (Invitrogen), following the manufacturer's instructions. Briefly, standard protein electrophoresis was carried out as described in Section 2.9.1; all washes and incubations were on a rotary shaker. The gel was fixed in Fix solution (50 % methanol and 5 % acetic acid in dH_2O) for 45 min. This was followed by two further washes in Wash solution (3 % glacial acetic acid in dH_2O) of 20 min each; the gel was then incubated in 25 ml of Oxidizing Solution (acetic acid/periodic acid) with gentle agitation for 30 min. After three further washes in Wash solution, the gel was stained using 25 ml of freshly prepared Staining Solution (Pro-Q® Emerald 300 stock solution 1:50 into Pro-Q® Emerald 300 staining buffer; both provided with the kit), incubated in the dark with gentle agitation for 120 min. The gel was then washed twice in Wash solution and visualized using a UV transilluminator.

4.2.10 PNGase-F and *O*-glycosidase removal of glycans

4.2.10.1 PNGase-F

The reactions were carried out as follows using PNGase-F enzyme and buffers supplied by New England Biolabs (Hitchin, UK): 225 ng (22.5 µl) of recombinant proteins in Phosphate Buffer (Appendix D) were mixed with 2.5 µl of 10 X Glycoprotein Denaturing Buffer. The proteins were denatured by heating at 100°C for 10 min; the reactions were allowed to cool then the following were added: 5 µl 10 X G7 Reaction Buffer, 5 µl 10% NP40, 5 µl PNGase F (1000 U), 10 µl dH_2O to a total volume of 50 µl. The reaction was incubated at 37°C for 1 h. To these, LDS Sample Buffer (Invitrogen) and reducing agent were then added for electrophoresis as described in Appendix B. The polyacrylamide gel was stained with Coomassie blue.

4.2.10.2 *O*-glycosidase

The reactions were carried out as follows using *O*-glycosidase and Neuraminidase enzymes and buffers supplied by New England Biolabs: 225 ng (22.5 µl) of recombinant proteins in Phosphate Buffer (Appendix D) were mixed with 2.5 µl of 10 X Glycoprotein Denaturing buffer. The proteins were denatured by heating at 100°C for 10 min in a heat block, then the following were added: 5 µl 10 X G7 Reaction Buffer, 5 µl 10% NP40, 5 µl Neuraminidase, 2.5 µl of a 1:40 dilution of *O*-glycosidase (1000 U) and 7.5 µl dH_2O to a total volume of 50 µl. The reactions were incubated at 37°C for 2.5 h. To these, LDS Sample Buffer (Invitrogen) and reducing agent were added for electrophoresis as described in Appendix B. The polyacrylamide gel was stained with Coomassie blue.

4.2.11 Lectin probes

Lectin binding experiments on native and recombinant versions of Tci-CF-1 were carried out as follows: proteins were electrophoresed and transferred onto nitrocellulose membrane as described in Appendix B. The membrane was then cut into strips using a clean scalpel blade and each strip was incubated with a different biotinylated lectin (Table 4.1, all supplied by Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:400 in TNTT for 1 h at RT (final concentrations were 12.5 µg/ml for all lectins except *Ulex europaea* agglutinin, UEA, which was 5 µg/ml). Following three 20 min washes in TNTT on a rocker, each strip was incubated for 1 h at RT with Streptavidin-Peroxidase Polymer, Ultrasensitive (Sigma) diluted 1:1000 in TNTT. After three further 20 min washes in

TNTT, all the strips were developed with 3,3′-Diaminobenzidine (DAB, SIGMA*FAST*™ DAB with Metal Enhancer, Sigma), following the manufacturer's instructions.

Table 4.1 The panel of lectins used in this study.

Lectin name	Abbreviation	Plant of origin	Residue specificity
Lentil lectin	LCH	*Lens culinaris*	Fucosylated core regions of bi- and tri-antennary complex N-glycans; α-D-mannose; α-D-gluocose
Concanavalin A	ConA	*Canavalia ensiformis*	Branched α-mannosidic structures; high mannose type, hybrid type and biantennary type N-glycans
Agglutinin	DBA	*Dolichos biflorus*	GalNAcα1-3GalNAc; GalNAcα1-3Gal
Agglutinin	UEA	*Ulex europaea*	Fucα1-2Gal-R
Soybean agglutinin	SBA	*Glycine max*	terminal α,β GalNAc; α,β Gal
Peanut agglutinin	PNA	*Arachis hypogea*	Galβ1-3GalNAcα1-S/T (T antigen)
Wheat germ agglutinin	WGA	*Triticum vulgaris*	Sialic acid
Agglutinin I	RCA I	*Ricinus communis*	βGal

Legend: GalNAc = D-*N*-Acetylgalactosamine; Gal = D-Galactose; Fuc = D-Fucose.

4.2.12 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.01 and GenStat 12th Edition. Arithmetic means with standard errors and replicate number (n) are shown throughout this chapter. Data were checked for normal distribution by analysing the histogram of frequencies and residual plots. Data normally distributed were analysis using a one-way Analysis of Variance (ANOVA) test was used, followed by the Tukey post hoc test for pair wise comparison of means; when only one pair of means was compared, an unpaired two-tailed *t*-test was used. *P* values of < 0.05 were considered significant.

4.3 Results

4.3.1 Comparison of Tci-CF-1 versions by inferred amino acid sequence alignment

Differing post-translational modifications (PTMs) between native and *P. pastoris*-expressed Tci-CF-1 (PiTci-CF-1) were hypothesised to be the cause of differences previously observed (Chapter 2 of this thesis) in antibody reactivity of sheep samples (serum, ASC probes and gastric lymph) to the two antigens. It was hypothesised that differences in the linear amino acid sequences may have been present between Tci-CF-1 versions, such as mutations or deletions in certain amino acid residues that might have modified the folding and/conformation of the active site. This was first investigated by sequence alignment and annotation; the newly generated *C. elegans*-expressed Tci-CF-1 (CeTci-CF-1, see Chapter 3) was included in the analysis. The amino acid sequence of Tci-CF-1 inferred from the published mRNA sequence [NCBI accession number ABA01328; (Redmond *et al.* 2006)] was compared to the amino acid sequences inferred from the cDNA of the two recombinant versions generated in this project (PiTci-CF-1 and CeTci-CF-1). The comparison was carried out by ClustalW sequence alignment. The results showed that amino acid sequence identity between Tci-CF-1 versions was > 98 %. The putative site of signal peptide cleavage had been previously identified during *in silico* analysis of the inferred amino acid sequence of Tci-CF-1 cDNA generated from *T. circumcincta* L4 worms RNA (Redmond *et al.* 2006). Although the sequence encoding the signal peptide had been omitted from the Tci-CF-1 construct expressed in *P. pastoris*, the signal peptide had been maintained for expression in *C. elegans* and it showed 100% identity with signal peptide from the inferred amino acid residues in the published sequence (Figure 4.1, closed circle). Comparison between the published sequence of Tci-CF-1 and both recombinant versions generated for this study showed that putative N-glycosylation sites were maintained (Figure 4.1, open triangles), as were the catalytic residues Cys27 and His161 (Figure 4.1, boxes). The differences in six amino acid residues shown between the publicly available Tci-CF-1 sequence (pavTci-CF-1, inferred from *T. circumcincta* L4 RNA) and the sequences of the two recombinant versions (PiTci-CF-1 and CeTci-CF-1) are likely to be due to polymorphisms within the *T. circumcincta* population.

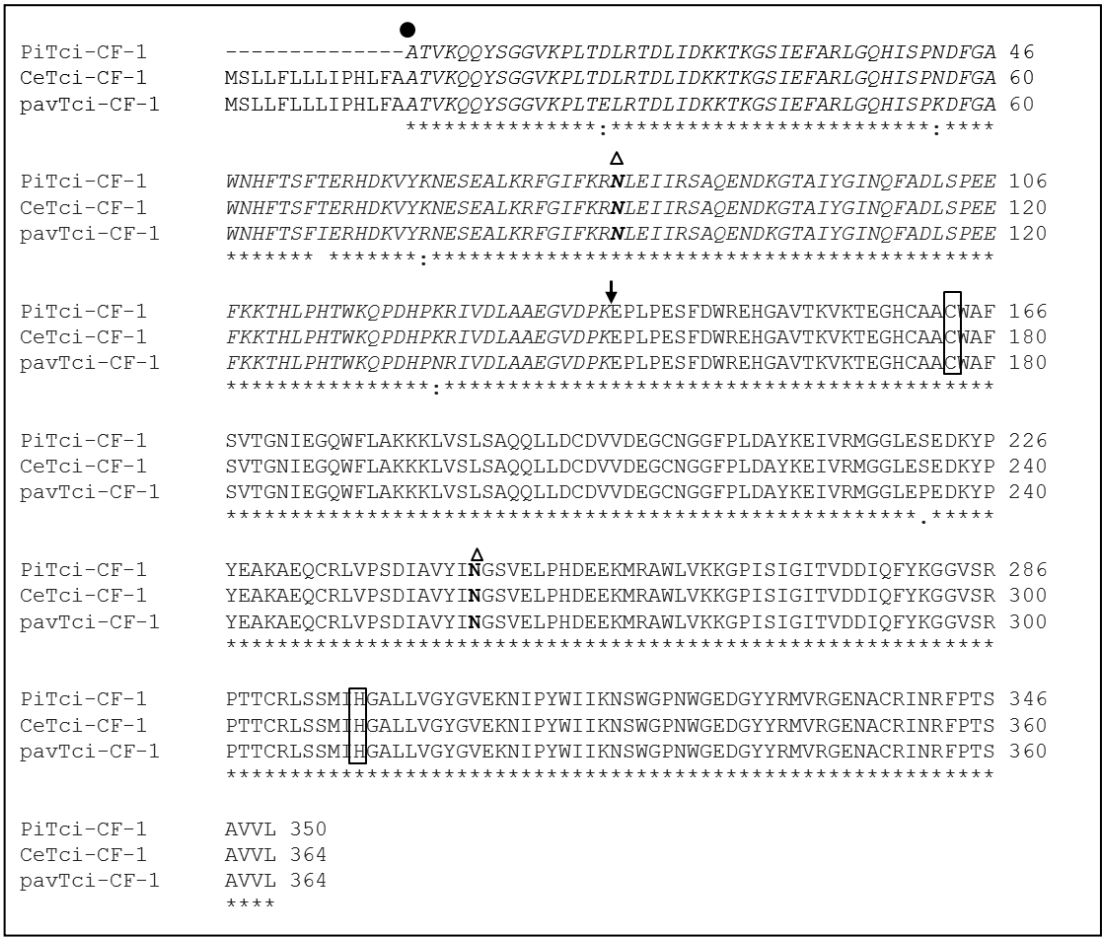

Figure 4.1 The alignment of inferred amino acid sequence of recombinant Tci-CF-1 versions generated in this project compared to the publicly available Tci-CF-1 amino acid sequence PiTci-CF-1: inferred (from L4 cDNA) *P. pastoris*-expressed Tci-CF-1 amino acid sequence; CeTci-CF-1: inferred (from L4 cDNA) *C. elegans*-expressed Tci-CF-1 amino acid sequence; pavTci-CF-1: publicly available nucleotide sequence (published with NCBI accession number ABA01328), inferred from *T. circumcincta* L4 cDNA, generated from RNA material. Identical amino acid residues are marked by asterisks, highly similar residues by a colon, less similar residues by a full stop and highly different residues by a space. The predicted signal peptide cleavage site is indicated by a closed circle; the pro-peptide sequence is in italics and the site of its cleavage from the mature enzyme is marked by an arrow; open triangles indicate the predicted N-glycosylation sites; the active site residues cysteine and histidine are boxed.

4.3.2 IgA Immune reactivity of gastric lymph to Tci-CF-1 versions

Following successful expression and purification of CeTci-CF-1, ELISA experiments were carried out to investigate its immune reactivity compared to PiTci-CF-1 and native Tci-CF-1 (see Chapter 2 of this thesis). Gastric lymph samples were collected during experiments detailed in Halliday *et al.* (2009, 2007) from: i) sheep that were previously trickle infected with *T. circumcincta* (2000 L3, three times a week for 8 weeks), then received a bolus challenge of 50 000 L3; and ii) sheep that were housed in helminth-free conditions, which did not receive a trickle infection, but only the bolus challenge (primary infection sheep).

Gastric lymph was collected from previously trickle infected sheep between +6 and +10 days post challenge (dpc); from primary infection sheep lymph was collected between -2 and +2 dpc.

IgA antibodies in the gastric lymph of sheep previously infected with *T. circumcincta* did not show significantly higher binding to CeTci-CF-1 (co-purified with Ce-LEC-8) than lymph from *T. circumcincta* primary infection animals (Figure 4.2, panel A).

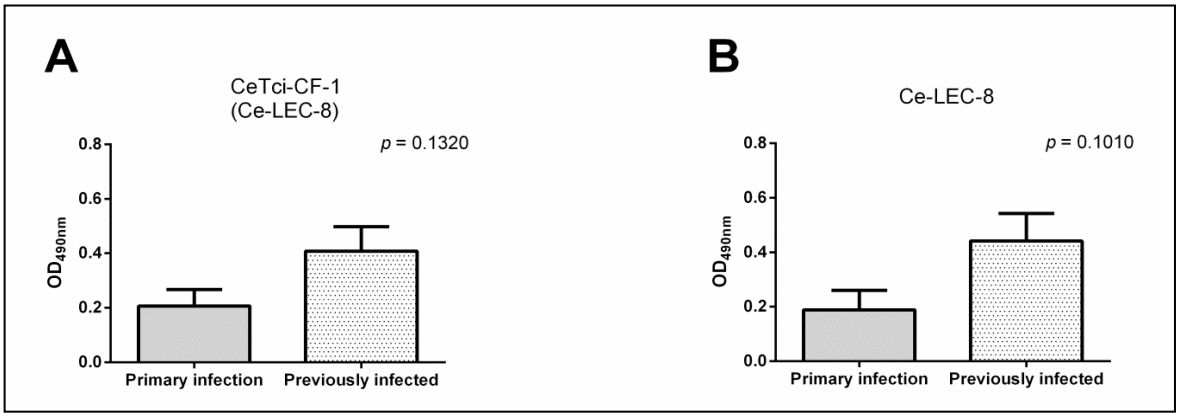

Figure 4.2 Ovine gastric lymph IgA binding to CeTci-CF-1 (co-purified with Ce-LEC-8) and to purified Ce-LEC-8.
Data represent mean ±SEM, n=5 (from primary infection sheep, grey bars) and 8 (previously infected sheep, dotted bars); all samples analysed in duplicate. Unpaired two-tailed *t*-test *p* value shown next to each graph. Panel (A): microplates were coated with 1 µg/ml CeTci-CF-1 (~2 µg/ml Ce-LEC-8). Panel (B): microplates were coated with 2 µg/ml purified Ce-LEC-8.

To characterise the effect of the presence of the co-purifying contaminant Ce-LEC-8 on the IgA binding in these experiments, Ce-LEC-8 was purified from *C. elegans* worms, strain N2 and used to coat microplates at the same concentration that would have been present in the CeTci-CF-1 preparations. The results showed that gastric lymph IgA binding to Ce-LEC-8 alone was approximately equal to that observed for CeTci-CF-1/Ce-LEC-8 (Figure 4.2, panel B), indicating no, or very little specific binding of IgA to CeTci-CF-1.

Both CeTci-CF-1 and Ce-LEC-8 purified from N2 worms were treated with periodate in further ELISA experiments, to investigate whether the antibody binding observed (Figure 4.2) was periodate sensitive. The results showed that following periodate treatment, ovine gastric lymph IgA reactivity decreased for both CeTci-CF-1 and Ce-LEC-8 (Figure 4.3), however the reductions in OD were not statistically significant.

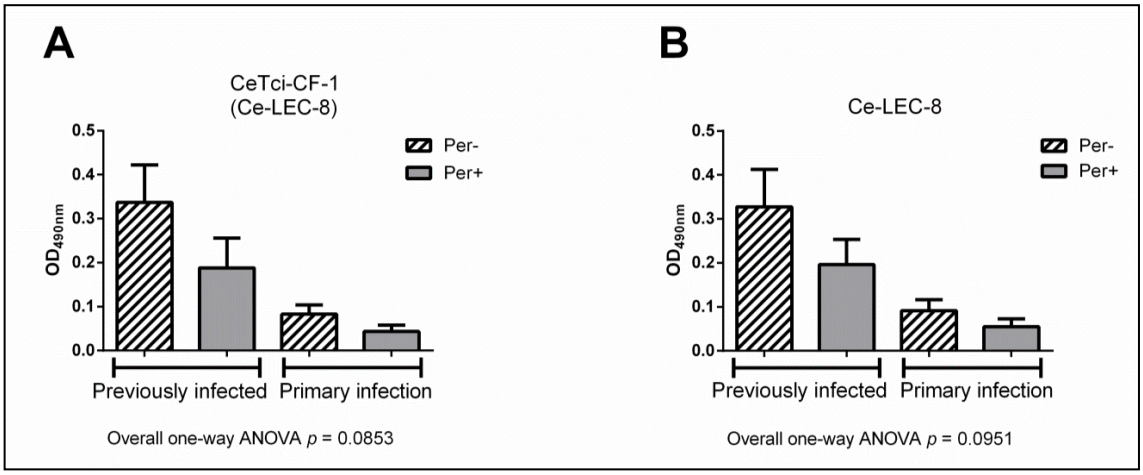

Figure 4.3 Ovine gastric lymph IgA binding to CeTci-CF-1 (co-purified with Ce-LEC-8) and purified Ce-LEC-8, before and after periodate treatment of the antigens by ELISA. Data represent mean ±SEM, n=3 (from primary infection sheep) and 7 (previously infected sheep); all samples analysed in duplicate. Overall ANOVA *p* value shown below each graph. Panel (A): microplates were coated with 1 µg/ml CeTci-CF-1 (~2 µg/ml Ce-LEC-8), then treated with periodate (grey bars) or left untreated (striped bars). Panel (B): microplates were coated with 2 µg/ml purified Ce-LEC-8, then treated with periodate (gray bars) or left untreated (striped bars).

Immunoblots were used to visually determine the binding of lymph IgA to Tci-CF-1 versions. Gastric lymph of individual sheep was used to probe each Tci-CF-1 version; lymph samples were divided in two sub-groups; those from previously infected animals and those from sheep that received a *T. circumcincta* primary infection. The results showed that gastric lymph IgA of previously infected animals was able to bind nTci-CF-1, whereas IgA in lymph collected from sheep exposed to the *T. circumcincta* challenge (i.e. still helminth-naive animals) only bound nTci-CF-1 weakly (Figure 4.4, panel A). A similar result was observed for each of the recombinant Tci-CF-1 versions; lymph IgA from previously infected animals showed stronger binding to both PiTci-CF-1 and CeTci-CF-1 than lymph IgA from naïve sheep (Figure 4.4, panels B-C).

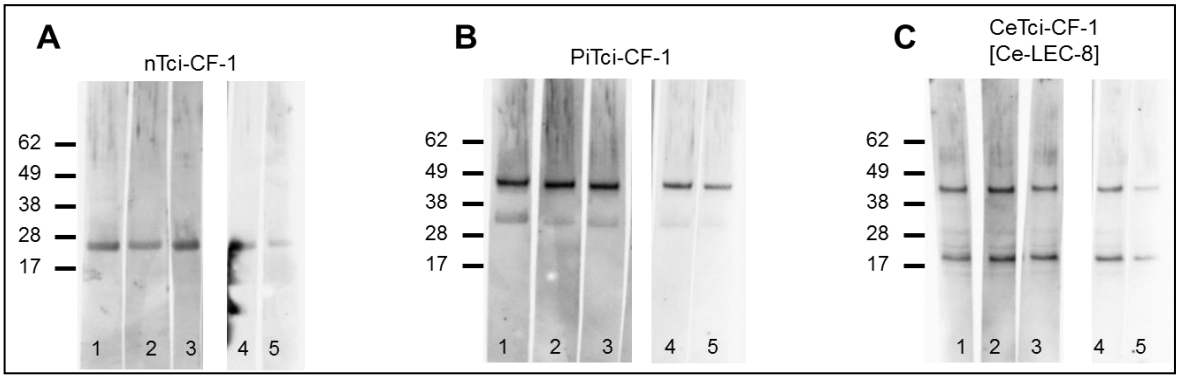

Figure 4.4 Immunoblots showing the gastric lymph IgA binding to Tci-CF-1 versions. Molecular weight markers (kDa) positioned to the left of each panel. Equal amount of proteins were loaded in each lane; (A) native Tci-CF-1; (B) *P. pastoris*-expressed Tci-CF-1; (C) *C. elegans*-expressed Tci-CF-1. Each strip was probed with gastric lymph from individual sheep (numbers at the bottom of each strip, same numbers across represent the same sheep), then IgA binding was detected with a secondary anti-sheep IgA antibody and developed by chemiluminescence. Lymph samples 1-3 were collected from "previously infected" animals between +6 and +10 days post bolus challenge (dpc): These "previously-infected" sheep had received a *T. circumcincta* trickle infection for 8 weeks (3000 L3, three times a week), then 7 days later, received a bolus challenge of 50 000 L3. Lymph samples 4-5 were collected between -2 and 0 dpc from helminth-naïve sheep prior to a primary bolus challenge.

Periodate-sensitive epitopes were identified in nTci-CF-1 in ELISA experiments, as defined by the IgA response induced in gastric lymph by *T. circumcincta* trickle infection (see Figure 2.10 in section 2.3.5 of this thesis). Furthermore, periodate-sensitive epitopes were also identified in PiTci-CF-1 during ELISA experiments, as defined by the IgG response from serum and pre-scapular ASC (see Figures 2.6 and 2.8, sections 2.3.3 and 2.3.4 of this thesis). In the current chapter, immunoblots were also treated with periodate to investigate whether the lymph IgA reactivity shown to each Tci-CF-1 version still retained characteristics of periodate-sensitivity following the denaturing conditions occurring during electrophoresis and blotting. In addition, as CeTci-CF-1 was co-purified with Ce-LEC-8, immunoblots allowed visualisation of the relative antibody binding to each of the two proteins. The results showed that following periodate treatment of immunoblots, lymph IgA binding to each Tci-CF-1 version from previously infected sheep was maintained, albeit to a lesser degree, as shown in periodate untreated immunoblots (Figure 4.5).

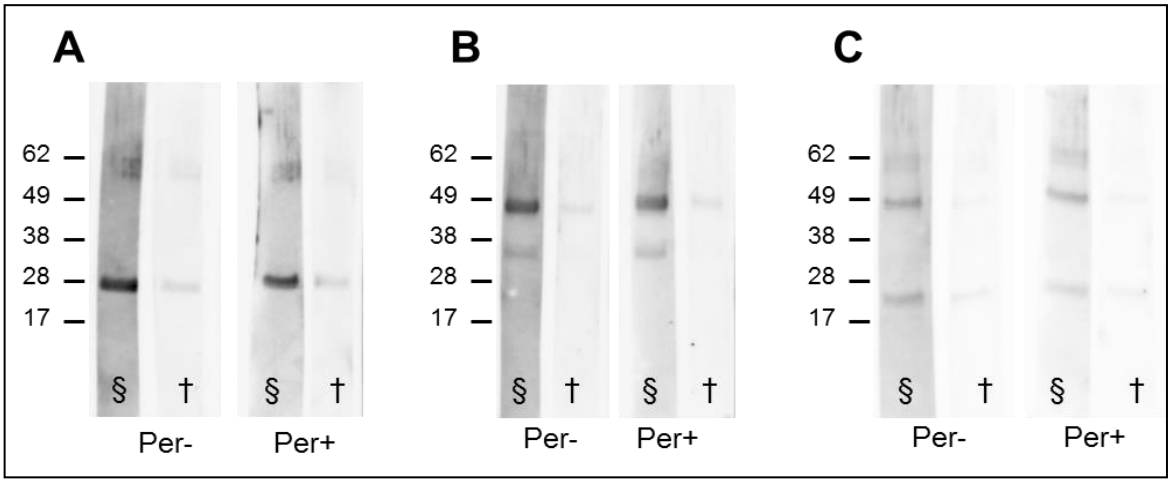

Figure 4.5 Immunoblots showing the gastric lymph IgA binding to purified Tci-CF-1 versions before and after periodate treatment of the antigens.
Molecular weight markers (kDa) positioned to the left of each panel. Equal amounts of proteins were loaded in each lane. Strips marked with § were probed with gastric lymph pooled from 4 sheep previously infected with a trickle infection and a *T. circumcincta* bolus challenge; strips marked with † were probed with lymph pooled from 3 sheep undergoing *T. circumcincta* primary infection, before bolus challenge. All strips were washed and incubated using the same antibodies and buffers, except those marked with Per-, strips that did not receive periodate treatment; Per+, strips that received periodate treatment. Panel (A): nTci-CF-1; panel (B): PiTci-CF-1; panel (C): CeTci-CF-1.

4.3.3 Cysteine protease activity of native and recombinant Tci-CF-1

Cysteine protease activity in Tci-CF-1 versions was investigated using the cysteine protease-specific substrates Z-FR-pNA and Z-FR-AMC. The positive control used throughout was a purified cathepsin L from human liver (hCath L). Specific cysteine protease activity was shown by failure of any of the enzymes to release chromogenic pNA and fluorescent AMC groups when the cysteine protease-specific inhibitor E-64 was added to the reactions.

Initial experiments were performed to determine whether *T. circumcincta* L4 ES and/or purified nTci-CF-1 were able to degrade the chromogenic substrate Z-FR-pNA across a range of pH values. The reaction rates expressed in μOD/min were calculated in the linear phase of the reactions. Enzyme activity was demonstrated for L4 ES and nTci-CF-1 at each pH evaluated, and a small degree of apparent activity only at pH 5.5 for both recombinant Tci-CF-1 versions (Figure 4.6). Neither of the two recombinant Tci-CF-1 versions was able to replicate the small amount of enzyme activity observed in Figure 4.6 and therefore they were subsequently considered not active.

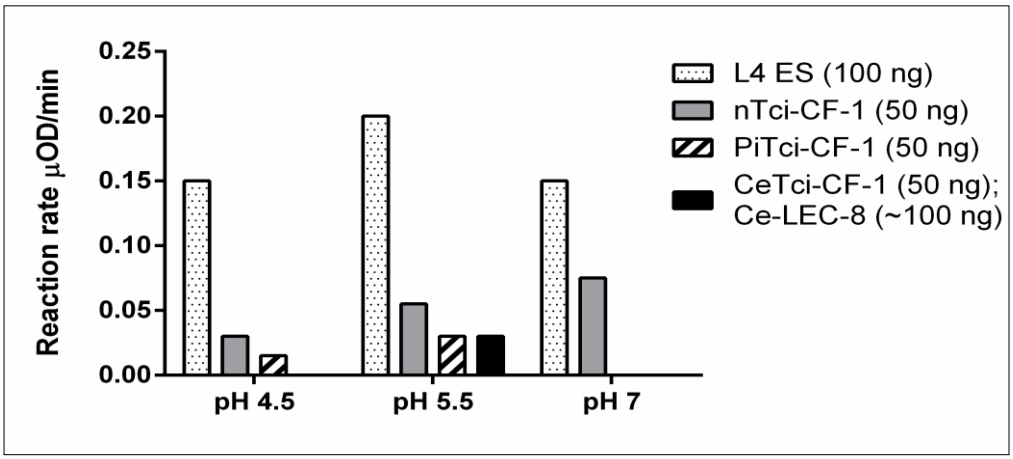

Figure 4.6 Rate of the linear phase of protease activity reactions using Z-FR-pNA for Tci-CF-1 native and recombinant versions.
An equal amount of protein (50 ng) was added in each reaction except L4 ES (100 ng) and CeTci-CF-1, where Ce-LEC-8 was also present. A blank OD reading (from wells without enzyme) was subtracted from each sample absorbance data. The rate of each reaction is shown as µOD/min during the linear phase. A range of pH was tested for the optimal reaction conditions. Two duplicate wells for each reaction. All enzyme activity was inhibited by the addition of E-64.

These results were very close to the sensitivity limits of the assay and therefore could not be reliably repeated. For this reason, a more sensitive fluorescent substrate (Z-FR-AMC) was used to determine the cysteine protease enzyme activity of native and recombinant Tci-CF-1 and L4 ES.

The results confirmed that *T. circumcincta* L4 ES and purified nTci-CF-1 were enzymically active over a range of pH values, with peaks of activity at pH 4.5 and 5.5 (Figure 4.7). Neither recombinant Tci-CF-1 version was able to degrade the substrate used at pH 3.5 and 4.5. Although a small amount of enzyme activity could be recorded at pH 5.5 and 7 (CeTci-CF-1), these results were thought to derive from the presence of Ce-LEC-8 in the purified protein preparation used, as control reactions containing purified Ce-LEC-8 showed a higher fluorescence background.

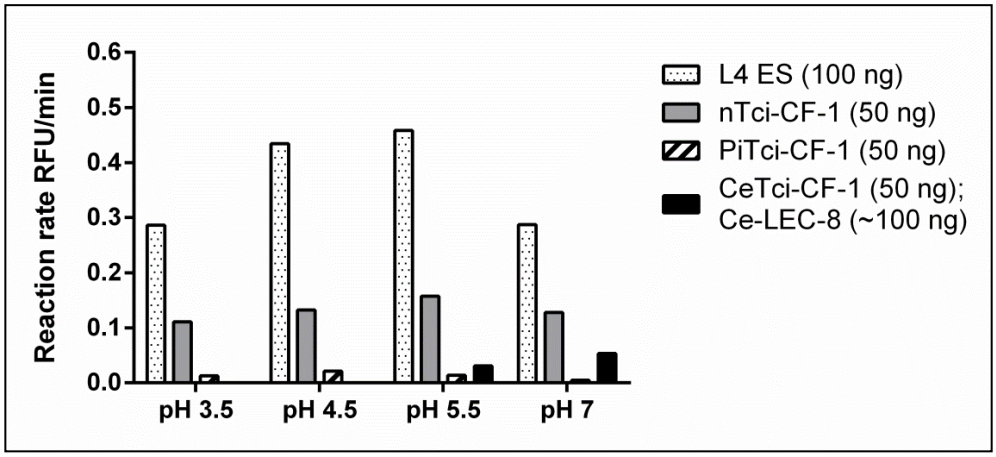

Figure 4.7 Rate of the linear phase of protease activity reactions using Z-FR-AMC for Tci-CF-1 native and recombinant versions.
An equal amount of protein (50 ng) was added in each reaction except L4 ES (100 ng) and CeTci-CF-1, where Ce-LEC-8 was also present. The fluorescence data was blank corrected. Two duplicate wells for each reaction. The rate of each reaction is shown as RFU/min during the linear phase. A range of pH was tested for the optimal reaction conditions.

Maximum protease activity in L4 ES and purified nTci-CF-1 preparations was observed at pH 4.5 and 5.5; all subsequent experiments were therefore conducted at pH 4.5.

Both initial experiments with Z-FR-pNa and Z-FR-AMC had shown that over a two hour incubation, neither recombinant Tci-CF-1 was able to degrade the substrates at low pH. Therefore, attempts were made to activate both PiTci-CF-1 and CeTCI-CF-1 recombinant versions by inducing cleavage of the pro-peptide by two methods. First, *self*-activation was attempted by incubating each recombinant Tci-CF-1 version in buffer at pH 4.5 (representing a drop in pH from the neutral conditions at which it was purified) in the presence of DTT and cysteine. Electrophoresis and Coomassie staining of PiTci-CF-1 incubated in buffer pH 4.5 with the addition of DTT and cysteine in the presence or absence of E-64 over a two hour period showed that *self*-activation was not a mechanism by which this enzyme could be activated. PiTci-CF-1 bands did not show a size shift (suggesting cleavage) at any of the time points analysed, and the presence of E-64 did not show differences compared to the reactions where it was absent (Figure 4.8). For CeTCi-CF-1, no protein bands were visible in gels with any treatment, even after silver staining, making it impossible to assess activation by this method.

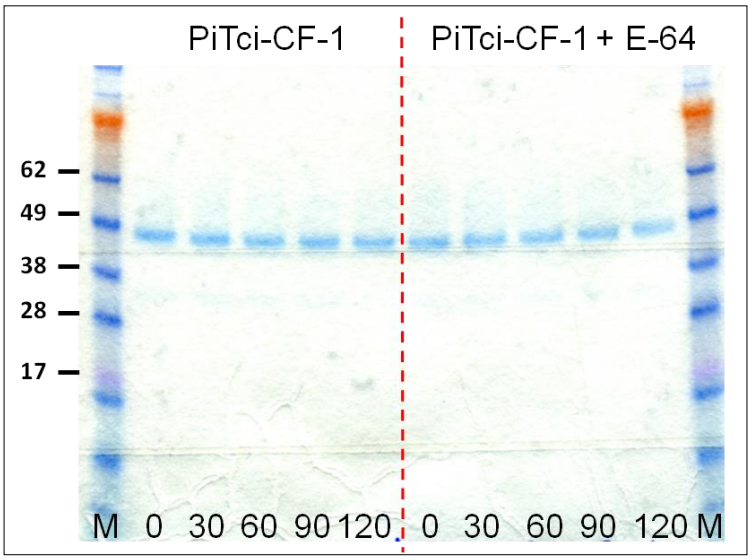

Figure 4.8 SDS-PAGE analysis of PiTci-CF-1 incubated at pH 4.5.
Equal amounts of protein were incubated at 37°C, pH 4.5 in the presence of DTT and absence (left side of the dotted line) or presence (right side) of E-64 for a maximum of 120 min; equal aliquots were collected every 30 min, heat inactivated and loaded on gel. The gel was stained with Coomassie blue. The time (min) at which each aliquot was collected is shown at the bottom of each lane. M: molecular weight marker (kDa).

As it appeared that PiTci-CF-1 could not be *self*-activated by a drop in pH and/or incubation with DTT and cysteine, an attempt at *trans*-activation was made by adding an exogenous enzyme (hCath L) to the reactions containing PiTci-CF-1. The results showed that, at pH 3.5, the pH at which hCath L was shown to be most active in preliminary experiments, a proportion of PiTci-CF-1 was cleaved by hCath L over a period of 140 min and a band of slightly smaller size (~38 kDa) appeared between the two PiTci-CF-1 bands (~48 and ~34 kDa) in silver stained polyacrylamide gels in the absence of E-64 after 20min of co-incubation (Figure 4.9, panel A). This additional band was not present in the reactions incubated in the presence of E-64 (Figure 4.9, panel B). Again, no bands were visible following either treatment of CeTci-CF-1.

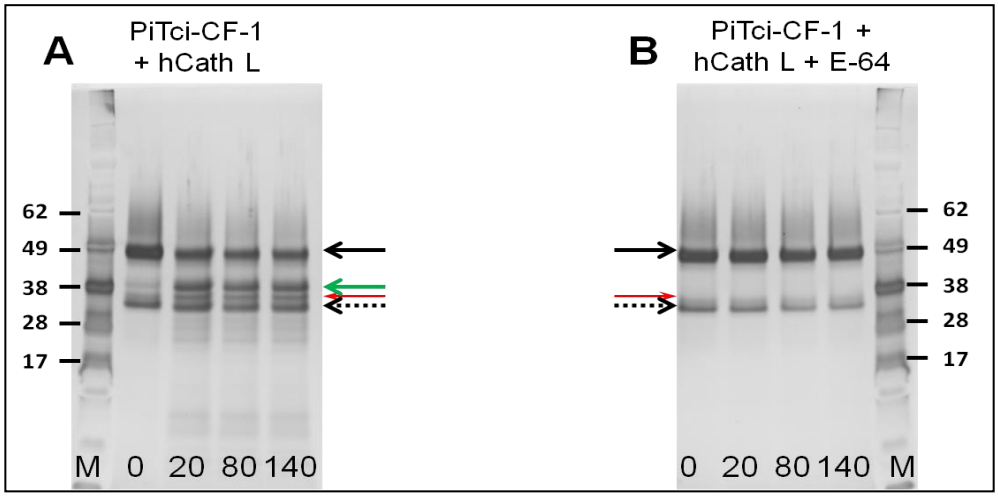

Figure 4.9 SDS-PAGE analysis of PiTci-CF-1 incubated over a period of time at pH 3.5 in the presence of human Cathepsin L.
Equal amounts of protein were incubated at 37°C, pH 3.5 for in the presence of h Cath L enzyme, in the absence (A) or presence (B) of E-64 for a maximum of 140 min; equal aliquots were collected at 20, 80 and 140 min, heat inactivated and loaded on gel. The gel was silver stained. The bands shown are: PiTci-CF-1 as expressed by the yeast cells (black solid and dotted arrows), hCath L (solid red arrow) and PiTci-CF-1 as cleaved in this experiment (solid green arrow). The time (min) at which each aliquot was collected is shown at the bottom of each lane. M: molecular weight marker (kDa).

It was not clear from SDS-PAGE analysis whether the addition of hCath L was effective in *trans*-activating PiTci-CF-1 by cleaving the pro-peptide or whether the band observed in Figure 4.9A (green arrow) was a degraded, but inactive version of PiTci-CF-1. For this reason, cysteine protease-specific assays were carried out using Z-FR-AMC, co-incubating PiTci-CF-1 or CeTci-CF-1 alone or in the presence of hCath L. By analysing the enzyme activity over 60 min periods at a range of substrate dilutions, it was shown that, at the highest substrate concentration used (476 μM), the reaction rates were significantly higher for both PiTci-CF-1 and CeTci-CF-1 co-incubated (for the whole assay duration, 20 + 120 min) with hCath L, compared to hCath L alone or the pre-*trans*-activated recombinant proteins alone (Figure 4.10).

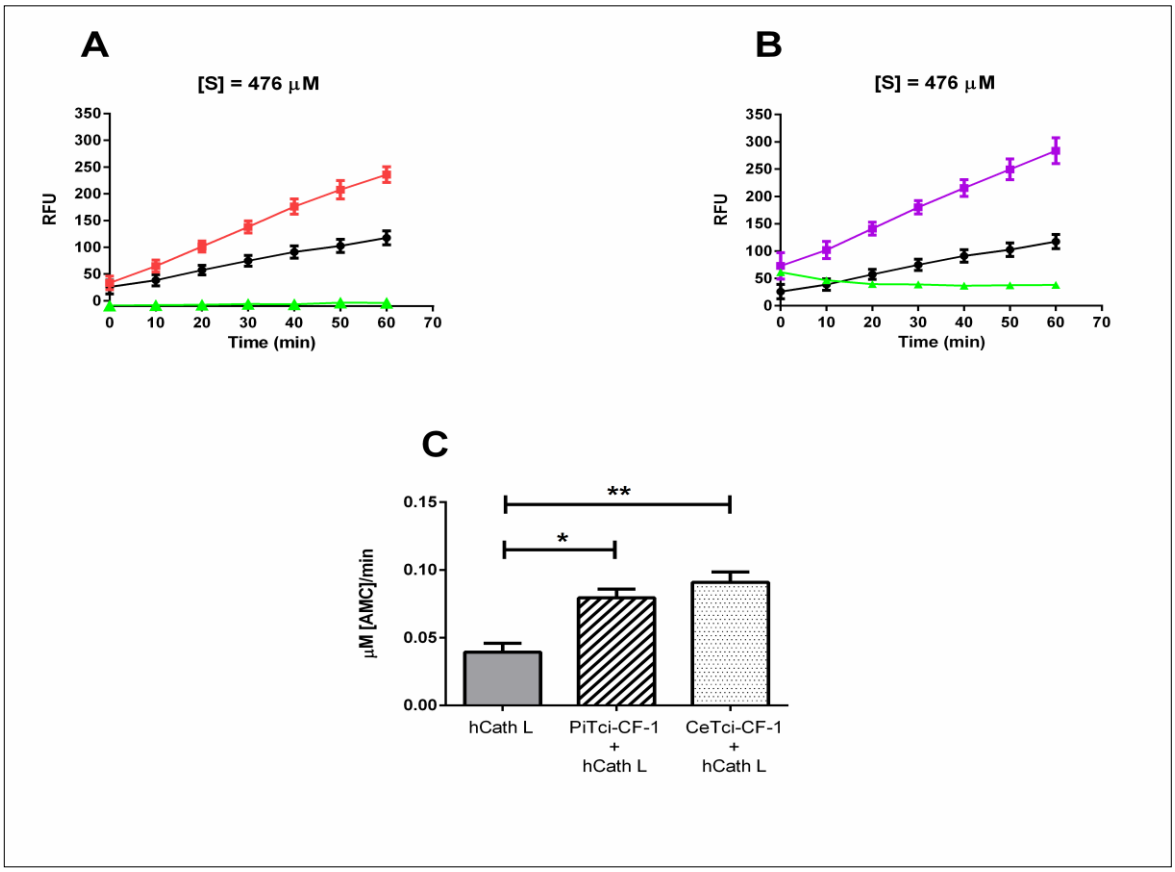

Figure 4.10 Graph showing the fluorescence emitted over 60min by enzyme digestion of Z-FR-AMC by PiTci-CF-1 and CeTci-CF-1 with or without hCath L and the rate of the enzyme reactions during the linear phase.
Relative fluorescence units (RFU) were plotted vs time (min). The final concentration of the substrate was 476 µM. (A): black closed circles, hCath L only wells (13.7 ng/well); red closed squares, purified PiTci-CF-1 (50 ng/well) co-incubated with hCath L (13.7 ng/well); green closed triangles, purified PiTci-CF-1 (50 ng/well); data represent mean±SEM, n=4 (except PiTci-CF-1 where n=1) (B): black closed circles, hCath L only wells (13.7 ng/well); purple closed squares, purified CeTci-CF-1 (50 ng/well) co-incubated with hCath L (13.7 ng/well); green closed triangles, purified CeTci-CF-1 (50 ng/well); data represent mean±SEM, n=4 (except CeTci-CF-1 where n=1). (C): The reaction rates expressed in µM AMC produced/min were calculated in the linear phase of the reactions (20 min). Data represent mean±SEM, n=3. Overall ANOVA *p* = 0.0042; post-ANOVA analysis: Tukey's multiple comparison test. ** = 0.001< *p* <0.01; * = 0.01< *p* <0.05.

When lower substrate concentrations were used (47.6-28.3 µM), the reactions containing the two recombinant Tci-CF-1 enzymes showed different rates, depending on whether PiTci-CF-1 or CeTci-CF-1 was present. The reaction rates for PiTci-CF-1 co-incubated with hCath L appeared lower than hCath L alone, but this difference was not significant (Figure 4.11, panel A). The reaction rates for CeTci-CF-1 co-incubated with hCath L appeared to coincide with hCath L alone at these lower substrate concentrations (Figure 4.11, panel B).

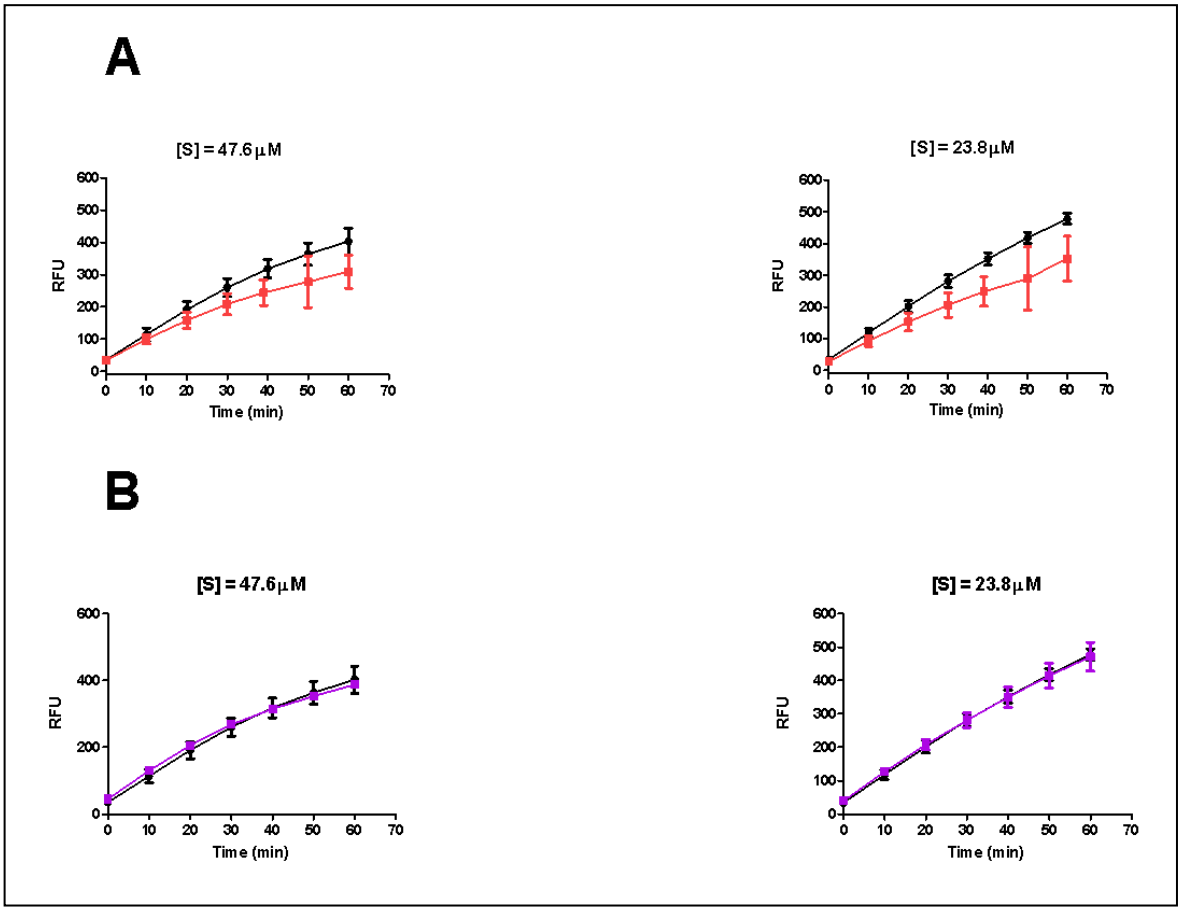

Figure 4.11 Graph showing the fluorescence emitted over 60 min by enzyme digestion of Z-FR-AMC by PiTci-CF-1 and CeTci-CF-1 with the addition of hCath L.
Relative fluorescence units (RFU) were plotted vs time (min). The final concentration of the substrate is shown above each graph. Data represent mean±SEM, n=4. (A): reaction rates of hCath L alone (black closed circles) or PiTci-CF-1 co-incubated with hCath L (red closed squares) at concentrations of Z-FR-AMC of 47.6 and 23.8 µM as shown. (B): reaction rates of hCath L alone (black closed circles) or CeTci-CF-1 co-incubated with hCath L (purple closed squares) at concentrations of Z-FR-AMC of 47.6 and 23.8 µM as shown.

By removing the pro-enzyme, epitopes of the mature enzyme may be more readily available for antibody binding. To investigate the hypothesis that enzyme activation by exogenous cleavage of the pro-peptide might enhance antibody binding to PiTci-CF-1, an ELISA experiment was carried out. PiTci-CF-1 alone or co-incubated with hCath L at different times (0 and 20 min) were used to coat microtitre plates to characterise the gastric lymph IgA reactivity to cleaved PiTci-CF-1. The gastric lymph used was a pool of samples from 5 previously infected sheep that received a *T. circumcincta* trickle infection and a bolus challenge (samples were collected between +6 and +10 days post-challenge, dpc). The results in Figure 4.12 showed that there was no significant increase in lymph IgA binding to hCath L-treated PiTci-CF-1, either at the moment of mixing (no incubation) or after a period of co-incubation at 37°C (20 min).

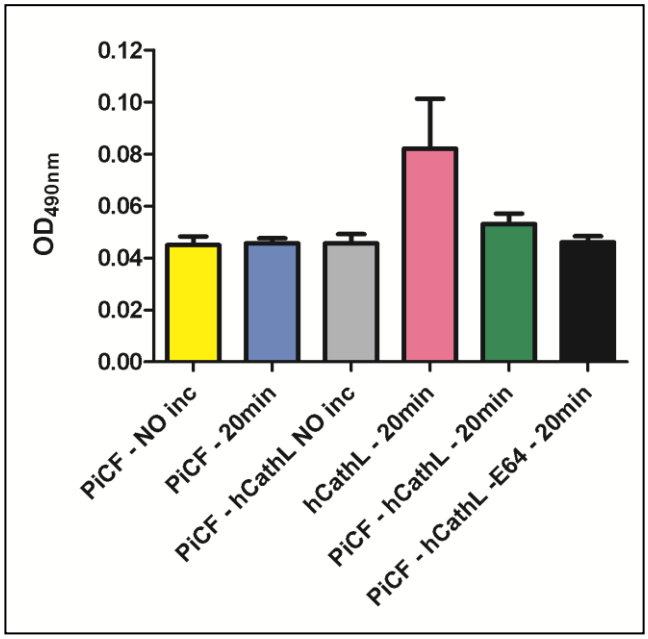

Figure 4.12 Ovine gastric lymph IgA binding to PiTci-CF-1 co-incubated at 37°C with or without addition of hCath L, in the presence or absence of E-64.
Equal amounts of PiTci-CF-1 (1 µg/ml) were used to coat a microtitre plate as follows: yellow bar, PiTci-CF-1 alone without incubation; blue bar, PiTci-CF-1 alone incubated at 37°C for 20min; grey bar, PiTci-CF-1 and hCath L were mixed but not incubated; pink bar, hCath L alone; green bar, PiTci-CF-1 and hCath L, mixed and incubated at 37°C for 20 min; black bar, as the green bar with the addition of E-64. Gastric lymph used was a pool of samples from 5 sheep that received a *T. circumcincta* trickle infection and challenge protocol; samples were collected between +6 and +10 dpc.

An immunoblot experiment was carried out to visually investigate the cleavage of PiTci-CF-1. This experiment was also used to compare the binding of lymph antibodies from *T. circumcincta* previously infected sheep with the binding of antibodies from PiTci-CF-1-immunised sheep, against hCath L-cleaved PiTci-CF-1. In this experiment, PiTci-CF-1 was incubated with hCath L for 80 min, with or without E-64, to induce cleavage of the pro-peptide and subsequently loaded in a polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane and were probed with two different pools of gastric lymph (from *T. circumcincta* previously infected or primary infection sheep), or a sheep anti-PiTci-CF-1 serum sample (samples provenance described in Section 4.2.4). The results showed that post-immunisation serum IgG from sheep immunised with PiTci-CF-1 was able to bind PiTci-CF-1, and that binding to PiTci-CF-1 was not observed for IgG from pre-immunisation serum (Figure 4.13, Ai-ii). To a lesser extent, sheep gastric lymph IgA from previously infected animals bound to PiTci-CF-1, however in this case the difference in intensity of the binding detected compared to primary infection sheep lymph was minimal (Figure 4.13, A1-2). Human CathL-mediated cleavage of PiTci-CF-1 pro-peptide did not greatly enhance binding to gastric lymph IgA from previously infected

sheep compared to primary infection sheep lymph, although a weak band corresponding to cleaved PiTci-CF-1 could be detected (Figure 4.13, B1-2). However, it was shown that post-immunisation serum IgG was able to bind an additional two bands (Figure 4.13, Bi), in the trans-activated PiTci-CF-1 compared to the unactivated PiTci-CF-1 lane alone (Ai), showing that hCath L was able to cleave the PiTci-CF-1 zymogen. This also showed that cleaved PiTci-CF-1 forms may still be bound by recombinant PiTci-CF-1-specific antibodies. The last panel of this experiment (Figure 4.13, C) showed that the addition of E-64 completely inhibited cleavage of PiTci-CF-1 by hCath L, and that the additional bands described were a product of the 80 min co-incubation of Pi Tci-CF-1 with hCath L, and not a previous autolysis of PiTci-CF-1. The same identical experiment was carried out using CeTci-CF-1 in the presence or absence of hCath L and E-64 in immunoblots probed with gastric lymph or serum; however no information could be gained as no protein was detected in either of the blots (Figure 4.14).

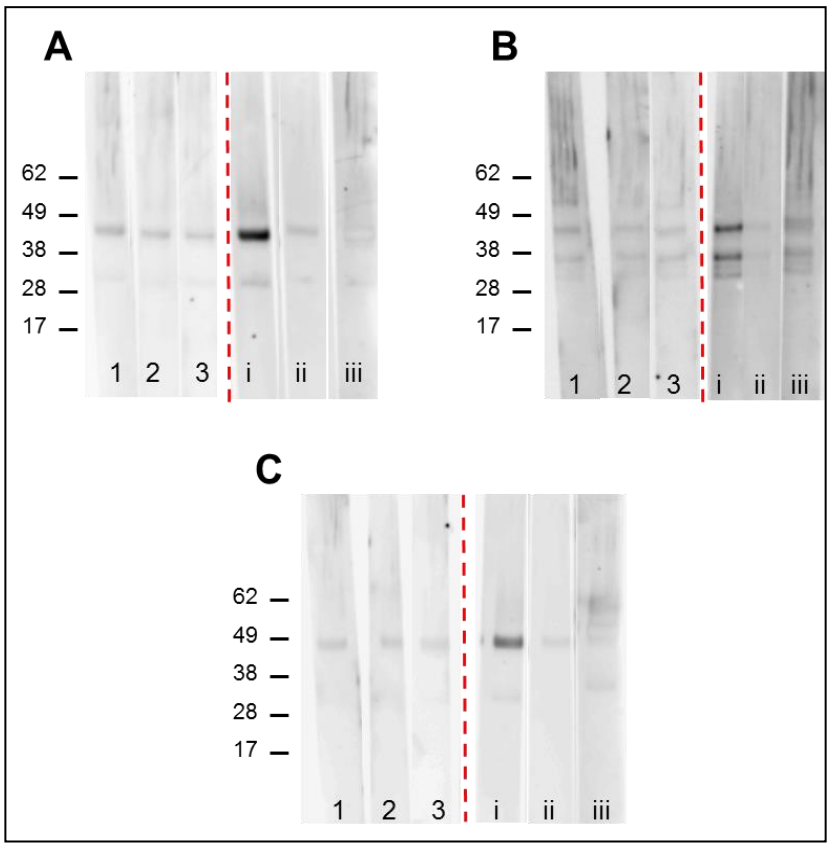

Figure 4.13 Immunoblots showing the antibody binding of gastric lymph IgA or serum IgG to PiTci-CF-1 cleaved with hCath L.
Molecular weight markers (kDa) on the left of each panel. An equal amount of PiTci-CF-1 incubated or not with hCath L (1:100 dilution), with or without E-64 was loaded in each lane. For all panels: lane 1, pool of gastric lymph from *T. circumcincta* previously infected sheep (collected between +6 and +10 days post bolus challenge); lane 2, pool of gastric lymph from *T. circumcincta* primary infection sheep (collected between -2 and +2 days post bolus challenge); lane 3, negative control incubated without primary antibody (lymph). In all panels, lane i) incubated with sheep anti *P. pastoris*-expressed Tci-CF-1 serum (post-immunisation); lane ii) incubated with sheep anti *P. pastoris*-expressed Tci-CF-1 serum (pre-immunisation); lane iii) negative control incubated without primary antibody (serum). Panel (A): PiTci-CF-1 alone was loaded in each lane; panel (B): PiTci-CF-1 was co-incubated with hCath L for 80 min before loading onto the gel; panel (C): PiTci-CF-1 was co-incubated with hCath L and E-64 for 80 min before loading onto the gel. In all lanes numbered 1-3, lymph IgA binding was shown with a secondary mouse anti-sheep IgA antibody, a tertiary rat anti-mouse IgG:HRP antibody. In all lanes numbered i-iii, serum IgG binding was shown with a secondary rabbit anti-sheep IgG:HRP antibody. Antibody binding in all blots was revealed with chemiluminescent substrate for HRP.

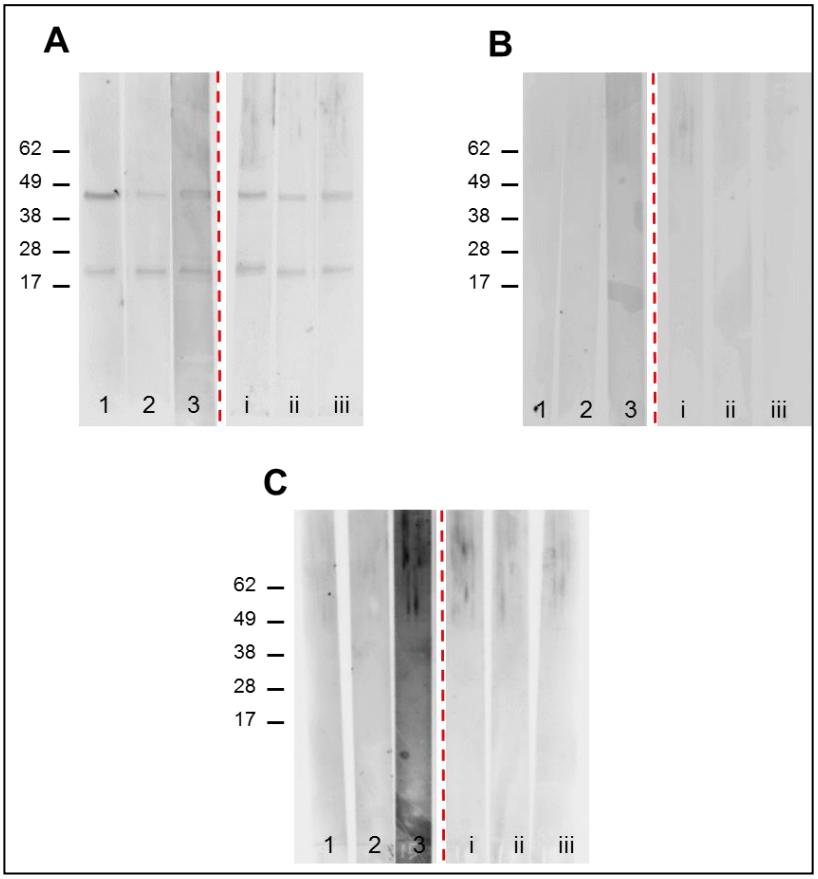

Figure 4.14 Immunoblots showing the antibody binding of gastric lymph IgA or serum IgG to CeTci-CF-1 cleaved with hCath L.
Molecular weight markers (kDa) on the left of each panel. An equal amount of CeTci-CF-1 incubated or not with hCath L (1:100 dilution), with or without E-64 was loaded in each lane. For all panels: lane 1, pool of gastric lymph from *T. circumcincta* previously infected sheep (collected between +6 and +10 days post bolus challenge); lane 2, pool of gastric lymph from *T. circumcincta* primary infection sheep (collected between -2 and +2 days post bolus challenge); lane 3, negative control incubated without primary antibody (lymph). In all panels, lane i) incubated with sheep anti *P. pastoris*-expressed Tci-CF-1 serum (post-immunisation); lane ii) incubated with sheep anti *P. pastoris*-expressed Tci-CF-1 serum (pre-immunisation); lane iii) negative control incubated without primary antibody (serum). Panel (A): CeTci-CF-1 alone was loaded in each lane; panel (B): CeTci-CF-1 was co-incubated with hCath L for 80 min before loading onto the gel; panel (C): CeTci-CF-1 was co-incubated with hCath L and E-64 for 80 min before loading onto the gel. In all lanes numbered 1-3, lymph IgA binding was shown with a secondary mouse anti-sheep IgA antibody, a tertiary rat anti-mouse IgG:HRP antibody. In all lanes numbered i-iii, serum IgG binding was shown with a secondary rabbit anti-sheep IgG:HRP antibody. Antibody binding in all blots was revealed with chemiluminescent substrate for HRP.

4.3.4 Glycosylation analysis of nTci-CF-1, PiTci-CF-1 and CeTci-CF-1

Each version of Tci-CF-1 generated and/or purified in this study was first confirmed to be glycosylated using a glycan-specific gel stain kit. By using the glycan-specific stain, nTci-CF-1 (mature enzyme) and CeTci-CF-1 (zymogen) showed fluorescent signals of similar intensity, whereas PiTci-CF-1 (zymogen) showed higher fluorescence (Figure 4.15).

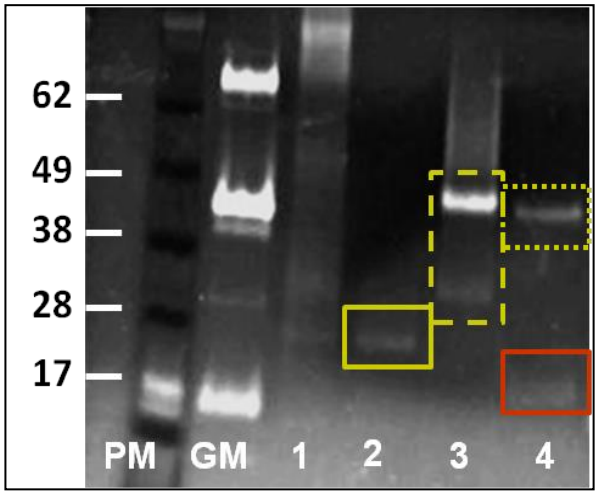

Figure 4.15 SDS-PAGE and glycan stain analysis of Tci-CF-1 versions.
PM: protein molecular marker (kDa); GM: glycan molecular marker (CandyCane, Invitrogen); lane 1: L4 ES; lane 2: purified nTci-CF-1 (solid line box); lane 3: purified PiTci-CF-1 (both bands within the dashed line box); lane 4: purified CeTci-CF-1 (dotted line box) and Ce-LEC-8 (solid line red box). Equal amounts of protein were loaded in each lane; the gel was stained using a glycan-specific stain (Invitrogen).

Subsequently, electroblots of each protein were probed with a panel of 8 different lectins, each with different glycan residue specificity (Figure 4.16).

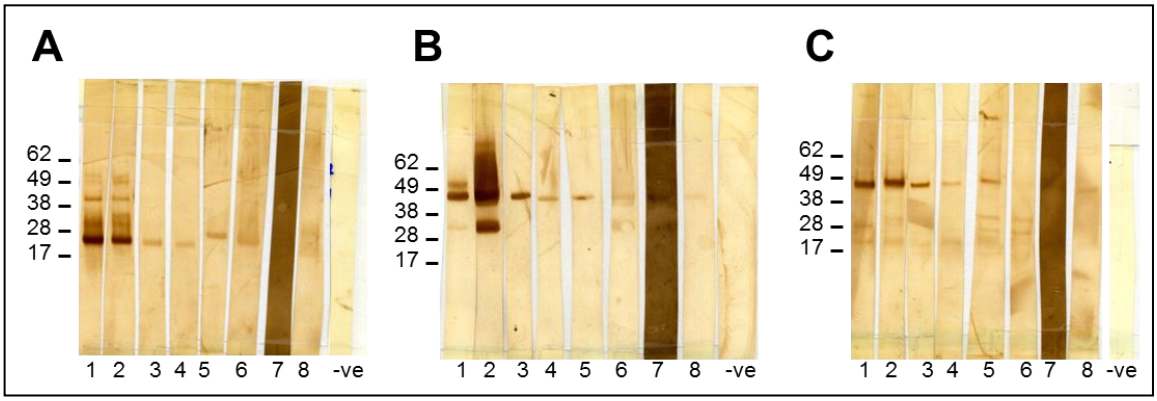

Figure 4.16 Electroblots showing lectin binding to Tci-CF-1 versions.
Molecular weight marker (kDa) positioned to the left of each panel. Panel (A): nTci-CF-1; panel (B): PiTci-CF-1; panel (C): CeTci-CF-1. Numbers at the bottom of each correspond to the lectins used: 1, LCH (Lentil lectin from *Lens culinaris*); 2, ConA (Concanavalin A from *Canavalia ensiformis*); 3, DBA (*Dolichos biflorus* agglutinin); 4, UEA (*Ulex europaea* agglutinin); 5, SBA (Soybean agglutinin from *Glycine max*); 6, PNA (Peanut agglutinin from *Arachis hypogea*); 7, WGA (Wheat germ agglutinin from *Triticum vulgaris*); 8, RCA I (*Ricinus communis* agglutinin I); -ve, electroblot of each protein incubated in buffer.

The lectin panel used to investigate the reactivity of glycans in each version of Tci-CF-1 highlighted similarities in the glycosylation of nTci-CF-1 and recombinant versions, although one notable difference was in the binding of Peanut agglutinin (PNA, Figure 4.16,

lane 6) to nTci-CF-1 and, to a lesser extent, to PiTci-CF-1 but not to CeTci-CF-1. It was apparent that most other lectins bound to PiTci-CF-1 in a similar pattern to nTci-CF-1, but to a much stronger degree. It was also shown that ConA and to a lesser extent LCH, bound to both PiTci-CF-1 bands (high and low molecular weight), however other lectins only bound to the higher molecular weight isoform. The results are summarized in Table 4.2.

Table 4.2 Panel of lectins used to probe the glycan composition of the structures present in each Tci-CF-1 versions purified for this thesis.

Lectin	PiTci-CF-1 (only higher molecular weight band if not specified)	nTci-CF-1 (mature enzyme)	CeTci-CF-1 (zymogen)
1. LCH	++++ (both bands)	++	++
2. ConA	++++ (both bands)	++	++
3. DBA	+++	+	+
4. UEA	++	+	+/-
5. SBA	++	+/-	+
6. PNA	+/-	++	-
7. WGA	+/-	-	-
8. RCA I	+/-	-	+/-

Lectins used were: LCH: Lentil lectin from *Lens culinaris*, ConA: Concanavalin A from *Canavalia ensiformis*, DBA: *Dolichos biflorus* agglutinin, UEA: *Ulex europaea* agglutinin, SBA: Soybean agglutinin from *Glycine max*, PNA: Peanut agglutinin from *Arachis hypogea*, WGA: Wheat germ agglutinin from *Triticum vulgaris*, RCA I: *Ricinus communis* agglutinin I.

Further characterisation of the glycan structures of Tci-CF-1 versions was carried out using PNGase F and *O*-glycosidase enzymes. The aim of this experiment was to investigate whether a shift in size could be induced by treatment of each Tci-CF-1 version with enzymes capable of removing glycan structures. Untreated Tci-CF-1 proteins (approximately 130 ng) were loaded in the gel (Figure 4.17, lanes 1-4-7), to ensure visualisation upon staining, and to act as positive control for the staining process (as this was the standard visualised amount used in SDS-PAGE gels throughout this thesis). However, due to an unavoidable dilution process during the enzymatic PNGase F and *O*-glycosidase reactions, only ~60 ng/well enzyme-treated Tci-CF-1 proteins could be loaded in the gel. For this reason, the untreated wells could not be directly compared to the treated ones. The results of this experiment showed that PiTci-CF-1 possessed PNGase F-sensitive glycans, as shown by the size shift, but not *O*-glycosidase-sensitive glycans (Figure 4.17, lane 5). The size of native Tci-CF-1 appeared unchanged following either enzymatic

treatment, as did CeTci-CF-1. This experiment suggests that PNGase F treatment was only successful in removing N-linked glycans from PiTci-CF-1. The presence of Ce-LEC-8 is shown in Figure 4.17 but no size shift was observed following treatment of the CeTci-CF-1/Ce-LEC-8 protein preparation.

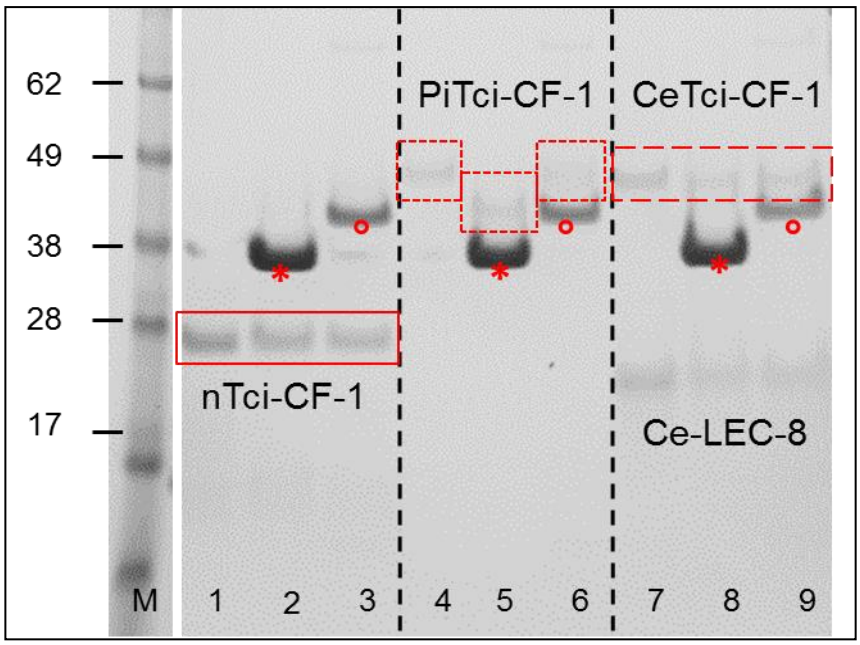

Figure 4.17 SDS-PAGE analysis of the size shift of Tci-CF-1 versions following PNGase F and *O*-glycosidase treatment.
Coomassie blue stain was used. M: molecular weight marker (kDa). Asterisks indicate PNGase F bands; open circles indicate *O*-glycosidase. The solid line box encloses nTci-CF-1, untreated (lane 1), PNGase F-treated (lane 2) and *O*-glycosidase-treated (lane 3). The dotted line box encloses PiTci-CF-1, untreated (lane 4), PNGase F-treated (lane 5) and *O*-glycosidase-treated (lane 6). The dashed line box encloses CeTci-CF-1, untreated (lane 7), PNGase F-treated (lane 8) and *O*-glycosidase-treated (lane 9). The presence of Ce-LEC-8 is shown.

4.4 Discussion

The results from this chapter showed that it was possible to identify conserved catalytic amino acid residues and N-linked glycosylation sites in amino acid sequence alignments of each Tci-CF-1 version; each clone had been generated by PCR on cDNA from L4 stage RNA material. The ability of each Tci-CF-1 version to degrade a substrate specific for papain-like enzymes (Z-FR-AMC) was demonstrated, suggesting that both PiTci-CF-1 and CeTci-CF-1 were correctly folded to the extent that they could form a functional active site. Additionally, evidence of the presence of glycosylation was provided for each of the Tci-CF-1 versions investigated, and immunoblots showed that antibody binding to recombinant Tci-CF-1 versions may be enhanced by denaturing conditions. Finally, a *C. elegans* galectin (Ce-LEC-8) consistently co-eluted with CeTci-CF-1 and showed a degree of non-specific binding with sheep IgA.

This chapter showed that, upon denaturing *T. circumcincta* recombinant Tci-CF-1 versions in immunoblots, gastric lymph IgA from sheep that received an immunising *T. circumcincta* trickle infection was able to bind these antigens. These results were in contrast to those described in Chapter 2 of this work, where it was shown by ELISA that *Pichia pastoris*-expressed Tci-CF-1 was not recognised by antibodies from *T. circumcincta* immune sheep. The differences observed had been attributed to failure of the yeast expression system to deliver correct protein folding and/or post-translational modifications (PTMs). The work shown in this chapter set out to answer these questions. First, antibody reactivity to each purified Tci-CF-1 version was investigated using immunoblots in addition to ELISA assays. Western blotting techniques allow investigation of the immune response to an antigen (or antigen mix) without the interference of tertiary structures, as standard protein electrophoresis techniques generally involve denaturing and reducing conditions which facilitate linearisation of amino acid sequences. For samples containing more than one protein, Western blotting gives the ability to visually determine which protein the antibodies bind to [reviewed by Harper *et al.* (1990) and Kurien and Scofield (2006)]. Western blotting experiments have been previously used to show the antibody immune response to complex preparations such as parasite somatic extracts, excretory-secretory products, but also to purified native and recombinant proteins (Collins *et al.* 2004, Geldhof *et al.* 2003, Jungersen *et al.* 2001, Vercauteren *et al.* 2004, Wedrychowicz *et al.* 1994). Here, immunoblots showed that denaturing conditions could reveal shared epitopes in native and recombinant Tci-CF-1 and confirmed that the previous differences in antibody binding shown in ELISA experiments between the native and recombinant Tci-

CF-1 were likely to be caused by differences in protein folding or PTMs. However it must be noted that non-specific antibody binding occurred in blots probed with lymph from previously infected sheep, resulting in more intense background staining (Figure 4.4). This might reflect a simple quantitative increase in non-specific IgA binding in lymph from previously infected sheep compared to lymph from primary infection sheep; for this reason it was not attempted to quantify the intensity of the Tci-CF-1-specific antibody binding in the immunoblots.

In periodate-treated immunoblots of each Tci-CF-1 version, a degree of lymph IgA binding was lost compared to untreated blots, suggesting the presence of periodate-sensitive epitopes, most probably glycans. These results, along with those from periodate-treated ELISAs (Chapter 2) in which it was shown that a significant ($p \leq 0.0002$) degree of antibody reactivity to native Tci-CF-1 is directed towards glycan epitopes, support the hypothesis that Tci-CF-1 glycans may not have been correctly replicated in the recombinant versions considered in this work. Hyperglycosylation of recombinant proteins has been reported previously in experiments with a *Pichia pastoris*-expressed ENV glycoprotein from human immunodeficiency virus (HIV; Scorer *et al.* 1993); in addition, yeast glycan structures might have differed from those of nematode glycans which present core fucose structures not identified elsewhere (Haslam *et al.* 2000, Haslam *et al.* 1998, Khoo *et al.* 1997, Poltl *et al.* 2007, van Die *et al.* 1999). The inappropriate glycosylation that is likely to have occurred using the yeast expression system could have negatively affected the ability of sheep antibodies to bind to Tci-CF-1 protein epitopes (see Chapter 2 discussion for more details).

It has been shown that protein conformation is essential to enzyme activity, as denatured proteins only retain partial activity compared to the native state (Anfinsen 1973). Recombinant expression systems have been variably successful in generating active proteases, as often the recombinant protein of interest is found in a denatured state within inclusion bodies and requires extraction techniques that may allow refolding [reviewed by Marston (1986) and Georgiou and Valax (1996)]. However, recombinant expression of active proteases can be achieved, particularly when baculovirus/insect cell expression systems are employed e.g. with the *H. contortus* protease H11 (Smith *et al.* 1997), a cathepsin D (Ac-APR-1) from the dog hookworm *Ancylostoma caninum* which was also expressed in active form using baculovirus (Williamson *et al.* 2002). Indeed *C. elegans* has also been shown to generate enzymically active proteases, such as another recombinant

version of *H. contortus* H11 and a cysteine protease (Hc-CPL-1) from the same parasite (Murray *et al.* 2007, Roberts *et al.* 2013).

In the generation of recombinant proteins and especially enzymes and/or proteins that will constitute vaccine antigens the importance of disulphide bonds should be noted. Disulphide bonds can occur between two cysteine residues in any amino acid chain, and can occur spontaneously or be dependent on the activity of another enzyme within the endoplasmic reticulum (ER) of cells (Anfinsen 1973, Frand *et al.* 2000). During this and a previous study (Redmond *et al*. 2006) of Tci-CF-1 versions it has been noted that the amino acid chain of this protein contains 6 cysteine residues which could contribute to the folding of the mature protein. It has been shown in Chapter 2 of this thesis that a fraction of native Tci-CF-1 can be purified using a thiol column; this suggests that a degree of disulphide bond activity can take place between cysteine residues in this protein and the –SH residues within the column. These results open the possibility that the native mature version of Tci-CF-1 (nTci-CF-1) may be folded by means of disulphide bonds and that could influence the antibody recognition of conformational epitopes. This could explain the differences observed between antibody binding to nTci-CF-1 and the two recombinant versions generated in this study. Reducing conditions used in SDS-PAGE and Western blotting would not allow studies of the potential disulphide bonds present in this protein. An alternative could be to use non-reducing conditions and, if further funding became available, a crystallography study of the native and recombinant versions of Tci-CF-1 would provide some useful information regarding the shape of this enzyme.

In this chapter it was shown that *T. circumcincta* L4 ES contains active cysteine proteases, as it was able to degrade the cysteine protease-specific substrates used and activity was inhibited by addition of the cysteine protease-specific inhibitor E-64 (Figures 4.6 and 4.7). Of the L4 ES proteases, the most abundant is Tci-CF-1 (Redmond *et al.* 2006); in purified form this protein was also able to degrade the substrates, albeit with weaker activity than L4 ES. This was initially thought to be caused by a dilution factor, as L4 ES was used in the assays at a concentration 20-fold higher than nTci-CF-1. However, the complete composition of *T. circumcincta* L4 ES is currently unknown; therefore it is possible that other less abundant cysteine proteases with higher affinity for the substrate could have been responsible for the activity observed. Another possibility is that nTci-CF-1 may have been partially degraded during purification from ES, therefore losing some enzyme activity. When each purified recombinant Tci-CF-1 version was examined for cysteine protease activity, neither showed the ability to degrade the substrates used. However, this

result could be explained as both recombinant proteins were expressed as zymogens and therefore the pro-peptide had not been cleaved to release the mature enzymes. Enzymatic activity of cathepsins B and L is inhibited by the pro-peptide lying in the substrate binding cleft, and it is thought that this mechanism may be a feature of all papain-like cysteine proteases (Coulombe *et al.* 1996). As only mature native Tci-CF-1 can be found in *T. circumcincta* L4 ES (suggested by the molecular size and the cysteine protease activity), it is likely that in the parasite, a cleaving process takes place before the enzyme is excreted or secreted.

Experiments to investigate the enzyme activity of Tci-CF-1 recombinant versions were carried out at pH 4.5, which represents a drop from the neutral pH at which the proteins were stored. The lack of activity observed in the unmodified recombinant proteins in experiments at pH 3.5, 4.5, 5.5 and 7 using the substrate Z-FR-AMC suggested that these enzymes were not able to *self*-activate. This was later confirmed by SDS-PAGE analysis, showing that prolonged incubation of the recombinant proteins (120 min) at pH 4.5 in the presence of DTT was not sufficient to induce protein activation by cleavage of the pro-peptide. These results were in contrast with the findings shown by different cysteine proteases, FhCL1 and FhCL3 from *Fasciola hepatica*, which were expressed in the yeast *Hansenula polymorpha* and only required a short incubation at 37°C to *self*-activate (Corvo *et al.* 2013). Another enzyme (BCP), the acid cysteine protease from the silk moth *Bombyx mori*, is able to *self*-activate when incubated in vitro at pH 4 in the presence of DTT (Takahashi *et al.* 1997).

However, this is not the only activation pathway for cysteine proteases. A recombinant *P. pastoris*-expressed cathepsin F, from the liver fluke *Opistorchis viverrini* (Ov-CF-1), could be *trans*-activated by addition of an exogenous cathepsin L [from the trematode *F. hepatica*, FhCL1 (Pinlaor *et al.* 2009)]. Another papain-like cysteine protease, recombinantly expressed in *P. pastoris*, is *Schistosoma mansoni* cathepsin B1 (SmCB1pm); this was activated by the presence of recombinant activated *S. mansoni* asparaginyl endopeptidase (SmAE), also expressed in *P. pastoris* (Sajid *et al.* 2003). Further investigations in this chapter showed that both recombinant Tci-CF-1 versions could be activated by *trans*-activation carried out by an exogenous protease (human Cathepsin L, hCath L). The *trans*-activation of both recombinant Tci-CF-1 versions by adding hCath L resulted in the enhanced ability of both PiTci-CF-1 and CeTci-CF-1 to degrade the fluorescent substrate Z-FR-AMC. Furthermore, PiTci-CF-1 secreted by *Pichia pastoris* cells has been shown to migrate as two bands during gel electrophoresis (see

Figure 2.4) indicating that the yeast may be able to generate a cleaved form of recombinant Tci-CF-1. However, recombinant PiTci-CF-1 is enzymatically inactive, suggesting that the cleaved version (of lower molecular weight) is not the active enzyme. This was supported by the finding in this chapter that when PiTci-CF-1 was activated by hCath L a 38 kDa band, presumably the active enzyme, was released; this cleaved form appeared of higher molecular mass than the cleavage product seen in the original inactive PiTci-CF-1. Moreover, the majority of PiTci-CF-1 was secreted as a zymogen (of higher molecular mass ~49 kDa) and therefore requiring activation. The differences in estimated molecular mass between the PiTci-CF-1 zymogen, the *P. pastoris*-cleaved smaller Tci-CF-1 and the hCath L-cleaved intermediate size PiTci-CF-1 may indicate that the exogenous enzyme hCath L cleaves the enzyme at a more appropriate site in the amino acid chain, leaving extra amino acids that might be vital to the correct activity of PiTci-CF-1. Further work may be carried out to investigate the site of cleavage in both *P. pastoris*-cleaved smaller Tci-CF-1 and the hCath L-cleaved intermediate size Tci-CF-1, and compared if possible to Tci-CF-1 purified from *T. circumcincta* L4 ES. This would shed more light on the mechanisms that lead to the activation of Tci-CF-1.

Pro-peptide cleavage experiments were also attempted with CeTci-CF-1. However, no protein bands were stained by either coomassie blue or silver staining in polyacrylamide gels, and immunoblotting also did not reveal protein bands corresponding to CeTci-CF-1. It was hypothesised that in these experiments the initial concentration of the protein (~10 µg/ml) and the subsequent dilutions necessary to carry out the experiments exceeded the sensitivity of the detection methods. As the amount of purified CeTci-CF-1 protein was limited, further attempts to detect the pro-peptide cleavage were abandoned. However, in future experiments a modification to the immunoblotting technique could be introduced. Experiments have previously reported that heating nitrocellulose membranes (by boiling in water at 100°C, autoclave at 120°C, or baking at > 80°C) following dot blots and protein transfer can increase the sensitivity of detection for certain antibody-protein pairs (Swerdlow *et al.* 1986).

At the highest Z-FR-AMC substrate concentration used (476 µM) the reaction rates were significantly higher for both PiTci-CF-1 and CeTci-CF-1 incubated with hCath L, compared to hCath L or the recombinant enzymes alone. This suggests that the co-incubation of hCath L with both recombinant Tci-CF-1 versions could activate the latter two. When the experiments were repeated at lower substrate concentrations (47.6-23.8 µM) the reaction rates for co-incubated hCath L and recombinant Tci-CF-1 were lower

than or similar to hCath L alone (Figure 4.11). This suggests competition for the substrates within this system, where both recombinant Tci-CF-1 and Z-FR-AMC were substrates for hCath L initially, followed by competition between activated recombinant Tci-CF-1 and hCath L for Z-FR-AMC substrate.

Native Tci-CF-1 is present in *T. circumcincta* L4 ES as a mature enzyme (as deduced from the estimated molecular weight in polyacrylamide gels). The mature Tci-CF-1, without the pro-peptide, would therefore have been an antigenic stimulus for those sheep that had been trickle infected with *T. circumcincta* L3; this means that antibodies specific for the pro-peptide were unlikely to be present in the serum, ASC probes or gastric lymph samples. For this thesis, samples from the *T. circumcincta* trickle infected sheep were used to investigate antibody binding to PiTci-CF-1 and CeTci-CF-1 and neither achieved statistically significant antibody binding. It is suggested here that the presence of the pro-peptide of PiTci-CF-1 and CeTci-CF-1 might have negatively affected binding of antibodies specific for the mature domain of Tci-CF-1. This might have occurred as the pro-peptide was positioned across the active site and could have masked epitopes present in the cleft. Another hypothesis is that the glycan group present on the pro-peptide of recombinant Tci-CF-1 could also have negatively affected antibody binding by masking protein epitopes. However, the real cause of the lack of antibody reactivity against PiTci-CF-1 remains unclear, as immunoblots and ELISA experiments of PiTci-CF-1 cleaved with hCath L showed that it was not possible to significantly enhance antibody binding by removing the pro-peptide. This could imply that infection-induced antibodies do not recognise the structure of recombinant CF-1 even after pro-peptide removal. Another hypothesis is that glycosylation on PiTci-CF-1 was too different from that on the native Tci-CF-1 from *T. circumcincta*, and antibodies were unable to recognise epitopes. Alternatively, hyperglycosylation of PiTci-CF-1 may have masked protein epitopes similar to nTci-CF-1that would otherwise have been bound by antibodies.

To further investigate the presence and relevance of PTMs in Tci-CF-1 versions, experiments were carried out first to confirm the presence of glycosylation and subsequently to try and characterise it. Glycosylation of native and recombinant Tci-CF-1 versions was confirmed by the use of a glycan-specific stain in polyacrylamide gels. As equal amounts of protein were loaded in each lane, the much higher fluorescence achieved by PiTci-CF-1 compared to nTci-CF-1 suggested that the former may have been hyperglycosylated by the yeast expression system. Yeast expression systems have been shown to hyperglycosylate recombinant proteins (Storer and Menard 1994). Engineering of

P. pastoris strains in order to generate the correct glycosylation patterns (if known) may provide a solution to this problem without significantly affecting yeast viability or protein expression (Vervecken *et al.* 2004). The higher fluorescence achieved by PiTci-CF-1 compared to nTci-CF-1 could also have been caused by the presence of two N-linked glycans on the PiTci-CF-1 as opposed to one on the mature native Tci-CF-1. As a tentative confirmation of this hypothesis, only the higher molecular size PiTci-CF-1 band showed the brightest glycan-dependent fluorescence, whereas the lower molecular weight band showed brightness comparable to nTci-CF-1 (Figure 4.15). CeTci-CF-1 was also expressed as a zymogen with two N-linked glycosylation sites, assuming that both sites carried a glycan structure. However, the glycan-specific fluorescence of CeTci-CF-1 was much lower than that of the high molecular mass PiTci-CF-1, possibly due to yeast-derived hyperglycosylation occurring at both predicted sites.

Further characterisation of the glycosylation of Tci-CF-1 versions was performed using a panel of lectins to determine which types of carbohydrate residues were present in each Tci-CF-1. Lectins have been used to detect, purify and characterise glycoproteins for many years by virtue of their specificity for certain carbohydrate residues (Bunkenborg *et al.* 2004, Glass *et al.* 1981). In studies of helminth parasites, lectins have been extensively used to isolate and purify *H. contortus* H11 and H-gal-GP antigens, subsequently used in immunisation studies (Smith *et al.* 1993, Smith *et al.* 1994). In another study, a 42 kDa antigen from *D. viviparus* adult ES bound to lentil lectin and ConA, and appeared to be synthesized in both a glycosylated and non-glycosylated form, both of which were recognised by serum antibodies from calves repeatedly infected with the parasite (Britton *et al.* 1993). Lectins have also been used to characterise antigens of the trematode *F. hepatica,* and highlighted the complexity of glycoconjugates of this parasite (McAllister *et al.* 2011). The results of the lectin binding experiments carried out in this chapter showed that each Tci-CF-1 version was glycosylated with a variety of carbohydrate residues; also that PiTci-CF-1 was hyper glycosylated, as shown by stronger binding of some lectins compared to either nTci-CF-1 or CeTci-CF-1. The three Tci-CF-1 versions did not show major differences in their glycan composition, with most of the lectins binding to a similar degree to each Tci-CF-1 version. However, one lectin (Peanut agglutinin, PNA) was able to bind to nTci-CF-1 but to a much lesser extent to PiTci-CF-1 and no binding was shown to CeTci-CF-1. This may indicate the presence of β-gal(1-3)galNAc residues in the glycosylation of nTci-CF-1, but not in the recombinant versions and could provide a partial explanation for the differences in immune recognition showed in previous results.

Here it was also shown that ConA and to a lesser extent LCH bound to carbohydrate residues on both isoforms of PiTci-CF-1, whereas other lectins only bound to the higher molecular weight protein band. This suggests some differences in the carbohydrate composition of glycans on the two PiTci-CF-1 forms expressed by the yeast cells. As *T. circumcincta* Tci-CF-1 had two predicted N-linked glycosylation sites, one on the pro-peptide and one on the mature enzyme (Redmond *et al.* 2006), differing structures in each of these glycans would change the reactivity of the molecule to lectins and antibodies after the pro-peptide was cleaved. If the lower molecular weight PiTci-CF-1 band was an inappropriately cleaved version (carried out by yeast cells but not enzymatically active), removal of all or part of the pro-peptide would also have removed the glycan structure linked to it. Therefore, if the two glycan structures of PiTci-CF-1 differed in composition, this was reflected in the slightly different pattern of lectin binding observed where only two of the lectins were able to bind both PiTci-CF-1 forms.

PNGase F and *O*-glycosidase enzymes were used to further investigate the glycosylation of Tci-CF-1 versions. PNGase F is an amidase frequently used to investigate carbohydrate structures, and releases most protein-bound N-linked carbohydrates except those with fucose attached to the 3 position of the Asn-linked GlcNAc residue (Morelle *et al.* 2009). *O*-glycosidase catalyzes the removal of Core 1 and Core 3 O-linked disaccharides from glycoproteins and it may also be used to characterise proteoglycan structures (Koutsioulis *et al.* 2008). The results of this chapter showed that a portion of PiTci-CF-1 N-linked glycans were cleaved, as the molecular mass of the protein band after PNGase F treatment was reduced, but native Tci-CF-1 or CeTCi-CF-1 were not. Indeed nTci-CF-1 protein appeared to be refractory to either PNGase or *O*-glycosidase treatment, as the molecular mass was unchanged. However, N-glycans with core fucose linked α1,3 to the Asn-bound N-acetylglucosamine have been shown to be resistant to the action of PNGase F (Tretter *et al.* 1991). Since these particular residues have been identified in helminth glycan structures previously (Haslam *et al.* 2000, Haslam *et al.* 1998, Khoo *et al.* 1997, Poltl *et al.* 2007, van Die *et al.* 1999), their presence cannot, at this stage, be excluded from Tci-CF-1 versions. In this situation, glycan analysis using PNGase A might have been more successful and could be included in further experiments of Tci-CF-1 glycan characterisation.

Further experiments would be required to repeat the glycosylation analysis with a larger amount of nTci-CF-1 and each recombinant version, testing various incubation times and conditions to investigate whether complete glycan removal is achievable. The results following *O*-glycosidase treatment were also unclear due to dilution of the proteins.

However neither protein showed predicted *O*-glycosylation sites in their amino acid sequences. These results agree with the study of Ov-CF-1 from *O. viverrini*, in which only a conserved N-linked glycosylation site was identified (Pinlaor *et al.* 2009). However, *O*-glycans are a common occurrence in helminths, as shown by Khoo *et al.* (1991). In this study, it was shown that *O*-glycans were major carbohydrate constituents of the *Toxocara canis* and *Toxocara cati* ES mixture. It was also reported that N-linked glycans were more commonly found on small molecular weight proteins, whereas *O*-glycosylation was more common in high molecular weight antigens (Khoo *et al.* 1991). Another study involved a number of helminth parasites, among which were *T. canis* and *Nippostrongylus brasiliensis*, which showed that a type of truncated *O*-glycosylation (GalNAc-*O*-Ser/Thr, termed Tn antigen) was commonly expressed in the helminth species examined (Casaravilla *et al.* 2003). These reports suggest that other *T. circumcincta* antigens are likely to present types of *O*-glycosylation and that they might play an important role in the immune response to the parasite.

Glycans have been shown to influence the immune response to antigens, either by masking epitopes or having higher immunogenicity than the protein backbone. These characteristics can mislead the antibody response and facilitate parasite escape from the immune system [reviewed by Harn *et al.* (2009) and Thomas and Harn (2004)]. For example, in a study carried out by Eberl *et al.* (2001) it was suggested that the antibody responses to *Schistosoma mansoni* larval glycans might be interpreted as a smoke screen to divert the host immune system [chimpanzees, *Pan troglodytes* (Eberl *et al.* 2001)]. The same mechanism may have played a role in the differences of antibody reactivity observed in this work to recombinant and nTci-CF-1. In the case of PiTci-CF-1, hyper-glycosylation may mask immunogenic protein epitopes and impair the antibody binding to amino acid structures. It was demonstrated here and Chapter 2 of this work that serum, ASC probes and gastric lymph antibodies were able to bind carbohydrate epitopes, as shown by the loss of antibody binding following periodate treatment of the *T. circumcincta* Tci-CF-1 versions considered. In the case of PiTci-CF-1, glycan-specific antibodies found in the lymph of *T. circumcincta* previously infected sheep may not have shown optimal recognition of the glycan structures on PiTci-CF-1 if these had a higher mass or very complex shape that hindered binding to the immunogenic glycans present on nTci-CF-1. Detailed glycan analysis of all three Tci-CF-1 versions is currently being carried out by Prof. Cornelis Hokke at the University of Leiden and results are expected to shed more light on any differences in the glycan structures of Tci-CF-1 versions.

The results of this Chapter have shown that denaturing conditions (as occur in SDS-PAGE/Western blot) allowed gastric lymph antibody to bind to each recombinant Tci-CF-1 version. This was in contrast with previous results from ELISA experiments (both in this Chapter and Chapter 2), where no antibody binding was shown to take place against PiTci-CF-1 or CeTci-CF-1. Further work showed that enzyme activity, as a measure of correct protein folding, was detected following activation with an exogenous enzyme for both recombinant Tci-CF-1 versions. The glycosylation of Tci-CF-1 versions was confirmed and investigated, showing a few differences between L4 ES-purified Tci-CF-1 and PiTci-CF-1/CeTci-CF-1, and also between the two recombinant versions; this may partially explain the differences in ELISA reactivity. This work has shown that *C. elegans* is a viable method to express *T. circumcincta* enzyme vaccine candidates which retain correct conformation and enzyme activity. In terms of immunological performance of a vaccine candidate, the ELISA reactivity should be considered more biologically relevant than Western blotting, as it is the folded version of the protein that will be targeted by B cells for the production of antibodies and not the denatured form. A possible alternative approach in the case of Tci-CF-1 may be to generate a recombinant version of this protein and denature it using the same conditions applied to Western blotting, before using it as denatured immunising agent. This may allow greater access of epitopes on the denatured form that are shared with the native version, and may therefore generate a more appropriate antibody response. Taken together these results imply that the ideal Tci-CF-1 protein to use as vaccine antigen has yet to be reached, and further work is required to identify the best version.

Chapter 5 – Characterisation of Enzyme Activity and Stimulatory Properties on Ovine Cells of Recombinant Tci-MIF-1 from *Teladorsagia circumcincta*

5.1 Introduction

A monocyte Migration Inhibitory Factor (MIF) was one of the first cytokines identified during studies on delayed hypersensitivity reactions in guinea pigs sensitised with *Mycobacteria* purified protein derivative [PPD; (Bloom and Bennett 1966, David 1966)]. In these studies, *in vitro* experiments showed that peritoneal exudate cells collected from guinea pigs (GP-PECs) that exhibited delayed hypersensitivity to specific *Mycobacteria* antigens were inhibited from migration through capillaries. A cell migration inhibitory factor which could not be dialysed and was hypothesised to be a protein, was present in the cell supernatant of lymphocytes isolated from GP-PECs and inhibited the migration of guinea pig peritoneal macrophages (Bloom and Bennett 1966, David 1966). The ability of MIF proteins to act as immune regulators was later confirmed; human MIF, secreted by peripheral lymphocytes, was demonstrated to possess the same migration inhibitory activity on guinea pig peritoneal exudate cells (Thor *et al.* 1968). Subsequently, cDNAs encoding human and mouse MIF were cloned and the expressed recombinant proteins characterised (Bernhagen *et al.* 1994, Weiser *et al.* 1989). Recombinant human MIF showed inhibition of the chemotactic migration of human peripheral blood monocytes stimulated by a gradient of monocyte chemotactic protein 1 (MCP-1), and was also able to inhibit the random migration of similarly isolated monocytes *in vitro* (Hermanowski-Vosatka *et al.* 1999). Since then, a number of MIF homologues have been identified in numerous species, including cattle, sheep, fish, nematode parasites and arachnids (Galat *et al.* 1993, Jaworski *et al.* 2001, Lopes *et al.* 2011, Sato *et al.* 2003, Vermeire *et al.* 2008); such widespread conservation suggests important biological functions among living organisms [reviewed by Calandra and Roger (2003)].

As well as the immunoregulatory functions described above, one striking characteristic of MIF that sets it apart from other cytokines is its enzymatic activity. The first report of the tautomerase activity of MIF was provided by (Rosengren *et al.* 1996), in which MIF purified from bovine eye lenses was able to catalyze the tautomerization of D-dopachrome

into 5,6-dihydroxyindole-2 carboxylic acid (DHICA). Another native MIF enriched from *T. circumcincta* L3 somatic extract was shown to possess the same type of enzyme activity (Nisbet *et al.* 2010b). Further studies on bovine MIF isolated from the eye lens showed that this protein also possessed oxidoreductase activity and was able to catalyse the keto-enol isomerization of p-hydroxyphenylpyruvate (HPP) and of phenylpyruvate (Rosengren *et al.* 1997). Moreover, studies on recombinant mutant versions of human MIF showed that a specific disulfide Cys_{57}-Ala-Leu-Cys_{60} motif (CALC) of the MIF amino acid sequence was responsible for thiol-protein oxidoreductase activity. When either cysteine residue was exchanged for serine, this enzyme activity was significantly lower compared to the wild type recombinant MIF (Kleemann *et al.* 1998). In the same study, the CALC motif was also shown to influence immunological functions: in a *Leishmania*-killing assay, mouse peritoneal macrophages showed significantly lower ability to kill parasitized cells when co-incubated with the mutant MIFs, compared to wild type [Kleemann *et al.* (1998) and reviewed by Thiele and Bernhagen (2005)].

In the free-living nematode *C. elegans*, four MIF genes have been identified; of these, Ce-MIF-2 and -3 transcripts were shown to be up-regulated in the dauer stage (Marson *et al.* 2001). Other evidence supports the hypothesis that in nematodes as well as mammals, MIF could be a multifunctional protein, with possible roles in response to environmental stressors and embryonic development (Marson *et al.* 2001, Pastrana *et al.*, 1998, Zang *et al.*, 2001). Indeed a previous study on *T. circumcincta* MIF has shown that the strongest expression of this gene was found in cDNA from L3 larval stages, suggesting that its functions lie within the metabolism of this pre-parasitic stage (Nisbet *et al.* 2010b).

In mammals, MIF is a pro-inflammatory cytokine expressed by a variety of cells of the immune and endocrine systems, and also by epithelial cells in direct contact with the "outside world", suggesting a role as a regulator of host responses to infection and stress [reviewed by Calandra and Roger (2003)]. For example, MIF was produced by a mouse anterior pituitary cell line following stimulation with *E. coli* LPS; furthermore, MIF secreted by mouse hypophysis *in vivo* was found to contribute to circulating MIF levels in the post-acute phases of endotoxaemia (Bernhagen *et al.* 1993). In other experiments, injections of recombinant murine MIF increased markedly the lethality of LPS-induced endotoxic shock in BALB/c mice and anti-recombinant MIF serum protected them fully from LPS challenge; this suggests that MIF proteins can interfere with and enhance the pro-inflammatory response observed following LPS challenge (Bernhagen *et al.* 1993, Calandra *et al.* 1995, Roger *et al.* 2001). Another study in mice showed that MIF could be

produced not only by activated T cells, but also by macrophages treated with LPS, TNFα and IFNγ; furthermore, MIF-activated mouse macrophages secreted TNFα in what appeared to be a pro-inflammatory loop (Calandra *et al.* 1994). MIF was also shown to counteract the protective effect of glucocorticoids during LPS challenge; in a model of endotoxic shock, mice that received recombinant murine MIF in addition to LPS and dexamethasone showed significantly lower survival rates (35 %) than the control mice which received LPS and dexamethasone only [80 %; $p < 0.005$ (Calandra *et al.* 1995)]. The addition of dexamethasone and neutralising anti-MIF IgG to human monocytes that had been stimulated *in vitro* with LPS inhibited the production of TNFα by 32.7 %, compared to only 9.4 % of cells stimulated with LPS in the presence of dexamethasone and control IgG; this suggests that during an inflammatory response MIF is able to override the anti-inflammatory effect of glucocorticoids (Calandra *et al.* 1995). Finally, it was demonstrated using macrophages from $MIF^{-/-}$ mice that MIF is required for the maintenance of activation-induced p53 induction and macrophage apoptosis and that, when MIF is lost, cell survival and function are compromised (Mitchell *et al.* 2002).

In recent years a novel subset of macrophages belonging to the Th2 arm of the immune response has been identified. These cells were named "alternatively" activated macrophages (AAMΦ), to differentiate them from other macrophages widely known as classically activated macrophages [CAMΦ (Goerdt and Orfanos 1999)]. CAMΦs are generated *in vivo* in pro-inflammatory environments and *in vitro* following stimulation with LPS or IFNγ. Upon activation, CAMΦs are able to express, amongst other cytokines and enzymes, TNFα, inducible Nitric Oxide Synthase (iNOS) and IL-12 (Coico *et al.* 2003, Goerdt and Orfanos 1999). Data gathered mostly from studies carried out in mice models showed that on the contrary, the novel macrophage phenotype, AAMΦ, occurs in strongly Th2-biased environments where IL-4 and IL-13 are present and are characterised by the production of IL-10, TGFβ, upregulated Mannose Receptor (CD206) and MHC II, markedly decreased TNFα and release of Nitric Oxide (NO) in favour of arginase-type enzymes [summarised in Figure 5.1; reviewed by Doyle *et al.* (1994), Kreider *et al.* (2007), Maizels and Yazdanbakhsh (2003), Noel *et al.* (2004), Stein *et al.* (1992)]. A defining characteristic of AAMΦ is the ability to inhibit T cell proliferation, in a contact-dependent manner (Loke *et al.* 2000). Most studies concur on the varied regulatory activity of AAMΦs on the immune response to infections, and on the inhibition of inflammatory reactions that can ultimately lead to tissue damage and pathology [reviewed by Jenkins and Allen (2010), Noel *et al.* (2004), Reyes and Terrazas (2007)].

Other genes have been found to be upregulated during alternative activation of mouse macrophages during parasitic infections (such as *Trichinella spiralis* and *Brugia malayi*), including Resistin-like molecules (RELMα), members of the chitinase family (acidic mammalian chitinase, AMCase), a lectin (Ym1) and arginase 1 (Arg1) (Chang *et al.* 2001, Loke *et al.* 2002, Nair *et al.* 2005, Raes *et al.* 2002). Interestingly, increased Arginase 1 activity inhibits the classical activation phenotype by competing with iNOS for the substrate L-arginine, and also helps inhibit T cell responses by depleting the environment of available arginine (Rutschman *et al.* 2001). In ruminants, it has been shown that adult *Fasciola hepatica* Excretory/Secretory (ES) products can elicit an alternatively activated phenotype in sheep and mouse macrophages *in vitro* (Donnelly *et al.* 2005, Flynn *et al.* 2007), and there is evidence that a chitinase-like molecule is upregulated in sheep abomasal tissue with *T. circumcincta* infections, suggesting that the presence of *T. circumcincta* may also induce AAMΦ *in vivo* (Knight *et al.* 2007).

Recently, a further role in the complex activity of MIF has been shown: *B. malayi* recombinant MIF was associated with the induction of AAMΦ in mouse models [(Falcone *et al.* 2001, Loke *et al.* 2002, Prieto-Lafuente *et al.* 2009); mechanism summarised in Figure 5.1]. It was also suggested that MIF produced by parasites and released during infection may be capable of activating an anti-inflammatory pathway through the recruitment of AAMΦ (Maizels and Yazdanbakhsh 2003). As numerous MIF orthologues have been identified in other nematodes (Vermeire *et al.* 2008), speculations were made about the anti-inflammatory role of parasite MIF when released in the host as a promoter of parasite survival in a hostile environment [reviewed by Kreider *et al.* (2007)].

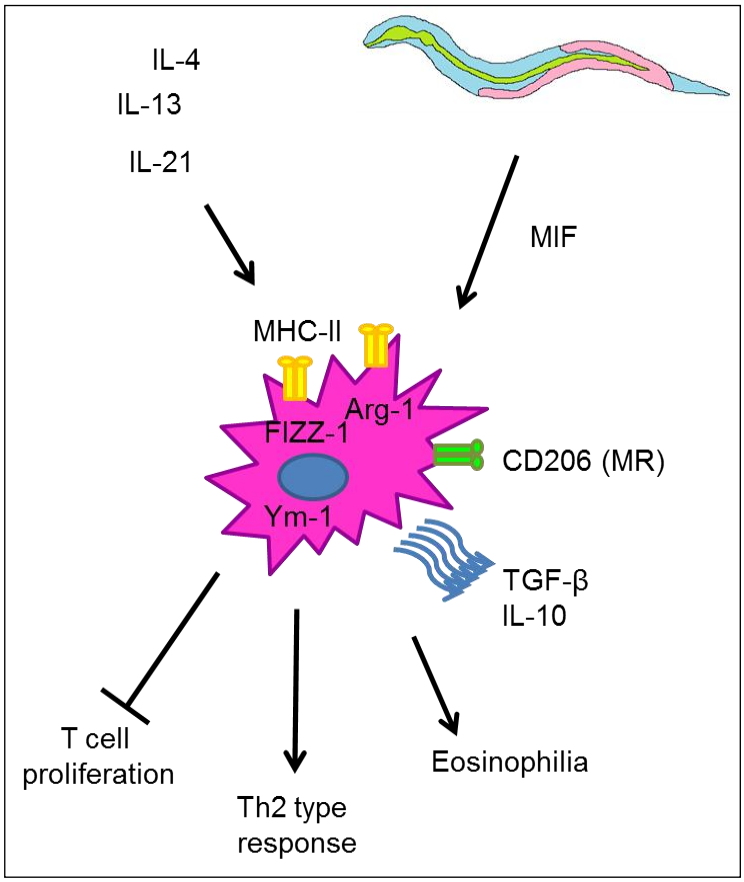

Figure 5.1 Diagram summarising the mechanisms of macrophage alternative activation and potential role of parasite-derived MIF in a mouse model.
Adapted from Vermeire et al. (2008). The cytokines IL-4, IL-13 and IL-21 synergise to induce alternative activation in macrophages, which is here shown as up-regulation of surface markers such as MHC-II and CD206 (Mannose Receptor). Other genes such as *Ym-1*, *Arg-1* and *FIZZ-1* are also up-regulated following exposure to IL-4 and IL-13. Alternatively activated macrophages initiate a Th2-biased immune response, by secreting TGF-β and IL-10. This can result in inhibition of T cell proliferation and promotion of eosinophil recruitment. It has been shown that parasite MIF could potentiate the mechanisms described when present at the same time as IL-4.

A MIF-like molecule (Tci-MIF-1) has been identified in *T. circumcincta* L3 extracts and it was shown that MIF mRNA was present in eggs, L3, L4 to a lower level and adult stages (Nisbet *et al.* 2010b). The protein content was highest in L3 extract and staining of the worms showed localization of the protein to the guts of L3, L4 and adult worms (Nisbet *et al.* 2010b). Although mammalian MIF acts as a pro-inflammatory cytokine, it has been demonstrated that MIFs derived from parasitic nematodes can induce alternative activation of macrophages, effectively acting as anti-inflammatory cytokines (Prieto-Lafuente *et al.* 2009). As Tci-MIF-1 might have an anti-inflammatory, immunosuppressing role facilitating parasite survival during *T. circumcincta* infection in sheep, it was decided to generate a recombinant version of Tci-MIF-1 and test its validity as vaccine candidate. Inhibition of monocyte migration is a function common to other recombinant or native

parasite MIFs (Pastrana *et al.* 1998, Tan *et al.* 2001). A recombinant version was expressed in *E. coli* and was shown to possess the dopachrome tautomerase enzyme activity associated with native MIF; however the results from monocyte migration inhibition assays on sheep cells did not convincingly show an inhibitory effect on monocyte migration (Nisbet *et al.* 2010b). The *E. coli*-expressed Tci-MIF-1 version was also included in immunisation trials where sheep received three immunisations with a cocktail of 8 *T. circumcincta* recombinant antigens, then were subsequently challenged with *T. circumcincta* L3 (2000 L3, three times a week for 4 weeks). The vaccine cocktail proved to be successful in reducing the faecal egg output by 59-70 % and worm burdens by 56-75 % in the immunised animals compared to the controls. The results also showed that *E. coli*-expressed Tci-MIF-1 was one of the least immunogenic antigens used in the vaccine (Nisbet *et al.* 2013). Although a component of a protective vaccine cocktail, it is currently unclear what role the Tci-MIF-1 generated in *E. coli* had on the overall levels of protection. In addition, although the enzymatic activity of recombinant Tci-MIF-1 was maintained, its inability to inhibit the migration of monocytes *in vitro* suggests that the recombinant version might have differed from the native *T. circumcincta* MIF. This may have occurred because of impaired post-translational modifications; therefore, in the work reported in Chapter 3 of this thesis, the nematode *C. elegans* was used to generate an alternative recombinant Tci-MIF-1 version in order to determine whether this expression system may be able to deliver a version of Tci-MIF-1 which maintains all its putative functions.

The overall aims of this, current, chapter were:

i) To express and purify recombinant Tci-MIF-1 using *E. coli*

ii) To characterise the enzymatic dopachrome tautomerase activity of recombinant CeTci-MIF-1 (generated in Chapter 3 of this thesis) and compare it with the enzyme activity of Tci-MIF-1 expressed in *E. coli*.

iii) To investigate the monocyte migration inhibitory factor activity of CeTci-MIF-1 and compare it with activity of Tci-MIF-1 expressed in *E. coli*.

iv) To use both recombinant Tci-MIF-1 versions to stimulate *in vitro* ovine macrophages and determine what type of activation, if any, this stimulation elicits.

5.2 Materials and Methods

5.2.1 Purification of recombinant Tci-MIF-1 in *E. coli* (EcTci-MIF-1)

A bacterial (*E. coli* BL21 codon (+) clone expressing Tci-MIF-1 was previously established and stored in 60 % glycerol at -80°C by Dr Alasdair Nisbet at MRI (Nisbet *et al.* 2010b). A scraping of the glycerol stock was incubated overnight in 10ml LB broth (Appendix D) at 37°C with shaking at 220 rpm. The process of induction of protein expression and purification of the bacterial recombinant Tci-MIF-1 had been previously described by (Nisbet *et al.* 2010b, 2013) and in Chapter 2, Section 2.2.4 of this thesis. The technique was reproduced for the experiments here described without modifications; the recipes for the buffers and solutions used are detailed in Appendix D.

5.2.2 Generation of recombinant Tci-MIF-1 in *C. elegans*

Recombinant Tci-MIF-1 was expressed using the free-living nematode *C. elegans* and purified as described in Chapter 3 of this thesis.

5.2.3 Dopachrome tautomerase activity assays

The procedure used in this assay was adapted from Nisbet *et al.* (2010b) and Pennock *et al.* (1998). Briefly, 720 µl of reaction buffer (Appendix D) were added to a 1 ml polystyrene disposable cuvette, followed by 4.8 µl of 0.1 M L-DOPA methyl ester and 3.2 µl of 0.2 M sodium *m*-periodate (both in Appendix D) to generate L-dopachrome methyl ester. The solution was mixed well by pipetting until the colour reached a uniform bright orange. Five microliters of each recombinant Tci-MIF-1 (diluted in PBS from stock solutions) or PBS as blank were added (approx. 250 ng protein/assay) and the loss of absorbance at 474 nm (A_{474}) was monitored for 2 min, recording reads at 15 sec intervals. Recombinant MIFs from the filarial parasite *Brugia malayi* ['Bm-MIF-1' (enzymically active) and 'Bm-MIF-1G' (enzymatically inactive; Zang *et al.* 2002)], provided by Prof. Rick Maizels (University of Edinburgh), were used as positive and negative controls. Once the absorbance data over 2 min were collected, a plot of time *vs* absorbance was generated and the slope of the linear phase was the rate of the reactions. Enzyme activity was expressed as loss of A_{474}/min/µg of enzyme. All assays were repeated in triplicate and repeated three times.

5.2.4 Stripping of LPS from purified recombinant Tci-MIF-1

LPS contamination was removed from recombinant proteins using the following protocol [adapted from Aida and Pabst (1990)]. Triton X-114 (Sigma-Aldrich, Gillingham, UK) was added to each sample to a final concentration of 1 %; this was left at 4°C rotating for at least 30 min or overnight to allow Triton X-114 to bind LPS. Samples mixed with Triton X-114 were incubated at 37°C for 10 min (samples became cloudy). These were centrifuged at 13 700 *x g* for 15 min at 30°C and the aqueous phase collected (temperatures above 23°C facilitated separation of the aqueous phase containing the LPS-free proteins). The aqueous phase was heated at 37°C for 5 min and centrifuged again at 13 700 *x g* for 15 min. The aqueous phase was again collected and ~20 µl were left at the bottom of each tube to avoid Triton X-114 contamination of the protein samples. This whole process was repeated 3 times for each protein sample, to ensure maximum LPS removal. Recombinant protein integrity was checked by polyacrylamide gel electrophoresis (Appendix B) and concentration was estimated using a BCA assay (Appendix B).

5.2.4.1 LPS quantification

LPS was quantified in protein samples used for cell stimulations using the Limulus amebocyte lysate (LAL) assay (Endpoint Chromogenic LAL Assay, Lonza, Basel, Switzerland) following the manufacturer's instructions. As the standard curve of the assay had a tight range (0.1-1 EU/ml), all samples were tested undiluted and diluted 1:10 in LPS-free dH_2O. All experiments were carried out using LPS-free reagents, glassware and polystyrene microplates. The wells were read using a microplate reader (Tecan, Sunrise) with filters set at 405 nm.

5.2.5 Differentiation of ovine macrophages from $CD14^+$ cells and bone marrow stem cells

5.2.5.1 Isolation of ovine $CD14^+$ cells from PBMC

Blood circulating monocytes in sheep are believed to amount to between 0-6 % of PBMC (Aiello 1998). Blood was collected by jugular venepuncture in EDTA (Vacutainer, BD Biosciences, Oxford, UK) or Alsever's solution (1:1 ratio, Appendix D) and PBMC isolated as previously described (Goddeeris and Morrison 1988). Briefly, blood was diluted 50 % with Alserver's solution or sterile PBS and carefully layered over 20 ml of Ficoll-Paque Plus (GE Healthcare, 2:3 ratio of Ficoll:blood) in 50 ml Falcon tubes. PBMC were isolated as described by Goddeeris and Morrison (1988), including cell washes.

After the last wash, PBMC were re-suspended in SCM (Appendix D) and counted in a haemocytometer by Trypan Blue exclusion. In order to carry out a positive selection on $CD14^+$ cells of PBMC, anti-human CD14 MicroBeads (Miltenyi Biotech, Bisley, Surrey, UK) were used following the manufacturer's instructions. The volumes of microbeads and buffers used were scaled up or down depending on the PBMC numbers isolated. The collected $CD14^+$ cells (labelled fraction) were washed once in MACS Buffer and re-suspended in SCM for counting as described above. The cells were then cultured *in vitro* as described below. Purity was assessed by morphology examination of cytospin slides of a 100 µl aliquot of cells (Section 5.2.7.1).

5.2.5.2 *In vitro* culture of ovine $CD14^+$ cells

$CD14^+$ ovine cells obtained as described in Section 5.2.5.1 were cultured at a concentration of 1×10^6 in flat bottomed 48-well or flat bottomed 96-well plates. The cells did not receive stimulation for 7 days and once the cells had adhered to the bottom of wells, half of the volume of the SCM was changed twice during this period, according to Flynn and Mulcahy (2008). This was to allow the differentiation of blood monocytes into mature macrophages (Mo-MΦ). Once cells appeared of the correct morphology, supernatant was harvested and stored at -20°C until use, and cell lysates obtained by re-suspending the adherent cells in 100 µl of 1 % Triton X-100 in PBS, shaken for 15 min at RT and stored at -20°C until use.

5.2.5.3 Isolation of ovine bone marrow stem cells

Bone marrow was collected from 7 sheep at post mortem. These animals were of a variety of ages (range 6 months to > 5 years old); the sheep were Scottish Blackface or crosses thereof. The animals had been part of control groups in experiments carried out by other research groups at MRI, with the exception of one animal (the oldest) which had been admitted from a commercial farm under suspicion of ovine pulmonary adenocarcinoma (OPA). Prior to use, all equipment was either autoclaved or sprayed with 70 % ethanol, or both, to sterilise it. The bone marrow-donor sheep were stunned and exsanguinated by a qualified person; during exsanguination, at least 500 ml of blood from each animal was collected into a sterile glass beaker for serum separation as previously described. Directly following exsanguination, the sterna were removed from each carcass and the excess adipose and muscular tissues removed with a sterile scalpel, until the periosteum was visible on the majority of the parts, which were also sprayed with 70 % ethanol. A clean, sterile handsaw was used to cut the sterna longitudinally along the median line and each

side was placed into the mouth of a cleaned vice lined with clean absorbent paper. Pressure was applied on the sternebrae until the bone marrow was squeezed out. The bone marrow was collected immediately using a sterile scalpel blade into a sterile universal containing ice cold Sheep Wash Medium (SWM, Appendix D), mixed gently and put on ice. The process was repeated with the other half of the sternum.

Under a tissue culture hood, the bone marrow cells were harvested from below the layer of fat that floated above the SWM using a sterile syringe and a 19G needle. The bone marrow cells were filtered through a double layer of sterile lens tissue paper into a glass beaker. The filtrate was centrifuged 550 *x g* for 10 min. The supernatant was discarded and the cell pellets resuspended and pooled in 50 ml of SWM. Subsequently, 25-30 ml of cell suspension was gently laid over 20 ml of Ficoll-Paque Plus (GE Healthcare, ratio Ficoll:cell = 2:3). The Ficoll-cell suspension was then centrifuged at 900 *x g* for 30 min without braking. The cells at the interface between the Ficoll and the SWM were collected and washed with SWM, then centrifuged at 550 *x g* for 10 min. The cells were resuspended in 1 ml SWM, then 1 ml of pre-warmed red blood cell Lysis Buffer (Appendix D) was added and the suspension was incubated at 37°C for 45 sec-1 min, after which fresh SWM was added and the cells centrifuged at 550 *x g* for 10 min. One last wash in SWM was carried out after which the cell pellet was resuspended in Bone Marrow Culture Medium (BM-CM, Appendix D) and a viable cell count (Trypan Blue exclusion) was carried out. The cells were then cultured *in vitro* as described below.

5.2.5.4 *In vitro* culture of ovine bone marrow stem cells

Bone marrow cells were re-suspended in BM-CM (Appendix D) at a concentration of 2 x 10^5 – 1 x 10^6 cells/ml and placed in a sterile non adherent T75 tissue culture flask (Sarstedt, Leicester, UK), with no other stimulation. All cells were cultured in the presence of 20 % FBS and 20 % autologous sheep serum (both heat inactivated) to allow macrophage maturation in the presence of Macrophage colony-stimulating factor (M-CSF)-like factors naturally present in serum (Francey *et al.* 1992, Francey *et al.* 1992). Bone marrow stem cells from one animal were cultured under different conditions to investigate the optimal incubation protocol to facilitate macrophage differentiation from the pool of monocyte and macrophage precursor cells. The bone marrow stem cells were cultured at the same concentration range (2 x 10^5 – 1 x 10^6 cells/ml) in either a Teflon-coated cell culture bag, a non-adherent polystyrene tissue culture flask, a polystyrene 6-well tissue culture plate or a polystyrene 24- well tissue culture plate, in order to

investigate the optimal differentiation rates and recovery of mature macrophages. In all cases, cells were left to differentiate for 15-18 days, by which point the majority of cells were adherent, with morphology consistent with macrophage type as assessed by staining of cytospin slides (described in Section 5.2.7). The adherent cells were detached using TrypLE™ Express 1X (Gibco, Life Technologies, Paisley, UK), collected in a 50 ml Falcon tube and centrifuged at 550 *x g* for 10 min, re-suspended in BM-CM, counted and characterised as described below prior to stimulation (BM-MΦ).

5.2.6 Collection of ovine alveolar macrophages

This procedure was carried out as described by McNeilly *et al.* (2008) without modifications. An average of 500 ml of SWM had been used for each set of lungs. The cell rich suspension was washed three times in SWM and the abundant, frothy surfactant removed by filtering the solution through lens tissue paper. Cells were finally re-suspended in SCM and counted. These cells (A-MΦ) were used in functional assays immediately.

5.2.7 Morphological and functional characterisation of ovine monocytes/macrophages

5.2.7.1 Fluorescence-Activated Cell Sorting (FACS) analysis

Sheep PBMC were isolated from 9 animals and flow cytometry carried out in order to determine the percentage of $CD14^+$ cells within the PBMC fraction. Aliquots of 1-2 x 10^5 cells in 100-150 µl were resuspended in 75 µl of FACS medium (Appendix D) in a 96-wells U-bottomed culture plate. To the cells, 25 µl of a mouse anti-human CD14:Alexa 647 antibody (dilution 1:1000; clone TUK4, IgG2a; MCA1568A647, Serotec) were added and incubated at 4°C for 30 min. Following incubation, the cells were washed 3 times in FACS medium (by centrifugation of the microplate at 1500 *x g* for 20 sec) and resuspended in 200 µl of 1X PBS. After one further wash, the cells were fixated in 200 µl of cell fixating medium (Appendix D) until analysis in a flow cytometer. The machine used was a Cyan™ ADP Flow Cytometer (Beckman Coulter Inc., Fullerton, CA, USA), using the manufacturer's acquisition software (Summit v4.3).

5.2.7.2 Cytospin of ovine cells

Ovine monocytes or macrophages were re-suspended at 1-2 x 10^5 in 100-150 µl standard culture medium or sheep wash medium (SCM and SWM, Appendix D) and smeared onto glass slides by centrifugation at 300 *x g* for 3 min using a Cytospin cytocentrifuge

(Shandon Thermo Scientific). The slides were left to dry at RT for 10 min before proceeding with staining procedures.

5.2.7.3 Hematoxylin-eosin stain

The air-dried slides were stained to visualize cell morphology using a hematoxylin-eosin based stain, Reastain Quick-Diff Kit (Reagena, Toivala, Finland) following the manufacturer's instructions. The slides were mounted with a glass coverslip using Consul-Mount mountant for glass cover slides (Shandon Thermo Scientific). Slides were prepared from at least 2 sheep and cells counted for the purposes of cell identification and descriptive statistics analysis. Cells from at least 5 fields in each slide were counted and differentiated on the basis of their morphology.

5.2.7.4 Non-specific esterase stain

Air-dried slides were subjected to the α-Naphthyl acetate esterase procedure (Kit 91A, Sigma-Aldrich, UK) as described by the manufacturer's instructions for the staining of non-specific esterase enzymes in monocytes. After the staining procedure was completed, slides were mounted with an aqueous mounting medium (Crystal Mount™ Aqueous Mounting Medium, Sigma-Aldrich). Slides were prepared from at least 2 sheep and cells counted for the purposes of cell identification and descriptive statistics analysis. Cells from at least 5 fields in each slide were counted and differentiated on the basis of the positive/negative non-specific esterase staining.

5.2.7.5 Immunocytochemistry (HCC) for CD68 receptor

The immunocytochemistry staining for CD68 receptor was carried out using an EnVision™ Kit (Dako, Ely, UK), following the manufacturer's instructions without modifications regarding dilutions and volumes of reagents used. Following an incubation in 3 % hydrogen peroxide (H_2O_2) in methanol for 20 min to remove endogenous peroxidase activity, the slides were washed with PBS. The reagents used subsequently were: for blocking, 100 μl of 25 % normal goat serum in PBS; primary antibody, a monoclonal mouse anti-human CD68 (clone EBM11 IgG1 κ, Dako); antibody isotype control, a mouse IgG1κ (clone MOPC-21, Sigma-Aldrich, Gillingham, UK). The secondary antibody used was a peroxidase-labelled anti-mouse EnVision™ with reagent (Dako; provided with the kit); the chromagen solution was DAB chromagen in substrate buffer (Dako; provided with the kit). At the end, slides were stained with haematoxylin in

an automatic slide stainer (Varistain 24-4, Shandon Thermo Scientific, Runcorn, UK) and mounted with Crystal Mount™ Aqueous Mounting Medium (Sigma-Aldrich). Slides were prepared from at least 2 sheep and cells counted for the purposes of cell identification and descriptive statistics analysis. Cells from at least 5 fields in each slide were counted and differentiated on the basis of their staining of CD68.

5.2.7.6 Phagocytosis assay

A cell phagocytosis assay using fluorescent FITC-labelled *E. coli* as target was adapted from Werners *et al.* (2004). Briefly, 1 x 10^5 ovine macrophages were placed in each well of a chamber slide and incubated at 37°C, 5 % CO_2 for 2 h. FITC-labelled *E. coli* cells (stock ~2 x 10^7/ml) were added at a range of macrophage:bacteria ratios (e.g. 10:1, 5:1, 1:1, no bacteria) and incubated at 37°C, 5 % CO_2 for 2 h. After 2 h the medium was removed and 1 ml of quencher solution (250 mg/ml trypan blue in citrate buffer, pH 4.4) added. The wells of the chamber slide were washed 1-2 times with PBS and fixed with 4 % paraformaldehyde (PFA) for 1 h at RT. After another 2 washes in PBS, the cell nuclei were stained with DAPI for 10-15 min, then the wells of the chamber slide were washed in PBS and the well partitions removed. The slide was mounted with Crystal Mount™ Aqueous Mounting Medium (Sigma-Aldrich) and clear nail varnish used to seal the edges. If not immediately visualized, the slides were stored at 4°C for a maximum of 24 h. Fluorescent cell nuclei and FITC-labelled *E. coli* were visualized using an Axiovert 200M inverted fluorescence microscope (Carl Zeiss Ltd., Welwyn Garden City, UK).

5.2.8 Monocyte migration assays

Ovine $CD14^+$ cells isolated as described in Section 5.2.4 were used in *in vitro* fluorimetric cell migration tests. The kit used was QCM Chemotaxis Cell Migration Assay, 96-well (5 μm; Millipore, Billerica, MA, USA), following the manufacturer's instructions with adaptations. The adaptations were as follows: the cells were counted and resuspended in monocyte migration assay quencher (MoMQ, Appendix D) to 1 x 10^6/ml. Two recombinant Tci-MIF-1 versions (EcTci-MIF-1 and CeTci-MIF-1) were added to a final concentration of 1 μg/ml. Two negative controls of *C. elegans* N2 extract and an extract of uninduced *E. coli* cells (BL21 (+) carrying the Tci-MIF-1 plasmid but where protein expression was not induced) were used throughout at the same final concentration as recombinant Tci-MIF-1. Cells (1 x 10^5 in 100 μl with or without stimulating molecules) were always placed in the top assembly compartments (inside the 5 μm-filter wells; see Figure 5.2). Monocyte migration assay medium (150 μl MoMM, Appendix D), with or

without stimulating molecules, was placed in the bottom compartments (migration feeder tray). Once the tray/filter assembly was complete with cells and stimulating molecules, it was placed at 37°C, 5 % CO_2 for 3 h to allow the cells to migrate through the 5 µm filter into the bottom compartment. After 3 h, the cells that migrated were harvested without modifications as described in the manufacturer's instructions for the QCM Chemotaxis Cell Migration Assay kit. Fluorescence emitted by the cells that had migrated through the filter was measured with a Cytofluor 4000 microplate reader (PerSeptive Biosystems, now Applied Biosystems, Foster City, CA, USA) using a 480 nm excitation/520 nm emission filter set. The fluorescence data were used to calculate the percentage of migration in each well, using the fluorescence emitted by wells containing cells incubated with medium only as 100 %. Positive controls were 7.5 x 10^5 cells lysed directly in the presence of CyQuant GR Dye in two wells of the black plate.

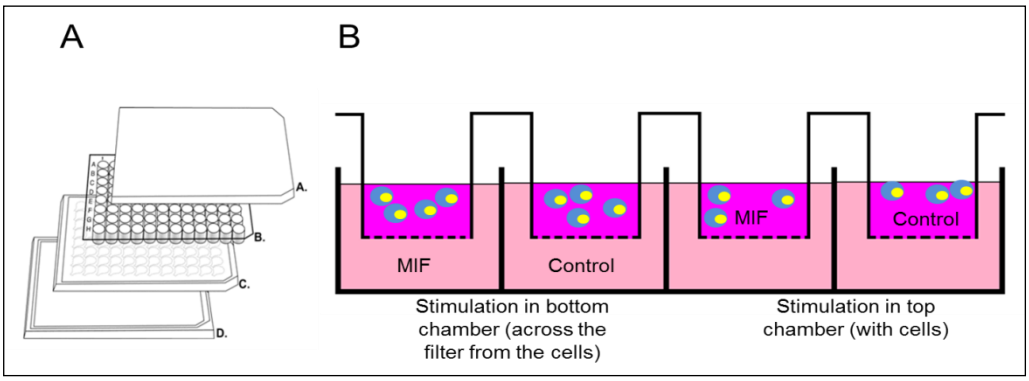

Figure 5.2 Diagrams of the filter-tray assembly used in the monocyte migration assays. Panel A: adapted from the kit instructions manual (ECM512, Millipore). A: lid; B: 5 µm -filter wells; C: 96-well feeder tray (2 provided); D: base. Panel B: a cross-section showing the placement of cells and stimulating MIF molecules or controls on relation to each other and the 5 µm filter.

5.2.9 Ovine macrophage *in vitro* stimulation protocols

5.2.9.1 Stimulation protocol for Mo-MΦ

Blood monocyte derived macrophages (Mo-MΦ) generated as previously described (Section 5.2.5.2) were used in stimulation assays as follows: 2 x 10^5 cells/well were placed in each well of a flat bottomed 96-well plate. To the cells, the following molecules were added: LPS A (*E. coli* O55:B5 LPS, Sigma-Aldrich); LPS B (*E. coli* O111:B4 LPS, Sigma-Aldrich); LPS C (*E. coli* O111:B4 LPS, Invivogen, San Diego, CA, USA), all to a final concentration of 20, 10 and 5 µg/ml. Wells that received 20 µg/ml of each LPS were harvested at 24 and 48 h; wells that received 10 or 5 µg/ml were only harvested at 48 h.

LPS was titrated to be used as positive control for classical activation of macrophages, whereas ovine recombinant IL-4 was used as inducer of alternative activation phenotype. Ovine recombinant IL-4 (ov rIL-4, a gift from Dr Sean Wattegedera, MRI) was diluted in SCM from a stock solution of 15.4 µg/ml and was added to final concentrations of 1.54, 0.308, 0.154 and 0.077 µg/ml; these wells were harvested at 48 h only. Treatments were administered to single wells only, due to constraints with cell numbers.

5.2.9.2 Stimulation protocol for BM-MΦ

Bone marrow-derived macrophages (BM-MΦ generated as previously described in Section 5.2.5.4) were stimulated as follows: 10^5 cells/well (for the first pilot experiment in which cells were tested with *E. coli* or *Rhodobacter sphaeroides* LPS (Invivogen), Figure 9.2, Appendix C) or 2 x 10^4 cells/well (for every other subsequent experiment) were added to flat bottomed 96-well plates. Cells were stimulated with the following: LPS B (*E. coli* O111:B4 LPS, Sigma-Aldrich) and *R. sphaeroides* LPS (Invivogen) at a final concentration of 10 µg/ml each. Recombinant ovine IL-4 was used at a final concentration of 0.3 µg/ml. *Escherichia coli*-expressed Tci-MIF-1 (EcTci-MIF-1) and *C. elegans*-expressed Tci-MIF-1 (CeTci-MIF-1) were both used at a final concentration of 10 µg/ml. All treatments were administered in duplicate wells and LPS contamination of recombinant proteins had not been evaluated at this stage.

5.2.9.3 Stimulation protocol for A-MΦ

Alveolar macrophages (A-MΦ) collected as described in Section 5.2.6 were stimulated as follows: 10^5 cells/well were placed in flat bottomed 96-well plates. All treatments of *E. coli* LPS (O111:B4, Sigma-Aldrich), EcTci-MIF-1 and CeTci-MIF-1 were used at a final concentration of 5 µg/ml. Recombinant ovine IL-4 was used at a final concentration of 0.3 µg/ml. All preparations had been de-contaminated from LPS as described in Section 5.2.4.1. All treatments were administered in duplicate wells.

5.2.10 Cytokine ELISA

Cell supernatant from all ovine macrophage cultures were used in ELISA assays to measure the release of the cytokines IL-10 and TNFα.

5.2.10.1 Ovine IL-10 ELISA

Secretion of IL-10 in the cell culture supernatant was investigated with a sandwich ELISA using the following adapted protocol (provided by Dr Tom McNeilly): high binding microplates were coated with 100 µl/well of a capture antibody (final concentration 4 µg/ml of monoclonal mouse anti-bovine IL-10, MCA2110, AbD Serotec), diluted in carbonate coating buffer (Appendix D), then incubated at 4°C overnight. The following day the plates were washed 4 times in PBST (Appendix D), wells were blocked with 200 µl/well of blocking buffer (3 % BSA in PBST) and after 1 h incubation at RT, plates were washed 4 times with PBST. A standard curve of recombinant ovine IL-10 was prepared by diluting a stock solution of recombinant ovine IL-10 (kindly provided by Sean Wattegedera, MRI) in Reagent Dilution Buffer (RDB, 1 % BSA in PBST) to obtain a concentration of 13.2 U/ml for the top standard. A 2-fold serial dilution in RDB was used to complete the standard curve (range 13.2-0.026 U/ml). Fifty microliters of either standards or cell culture supernatants was added to each well and incubated for 1 h at RT. Wells were washed 4 times in PBST and 50 µl of biotin-conjugated detection antibody (final concentration 1 µg/ml of monoclonal mouse anti-bovine IL-10, MCA2111B, AbDSerotec) were added to each well; the plate was then incubated for 1 h at RT. Plates were washed 4 times in PBST and 50 µl of Streptavidin polymer-HRP (Sigma-Aldrich, 1:1000 dilution in RDB) were added to each well; plates were incubated at RT for 45 min, then washed 4 times in PBST. One hundred microliters of TMB substrate were added to each well (SureBlue™ TMB Microwell Peroxidase Substrate, KPL Inc., Gaithersburg, MA, USA) and incubated for 5-10 min. Reactions were stopped using 100 µl/well of TMB Stop Solution (KPL, Inc.) and plates were read using a Sunrise microplate reader (Tecan, Reading, UK) with a 450 nm filter.

5.2.10.2 TNFα ELISA

Investigation of the release of TNFα from ovine macrophage cultures was carried out using a commercially available kit (Bovine TNFα Vet Set, Kingfisher Biotech, St. Paul, MN, USA). The manufacturer's instructions were followed to carry out the experiments. This kit uses the sandwich ELISA technique and provided microplates coated with an anti-bovine TNFα antibody (cross-reactive with sheep TNFα), pre-blocked and ready to use. A bovine TNFα standard was provided to generate a standard curve, together with an anti-bovine TNFα detection antibody (cross-reactive with sheep TNFα). Biotin-conjugated streptavidin (provided with the kit) and TMB substrate (SureBlue™ TMB Microwell

Peroxidase Substrate, KPL Inc.) were used to detect antibody binding. A Sunrise microplate reader (Tecan) was used with a 450 nm filter.

5.2.11 Analysis of macrophage transcripts by quantitative RT-PCR

5.2.11.1 Extraction of total RNA from ovine macrophages

The Qiagen RNeasy Plus Mini kit was used each time. Briefly, between 1-2 x 10^5 cells were harvested from flat-bottomed 96-well plates following stimulation by adding 200µl of TrypLE™ Express (Invitrogen, Paisley, UK) in each well and incubating the cells at 37°C, 5% CO_2 for 10 min. The cells were detached by pipetting and transferred into sterile, RNAse-free Eppendorfs and pelleted by centrifuged at 300 *x g* for 5 min. The supernatant was discarded and the cell pellet either frozen at -80°C prior to processing, or processed immediately using an RNEasy Plus Mini kit (Qiagen) to extract total RNA according to the manufacturer's instructions. As part of the kit protocol, samples were passed through a gDNA Eliminator column (Qiagen) to eliminate genomic DNA. The concentration of RNA was estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific) and stored at -80°C until use.

5.2.11.2 Generation of cDNA from mRNA

Complementary DNA (cDNA) was generated following two procedures, for logistical reasons of different kits available at the time of the cDNA generation. The first method used reagents supplied as *Precision*™ nanoScript Reverse Transcription kit by PrimerDesign Ltd. (Southampton, UK) and it was carried out following the manufacturer's instructions (for details of each reaction refer to appendix A). The second method used to generate cDNA was carried out using reagents already present in the laboratory, following the method detailed by McNeilly *et al.* (2008); the reaction volumes and conditions are also detailed in Appendix A. Complementary DNA thus generated was stored at -20°C until use.

5.2.11.3 Oligonucleotides

Primers used in qRT-PCR reactions for ovine *iNOS-2*, *Chit3-L1*, *Arg-1*, *YWHAZ* were designed and optimized by PrimerDesign Ltd. (Gold Sponsorship Student Award). The primers were supplied as pre-mixed solutions of forward and reverse primers, at a working concentration of 6 pmol each. Primers for ovine *TNF-α*, *IL-10*, *IL-8*, *IL-12p40* and *GAPDH*

were as described by McNeilly *et al.* (2008) and were supplied by Eurofins MWG Operon (Ebersberg, Germany) and diluted to a stock concentration of 100 pmol. The working concentration of all primers was 10 pmol. All primer sequences and the gene accession numbers are reported in Appendix A.

5.2.11.4 Standard qPCR reactions

For the *iNOS-2*, *Chit3-L1*, *Arg-1*, *YWHAZ* qRT-PCR assays, each standard reaction contained reagents provided by PrimerDesign Ltd. (see Appendix A for details of the reaction conditions). For all other assays (*IL-10*, *IL-8*, *IL-12p40*, *TNFα*, *GAPDH*), each standard reaction also contained PrimerDesign Ltd. reagents, however the cycling conditions were maintained as described by McNeilly *et al.* (2008) (see Appendix A for details).

Normalisation of data (copy number/µl) was carried out using the following calculation:

$$C = \frac{\text{Geomean [A1; A2]}}{B}$$

And the following:

$$E = \frac{D}{C}$$

Where: C was the normalisation coefficient; A was the geometric mean of the copy number/µl in each of the two housekeeping (HK) genes *YWHAZ* and *GAPDH* for each cDNA sample considered; B was the average value of the housekeeping genes geometric means across all cDNA samples; E was the normalised copy number/µl of each test gene and D was the copy number/µl in each cDNA sample for each of the test genes investigated. Values E were plotted and used for statistical analysis of differences in gene expression.

5.2.11.5 GeNorm analysis

Appropriate HK sheep genes were chosen from a panel of 12 provided in a geNorm kit by PrimerDesign Ltd. Reactions were prepared with cDNA derived from six BM-MΦ cell cultures stimulated with a series of molecules (*E. coli* O111:B4 LPS, *R. sphaeroides* LPS, EcTci-MIF-1, ov rIL-4, and unstimulated cells control; see section 5.2.10 for details) and

cDNA from 15 A-MΦ cultures that had been stimulated with the molecules detailed above for BM-MΦ, with the addition of CeTci-MIF-1. C_t values were collected for all cDNA samples across the candidate HK genes in the panel and analysed with the software qBase (Biogazelle, Belgium) to determine the most stable genes for this type of ovine cell culture.

5.2.12 Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 5.01 and GenStat 12th Edition. Arithmetic means with standard errors and replicate number (n) are shown throughout this chapter. Data were checked for normal distribution by analysing the histogram of frequencies and residual plots. If data were not normally distributed, a Box-Cox transformation (Box 1964, Peltier *et al.* 1998) was used to choose the appropriate transformation method; the details of each transformation are provided for the data sets in each figure throughout this chapter. For data normally distributed (with or without transformation), a one-way Analysis of Variance (ANOVA) test was used, followed by the Tukey post hoc test for pair wise comparison of means; when only one pair of means was compared, a two-tailed unpaired t-test was used. If data were not normally distributed and Box-Cox analysis failed to suggest an appropriate transformation method, the non-parametric Mann-Whitney test and Kruskal-Wallis test were used (details are provided for the data sets in each figure). *P* values of < 0.05 were considered significant.

5.3 Results

5.3.1 Purification of *E. coli*-expressed recombinant Tci-MIF-1

Recombinant Tci-MIF-1 expression was induced in an *E. coli* BL21 (+) codon clone provided by Dr Alasdair Nisbet. The recombinant protein was purified from the cell pellets as a soluble fraction and the identity confirmed by MALDI-ToF. Figure 5.3 shows the pattern of elution of the protein from the bacterial cell lysate. The recombinant EcTci-MIF-1 was purified using a Ni^{2+} IMAC system (as described in Section 5.2.1) and the protein was visualised in polyacrylamide gels using a Coomassie blue-based stain.

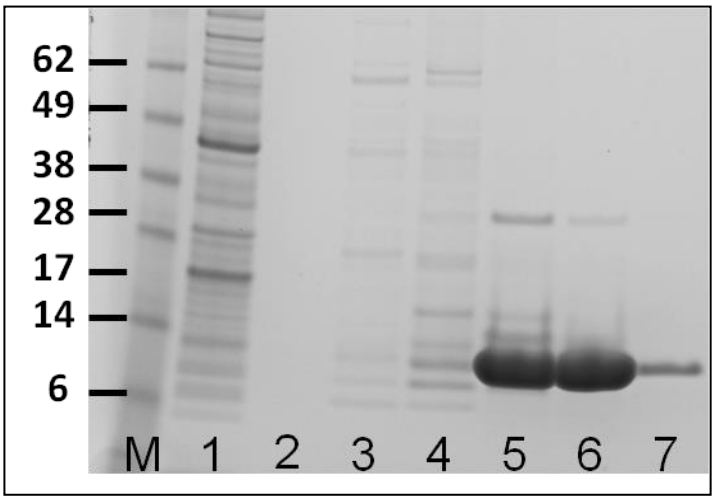

Figure 5.3 SDS-PAGE analysis of the fractions eluted by Ni^{2+} IMAC from *E. coli* BL21 (+) cell pellet expressing Tci-MIF-1.
Coomassie stained gel. Lane M: molecular weight marker (kDa); lane 1: Flow through; lane 2: fraction eluted at 20 mM imidazole; lane 3: fraction eluted at 50 mM imidazole; lane 4: fraction eluted at 200 mM imidazole; lane 5: fraction eluted at 350 mM imidazole; lanes 6-7: proteins eluted at 500 mM imidazole. Tci-MIF-1 is the prominent band in both lane 5 and 6.

5.3.2 Dopachrome tautomerase activity of recombinant Tci-MIF-1 versions

The ability of recombinant Tci-MIF-1 versions (EcTci-MIF-1 and CeTci-MIF-1) to tautomerise the substrate l-dopachrome methyl ester was assessed in a dopachrome tautomerase enzyme activity assay. The results of the experiment are shown in Figure 5.4. CeTci-MIF-1 was able to tautomerise the substrate l-dopachrome methyl ester indicating it possessed dopachrome tautomerase activity; however, CeTci-MIF-1 showed significantly lower dopachrome tautomerase activity compared to EcTci-MIF-1 ($p < 0.01$). The enzyme activity was significantly higher than the PBS negative controls for both EcTci-MIF-1 and CeTci-MIF-1 ($p < 0.001$). The enzyme activity of EcTci-MIF-1 was also significantly

higher than the extract of BL21 (+) cells ($p < 0.001$) and the enzyme activity of Ce Tci-MIF-1 was significantly higher than the extract of N2 *C. elegans* worms ($p < 0.001$). The enzyme activity of both recombinant *T. circumcincta* Tci-MIF-1 proteins was compared with a positive and a negative control provided by Prof. Rick Maizels; these were a recombinant version of wild type *Brugia malayi* MIF-1 and a mutant of the same protein with a Gly residue instead of Pro at amino acid position 2, both expressed in *E. coli* (Zang *et al.* 2002). The results showed that EcTci-MIF-1 enzyme activity was not significantly different from Bm-MIF-1; while CeTci-MIF-1 showed dopachrome tautomerase activity, this was significantly lower than Bm-MIF-1 ($p < 0.001$) based on the same amount of total protein assayed.

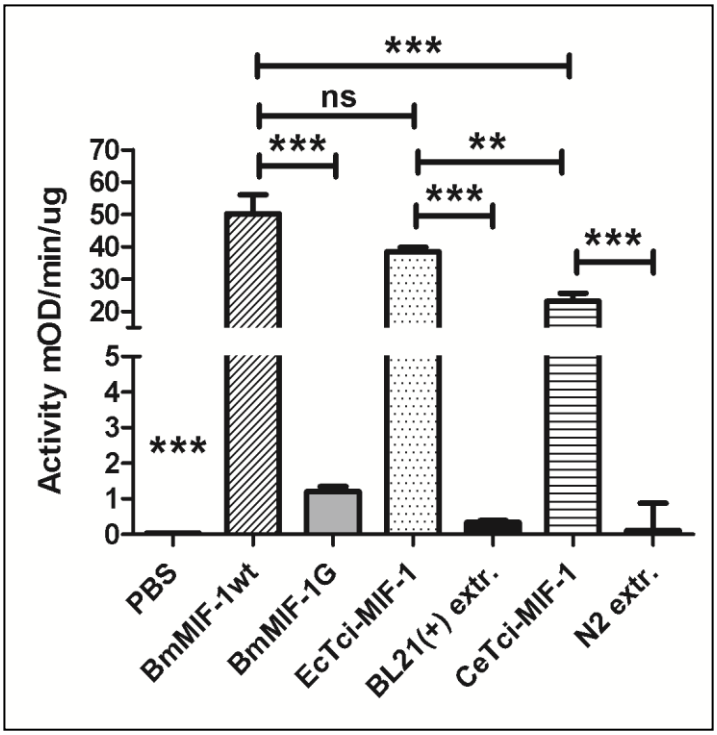

Figure 5.4 Dopachrome tautomerase activity of two recombinant Tci-MIF-1 versions and two recombinant Bm-MIF-1 versions, measured in the linear phase of the reaction.
The enzyme activity was calculated as Δ mOD (A_{474}) per minute per µg of protein. Spontaneous decay of L-dopachrome methyl ester as a result of decarboxylation of the substrate was measured by adding an equal volume of PBS instead of enzyme to the assays. Data were transformed with the formula $y = x^{0.25}$ to carry out statistical analysis. One-way overall ANOVA *p* value relative to transformed data is shown below the graph; *** $p < 0.001$; ** $0.001 < p < 0.05$; ns, not significant. Untransformed data are shown, representing mean±SEM of 3 experiments in which samples were analysed in triplicate (n=3). Negative controls were: PBS (used instead of enzyme), Bm-MIF-1G (a recombinant *B. malayi* MIF-1 version in which the Pro-2 residue was mutated to a Gly), *E. coli* BL21 (+) cell extracts (eluted at the same imidazole conditions as EcTci-MIF-1 from cells carrying the Tci-MIF-1 plasmid, but not induced for protein expression), and N2 *C. elegans* worms extracts (eluted from worms under the same conditions used to purify CeTci-MIF-1). Recombinant Bm-MIF-1wt was a positive control.

5.3.3 Removal of LPS contamination from purified recombinant Tci-MIF-1

In order to correctly interpret results from the *in vitro* stimulation of monocytes (in migration assays) and alveolar macrophages, LPS was removed from the recombinant Tci-MIF-1 versions. Initial LPS contamination was quantified by LAL assay and shown to be 0.12 EU/μg of EcTci-MIF-1 and 2.26 EU/μg of CeTci-MIF-1. Following 2-3 rounds of LPS removal using Triton X-114, the LPS contamination was quantified again by LAL assay and estimated between 1.6-9.7 mEU/μg protein (1 pg LPS = 10 EU). Levels of LPS < 0.05 pg/ml LPS per ng/MIF proteins were considered negligible in an *in vitro* experiment using human monocytes and recombinant human and murine MIF (Hermanowski-Vosatka *et al.* 1999). Therefore, although it was not certain whether cells from different species might show similar negligible reactivity to low levels of LPS, it was assumed that the remaining LPS contamination (≤ 9.7 fg of LPS per ml of MIF) would not have negatively affected or altered results in the sheep monocyte migration and macrophage stimulation experiments carried out in the study described here. As the recombinant Tci-MIF-1 proteins had to be heated at 37°C for the process involving Triton X-114, an aliquot of the LPS-free Tci-MIF-1 proteins was tested for maintained dopachrome tautomerase activity after being heated (as measure of correct folding of the active site). Following decontamination, both Tci-MIF-1 versions maintained dopachrome tautomerase activity in the LPS-free protein aliquots (data not shown). This suggested that no major denaturation of these proteins occurred during LPS-decontamination treatment and that therefore they could be used in cell stimulation experiments.

5.3.4 Monocyte migration inhibitory factor activity of recombinant Tci-MIF-1 versions

Two versions of recombinant *T. circumcincta* MIF, EcTci-MIF-1 and CeTci-MIF-1, were used in a *trans*-filter migration assay to investigate the effect of each on the migration of blood-derived ovine $CD14^+$ monocytes. To test the ability of Tci-MIF-1 to inhibit cell migration, cells were placed in the top compartment of the filter/tray assembly (above the 5μm filter) in the presence of either recombinant Tci-MIF-1 version or their controls [BL21 (+) *E. coli* cells containing the Tci-MIF-1 plasmid where protein expression had not been induced, and *C. elegans* N2 extract fraction eluted from cleared lysate at the same imidazole concentration as CeTci-MIF-1 (N2)]. To test the ability of Tci-MIF-1 to induce enhanced migration of cells through the filter, in some experiments cells were placed in the top compartment and recombinant Tci-MIF-1 versions or their controls were placed in the

bottom compartment (across the filter from the cells). The results from three experiments showed a trend of CeTci-MIF-1 to inhibit the migration of ovine $CD14^+$ monocytes through the filter compared to cells incubated in the absence of MIF (*C. elegans* N2 extract or uninduced *E. coli* BL21 (+) extract). However, no significant difference was observed in the inhibition of the migration of cells between treatments (Figure 5.5).

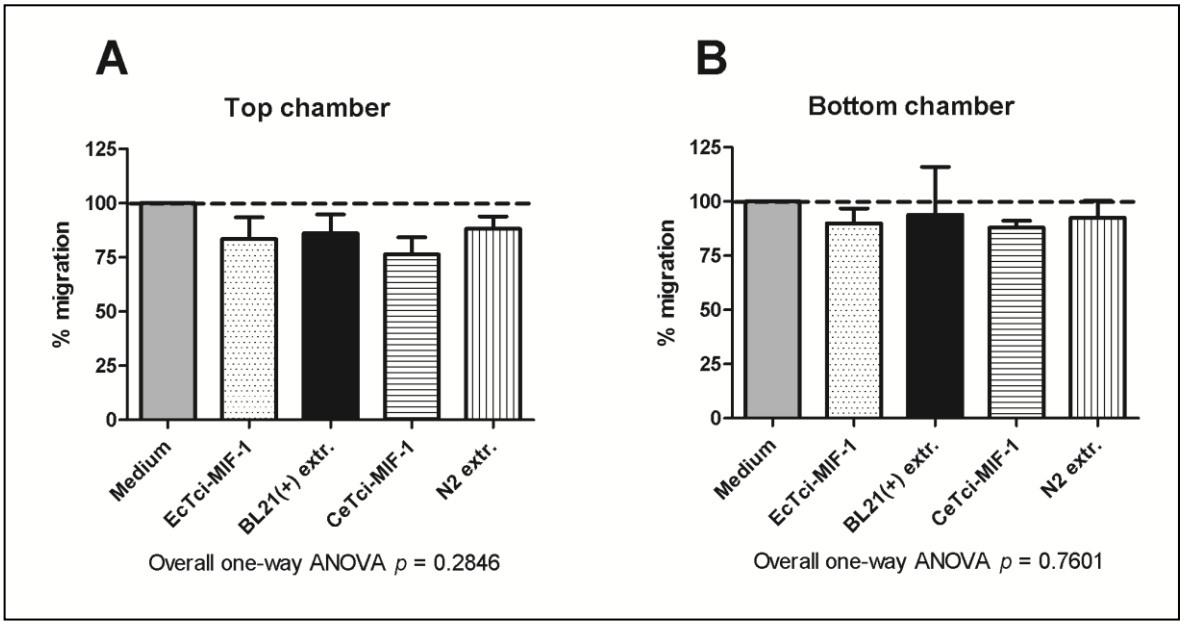

Figure 5.5 Monocyte migration inhibitory activity of purified recombinant Tci-MIF-1 versions on sheep $CD14^+$ cells.
The inhibition of migration is represented as percentage of the medium control (set as 100 %) Data were transformed before carrying out statistical analysis, using the formulae $y = x^3$ (panel A) and $y = 1/x^2$ (panel B), where x = percentage of medium control migration. Untransformed data is represented as mean±SEM (n=3), with monocytes isolated from the same donor sheep. Overall one-way ANOVA *p* values are shown below each graph; $p > 0.05$, not significant. 1 x 10^5 sheep $CD14^+$ cells were treated with 1 µg/ml of each recombinant Tci-MIF-1 or negative controls, which were: *E. coli* BL21 (+) cell extracts (eluted at the same imidazole conditions as EcTci-MIF-1 from cells carrying the Tci-MIF-1 plasmid, but not induced for protein expression), and N2 *C. elegans* worms extracts (eluted from worms under the same conditions used to purify CeTci-MIF-1). All treatments were stripped of LPS using Triton X-114. Panel (A): cells and recombinant Tci-MIF-1 versions or their controls were placed in the top compartment for 3 h. Panel (B): cells were placed in the top compartment and stimulating recombinant Tci-MIF-1 versions or their controls in the bottom compartment, incubated for 3 h.

5.3.5 Pilot study on monocyte- and bone marrow-derived ovine macrophages

In order to investigate the ability of recombinant Tci-MIF-1 versions to induce ovine cell activation, a reproducible and robust ovine macrophage culture system needed to be optimised. Three different approaches were taken in the search for the most reliable and practical technique (from a cell collection logistics point of view) to induce sheep

macrophage maturation *in vitro*. The results are presented in chronological order and the reasons for changing the approach are provided in each section. Sheep $CD14^+$ cells were isolated from the PBMC of two animals and cultured *in vitro* with the aim of harvesting differentiated macrophages to be subsequently stimulated with recombinant Tci-MIF-1. The sheep $CD14^+$ cells differentiated into macrophage-like cells (Mo-MΦ) over a period of 7-days *in vitro* culture. The identity of these newly differentiated macrophage-like cells was assessed by morphology and cytokine release upon stimulation (see Appendix C, Section 9.1). The preliminary results showed that after 48 h stimulation with *E. coli* LPS, the cells had responded with production of both IL-10 and TNFα, suggesting that the culture method was a viable technique in generating macrophages *in vitro*.

However, data could only be collected from Mo-MΦ derived from one sheep, as a large proportion of monocytes isolated from other sheep died during the culture period prior to stimulation, as assessed by Trypan Blue exclusion stain. Less than 1×10^6 viable cells could be recovered for subsequent stimulation assays and after a further unsuccessful attempt to generate sufficient ovine macrophages from $CD14^+$ cells this culture method was deemed unsatisfactory and collection of bone marrow cells was initiated.

Bone marrow stem cells were collected from the sternum of 7 sheep; cells from 4 of these animals were subsequently cultured *in vitro* for at least 15 days (up to 18 days) to allow macrophage maturation. The number of bone marrow cells isolated from each animal could not be consistently repeated (range between 2.8×10^6 and 8.8×10^7 cells/sternum of individual sheep). Cytospins slides were prepared at regular intervals during the *in vitro* culture of bone marrow cells to follow the maturation process of bone marrow-derived macrophages (BM-MΦ). Bone marrow cells were cultured from one animal using different tissue culture plates or flasks to investigate the optimal culture conditions. The purest (95 %) and more reliably reproduced macrophage culture was obtained using non-adherent tissue culture flasks (see Appendix C and Table 9.1 for details). At day 15 of *in vitro* culture in the above mentioned flasks, monocyte- and macrophage-like appearance (large nucleus with chromatine granules and large cytoplasm rich in vacuoles, "foamy cell" appearance) was predominant in the cells (Figure 5.6).

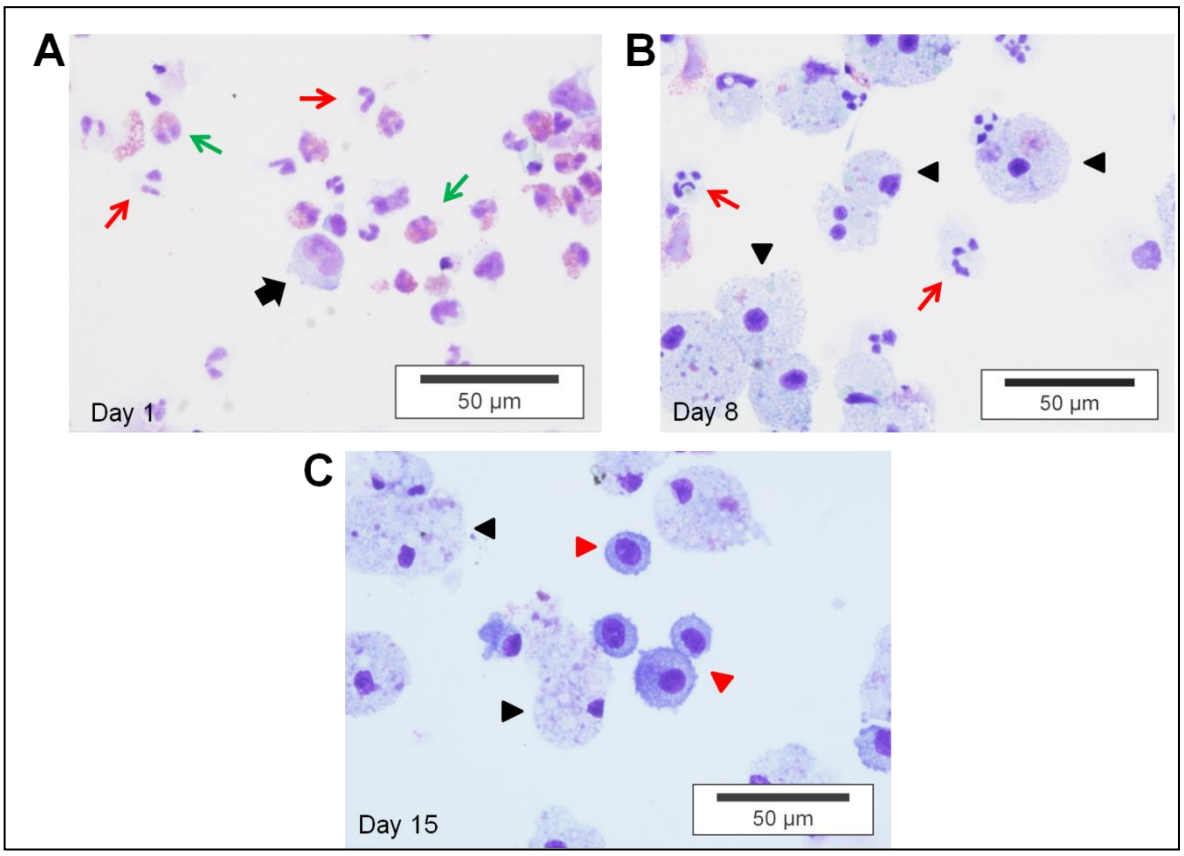

Figure 5.6 Staining with haematoxylin-eosin of ovine BM-MΦ cytospin slides at different stages of maturation.
All cells were collected from the adherent fraction of the polystyrene flask at day 1 (A), 8 (B) and 15 (C) of *in vitro* culture. The bar shows the size of cells relative to 50 µm. Magnification 400x in each panel. Panel A, day 1: red arrows indicate the presence of neutrophils, green arrows for eosinophils; block black arrow show a cell with monocyte-like appearance; panels B and C, day 8-15: red arrows show the presence of neutrophils; black arrow heads indicate mature macrophages with a large cytoplasm rich in vacuoles ("foamy cells"); red arrow heads indicate cells of the monocyte/macrophage lineage at a less differentiated stage.

The cell populations thus generated showed morphological and phenotypical characteristics consistent with mature macrophages (BM-MΦ), as shown by the staining of CD68, positive staining of the monocyte/macrophage-specific non-specific esterase enzymes and the ability to phagocytise fluorescent labelled *E. coli* cells (see Appendix C for details). The BM-MΦ cultures were able to initiate an inflammatory immune response following stimulation with two different types of LPS (*E. coli* O111:B4 LPS or *R. sphaeroides* LPS), as shown by the production of TNFα (Figure 9.2, Appendix C). Furthermore, the BM-MΦ cells generated *in vitro* were able to secrete detectable levels of IL-10 in the culture supernatant following LPS stimulation. Although the amount of cytokine produced by BM-MΦ was not significantly higher than unstimulated cells, it presented encouraging preliminary data. The results described showed that the *in vitro* culture of sheep bone marrow stem cells is able to generate morphologically and

functionally mature macrophages. However, the number of cells thus generated was consistently too low (< 1 x 10^6) to complete a thorough investigation of the cell responses to recombinant Tci-MIF-1 versions. Therefore this culture method was also abandoned in favour of the collection of alveolar macrophages, a cell population which is mature and fully differentiated.

5.3.6 Collection and characterisation of macrophages from sheep lungs

Alveolar macrophages (A-MΦ) were collected from each lung lobe of 7 sheep. Cytospins of cells were stained with a hematoxylin-eosin based stain (Diff-Quick). Cell counts on at least 5 fields/slide and differential morphology of the adherent cells harvested after 2 h incubation of the mixed population found in the lung lavages, showed that > 90 % of the cells were differentiated macrophages (black arrow heads, Figure 5.7). A small number of PMNs and lymphocytes could also be observed (Figure 5.7, red arrow head and arrow, respectively). Total cell counts of the adherent populations ranged from 2.5 x 10^5 to 2.25 x 10^7 (average 7.23 x 10^6).

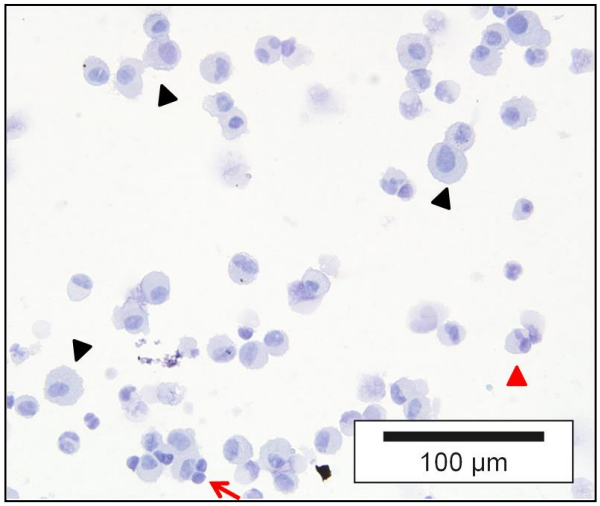

Figure 5.7 Staining with hematoxylin of ovine A-MΦ cytospin slides at the time of collection. These cells were the adherent fraction of the cells collected by lung lavage from one sheep. The bar shows the size of cells relative to 100 µm. Magnification 200x. Red arrow head indicates the presence of PMNs (neutrophil cell); red arrow indicates two lymphocytes; black arrow heads indicate examples of mature macrophages with a large cytoplasm. The figure is representative of cells from one of seven sheep used in subsequent experiments.

5.3.6.1 Non-specific esterase staining of A-MΦ

Non-specific esterase enzymes can be detected primarily in monocytes, macrophages and histiocytes; this type of enzyme is considered virtually absent in granulocytes, thus they are considered specific markers of the monocyte/macrophage cell lineage (Miller 1981). Cytospin slides of sheep A-MΦ carried out shortly after isolation from lung lavage showed a macrophage population with heterogeneous non-specific esterase staining. Some A-MΦ cells showed a more diffuse staining of the cytoplasm (slim arrow, Figure 5.8), other cells appeared with diffuse grey cytoplasm and darker granules (thick arrow, Figure 5.8) and cells with a diffuse black staining and undistinguishable black granules were also observed (arrow head, Figure 5.8).

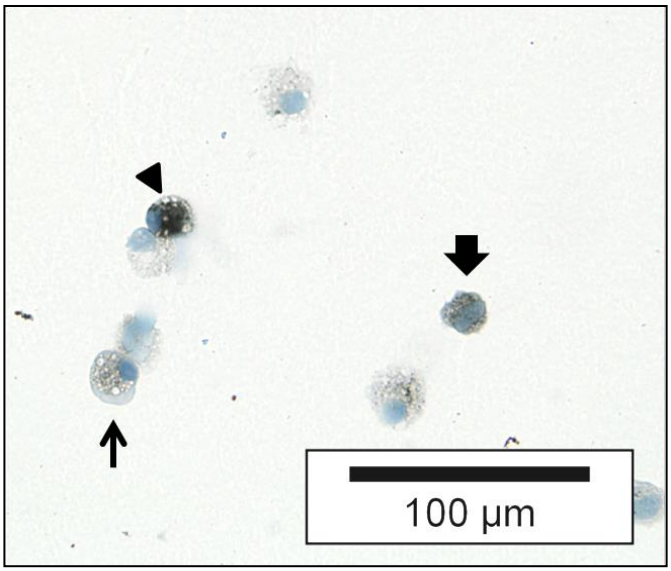

Figure 5.8 Staining for non-specific esterase in ovine A-MΦ cells on the day of isolation from sheep lungs.
The bar shows the size of cells relative to 100 µm. Magnification of 200x was used to visualise stained cells. Slim arrow: cell with more diffuse cytoplasmic staining; thick arrow: cell with diffuse cytoplasmic staining and darker granules; arrow head: cell with dark coloured cytoplasm where granules of non-specific esterase are not distinguishable. The figure shows representative alveolar cells harvested from one of three sheep used in this experiment.

5.3.6.2 CD68 staining of A-MΦ

The cell surface marker CD68 (also known as macrosialin) is a glycoprotein of intracellular membranes, and was first identified in mouse macrophages as a specific surface marker for this type of cell (Rabinowitz and Gordon 1991, Smith and Koch 1987). CD68 has been shown to bind low density lipoproteins (LDL), suggesting a potential role as a lipoprotein uptake receptor (Van Velzen *et al.* 1997). Cytospins of A-MΦ showed that

although ~100 % cells were characterised by macrophage morphology, on average only 21 % of cells (cells from two sheep, over 5 fields each, range 0-46 %) stained for CD68. A-MΦ that were positive for CD68 showed a uniform pattern of labelling, occurring mostly in defined cytoplasmic areas (Figure 5.9). Negative control slides of A-MΦ incubated with an isotype control antibody and without primary antibody did not show CD68 stain.

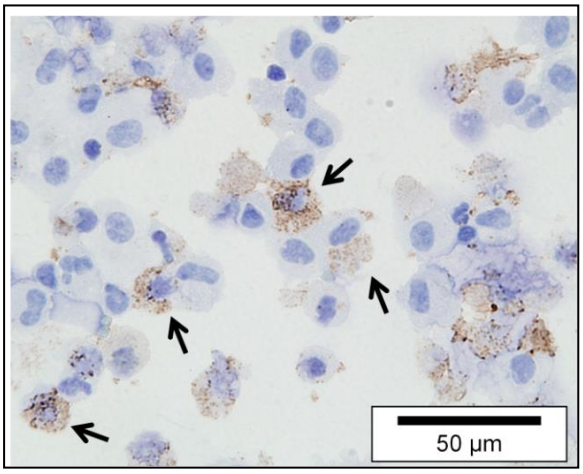

Figure 5.9 Immunocytochemical (ICC) labelling of CD68 in ovine A-MΦ.
The bar shows the size of cells relative to 50 µm. Magnification of 400x was used to visualise positively labelled cells. Black arrows: A-MΦ showing various degrees of CD68 staining.

5.3.6.3 In vitro phagocytosis activity of A-MΦ

The phagocytic activity of A-MΦ was investigated by using fluorescent (FITC-labelled) *E. coli* cells in macrophage feeding assays. The results showed A-MΦ were able to phagocytise the FITC-labelled *E. coli* cells. Dual fluorescent microscopy showed that co-localisation of green fluorescent particles (*E. coli*) occurred within the area of macrophage cytoplasm and next to the blue DAPI-stained nucleus (Figure 5.10). A-MΦ incubated without FITC-labelled *E. coli* cells did not show any fluorescent labelling (data not shown).

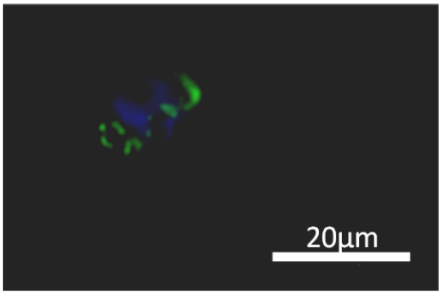

Figure 5.10 Fluorescent labelling of ovine A-MΦ following phagocytosis of FITC-labelled *E. coli cells*.
Green fluorescent labelling within cells represents the phagocytosed FITC-labelled *E. coli cells* (both panels). Dual fluorescence staining demonstrating FITC-labelled *E. coli* cells (green) adjacent to the DAPI-labelled cell nuclei (blue). Ratio of A-MΦ to *E. coli* cells was 1:5. Magnification used was 400x; the bar shows the size of cells relative to 20 µm.

5.3.6.4 Cytokine production of A-MΦ upon stimulation

The production of IL-10 and TNFα was investigated in *in vitro* cultures of ovine alveolar macrophages (A-MΦ). Alveolar macrophages were collected from sheep by bronchial lavages and immediately stimulated for 6, 24 or 48 h to investigate the cytokine responses to stimulation. IL-10 release was measured in the cell supernatant of sheep A-MΦ harvested at 6, 24 and 48 h following stimulation with LPS B (from *E. coli* O111:B4), both recombinant Tci-MIF-1 versions and ov rIL-4. Although an increase in IL-10 was shown to occur in cell supernatants 24 h post stimulation with LPS B, the IL-10 release measured both at 6 h and 24 h post-LPS stimulation did not show significant differences between unstimulated and stimulated cells (Figure 5.11, panels A and B). At 48 h post-stimulation, the IL-10 release in the supernatant of cells stimulated with LPS B was significantly higher than supernatant from cells stimulated with either CeTci-MIF-1 ($0.01 < p < 0.05$) or ov rIL-4 ($p < 0.001$). However, neither LPS, CeTci-MIF-1 or ov rIL-4 stimulation induced an IL-10 release significantly different from unstimulated cells (Figure 5.11, panel C).

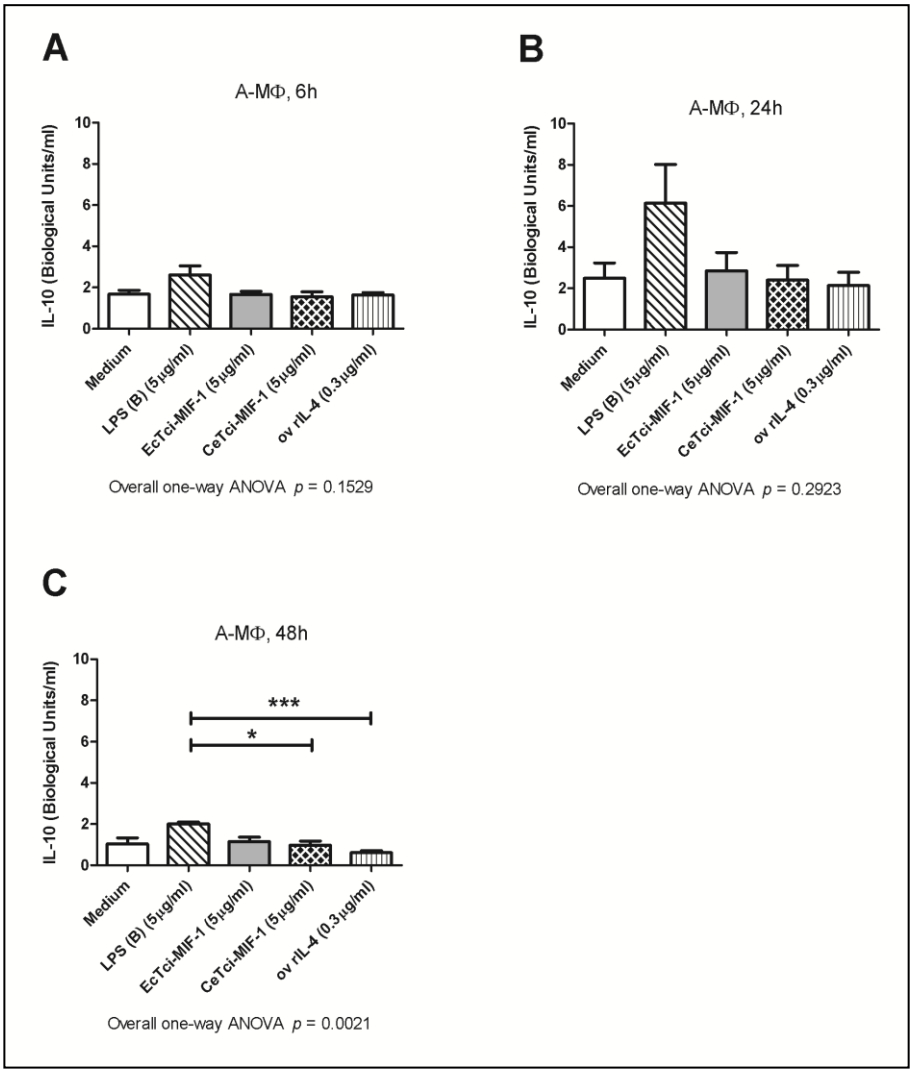

Figure 5.11 IL-10 detection in the cell supernatant of ovine AM-MΦ cultures stimulated with a range of molecules.
Cells were plated at 10^5/well and supernatants harvested after 6-24-48h co-incubation with the molecules indicated below each graph (final concentrations), or with medium alone (unstimulated control). Data was transformed prior to carrying out statistical analysis to reduce skew using the formulae y = 1/x, where x = IL-10 U/ml (panels A, B) and y = 1/sqrt x, where x = IL-10 U/ml (panel C). Mean±SEM of untransformed data is shown [panel A, n=4; panel B, n=7; panel C, n=6). Overall one-way ANOVA *p* value of transformed data is shown below each graph; $p > 0.05$, not significant; * indicates $p < 0.001$; * indicates $0.01 < p < 0.05$. Panel A: IL-10 release in A-MΦ supernatant after 6 h incubation in the presence of the molecules indicated below the graph; panel B: IL-10 release in A-MΦ supernatant after 24 h incubation in the presence of the molecules indicated below the graph; panel C: IL-10 release in A-MΦ supernatant after 48 h incubation in the presence of the molecules indicated below the graph.**

The TNFα release of A-MΦ cultures was also investigated at 6, 24 and 48 h following stimulation with the molecules previously indicated. At 6 h post-stimulation, cells that received LPS B (from *E. coli* O111:B4) stimulation secreted a significantly higher level of TNFα than cells stimulated with ov rIL-4 ($0.01 < p < 0.05$, Figure 5.12, panel A). However, neither stimulation protocol induced a significant change in TNFα secretion compared to unstimulated cells. Although LPS B (from O111:B4 *E. coli)* appeared to

induce the highest TNFα release after 24 and 48 h stimulation, the change was again not significant compared to unstimulated cells. Neither recombinant Tci-MIF-1 version was able to induce a significant change in TNFα secretion compared to supernatant from unstimulated cells, at any of the time points investigated (Figure 5.12).

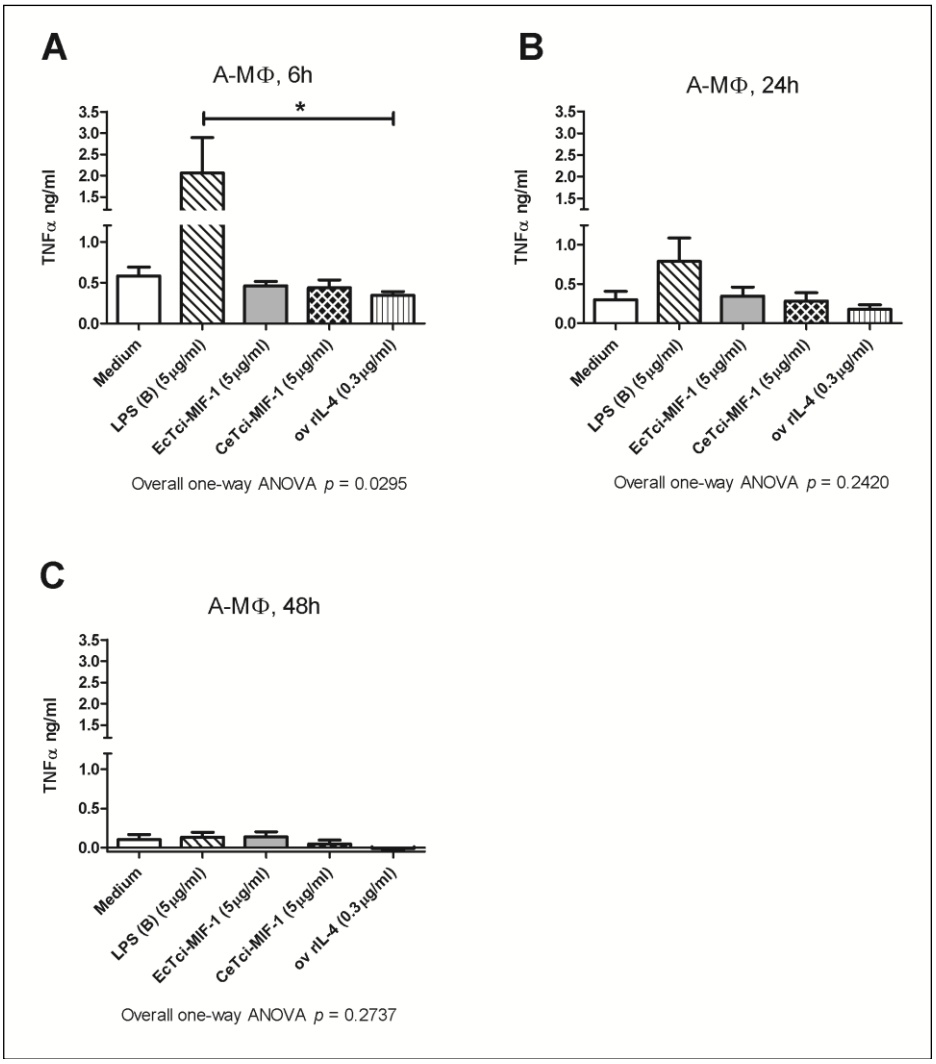

Figure 5.12 TNFα detection in the cell supernatant of ovine AM-MΦ cultures stimulated with a range of molecules.
Cells were plated at 10^5/well and supernatants harvested after 6-24-48 h co-incubation with the molecules indicated below the graph (final concentrations), or with medium alone (unstimulated control). Data was transformed to reduce skew prior to statistical analysis using the formulae y = 1/x (panel A) and y = $\log_{10}$ x (panel B), where x = TNFα ng/ml. Overall one-way ANOVA *p* value of transformed data is shown below each graph; *p* > 0.05, not significant; * indicates 0.01 < *p* < 0.05. Untransformed data is represented as mean±SEM [panel A, n=4, panel B, n=7 and panel C, n=6). Panel A: TNFα release in A-MΦ supernatant after 6 h incubation in the presence of the molecules indicated below the graph; panel B: TNFα release in A-MΦ supernatant after 24 h incubation in the presence of the molecules indicated below the graph; panel C: TNFα release in A-MΦ supernatant after 48 h incubation in the presence of the molecules indicated below the graph.

Although the cytokine production of A-MΦ cultures stimulated with LPS, ov rIL-4 or indeed recombinant Tci-MIF-1 was not significantly higher than unstimulated cells, the

significant differences observed between stimulation protocols suggest that these cells may be able to initiate an immune response. In addition, A-MΦ are fully differentiated cells which are logistically easier to collect and handle than Mo-MΦ and BM-MΦ. A-MΦ also show the macrophage-specific expression of non-specific esterase enzymes, CD68 surface marker and the ability to phagocytise *E. coli* cells. For these combined reasons, the experiments on gene expression described below were carried out using only cDNA from A-MΦ, with the exception of the initial GeNorm algorithm analysis.

5.3.7 Gene expression analysis of stimulated ovine macrophages by qRT-PCR

5.3.7.1 Selection of candidate reference genes by GeNorm analysis

The geNorm algorithm (Vandesompele *et al.* 2002) was used to analyse the stability of candidate reference genes (i.e. genes of which the mRNA expression did not change significantly when different cell stimulations were used). This analysis was carried out on C_t values from cDNA of both BM-MΦ and A-MΦ; cDNA was generated from stimulated (with LPS, ov rIL-4 or recombinant Tci-MIF-1 versions) and unstimulated cells of both culture systems, to compare which may have been the cell type most suited to this analysis. The results showed that the candidate house-keeping (HK) genes *YWHAZ* and *GAPDH* were the two most stable in the experiment carried out using cDNA generated from BM-MΦ (Figure 5.13). When cDNA from A-MΦ was analysed, *GAPDH* was the most stable gene for this type of cell, with *YWHAZ* being the fifth (out of 6) most stable (Figure 5.14). In this instance, it was expected to use cDNA from both BM-MΦ and A-MΦ for all subsequent RT-qPCR experiments, therefore the choice of HK genes fell on the most stable for each cell type considered (*YWHAZ* and *GAPDH*). However, due to cell numbers, reagents and time constraints it was later decided to only use cDNA from A-MΦ in the qPCR experiments and the choice of HK genes was maintained.

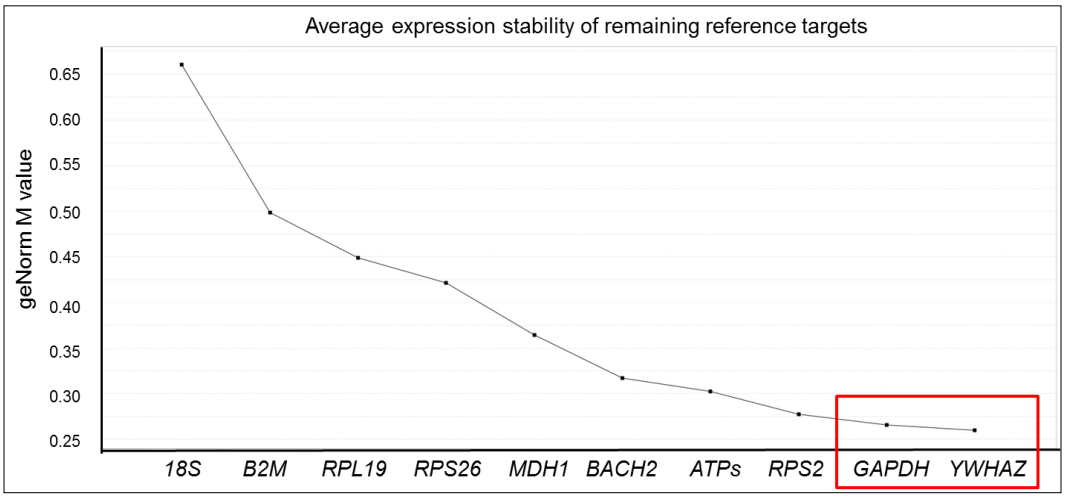

Figure 5.13 GeNorm analysis results from ovine cDNA of 6 BM-MΦ cultures.
The x axis shows the 12 candidate HK genes considered in this assay, with the most stable at the right end and the less stable at the left. The y axis shows the M coefficient, calculated with the geNorm algorithm on the basis of the variation of a gene compared to all other candidates. The box shows the two genes considered most stable (*YWHAZ* and *GAPDH*) on the basis of the Ct values achieved from 6 BM-MΦ cDNA samples from cells which were stimulated as described in Section 5.2.10. The box shows the position of the relative stability ranking of both genes in cDNA from the BM-MΦ cultures considered.

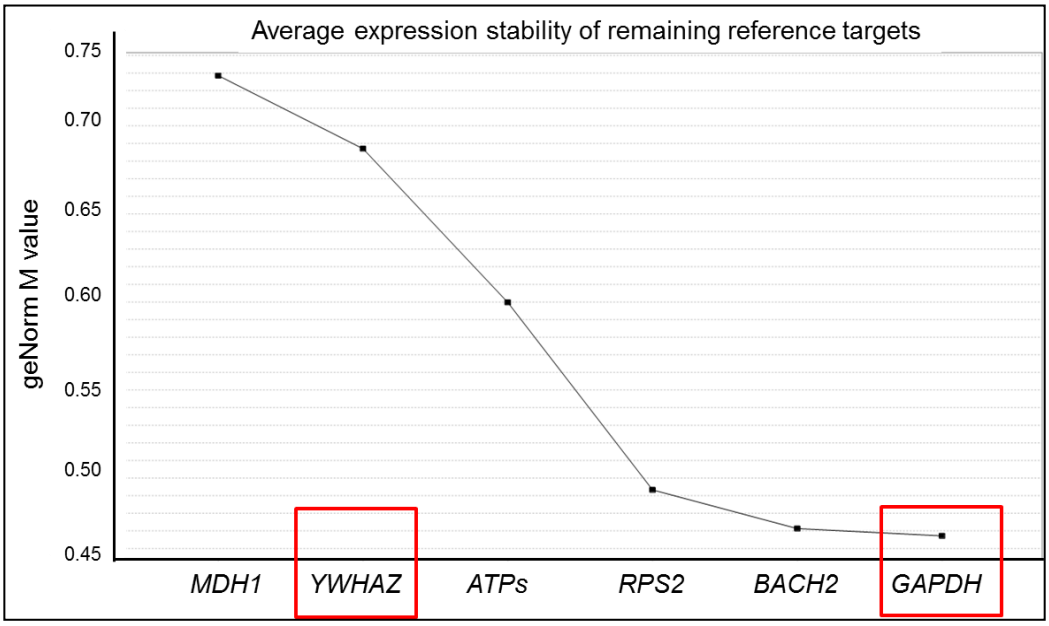

Figure 5.14 GeNorm analysis results from ovine cDNA of 15 A-MΦ cultures.
The x axis shows the 12 candidate HK genes considered in this assay, with the most stable at the right end and the less stable at the left. The y axis shows the M coefficient, calculated with the geNorm algorithm on the basis of the variation of a gene compared to all other candidates. The boxes shows the position of the genes considered (*GAPDH*, right box and *YWHAZ*, left box) on the basis of the Ct values achieved by 15 A-MΦ cDNA samples from cells which were stimulated as described in Section 5.2.10. The boxes show the position of the relative stability ranking of both genes in cDNA from the A-MΦ cultures considered.

5.3.7.2 Essential information on qPCR reactions

A list of essential information that must be included in studies carried out using qPCR experiments has been published by Bustin *et al.* (2009). For the purposes of this thesis, all available information on validation and analysis of data is reported below. Section 5.2 (Materials and Methods) and Appendix A can be consulted for details of the protocols and sequences of primers used.

Reaction efficiencies were calculated by the ABI 7500 v2.0.6 software for each plate and each gene investigated in this study. The results are summarised in Table 5.3 below. Reaction efficiency of 100 % indicates that for each of the 40 cycles used there was doubling of the amplified product. Reaction efficiency below 80 % suggested that data interpretation should be carefully carried out, as reaction artefacts were likely to have affected the estimated copy number of the genes examined.

Table 5.1 The RT-qPCR reaction efficiency calculated as mean (±SEM) of 4 PCR plates, for each of the genes included in this study.

Genes	Mean % of reaction efficiency (±SEM; n=4)
YWHAZ	90.68 (1.61)
GAPDH	76.07 (3.22)
iNOS	92.33 (1.54)
Chit3-L1	91.47 (2.20)
Arg-1	89.97 (9.41)
TNFα	88.90 (2.52)
IL-10	78.61 (2.88)
IL-8	83.62 (2.86)
IL-12p40	86.21 (1.70)

5.3.7.3 Identification of qRT-PCR target genes

A panel of 7 ovine genes was selected for investigation of expression in sheep A-MΦ cultures. Genes that were chosen as representatives of a classical inflammatory macrophage response were *TNFα*, *IL-8*, *iNOS* (or NO as the product of iNOS enzymatic activity) and *IL-12p40* [as inducer of the Th1 cytokine IFNγ (Finkelman *et al.* 2004, Flynn *et al.* 2007, Harada *et al.* 1994, Raes *et al.* 2002)]. Representative genes tested as markers of alternative activation status were *IL-10*, Chitinase 3-like molecule (*Chit3-L1*) and

Arginase-1 (*Arg-1*) (Flynn *et al.* 2007, Gordon 2003, Knight *et al.* 2007). Assays for a number of these genes (*TNFα*, *IL-8*, *IL-12p40*, *IL-10*) had been previously validated (McNeilly *et al.* 2008). The remaining three qRT-PCR assays (*iNOS*, *Chit3-L1* and *Arg-1*) had been developed *de novo* in collaboration with PrimerDesign Ltd. In order to produce and validate the assays, this company requested sequences of the genes of interest (GOIs) which had been sourced during the course of this study. Briefly, ovine *iNOS* nucleotide sequence was identified in NCBI as a partial coding sequence of inducible nitric oxide synthase mRNA [accession number AF223942.1 (Mershon *et al.* 2002)]. Ovine chitinase 3-like molecule *Chi3-L1* had been previously described as a signal processing protein; mRNA partial coding sequence was available in NCBI [accession number AY392761.1 (Knight *et al.* 2007)]. Both iNOS and Chi3-L1 sequences had been submitted to PrimerDesign Ltd in order to design and validate appropriate primers to be used in qPCR assays.

An EST sequence for ovine *Arg-1* was identified in autumn 2012 in NCBI (accession number EE768328.1) by BLAST search using the nucleotide sequence encoding *Bos taurus* liver Arginase-1 (accession number NM_001046154), mouse Arginase-1 (*Mus musculus*, accession number EDL04794.1) and goat Arginase-1 (*Capra hircus*, accession number ACS34711.1). Nucleotide sequence identity between the ovine EST sequence thus identified and the above bovine, mouse and goat arginase-1 sequences is shown in Table 5.4. As the nucleotide sequence identity between bovine, mouse, goat and putative ovine Arginase-1 was shown to be at least 82 % (Table 5.4), the identified ovine EST (EE768328.1) was considered an adequate representative of ovine Arginase-1 gene and was therefore also submitted to PrimerDesign Ltd in order to design and validate appropriate qPCR primers. At the time of writing, a BLAST search of the ovine EST sequence EE768328.1 within the NCBI nucleotide collection identified two predicted *Ovis aries* liver Arginase-1 sequences, named variant 1 and 2 (accession numbers XM_004011323.1 and XM_004011324.1 respectively). These had been predicted from the *Ovis aries* (Texel) chromosome 8 genomic scaffold (NCBI accession number NW_004080171.1) and both showed > 99 % nucleotide sequence identity with the previously identified EST sequence EE768328.1.

Table 5.2 The nucleotide sequence identity of 4 mammalian *Arg-1* genes.

Species	NCBI Accession number	% identity			
		Bos taurus	*Ovis aries*	*Capra hircus*	*Mus musculus*
Bos taurus	NM_001046154	100	97.32	96.90	82.25
Ovis aries	EE768328.1	97.32	100	99.26	82.25
Capra hircus	ACS34711.1	96.90	99.26	100	82.87
Mus musculus	NM_007482.3	82.25	82.25	82.87	100

5.3.7.4 Gene expression analysis in ovine A-MΦ cultures

Sheep A-MΦ cultures were stimulated with a range of molecules to induce activation (*E. coli* O111:B4 LPS as inducer of classical activation, ov rIL-4 as inducer of alternative activation and EcTci-MIF-1 or CeTci-MIF-1). RNA was purified at 6 and 24 h post-stimulation and qRT-PCR carried out to determine the expression levels of genes indicative of classical activation (*TNFα, IL-8*, *IL-12p40* and *iNOS*) or alternative activation (*Arg-1*, *Chit3-L1* and *IL-10*). Levels of *Arg-1* and *IL-12p40* mRNA were below the detection threshold of the assay for all samples and these genes were therefore not represented in the analysis shown. Ovine A-MΦ which were stimulated for 6 h did not show significant differences in gene expression compared to the unstimulated cells, with the exception of cultures stimulated with ov rIL-4 which resulted in significantly lower *iNOS* expression compared to unstimulated cultures (Figure 5.15).

The gene expression levels in A-MΦ at 24 h were also investigated, to detect later changes in cell responses to activating stimuli. Similar to the results shown at 6 h post stimulation, no major changes in gene expression were observed after 24 h except for *iNOS* and *Chit3-L1*. Ovine rIL-4 stimulation of cells induced significantly lower levels of *iNOS* expression compared to stimulation with LPS and CeTci-MIF-1 and unstimulated controls. Surprisingly, ov rIL-4 was able to significantly down-regulate *Chit3-L1* expression levels compared to unstimulated cells or cells after 24 h of LPS stimulation (Figure 5.16).

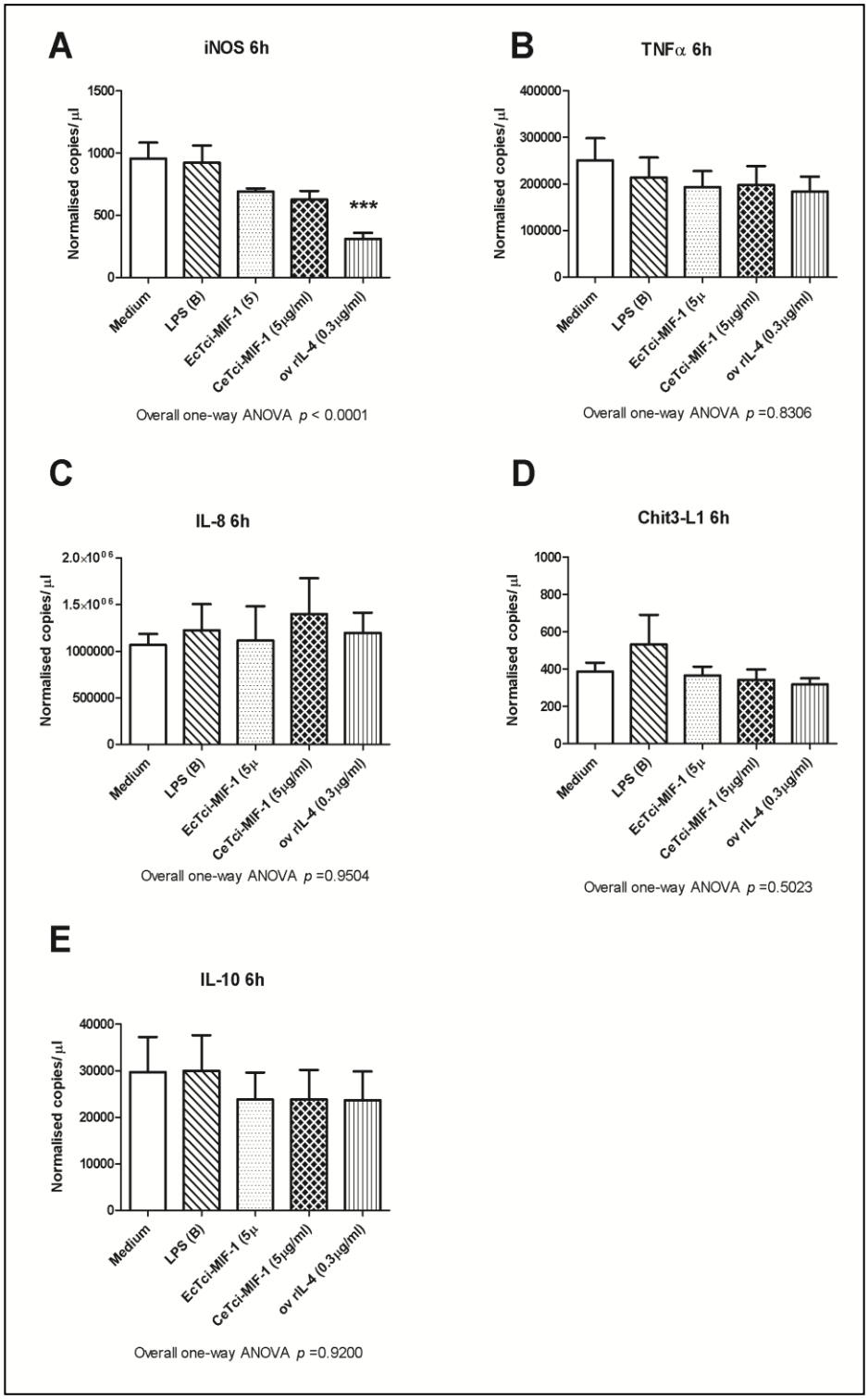

Figure 5.15 The gene expression analysis of ovine A-MΦ cultures after 6 h in incubation with a range of molecules.
Complementary cDNA samples of A-MΦ cultures stimulated with *E. coli* O111:B4 LPS (LPS B), EcTci-MIF-1, CeTci-MIF-1 and ov rIL-4 were analysed for expression of the genes *iNOS*, *TNFα*, *IL-8*, *Chit3-L1* and *IL-10*. Cells stimulated with medium alone were unstimulated controls. Data was normalised against the two housekeeping genes *GAPDH* and *YWHAZ*; normalised copies/µl values were transformed to reduce skew using the formulae $y = \log_{10} x$ (panels A, B), $y = x^{0.5}$ (panels C, E), and $y = 1/x$ (panel D); in each formula x = normalised copies/µl. Overall one-way ANOVA *p* value of transformed data is shown below each graph; *** $p < 0.001$. Untransformed data is shown as mean±SEM of normalised copies/µl (n=4).

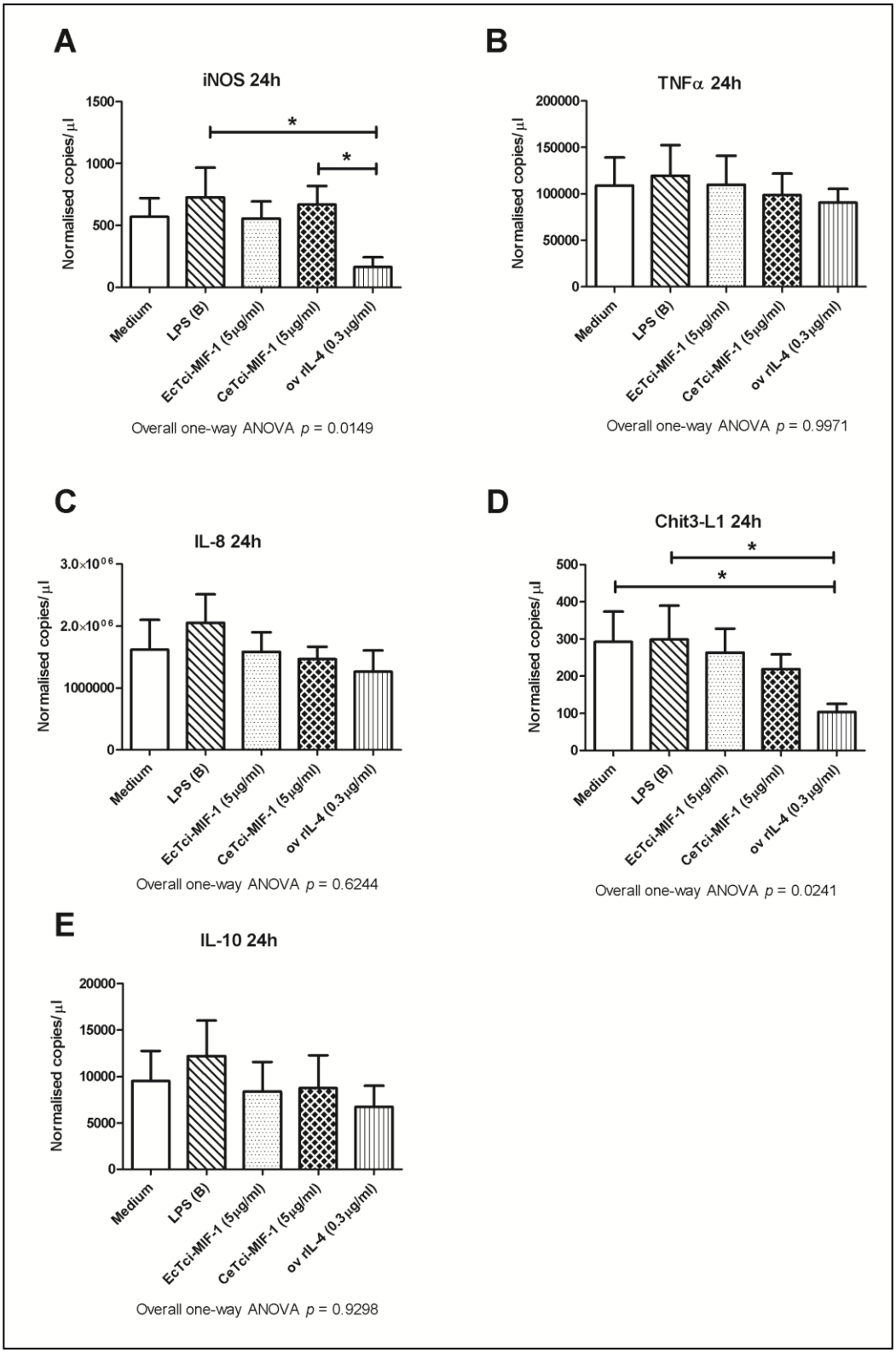

Figure 5.16 The gene expression analysis of ovine A-MΦ cultures after 24 h in incubation with a range of molecules.
Complementary cDNA samples of A-MΦ cultures stimulated with *E. coli* O111:B4 LPS (LPS B), EcTci-MIF-1, CeTci-MIF-1 and ov rIL-4 were analysed for expression of the genes *iNOS*, *TNFα*, *IL-8*, *Chit3-L1* and *IL-10*. Cells stimulated with medium alone were unstimulated controls. Data were normalised against the two housekeeping genes *GAPDH* and *YWHAZ*; normalised copies/µl values were transformed to reduce skew using the formulae $y = x^{0.5}$ (panels A-C) and $y = \log_{10} x$ (panels D, E); in each formula x = normalised copies/µl. Overall one-way ANOVA *p* value of transformed data is shown below each graph; ** $0.001 < p < 0.01$; * $0.01 < p < 0.05$. Untransformed data is shown as mean±SEM of normalised copies/µl (n=7).

5.3.8 MIF proteins alignment

Previous results in this chapter have shown that Tci-MIF-1 did not convincingly inhibit the migration of sheep monocytes, nor activate sheep alveolar macrophages. As a preliminary investigation of these results, a comparison of the amino acid sequences of Tci-MIF-1, Bm-MIF-1 (Zang *et al.* 2002), ovine MIF (Lopes *et al.* 2011) and human MIF (Bernhagen *et al.* 1994) was carried out.

The results showed that mammalian MIF protein sequences showed higher sequence similarity to each other compared to parasite MIF sequences (92.17 % amino acid sequence identity between human and sheep MIF, compared to 49.57 % ID between *T. circumcincta* Tci-MIF-1 and *B. malayi* MIFs). In addition, both mammalian MIFs showed between 40 and 42 % amino acid sequence identity with each of the parasite MIFs. Only the human and sheep MIF sequences possess the CALC motif (solid line box, Figure 5.17), which had been implicated in the ability of human MIF to show oxidoreductase enzyme activity (Kleemann *et al.* 1998). Residues involved in the formation of the dopachrome tautomerase function active site are highlighted in each version (yellow boxes, Figure 5.17), as described by Nisbet *et al.* (2010b). All the MIF proteins investigated here showed the presence of a conserved Pro residue in position 2 of the amino acid chain (Pro_2), which had been previously indicated as pivotal for the tautomerase activity of this protein (Hermanowski-Vosatka *et al.* 1999, Swope *et al.* 1998, Zang *et al.* 2002).

```
Hs-MIF-1        MPMFIVNTNVPRASVPDGFLSELTQQLAQATGKPPQYIAVHVVPDQLMAFGGSSEPCALC 60
Oa-MIF-1        MPMFVVNTNVPRASVPDGLLSELTQQLAQATGKPAQYIAVHVVPDQLMTFGGSSEPCALC 60
Tci-MIF-1       MPVFSFHTNVSADKVTPDLLKQISSVVARILHKPESYVCVHVVPDQQMIFDGTDGPCGVG 60
Bm-MIF-1        MPYFTIDTNIPQNSISSAFLKKASNVVAKALGKPESYVSIHVNGGQAMVFGGSEDPCAVC 60
                ** * ..**:.  .:.  :*.: :. :*:   ** .*:.:**  .* * *.*:. **.:

Hs-MIF-1        SLHSIGKIGGAQNRSYSKLLCGLLAERLRISPDRVYINYYDMNAANVGWNNSTFA 115
Oa-MIF-1        SLHSIGKIGGAQNRSYSKLLCGLLTERLRISPDRIYINFCDMNAANVGWNGSTFA 115
Tci-MIF-1       VLKSIGGVGGSKNNEHAKALFALIKDHLGIAGNRMYIEFIDIGAADIAFNSRTFA 115
Bm-MIF-1        VLKSIGCVGPKVNNSHAEKLYKLLADELKIPKNRCYIEFVDIEASSMAFNGSTLG 115
                *:*** :*   *..::: *  *: :.* *. :* **:: *: *:.:.:*. *:.
```

Figure 5.17 Alignment of deduced amino acid sequence of different MIF versions. Human MIF (Hs-MIF-1, NCBI accession number P14174), ovine MIF (Oa-MIF-1, accession number NP_001072123), *T. circumcincta* MIF (Tci-MIF-1, accession number FN599526) and *Brugia malayi* MIF (Bm-MIF-1, accession number AAB60943) were compared. Green letters indicate the conserved residues; bold letters indicate the CALC motif; yellow highlights indicate the active site residues. The Pro_2 residue is shown in bold red letters.

5.4 Discussion

This chapter reports the characterisation of recombinant Tci-MIF-1 with respect to its enzymatic activity as a dopachrome tautomerase and its biological activities on ovine cells both as an inhibitor of monocyte migration and as an activator of sheep macrophages. The experiments were carried out using two versions of Tci-MIF-1, one generated using a traditional *E. coli* expression system and the other purified from transgenic *Tci-mif-1*-expressing *C. elegans* worms. The comparisons between the two recombinant Tci-MIF-1 versions showed that both maintained dopachrome tautomerase activity and that *C. elegans*-expressed Tci-MIF-1, similarly to *E. coli*-expressed Tci-MIF-1, was not able to inhibit the migration of ovine monocytes. To investigate the ability of either recombinant Tci-MIF-1 version to activate ovine macrophages, *in vitro* cultures were established. Three different methods were tested: macrophages derived from blood $CD14^+$ monocytes (Mo-MΦ), those derived *in vitro* from bone marrow (BM-MΦ) and macrophages isolated from lungs (alveolar macrophages, A-MΦ). Each of these culture methods proved successful in generating macrophages and characterisation of these cells showed that they maintained features typical of this cell type such as cytokine production (TNFα and IL-10), phagocytosis, CD68 expression and non-specific esterase enzymes contained in cytoplasmic granules. Stimulation of BM-MΦ and A-MΦ with different types of LPS, both recombinant Tci-MIF-1 versions and recombinant ovine IL-4 (ov rIL-4) showed that these cells were able to secrete TNFα and IL-10 into the culture supernatant in response to *in vitro* culture. It is possible that collection and *in vitro* handling of these cells may initiate a low background level of cell activation, which was detected by cytokine ELISA experiments here carried out. However investigation of gene expression in A-MΦ cultures did not show major changes in expression between the LPS and ov rIL-4 stimulated cells (used as controls of classical and alternative activation, respectively) and the recombinant Tci-MIF-1 stimulated cultures.

MIF cytokines have been shown to possess different types of enzyme activity, such as dopachrome tautomerase, keto-enol isomerase and oxidoreductase (Kleemann *et al.* 1998, Nisbet *et al.* 2010b, Rosengren *et al.* 1997, Rosengren *et al.* 1996). Experiments using mutated recombinant MIF versions have shown that the dopachrome tautomerase activity of mammalian MIF is dependent on a Proline residue in position 2 of the amino acid chain, which becomes the N-terminal residue once the initiating Met residue is cleaved post-translationally (Bernhagen *et al.* 1993, Swope *et al.* 1998). Furthermore, it was shown that the P1G mutant MIF (i.e. a recombinant human MIF version in which the residue Pro2 was

mutated to Gly) had a significantly lower ability to induce superoxide in a human neutrophil priming assay, compared to wild type recombinant MIF (Swope *et al.* 1998). However, in the study carried out by Swope *et al.* (1998) the cell migration inhibitory activity was not investigated, therefore a complete assessment of the cytokine activity could not be established. In the parasitic filarioid *Brugia malayi*, mutant recombinant versions of MIF-1 and MIF-2 in which Pro was substituted with Gly showed significantly reduced dopachrome tautomerase activity across a range of substrates compared to wild type Bm-MIF-1 and -2; therefore, as in mammalian MIF, a Pro residue in position 2 is required for the enzyme activity of Bm-MIF-1 and -2 (Zang *et al.* 2002). Furthermore, although significantly reduced compared to wild type Bm-MIF-1 and -2, the two Gly mutants were still able to partially inhibit the migration of human monocytes (Zang *et al.* 2002). In another study, on mouse recombinant MIF, two mutant versions in which a Phe or Ser residue substituted Pro1 (P1F and P1S) showed no phenylpyruvate tautomerase activity but retained full ability to inhibit both the chemotactic and random migration of human monocytes (Hermanowski-Vosatka *et al.* 1999). These results showed that Pro in position 2 of the amino acid chain has a pivotal role in conferring MIF tautomerase activity; however the cytokine functions appear to be only partially dependent on the presence of this residue. Results from this chapter confirmed that both recombinant *T. circumcincta* Tci-MIF-1 versions possess dopachrome tautomerase activity, as previously shown for *E. coli*-expressed Tci-MIF-1 (Nisbet *et al.* 2010b). Both these proteins have a Pro residue in position 2 of their amino acid chain; however, it was not investigated whether the N-terminal Met had been post-translationally cleaved, as is the case in mammalian MIF.

Neither of the *T. circumcincta* recombinant Tci-MIF-1 versions used in this chapter was able to inhibit migration of sheep $CD14^+$ monocytes; the results from three experiments showed a trend of the CeTci-MIF-1 preparations used to inhibit the migration of cells through the filter compared to the relevant unstimulated control cells. However, the differences observed were not statistically significant. Although previous studies have shown the ability of two recombinant MIF from the parasite *Brugia malayi* to inhibit the migration of human monocytes (Pastrana *et al.* 1998, Zang *et al.* 2002), the results of this chapter agree with the findings reported by (Nisbet *et al.* 2010b), which showed that *T. circumcincta* recombinant MIF was unable to inhibit the migration of sheep monocytes. Lipopolysaccharide (LPS) is capable of inhibiting monocyte chemotaxis at concentrations > 0.5 ng/ml (Hermanowski-Vosatka *et al.* 1999). For this reason, all Tci-MIF-1 preparations used in the monocyte migration experiments were treated with Triton X-114

to reduce the LPS contamination below the level mentioned above, (which equates in the experiments here described to < 9.7 fg LPS/μg protein). For the experiments described in this thesis it was not possible to purify adequate amounts of Tci-MIF-1 from *T. circumcincta* L3 stages to study the effect of the native protein on these cells. A possible explanation for the lack of function of recombinant Tci-MIF-1 on sheep monocytes could be that Tci-MIF-1 might not be involved in the immune interplay between *T. circumcincta* and sheep host, and that the real functions of this enzyme lie in the survival of L3 larvae on pasture, as suggested previously (Nisbet *et al.* 2010b). Future work could further investigate the ability of Tci-MIF-1 to inhibit sheep monocyte migration, with the addition of i) an inhibitor of monocyte migration [such as LPS (Hermanowski-Vosatka *et al.* 1999)]; or ii) an enhancer of monocyte migration [such as vascular endothelial growth factor, VEGF (Barleon *et al.* 1996), plateled-derived growth factor, PDGF (Deuel *et al.* 1982), or macrophage colony stimulating factor M-CSF (Wang *et al.* 1988)]; iii) native L3-purified Tci-MIF-1 (Nisbet *et al.* 2010b); iv) recombinant and v) native ovine MIF as controls [described by Lopes *et al.* (2011)]. Moreover, it should be possible to generate mutants of both sheep and *T. circumcincta* MIF in order to investigate whether the N-terminal Pro residue is as important for MIF functions as shown previously in other species.

In this chapter, three different methods of obtaining ovine macrophages have been demonstrated. Preliminary data has shown that each method proved successful in delivering differentiated macrophage cultures; however, the efficiency and repeatability were very variable for each method considered and the final decision was taken to only use alveolar macrophages in order to be able to carry out complete experiments. Blood-derived monocytes from cattle had been previously used to generate macrophage cultures *in vitro* (Flynn *et al.* 2007). In the veterinary literature, cattle circulating monocytes are estimated at between 0 and 8 % of PBMC (averaging around 5-6 %, personal observation from previous MSc studies), whereas in sheep values of between 0-6 % are reported (Aiello 1998). In these conditions, finding individual animals that could be used as monocyte donors would involve screening of the wider population, with considerable use of time and resources. Moreover, the culture method previously described by Flynn *et al.* (2007) to generate macrophages from blood monocytes appeared to be less effective in the ovine species, as cells showed scarce viability after 7 days of *in vitro* culture. For these reasons and the low number of cells available following 7 days in culture ($<1 \times 10^6$), it was decided to attempt culture from a different source of cells.

Bone marrow has been previously used as a source of stem cells from which to generate fully differentiated macrophages. In mice, this process involves the use of macrophage colony stimulating factor (M-CSF), often available as supernatant of L929 fibroblast cell cultures (Cho *et al.* 2012, Corraliza *et al.* 1994, Flesch and Kaufmann 1987). In cattle, bone marrow-derived macrophages had been previously generated *in vitro* from foetal bone marrow without additional cytokines and the addition only of FBS (Adler *et al.* 1994); presumably FBS contains M-CSF. In sheep, dendritic cells (DCs), a cell population that may share features with macrophages (Coico *et al.* 2003), have been previously generated from blood-derived monocytes and bone marrow, using recombinant ovine GM-CSF and IL-4 (Chan *et al.* 2002, Foulon and Foucras 2008). Ovine macrophage generation from bone marrow had been previously achieved with the use of a high concentration of FBS and autologous serum (Francey *et al.* 1992, Francey *et al.* 1992). In one of these studies, a number of recombinant cytokines (including recombinant mouse, human and bovine GM-CSF, pure mouse M-CSF and recombinant human M-CSF) had been tested for their ability to induce macrophage proliferation. Although all cytokines tested induced macrophage proliferation, the cells had been cultured in Teflon-coated bags and this did not allow for separation of BM-MΦ from cells of other lineage or undifferentiated myeloid cells present in culture (Francey *et al.* 1992).

Ovine recombinant IL-4 is not widely available as a single reagent and titrations of its biological activity were not available at the time it was used for the experiments described here. Similarly, a purified native or recombinant ovine M-CSF has not yet been developed, and therefore the approaches described above could not be used. In order to achieve macrophage differentiation, separation from other cell types and a certain degree of proliferation, high concentrations of serum were used in the culture medium in the current study. In addition to M-CSF, the amount of which will naturally vary, it is hypothesised that serum may contain other molecules (as yet unknown) contributing to macrophage differentiation and proliferation (Francey *et al.* 1992, Yen and Stewart 1982). Relying on unknown growth factor(s) in serum to induce cell proliferation/differentiation for lack of biologically active recombinant ovine M-CSF may present some problems; notably that these factors are not only of unknown identity but also not titrated, and therefore their amount and effect would be variable with each culture. This was empirically shown by the results of this chapter, as the number of cells available for stimulation was not only affected by the amount of bone marrow cells that could be isolated (ranging between 2.8 x 10^6 and 8.8 x 10^7 total bone marrow cells from individual animals), but also by the incubation method (flask, Teflon bag or tissue culture plates). As all other factors were

identical across cultures, the only variable introduced (except from the sheep itself) was the composition of the FBS and autologous serum used.

All results considered, *in vitro* sheep macrophage culture from bone marrow stem cells proved to be a successful method in delivering completely differentiated macrophages, as shown by positive CD68 and non-specific esterase staining of these cells, and also by their ability to phagocytose *E. coli* cells. Although CD68 could not be considered a definitive, specific macrophage marker (Kunisch *et al.* 2004), in combination with other markers such as staining of non-specific esterase enzymes, the use of CD68 can be considered a good compromise (Edwards *et al.* 1993). The BM-MΦ cultures characterised here were able to respond to stimulation with *E. coli* LPS by producing detectable levels of TNFα and IL-10, although it was not possible to demonstrate significant changes compared to the unstimulated cells. Further work may include more numerous repeats of stimulation wells and higher cell numbers/well. In order to be able to correctly assess cell responses to recombinant Tci-MIF-1, a reliable positive control of macrophage stimulation using LPS or other molecule [such as poly(I:C), a TLR3 agonist that imitates viral infections (Alexopoulou *et al.* 2001), or thioglycollate, used to elicit peritoneal macrophages in mice (Iqbal *et al.* 2013)], must first be established. A further possibility in designing a positive control could be found in the use of combinations of Pathogen-Associated Molecular Patterns (PAMPs) to stimulate cells into *in vitro* activation, as described in a murine model of DCs and cytotoxic T cells by (Warger *et al.* 2006). Indeed, recent unpublished work suggests that ruminant immune cells' hyporesponsiveness could be overcome by using a combination of PAMPs in order to elicit activation (Tom McNeilly, unpublished data). It is also possible that bone marrow-derived ovine macrophages do not fully activate in response to certain types of LPS, and that they might use different activation pathways in their response to it. As convincing classical activation of BM-MΦ was not achieved for this thesis, it would be favourable for future experiments to optimise this aspect of ovine macrophage culture before proceeding to further stimulate cells with other molecules. In this chapter, although BM-MΦ were successfully generated and reliably characterised, the cell numbers available for stimulation experiments were unpredictably low (< 1 x 10^6/culture). Furthermore, results from stimulation of BM-MΦ described here must be carefully interpreted as the stimulation protocol had not involved LPS decontamination of proteins. For these reasons, it was decided to use alveolar macrophages (A-MΦ) instead, which are already differentiated and can be readily isolated from lung lavage.

A-MΦ cells do not need any specific isolation technique and can be used as soon as separated from the other cell populations present in the lung lavage. Ovine A-MΦ were shown here to stain for CD68 and the enzyme non-specific esterase, and were able to phagocytose FITC-labelled *E. coli* cells. Their morphology and functionality confirmed, these cells were stimulated following a protocol similar to that used for BM-MΦ. The results showed that A-MΦ were also able to produce the cytokines TNFα and IL-10 following stimulation with LPS; however, again the difference between stimulated cells and unstimulated controls was not significant. One of the main receptors involved in LPS recognition, TLR4, has been shown to possess various degrees of polymorphism in vertebrates (Matsushima *et al.* 2007), with varied responses to LPS challenges between species [reviewed by Miller *et al.* (2005) and Werling *et al.* (2009)]. In humans, it has been shown that activation of TLR4 by *E. coli* lipid A was found to be host species-independent, whereas recognition of *Salmonella enterica* lipid A depended on the TLR4 molecule of the host (Muroi and Tanamoto 2002). In this respect, it has been shown that sheep TLRs present more similarity to cattle and humans, than mice (Nalubamba *et al.* 2007). TLR4 mRNA, together with TLR1, -2, -3 and -10, has been shown to be relatively highly expressed in sheep alveolar macrophages, compared to other TLRs (Chang *et al.* 2009). Although it was not investigated for this thesis, it is unlikely that the A-MΦ used in this study did not express TLR4, and therefore they should have been able to bind LPS. However, based on observations on sheep keratinocytes, it appeared that ovine TLR4 may preferentially recognise under-acetylated forms of LPS such as that derived from *Rhodobacter sphaeroides*, being only poorly responsive to *E. coli* LPS (Tom McNeilly, personal communication). In cattle, it has been shown that LPS-dependent activation of cells could not be fully achieved unless both the co-receptors CD14 (in cell-bound or soluble form) and MD-2 were present (Lizundia *et al.* 2008). Moreover, it has been previously shown that *R. sphaeroides* LPS acted as an agonist for cells expressing equine TLR4/CD14/MD-2 complex, but not for cells expressing the human homologues (Lohmann *et al.* 2007). Interestingly, when ovine BM-MΦ generated in this chapter were stimulated with both *E. coli* and *R. sphaeroides* LPS versions, it was possible to elicit similar results in terms of TNFα secretion in the cell supernatant. This could be explained by the use of autologous sheep serum for the BM-MΦ *in vitro* culture, which may have contained soluble sheep CD14. As sheep serum had not been added to the A-MΦ cultures, a lack of co-receptor such as CD14 might have induced sub-optimal TLR4 activation. It would have been interesting to investigate the activation of sheep A-MΦ following stimulation with *R. sphaeroides* LPS, had the cell numbers available for pilot experiments been higher. Another explanation for the sub-optimal activation observed may be that

sheep A-MΦ were hyporesponsive to the particular *E. coli* O111:B4 LPS used in this study, possibly due to cell signalling independent from the TLR4/CD14 complex.

For both IL-10 and TNFα production, the addition of ov rIL-4 showed a significant decrease in the levels of cytokines produced compared to LPS-stimulated cells, suggesting that IL-4 may have generated an alternatively activated macrophage phenotype, inhibiting release of these cytokines. However, as the cytokine release in response to IL-4 stimulation was not significantly reduced compared to unstimulated cells, these results should also be carefully interpreted. MIF alone has been reported to have an inflammatory effect [reviewed by Calandra and Roger (2003)], and therefore a phenotype closer to classical activation might have been observed when either recombinant Tci-MIF-1 was used. However, the results from this chapter did not show a significant effect due to either recombinant Tci-MIF-1 version. A more pronounced alternative activation phenotype might have been observed if combined stimulation protocols were used, such as MIF in association with IL-4 as previously described in mice (Prieto-Lafuente *et al.* 2009). However, inconsistencies in the numbers of cells viable for stimulation meant that the stimulation protocols used in this work had to be curtailed and only single molecules could be used at any one time.

The gene expression in A-MΦ cultures was investigated with the aim of identifying markers of classical (CAMΦ) or alternative activation (AAMΦ) in ovine cells following stimulation with either recombinant version of Tci-MIF-1 generated in this thesis. Alternative activation of macrophages following stimulation with MIF proteins has not been shown before in sheep, although previous work showed that a predominant Th2 environment was indicative of AAMΦ and characteristic of parasitic infections in general [reviewed by Jenkins and Allen (2010)], and *T. circumcincta* infection in sheep in particular (Craig *et al.* 2007). Following the example of macrophage stimulation in cattle systems (Flynn *et al.* 2007), a number of sheep genes had been selected for this purpose [*iNOS, IL-8, IL-12p40* and *TNFα* for classical activation; *Arg-1, Chit3-L1* and *IL-10* as measure of alternative activation (Knight *et al.* 2007, McNeilly *et al.* 2008, Mershon *et al.* 2002)]. Although the qPCR data failed to highlight significant differences in the gene expression of the macrophage cultures examined, a few observations could be made from the results gained. In sheep, *Arg-1* and *iNOS* genes had not been previously linked to macrophage alternative activation. The work shown here aimed to provide some preliminary data on these aspects of macrophage biology and it was achieved with respect to *iNOS*: this gene showed significant down-regulation following stimulation with ov rIL-

4. IL-4 is a Th2 cytokine that drives alternative activation by switching the use of L-Arginine by iNOS to Arg-1, therefore inhibiting nitric oxide (NO) release (Modolell *et al.* 1995). Theoretically, expression of *iNOS* would exclude *Arg-1*, and vice-versa. In the work described here, expression of *Arg-1* was below detection levels in all cell cultures examined and it was not possible to determine whether this gene was an appropriate choice for alternative activation status, or whether the assay needed further optimisation. For this reason, the results involving *iNOS* gene expression should also be carefully interpreted. IL-8, IL-12p40 and TNFα are classical pro-inflammatory cytokines that are secreted by macrophages upon classical activation [generally by LPS or viral activation, although methamphetamine has also been shown to increase release of pro-inflammatory cytokines (Liu *et al.* 2012, McNeilly *et al.* 2008)]. Following *T. circumcincta* infection, the cytokines IL-10, IL-12p40 and TNFα have been shown to be up-regulated in abomasal lymph nodes (Craig *et al.* 2007). Mammalian acidic chitinase (AMCase) molecules have been previously described in mouse models of alternative activation (Prieto-Lafuente *et al.* 2009). In mice the gene *Ym1* (also known as Chit3-L3), was significantly up-regulated in the lung, small intestine and peritoneal macrophages of mice infected with the nematode *Nippostrongylus brasiliensis* or the protozoan *Trypanosoma brucei*, and was found to be predominantly produced by alternatively activated macrophages in an IL-4-dependent manner (Nair *et al.* 2005, Raes *et al.* 2002). In sheep, primers to murine *Ym1* and *Ym2* failed to amplify any product; however Chit3-L1 (belonging to the same family of mammalian chitinases of Chit3-L3 or Ym1) had been shown to be up-regulated in the abomasal mucosa of sheep infected with *T. circumcincta* and therefore appeared a likely candidate to study the alternative activation of sheep macrophages (Knight *et al.* 2007). However, it was shown in this chapter that *Chit3-L1* was significantly down-regulated in A-MΦ stimulated with ov rIL-4 compared to unstimulated cells. This result contrasts with the previous observations of this gene's IL-4 dependent expression in murine macrophages. Indeed the gene choice was broadly based on mouse models, and therefore it is possible that sheep macrophages may respond differently to IL-4 and/or predominantly Th2 environments. Another possibility may be that the observed up-regulation of *Chit3-L1* in the abomasal mucosa during *T. circumcincta* infection [as described by Knight *et al.* (2007)] involved cell-types other than macrophages; it is also possible that macrophages might up-regulate different chitinase genes in response to IL-4 stimulation, and that *Chit3-L1* is not the major gene involved in this activity.

The qPCR data were acquired using mRNA samples extracted from the A-MΦ cells stimulated with *E. coli* LPS, ov rIL-4 and two Tci-MIF-1 recombinant versions (EcTci-

MIF-1 and CeTci-MIF-1). In this occasion, RNA quantification and quality control was carried out using the Nanodrop method. Acquisition of a RIN (RNA integrity number) had not been possible at this time, as the amounts of mRNA extracted from the cells were too small to allow further characterisation of the samples. However, in further experiments a better approach should include higher cell number/well, in order to allow higher quality and quantity of mRNA available for qPCR assays. Housekeeping genes are used as internal reference in qPCR assays, as it is assumed that their expression levels would remain stable throughout the tissue culture experiment. Traditionally, it was believed that once a housekeeping gene was chosen for a particular species or cell type, it was not necessary to review the choice (Thellin *et al.* 1999). However, experiments have shown that candidate housekeeping genes transcription levels can vary (Thellin *et al.* 1999). To avoid errors caused by the use of inappropriate housekeeping genes, a new algorithm was developed (geNorm); this enables analysis of a number of reference genes and allows choice of the most stable for any specific experiment, cell type or organism (Vandesompele *et al.* 2002). This software has been used previously to identify the best housekeeping gene candidates in various species, such as dog, sheep, cattle and horse (Bogaert *et al.* 2006, Etschmann *et al.* 2006, Nalubamba *et al.* 2007, Robinson *et al.* 2007). In this chapter, insufficient cell viability and low quantities of mRNA available from each sample meant that the geNorm analysis could not be carried out on every single cDNA sample derived from A-MΦ cultures. Therefore, the candidate housekeeping genes *YWHAZ* and *GAPDH* had been chosen on the basis of a partial analysis carried out on both A-MΦ and BM-MΦ cultures. In future experiments, it would be advisable to test every cDNA sample for expression of each candidate housekeeping gene, in order to obtain a more accurate estimate of the most stable genes to use as reference.

Further work should also be carried out on the reliability of efficient qPCR reactions; the results of this chapter have shown that acceptable reaction efficiency between 90-100 % (Bustin *et al.* 2009) could only be achieved for 4 of the target or house-keeping genes considered (see Table 5.1). As A-MΦ cell numbers used in the stimulation experiments and the qPCR reaction set-up had both been consistent, the variable reaction efficiency may have been a reflection of the two different methods used to generate cDNA from mRNA extracted from the cell cultures. A PCR reaction with lower efficiency will have lower sensitivity (Bustin *et al.* 2009). For example, compared to a reaction in which efficiency is 100 %, the copies of PCR product/µl will be underestimated in a sample where transcript is relatively abundant, and conversely, will be overestimated in a sample

where the transcript has low abundancy (Bustin *et al.* 2009). Therefore, the results here shown should be carefully interpreted and further validation is required.

In conclusion, ovine macrophages showed the ability to be partially stimulated by LPS, recombinant ovine IL-4 and recombinant *T. circumcincta* MIF versions; however, the preliminary data presented here showed that further work is required to reach full protocol optimisation. The inconsistencies shown in this chapter regarding the activation of sheep macrophages indicate that data obtained from stimulation protocols (especially from either recombinant Tci-MIF-1 version) should be carefully interpreted. Further stimulation experiments of ovine macrophages should involve a molecule able to reliably and consistently activate the cell response in the classical way [such as poly(I:C) or thioglycollate (Alexopoulou *et al.* 2001, Calandra *et al.* 1994, Iqbal *et al.* 2013)], to be able to distinguish the classical and alternative phenotype. Titration of stimulating molecules should also be investigated, as in this chapter only one set concentration of each was tested. Production of endogenous ovine MIF from the *in vitro* macrophage cultures was not investigated in this occasion; it has been previously shown in a mouse model that only low concentrations (10-100 pg/ml) of MIF may induce a positive loop of further MIF release from macrophages (Calandra *et al.* 1994). Indeed in their experiment, it was shown that concentrations of 1-10 µg MIF had significantly decreased macrophage MIF production compared to the lower MIF concentrations (Calandra *et al.* 1994). In this chapter, stimulation assays used 5 µg/ml of both recombinant Tci-MIF-1, therefore the likelihood of detecting an increase in sheep MIF produced by the A-MΦ was greatly reduced. However, ovine MIF, if produced by activated macrophages, would be an interesting product to investigate in further experiments as this could provide another measure of the activation status of these cells. The results reported in this chapter have shown that careful interpretation of data is essential when analysing sheep macrophage responses to stimuli. Moreover, ruminants (and sheep in particular) may show distinct immunological responses from other mammalian species, and therefore species-specific work is required to better understand the immune response of these animals. Indeed mouse models cannot always be proficiently used in comparative studies to infer data upon other species, and careful planning must always be considered when testing hypotheses previously confirmed in other host species.

The inability of either recombinant Tci-MIF-1 versions used in this chapter to inhibit cell migration is consistent with (Nisbet *et al.* 2010b) and suggested that Tci-MIF-1 might not be involved in the immunomodulation of the sheep immune response against *T.*

circumcincta. This hypothesis was further corroborated by the subsequent findings that neither recombinant Tci-MIF-1 version was able to induce consistent *in vitro* activation of sheep macrophages, either by cytokine production or gene expression. It would however be important to test activity of native Tci-MIF-1 from the parasite, before concluding that this molecule has no effect on sheep cells. *T. circumcincta* MIF may have evolved to mediate parasite development and may be used during the process of melanisation, as suggested by (Nisbet *et al.* 2010b). In support of this hypothesis, the amino acid sequence alignment shown in this Chapter revealed that sheep MIF did not share the same similarities with Tci-MIF-1, as it did with human MIF. This suggests that the sheep host version of this cytokine may have retained different functions from Tci-MIF-1, and that *T. circumcincta* had not evolved an immunomodulatory function for this cytokine, as opposed to the immunomodulatory activity achieved by *B. malayi* MIFs (Pastrana *et al.* 1998, Zang *et al.* 2002). Indeed in the free-living nematode *C. elegans*, Ce-MIF-2 and -3 transcripts were shown to be up-regulated in the dauer stage (Marson *et al.* 2001). The dauer stage is characterised by arrested metabolism and growth until more favourable conditions become available (Hu 2007), and suggestions have been made that L3 stages of parasitic nematodes may represent a dauer-like stage (Brand *et al.* 2005). This aspect of the *T. circumcincta* life cycle is currently still unclear, and therefore further work is required to shed light on the biological functions of Tci-MIF-1.

Taken together, the results shown in this chapter have provided an insight into the biological activity of Tci-MIF-1 as an enzyme and as a cytokine. The aims of this chapter were achieved and two recombinant versions of Tci-MIF-1 were compared in respect to their enzymatic activity and biological effect on sheep cells. It was shown that both recombinant Tci-MIF-1 proteins maintained the putative dopachrome tautomerase activity, each being capable of degrading the substrate L-dopachrome methyl ester. This confirmed once again that *C. elegans* is a viable means of expressing active recombinant enzymes, as previously shown by Murray *et al.* (2007) and Roberts *et al.* (2013). As neither recombinant Tci-MIF-1 version had a significant effect in the monocyte migration assays or the macrophage stimulation experiments, it is possible that this molecule is not involved in the immunomodulation of the sheep immune response as other parasite MIFs have been shown to be (Loke *et al.* 2000). Furthermore, *C. elegans* was used as an alternative expression system in an attempt to generate a recombinant version of Tci-MIF-1 that might show improved enzymatic activity or biological effects on sheep cells, compared to the *E. coli*-expressed version. However, the two enzymes showed similar activity in all fields examined. It is therefore concluded that *C. elegans*-expressed Tci-MIF-1 did not

substantially differ from its *E. coli*-expressed counterpart and that both proteins were equally valid in *in vitro* experiments. Moreover, it is suggested that the lack of convincing monocyte/macrophage activation may not be due to experimental artefacts or PTM defects of the recombinant Tci-MIF-1 versions, but perhaps to a different biological function that Tci-MIF-1 carries out within its natural host, *T. circumcincta*.

Chapter 6 – General Discussion

Teladorsagia circumcincta is a parasitic pathogen of sheep in temperate areas and is the principal causative agent of parasitic gastroenteritis (PGE) in growing lambs (Bartley *et al.* 2003, Taylor M. A. *et al.* 2007). *T. circumcincta* causes major production losses due to clinical PGE, with a huge economic impact on the sheep industry calculated as > 80 millions of pounds sterling (Nieuwhof and Bishop 2005). The associated clinical signs of PGE range from suppressed appetite to diarrhoea, quickly followed by dehydration and sometimes death in heavy infections (i.e. a mixed species parasite burden of ~10 000 worms in sheep; Radostits O.M. *et al.* 2007). The sum of the clinical signs of PGE results in weight gain reduction in the first few months of life in store lambs, often causing delays in reaching target slaughter weights (Gibson and Everett 1976, Miller *et al.* 2012). Studies in which corticosteroid drugs (with a potent immunosuppressive effect) were administered to sheep infected with *T. circumcincta* have shown that the resulting growth check may not only be due to the parasite infection *per se*, but can also arise from immune responses of the sheep. This may happen through redirection of nutrients from the processes required for meat and wool production towards the immune response (Greer *et al.* 2008).

Control of teladorsagiosis relies heavily on the use of anthelminthic drugs. Three major classes of chemicals are used in this respect: benzimidazoles (BZs or white drenches), levamisole (yellow drenches) and macrocyclic lactones (MLs or clear drenches). Recently, two new classes of anthelmintics have been introduced: the amino-acetonitrile derivatives (AADs or orange drenches) and the spiroindoles (purple drenches). However, despite the number of drugs available to treat sheep against GI nematodes, heavy or inappropriate uses of all classes have resulted in widespread anthelmintic resistance. Anthelmintic resistant parasites have been known since the late 1950s (Drudge *et al.* 1957) although, until more recent years, isolates that showed resistance to more than one type of drug had been relatively rare. However, *T. circumcincta* isolates showing multiple resistance to the three most commonly used drug classes have been identified (Bartley *et al.* 2003, Bartley *et al.* 2005, Sargison *et al.* 2005, Sargison *et al.* 2010, Sutherland *et al.* 2008). In some cases, these parasites represented a threat so great to the local sheep industry that farms were forced to stop all sheep operations until clear of the offending parasites (Blake and Coles 2007, Sargison *et al.* 2005).

For these reasons, alternative means of controlling these parasites are urgently needed. Research has focused not only on developing new anthelmintic drugs (against which

resistance is inevitable in the long term), but also on alternative measures such as pasture management, breeding resistant or resilient breeds of sheep, targeted treatments of only certain individuals in a flock, establishing a *refugia* worm population, and more importantly, studying the sheep immune response against the parasite (Bahirathan *et al.* 1996, Behnke *et al.* 2008, Besier 2012, Bisset *et al.* 2001, Golding and Small 2009, Good *et al.* 2006, Kenyon and Jackson 2012, McNeilly *et al.* 2009, Miller and Horohov 2006, Nisbet *et al.* 2013, Sargison *et al.* 2005, Stepek *et al.* 2005, Stepek *et al.* 2010b, Stepek *et al.* 2010a).

It has been established that immunity to *T. circumcincta* can be induced by natural infection on infected pasture or experimental trickle infection (Seaton *et al.* 1989, Taylor M. A. *et al.* 2007). Immunity against this parasite occurs through different mechanisms such as exclusion/expulsion of L3 stages, production of IgA in the gastric environment and local lymph nodes and circulating IgE resulting in impaired larval development at the L4 stage and reduced fecundity in adults (Halliday *et al.* 2007, Huntley *et al.* 2001, Pettit *et al.* 2005, Smith *et al.* 2009, Smith *et al.* 1985, Stear *et al.* 1995, Stear *et al.* 1999, Strain *et al.* 2002). However, antibody responses alone may not be sufficient to clear the infection and recruitment of specialised cells such as DCs has been documented [reviewed by McNeilly *et al.* (2009)].

As protective immunity against *T. circumcincta* can be elicited in sheep over a period of time, it may be possible to quicken this natural process by administering vaccinations against the parasite, in order to protect the most susceptible animals (Knox 2000). Vaccinations could reduce the widespread use of anthelmintic drugs, therefore reducing costs and handling procedures for the farmers, increasing the number of lambs reaching slaughter weights within time targets expected, with a further beneficial effect due to reduced or nil withdrawal times on the meat products and slowing down the establishment of anthelmintic resistance in the parasite populations (Knox 2000, Smith and Zarlenga 2006). Vaccinating animals against gastrointestinal parasites is therefore a desirable alternative control strategy to treating animals with anthelmintic drugs; firstly because of the ever-rising drug-resistant parasite population, and secondly due to consumer concerns about chemical residues in the food and environment (Science for Environment Policy, 2008). However, there have been limited successes in developing vaccines against helminth parasites, especially when recombinant proteins were used (See Tables 1.1 and 1.2 of this thesis). For example, immunisation of dogs with a *P. pastoris*-expressed Ac-CP-2 antigen (a cysteine protease) from the hookworm *Ancylostoma caninum* achieved

significant reductions in the faecal egg output [approximately 13 000 eggs/g faeces (epg) and ~ 5000 epg in controls and immunised dogs, respectively] following intradermal challenge with *A. caninum* L3 (Loukas *et al.* 2004). However, in other cases when native proteins such as *Ostertagia ostertagi* ASP (activation-associated secreted proteins) afforded protection against homologous challenge (Meyvis *et al.* 2007), trials carried out using recombinant versions (baculovirus-expressed Oo-ASP-1) showed that this version did not confer protection against *O. ostertagi* challenge when used to immunise calves (Geldhof *et al.* 2008). Several studies have shown that certain *Haemonchus contortus* proteins such as H11 and H-Gal-GP are highly protective against homologous challenge in their native form (Roberts *et al.* 2013, Smith *et al.* 1994, Smith *et al.* 2000). However, when the same proteins have been expressed in recombinant form, using different expression systems, they have not been able to achieve protection against *H. contortus* challenge (Cachat *et al.* 2010, Roberts *et al.* 2013), suggesting that the process of recombinant expression reduces potential to induce a protective immune response. Recently, a study published by Nisbet *et al.* (2013) has shown that a vaccine against *T. circumcincta* which contained 8 recombinant antigens protected sheep from trickle challenge. The reductions in faecal egg counts and worm burdens were significantly lower in the immunised sheep compared to the controls in both trials that were carried out (Nisbet *et al.* 2013). These are promising results and show that recombinant antigen immunisation can achieve protection in vaccine trials in an experimental setting. However, the road to a commercial *T. circumcincta* vaccine is still long and further refinement is required, initially to determine which components of the vaccine were crucial in the protection elicited. No less importantly, it should be determined whether a vaccine of this type could still be effective in field conditions where sheep are subjected to different challenges (i.e. mixed worm populations, the pressure of intensive farming, etc.) than in an experimental setting. For these purposes, a better understanding of the interactions between the sheep host, the parasite and the vaccine antigens is required. This can be achieved by investigating the sheep immune response to both *T. circumcincta* and the recombinant vaccine, and by characterising the relative biological properties of native and recombinant antigens. These requirements set the origins of the study here presented, with the assumption that a more intimate knowledge of the vaccine candidates used and their interactions would lead to an improved prototype of the vaccine.

The main aim of this thesis was to establish a nematode expression system that would allow the generation of *T. circumcincta* recombinant antigens which could be compared to the exisiting vaccine candidates. For this purpose, two *T. circumcincta* antigens which had

already been expressed in recombinant form and used in the successful vaccine (Nisbet *et al.* 2013) were chosen for further characterisation; these were an immunogenic cysteine protease, Tci-CF-1 (Redmond *et al.* 2006), and a putative immunoregulatory monocyte migration inhibitory factor, Tci-MIF-1 (Nisbet *et al.* 2010b). The sheep antibody response to the existing recombinant (expressed in *P. pastoris*) and native antigens was first studied, in order to have data with which to compare the antibody response to the *C. elegans*-generated recombinant version. The results of this work (Chapter 2 of this thesis) showed that a different antibody response is generated in sheep following the immunisation with the existing, yeast- and bacteria-generated recombinant antigens (Tci-CF-1 and Tci-MIF-1) than that observed during trickle challenge. In these studies [and those described by Nisbet *et al.* (2013)] it was evident that although the antigens present in the vaccine cocktail induced an antibody response in the serum and abomasal mucus of immunised sheep which was able to bind native antigens, the antibodies generated by trickle infection in control sheep (non–immunised) did not bind the recombinant Tci-CF-1 and Tci-MIF-1 used in the vaccine, suggesting that the recombinant antigens differed somewhat from the native versions produced by the parasite to which the sheep host is exposed to. This was in contrast to the antibody binding demonstrated to occur to native *T. circumcincta* ES antigens (Halliday *et al.* 2007, Redmond *et al.* 2006) and more particularly, to Tci-CF-1 purified from ES (Chapter 2 of this thesis).

In an attempt to generate recombinant antigens that would be recognised by sheep antibodies in a manner more similar to native antigens, the free-living nematode *C. elegans* was used. The work reported here showed that *C. elegans* is a viable expression system for the generation of *T. circumcincta* vaccine candidates, although it may require some optimisation regarding expression yields. Two different methods have been used in order to generate a *C. elegans*-expressed *T. circumcincta* protein: firstly, transformation of wild type *C. elegans* worms with an engineered plasmid encoding *T. circumcincta* Tci-CF-1 and, subsequently, phenotype rescue of a mutant *C. elegans*-strain (DR96) with a plasmid encoding *T. circumcincta* Tci-MIF-1. Both methods involved the use of marker genes to enable identification of the transformed *C. elegans* worms. Both methods were successful in delivering *C. elegans*-expressed *T. circumcincta* antigens and these could be purified in stable, soluble forms in order to carry out further characterisation.

Characterisation of the biological functions of both *Pichia pastoris*- and *C. elegans*-expressed Tci-CF-1 and comparison with the native ES-purified version showed that indeed some differences do exist. The enzymatic activity of either recombinant version

could be analysed only following *trans*-activation with another enzyme (exogenously added human, liver cathepsin L). Together with the observation that native Tci-CF-1 is present in *T. circumcincta* L4 ES as a mature enzyme, these results suggest that in order to be correctly recognised by *T. circumcincta* infection-induced antibodies the recombinant antigens should be cleaved in a similar manner to the native Tci-CF-1. Furthermore, the results showed that glycosylation of this antigen appears to have a role in the induction of an appropriate antibody response in sheep, as shown by the sodium-periodate ELISA experiments of Chapters 2 and 4 of this thesis. Moreover, the recombinant version expressed in the yeast *P. pastoris* is likely to be hyperglycosylated with carbohydrate residues that differ in size and conformation to those of the native protein. The results from the initial lectin binding experiments also showed that the glycan composition *C. elegans*-expressed version appeared similar to the *P. pastoris* recombinant Tci-CF-1; however, detailed comparative analysis of the defined glycan structures on each will provide more information (glycan analysis of native and recombinant Tci-CF-1 versions is currently in progress in collaboration with Prof. Cornelis Hokke, University of Leiden). Further work could be carried out to investigate the hypothesis of the presence of disulphide bonds within the cysteine residues of the Tci-CF-1 amino acid chain; if differences in folding were identified (i.e. bonds were present in certain positions within nTci-CF-1 but not present or scrambled within either recombinant version), it could provide an explanation as to why in this study antibody binding differed between Tci-CF-1 versions.

The putative immunoregulatory functions of *T. circumcincta* Tci-MIF-1 had been previously listed (Nisbet *et al.* 2010b). This protein in its native state was thought to have a role both in the maturation of *T. circumcincta* larval stages and also to affect the immune response of sheep mononuclear cells, more specifically to inhibit their ability to migrate. These functions had been hypothesised on the basis Tci-MIF-1 enzyme activity [as dopachrome tautomerase (Nisbet *et al.* 2010b)] and studies carried out in other nematode species, such as *Brugia malayi* and *Trichinella spiralis*, which showed the ability of parasite MIFs to inhibit the random migration of host monocytes (Pastrana *et al.* 1998, Tan *et al.* 2001, Zang *et al.* 2002). The characterisation of recombinant Tci-MIF-1 versions carried out for this thesis has shown that although both of these proteins maintain the dopachrome tautomerase enzymatic activity previously shown, neither was able to significantly influence the migration of sheep monocytes in *in vitro* assays. This confirmed the results reported by (Nisbet *et al.* 2010b), and suggests that perhaps the main biological functions of Tci-MIF-1 do not involve interaction with sheep immune cells but instead are involved in larval development of the parasite. Nonetheless, the hypothesis that Tci-MIF-1

might modulate the sheep immune response was supported by studies of other parasite MIFs (Pastrana *et al.* 1998, Prieto-Lafuente *et al.* 2009, Tan *et al.* 2001, Vermeire *et al.* 2008), and it was thought that although it did not have an effect on cell migration, it may have been involved in the induction of activated macrophages. MIF has been known as pro-inflammatory cytokine expressed by a variety of cells of the immune and endocrine systems (Calandra and Roger 2003). For example, a previous study showed that mouse macrophages stimulated with MIF molecules secreted TNFα in a pro-inflammatory loop (Calandra *et al.* 1994). In contrast to the pro-inflammatory function of MIF, other experiments showed that this molecule can also act in an anti-inflammatory fashion, and it is able to induce alternatively activated macrophages, which have been reported to occur during parasite infections (Allen and Loke 2001, Loke *et al.* 2000, Vermeire *et al.* 2008). To investigate this hypothesis, Tci-MIF-1 was further tested by stimulating *in vitro*-generated sheep macrophages. The results of these experiments have been largely consistent with the lack of migration inhibitory capacities of recombinant Tci-MIF-1, as although sheep macrophages were able to produce detectable levels of cytokines (TNFα and IL-10) in response to Tci-MIF-1, these were not significantly different from the unstimulated cells. Furthermore, when gene expression following exposure to LPS and both recombinant Tci-MIF-1 versions was investigated, few significant changes were noted at 6 and 24 hours post-stimulation in the expression of pro-inflammatory and alternatively activation-associated gene expression (Chapter 5 of this thesis). It is not clear whether sheep macrophages might respond differently than human and mouse cells to various types of infectious insult through their TLRs, although it is possible. Werling *et al.* (2009) and Zhou *et al.* (2007) showed with phylogenetic trees the differences in structure of the extracellular TLRs domains between livestock species, rodents and primates. Differences in structure might suggest differences in binding ligands, or the affinity with which a ligand is bound (Werling *et al.* 2009). Differences in macrophage activation were shown by experiments in which bovine and murine macrophages were stimulated *in vitro* with *Salmonella dublin* and/or homologous recombinant IFNγ, TNFα and IL-1 (Adler *et al.* 1995). Although both mouse and bovine macrophages showed expression of iNOS following *S. dublin* stimulation, neither of the recombinant cytokines was able to induce iNOS in the bovine macrophages, nor priming of these cells, compared to mouse macrophages (Adler *et al.* 1995). Furthermore, there are profound differences in the physiology of these species (i.e. monogastrics *vs* ruminants, omnivores *vs* strict herbivores). The environment in which sheep live constantly exposes them to a large variety of immunological challenges. Moreover, ruminants survive thanks to the presence of the rumen, which is, put simply, a very large incubator of microrganisms. It is possible

that ruminants might have evolved mechanisms of tolerance or hypo-responsiveness to certain bacterial stimuli, and this would be reflected in the results of the experiments here described. Another possibility could be that ruminants, and sheep in particular, may adopt different immunological strategies to combat infection compared to monogastric mammals such as mice. Innate immune responses, for example, may show a degree of antigen specificity, due to the interactions between Pattern Recognition Receptors [PRRs, such as Toll-like Receptors (TLRs)] and generic Pathogen-Associated Molecular Patterns (PAMPs); thus, different exposure to PAMPs and different host usage of TLRs may ultimately achieve the same cell response (McNeilly 2008). In this respect, it has been shown that sheep TLRs present more similarity to bovine and human receptors than those present in mice (Nalubamba *et al.* 2007). Moreover, a perceived hyporesponsiveness (or higher activation threshold) of ruminant cells may be overcome by using a combination of two or more PAMPs to achieve activation and allow investigations (Dr Tom McNeilly, personal communication).

Taken together, the results from Chapter 5 of this thesis confirm the hypothesis that Tci-MIF-1 might be mostly involved in processes related with *T. circumcincta* larval development, rather than influencing the sheep immune response. This does not mean that Tci-MIF-1 may be a less valid candidate for the investigation of the biology of *T. circumcincta*. (Nisbet *et al.* 2010b) have shown that although the highest expression of Tci-MIF-1 (both mRNA and protein) was in the L3 stages, protein was also detectable in somatic extracts of L4 and adult stages. This suggests that, although to a lesser extent, Tci-MIF-1 is present in host-dependent stages. If the functions of this protein were primarily involved in larval development, this could make Tci-MIF-1 a candidate for drug targeting still worth investigating. Furthermore, the role of Tci-MIF-1 in the vaccine cocktail developed by Nisbet *et al.* (2013) is still to be defined and, not being a particularly abundant or immunogenic protein in its native state, it is harder to characterise its putative protective effect during *T. circumcincta* infection compared to Tci-CF-1.

Overall, the results of this thesis have answered the main aim: the generation of alternative *T. circumcincta* recombinant vaccine candidates has been achieved using a novel nematode expression system, *C. elegans*. The proteins were purified and characterised alongside the existing recombinant versions of Tci-CF-1 (expressed in the yeast *P. pastoris*) and Tci-MIF-1 (expressed in *E. coli*), and showed similar characteristics. In the greater picture of vaccine development, considering that the existing Tci-CF-1 and Tci-MIF-1 versions had already been used in a successful vaccine cocktail against *T. circumcincta* (Nisbet *et al.*

2013), it appears that the *C. elegans*-expressed versions here generated might be equally successfully used in vaccination studies, given their high degree of similarity in *in vitro* studies. However, minute differences between the exisiting recombinant versions and those expressed in *C. elegans* may nonetheless exist and influence the host immune response; these questions could only be answered by further immunisation trials.

To date, there have been no immunisation studies that used *C. elegans*-expressed *T. circumcincta* antigens in sheep, and this would be an interesting avenue to explore. An initial pilot study may involve immunisation of a small number of sheep with a traditionally expressed antigen (generated using *E. coli* or *P. pastoris*), and including both sheep immunised with a *C. elegans*-expressed antigens, and unvaccinated animals. Serum samples collected from each group could then be tested for their efficacy in reducing protein functions (e.g. loss of enzyme activity if exposed to immune serum) and recombinant systems thus compared. However, given the relatively small amounts of recombinant protein obtained in this study from *C. elegans* compared to yeast and bacterial cultures, this option would require further optimisation, especially with views of producing the vaccine on a large scale for commercialisation. A recent study has shown that *C. elegans* can be successfully and productively employed to generate amounts of nematode (*H. contortus*) recombinant proteins sufficient to immunise sheep for a vaccine trial (Roberts *et al.* 2013). This further confirms that *C. elegans* is a viable alternative to other expression systems and that it may be used to generate recombinant vaccine antigens, particularly where expression of soluble and/or active proteins is challenging. However, further validation work is required to establish whether the recombinant proteins expressed using *C. elegans* are sufficiently antigenically similar to the native versions than those expressed using traditional methods (such as *E. coli* and yeast cultures), in order to afford the ultimate protective nematode vaccine.

Appendix A – Primers, cDNA and PCR reactions

7.1 Semi-quantitative RT-PCR primers

Unless otherwise stated, oligonucleotide primers used throughout this project were designed using Eurofins MWG Biotech and Primer3 tools (www.eurofinsdna.com and http://frodo.wi.mit.edu/, respectively) to design the primers and calculate melting temperatures (Tm). Primers were ordered from Eurofins MWG Operon (Ebersberg, Germany) and diluted to a stock concentration of 100 pmol. The working concentration of all primers for PCR was 10 pmol. Primers used to generate PCR products in Chapter 2 are listed in Table 7.1; the primers used for reactions in Chapter 3 are listed in Table 7.2. Details of the reaction conditions used are available in each individual results chapter.

Table 7.1 Primers used for cloning Tci-CF-1 into *Pichia pastoris*.

Set number	Name	Forward (F) 5' → 3'	Reverse (R) 5' → 3'	Product Size (bp)	Average pair Tm (°C)
1*	FLCF-1	ATGTCTCTTTTGTTCCTGCTTCTC	TAGGACAACAGCTGAAGTGG	1095	60
2*	CathFPICz	AATTCACGTGGCGCTACTGTAAAGCAGCAATACTC	TTTGTTCTAGAAGGACAACAGCTGACGTGG	1052	60
3	*tci-cf-1*-specific	ForIN AGGAAAACGATAAGGGAACAG	RevIN CGCCTTTATAGAACTGTATGTC	594	56
4^	pPICZαC-specific	5' AOX1 GACTGGTTCCAATTGACAAGC α-factor TACTATTGCCAGCATTGCTGC	3' AOX1 GCAAATGGCATTCTGACATCC	(empty vector) **5'AOX1** 593 **αfactor** 304	Annealed at 52

The underlined nucleotides represent the recognition sites of restriction enzymes: dashed line: *Pml*I, solid line: *Xba*I. Primer sets noted with * were provided by Dr Alasdair Nisbet (MRI). Primers noted by ^ were included in the EasySelect™ *Pichia* Expression kit (Invitrogen).

Table 7.2 Primers used for cloning Tci-CF-1 and Tci-MIF-1 into *Caenorhabditis elegans*.

Set number	Name	Forward (F) 5' → 3'	Reverse (R) 5' → 3'	Product Size (bp)	Average pair Tm (°C)
1	Tci-[CF1]	GCGGCCGCATGTCTCTTTTGTTCCTGCTTCTC	GTCGACCTAAGCGCTTAGGACAACAGCTGACGTGG	1115	60
2	[CF1]-His	GCGGCCGCATGTCTCTTTTG	Rev1: *GTGGTGGTGGTGGTGGT*TAGGACAACAGCTGAGCTGG	1118	57
			Rev2: GTCGACCTA*GTGGTGGTGGTGGTGGTGGTGGTGGT*AGGACAAC	1139	60
3	Sequencing ABR-09-specific	ForOut: ATCGCTCTCTGTTTCTCTCC	RevOut: TACGAGCTAATTATATGCTCTTAC	1586	56.5
4*	Ce-cpl-1	TTGGTCGACATTTGAACTTAATAATTAA	CCAGAGCTCCCACCGAGATTTGAAC	512	55
5	[MIF]+His	AATTGCGGCCGCATGCCGGTTTTCTCATTCCAC	Rev1: *GTGGTGGTGGTGGTG*GGCAAAAGTTCTGCTGTTGAAG	375	57.9
			Rev2: AATTCTCGAGCTA*GTGGTGGTGGTGGTGGTGGTGGTGGTG*GGCAAAAGTTC	403	62
6	Sequencing TciMIF	ForIN: ATGCCGGTTTTCTCATTCCAC	RevIN: CAAAAGTTCTGCTGTTGAAGG	343	57

The underlined nucleotides represent the recognition sites of restriction enzymes: dashed line: *Not*I, solid line: *Sal*I; double line: *Xho*I; nucleotides in *italics* represent the poly-histidine tag. An aliquot of the primer set noted with * was provided by Dr Brett Roberts at the University of Glasgow (Britton and Murray 2002).

7.2 Generation of cDNA from ovine macrophages

For the generation of cDNA from ovine macrophages, one method used the kit *Precision*™ nanoScript Reverse Transcription kit by PrimerDesign Ltd. (Southampton, UK) was used in the following reactions: 1 µl of Oligo-dT Primers was added to ~120 ng of RNA previously diluted to the desired concentration in dH_2O in sterile, RNAse free 0.2 ml thin-walled PCR tubes. The samples were then heated at 65°C for 5 min and immediately cooled on ice. A master mix was prepared, containing 2 µl of nanoScript 10X Buffer, 1 µl of dNTP mix (10 mM of each), 2 µl of DTT (100 mM), 4 µl of RNAse/DNAse free dH_2O and 1 µl of nanoScript enzyme for each reaction. Ten microliters of the master mix were aliquoted into each RNA samples on ice, which were then incubated at 55°C for 20 min, then heat inactivated at 75°C for 15 min.

The second method used for the generation of cDNA was as follows: a master mix containing 1 µl of Oligo(dT)23 Primers (anchored) (Sigma-Aldrich) was added to 1 µl of dNTP mix (10 mM) and 2 µl of this solution was aliquoted into each 10 µl RNA sample (diluted in RNAse-free dH_2O as previously described). The samples were incubated at 65°C for 5 min, then immediately transferred on ice. Another master mix was prepared containing 4 µl of 5X First strand buffer (Invitrogen, Paisley, UK), 2 µl of 0.1 M DTT (Invitrogen) and 1 µl of RNaseOUT (Invitrogen) for each reaction. Seven microliters of this master mix was added to each RNA sample and incubated at 42°C for 2 min; 1 µl of Superscript™ II Reverse Transcriptase (Invitrogen) was added to each sample, mixed by pipetting and incubated at 42°C for 50 min, then heat inactivated at 70°C for 15 min.

7.3 Quantitative RT-qPCR primers

Primers used in qRT-PCR reactions for ovine *iNOS-2*, *Chit3-L1*, *Arg-1*, *YWHAZ* were designed and optimized by PrimerDesign Ltd, courtesy of a Gold Sponsorship Student Award. The primers were supplied as pre-mixed solutions of forward and reverse primers, at a working concentration of 6 pmol/µl each.

Primers sequences for ovine *TNF-α*, *IL-10*, *IL-8*, *IL-12p40* and *GAPDH* were previously detailed by McNeilly *et al.* (2008) and supplied by Eurofins MWG Operon (Ebersberg, Germany); upon arrival, the primers were diluted to a stock concentration of 100 pmol. The working concentration of all primers was 10 pmol. Details of all primers are summarised in Table 7.3.

Table 7.3 Primers used in this study in RT-qPCR experiments.

Gene product	Accession number	Sequences	Product size	Annealing temperature (°C)
iNOS	AF223942	F, GCC TCT GGA CCT CAA CAA AG R, GTG GTG CGG CTG GAT TTC	108	60
Chit3-L1	AY392761	F, CCA ACA TAA GCA ACA ATG AGA TC R, CCA AAG TTC CAT CCT CCA ACA	130	60
Arg-1	EE768328	F, CAT ATC TGC CAA AGA CAT TGT G R, ACC TTG CCA ATT CCC AGT T	129	60
YWHAZ	N/A	N/A	N/A	60
*TNFα^*	X55152	F, GAA TAC CTG GAC TAT GCC GA R, CCT CAC TTC CCT ACA TCC CT	238	57
*GAPDH^*	AF030943	F, GGT GAT GCT GGT GCT GAG TA R, TCA TAA GTC CCT CCA CGA TG	265	57
*IL-10^*	NM_001009327	F, TGA AGG ACC AAC TGA ACA GC R, TTC ACG TGC TCC TTG ATG TC	160	55
*IL-8^*	NM_001009401	F, ATG AGT ACA GAA CTT CGA R, TCA TGG ATC TTG CTT CTC	222	55
*IL-12p40^*	AF209435	F, TCA GAC CAG AGC AGT GAG GT R, GCA GGT GAA GTG TCC AGA AT	243	57

N/A: data not available for commercial reasons (property of PrimerDesign Ltd., Southampton, UK), as included in sheep Genorm kit. ^ = primer sequences provided by Dr Tom McNeilly (MRI), as detailed by McNeilly *et al* (2008).

7.4 Standard qPCR reactions

Assays for the detection of *iNOS-2, Chit3-L1, Arg-1, YWHAZ* gene products were carried out as follows: 4 µl of dH_2O, 10 µl of Precision 2x SYBR green MasterMix (PrimerDesign) were added to 1 µl of primers mix (a solution prepared adding 10 µl of forward primer to 10 µl of reverse primer; Table 7.3) and 5 µl of cDNA template diluted 1:10 in dH_2O. Cycling conditions were: 95°C for 10 min, then 40 cycles of (95°C for 30 sec, 60°C for 1 min); fluorescent data was collected during the 60°C for 1 min step. The cycling was completed with the addition of dissociation steps for melting temperature data collection. Negative control reactions (NTC) contained 5 µl of dH_2O instead of template. A standard curve was prepared using serial dilutions of positive control plasmids (PrimerDesign Ltd); the undiluted plasmid was 2 x 10^5 copies/µl (5 µl/reaction = 10^6 copies/reaction), diluted ten-fold serially (range of the standard curve: 10^6-10^1

copies/reaction). The above reaction volumes and cycling conditions were also used for generation of qPCR data used in the GeNorm analysis described in Chapter 5.

Other assays (*IL-10, IL-8, IL-12p40, TNFα, GAPDH*) used the following conditions: 4 µl of dH_2O, 10 µl of Precision 2x SYBR green MasterMix (PrimerDesign) were added to 0.5 µl of forward and 0.5 µl of reverse primers (see Table 7.3), 5 µl of cDNA diluted 1:10 in dH_2O. Cycling conditions for all assays were: 50°C for 2 min, 95°C for 10 min, then 40 cycles of (95°C for 30 sec, X°C for 30 sec and 72°C for 32 sec, where X was the annealing temperature specific of each primer set); fluorescent data was collected during the 72°C for 32 sec step. The cycling was completed with the addition of dissociation steps for melting temperature data collection. Negative control reactions (NTC) contained 5 µl of dH_2O instead of template. A standard curve was prepared using serial dilutions of positive control plasmids (provided by Dr Tom McNeilly, MRI); the undiluted plasmid was 10^9 copies/µl, diluted ten-fold serially (range of the standard curve: 10^7-10^2 copies/reaction).

Appendix B – Other Laboratory Techniques

8.1 Electrophoresis

8.1.1 DNA electrophoresis

PCR products were visualized in 1 % agarose gels prepared with 1 X TAE Buffer; 2 g agarose (Bioline, London, UK) were dissolved in 200 ml of 1 X TAE Buffer (Appendix B), heated on full power in a microwave for approximately 3 min (until boiling temperature was reached and the solution turned clear) and then cooled before use. Twenty microliters of 10 000 X Gel Red (Biotium, Hayward, CA, USA) were added and mixed. The gel was poured into a gel electrophoresis tank with a comb to leave wells during the setting of the gel. Once the gel had set, the comb was removed and 1 X TAE poured in the tank to submerge the gel. Six microliters of samples were mixed with 2 µl of DNA loading buffer (Appendix B); one or two wells at either end of the gel was loaded with Ready-Load™ 1 kb DNA Ladder (Invitrogen, Life Technologies Ltd, Paisley, UK) and the tank attached to a power supply. One hundred Volts (V) were applied for approximately 35 min and gels were visualized using an UV transilluminator.

8.1.2 Protein electrophoresis

Unless otherwise stated, a standard amount of 130 ng (13 µl of a 10 µg/ml solution) of protein sample was added to 5 µl of NuPAGE® LDS Sample Buffer (4X) with the addition of 2 µl of NuPAGE® Reducing agent (all from Invitrogen), for a total volume of 20 µl, heated at 70°C for 10 min and loaded into each well of NuPAGE® Bis-Tris 4–12 % polyacrylamide gels, with the addition of running buffer into the chambers of the electrophoresis assembly. This was prepared by mixing 50 ml of 20 X NuPAGE® running buffer (Invitrogen) with 950 ml distilled water; 200 ml were removed, poured in the inner chamber of the gel/clamp assembly, and 500 µl of NuPAGE® Antioxidant (Invitrogen) were added. Further 500-600 ml of diluted running buffer were poured into the outer chamber. Eight microliters of SeeBlue® Plus2 Pre-Stained standard (Invitrogen) were added in the first and last well of each gel for protein size estimation. Gel electrophoresis conditions were 200 Volts for 35 min. Gels were routinely stained with Coomassie-based dye (SimplyBlue™ SafeStain, Invitrogen) and when more detection sensitivity was required, silver staining was also used (SilverQuest™ Silver Staining Kit, Invitrogen).

8.2 Western blotting

Following SDS-PAGE, protein samples were transferred onto a nitrocellulose membrane using iBlot System (Invitrogen), stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) for protein band identification and membranes cut into strips with a scalpel blade. All steps from hereon were conducted on a rotary shaker. The strips were blocked overnight in 20 ml TNTT (10 mM Tris, 0.5 M NaCl, 0.05 % Tween 20); the same were used for washes between incubations. TNTT/5 % dried skimmed milk was also used for blocking and washes when probing blots with an anti-His antibody (Invitrogen). The strips were then incubated with serum, gastric lymph or commercial antibodies detailed in each chapter of this work. Strips were washed three times for 20 min, and subsequently incubated with the appropriate secondary antibody (detailed in each individual chapter). After three 20 min washes, the strips were incubated with a tertiary antibody, where appropriate, or left rocking in TNTT for two hours/overnight for a final wash to reduce the background binding. The strips were developed with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) following the manufacturer's instructions. Occasionally strips were also developed with 3,3′-Diaminobenzidine (DAB, SIGMA*FAST*™ DAB with Metal Enhancer, Sigma-Aldrich).

8.3 Protein concentration estimations

8.3.1 BCA assays

These assays based on the use of bicinchoninic acid were carried out following the manufacturer's instructions (Pierce™ BCA Protein Assay Kit, Thermo Scientific, Loughborough, UK). Twenty-five microliters of BSA standards or protein preparations were placed in duplicate or triplicate wells of a flat bottomed, clear 96-well microplate. Blanks were prepared using 25 µl of PBS instead of protein preparations. The working reagent (WR), prepared with the ratio 50:1 or reagent A:B, was added to each well (200 µl). The plate was incubated at 37°C for 30 min and when cooled at RT; the absorbance was read using a microplate reader with a 562 nm filter. The average blank reading was subtracted from every other well reading and standard curve calculated by plotting the blank-corrected BSA well readings *vs.* the respective concentrations. The protein concentration of unknown protein samples was derived from the equation of the standard curve.

8.3.2 ImageQuant method

Another method used in this study for the estimation of protein concentration used polyacrylamide gels and the ImageQuant LAS 4000 digital imaging system (GE Healthcare). A SDS-PAGE of the protein of interest and a serial dilution of a BSA standard curve was carried out as previously described (Section 8.1.2) and stained used coomassie blue- or silver-based stains. A digital image of the gel was acquired and analysed using the software ImageQuant TL (GE Healthcare), using the default settings for image acquisition and background stain removal. The software calculated the pixel density of each band of BSA standard curve, and after the operator specified each individual concentration of the BSA bands, the software provided an estimate of the concentration of the unknown protein band of interest.

Appendix C – Additional data

9.1 Generation and characterisation of ovine macrophages from blood monocytes

On average, $CD14^+$ cells represented 3.54 % of the total PBMC isolated, with a wide inter-donor range between 0.46-5.8 % [normal range of ovine monocytes was reported by The Merck Veterinary Manual , ed. Aiello (1998) as 0-6 % of total PBMC]. PBMC were subsequently collected from 2 animals with the highest number of PBMC/ml blood and circulating $CD14^+$ cells above 3 % to attempt in vitro culture once the $CD14^+$ fraction had been isolated. Cultures of the $CD14^+$ cell fraction from these two animals proved partly successful. Sufficient macrophages were recovered from the 7-day culture of $CD14^+$ cells from one sheep to investigate the effect of three types of LPS [LPS preparations A (*E. coli* O55:B5, Sigma-Aldrich), B (*E. coli* O111:B4, Sigma-Aldrich) and C (*E. coli* O111:B4 LPS, Invivogen), as detailed in Section 5.2.9.1] and ovine recombinant IL-4 (ov rIL-4), for induction of classically activated (LPS) and alternatively activated (ov rIL-4) macrophages. The results are summarised in Figure 9.1.

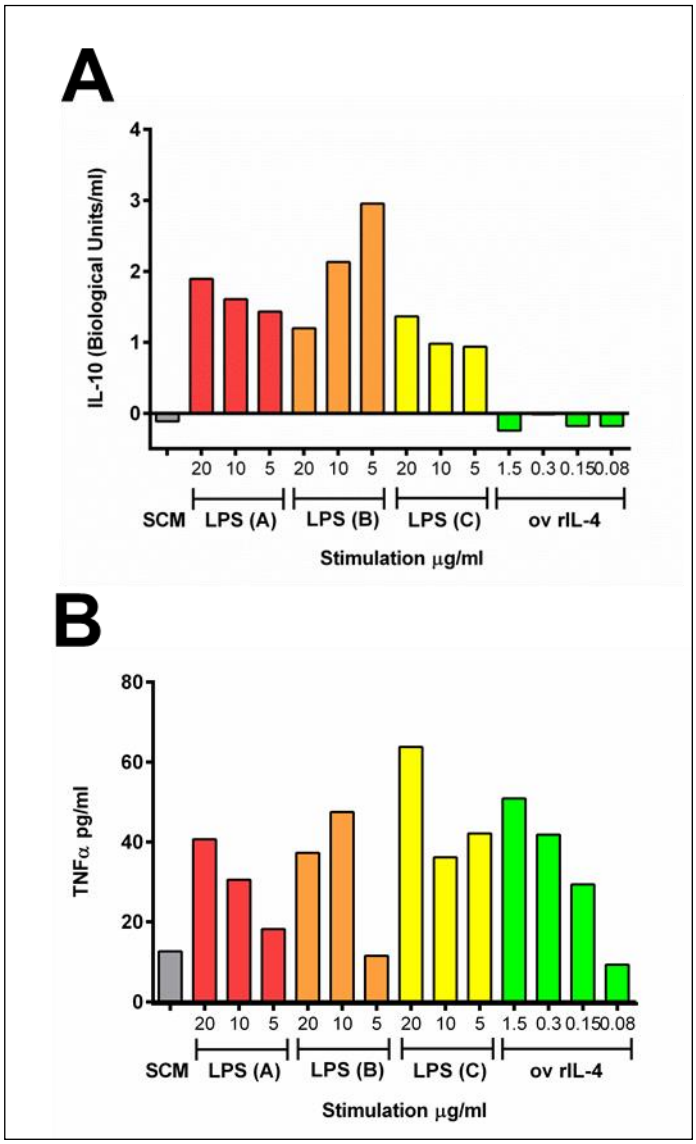

Figure 9.1 IL-10 and TNFα detection in the cell supernatant of sheep monocyte-derived macrophages stimulated with a range of molecules.
Data represent one experiment in which supernatant from 3 wells in each cell culture was pooled and analysed. Cells were stimulated with a range of concentrations of the molecules shown below each graph: SCM, unstimulated cells; LPS (A), O55:B5 *E. coli* LPS; LPS (B), O111:B4 *E. coli* LPS (Sigma-Aldrich); LPS (C), O111:B4 *E. coli* LPS (Invivogen); ov rIL-4, recombinant ovine IL-4 provided by Dr Sean Wattegedera at MRI. Panel (A): IL-10 represented as Biological Units/ml, as calculated from a standard curve of recombinant ovine IL-10; data represent one ELISA well analysed. Panel (B): TNFα concentrations shown in pg/ml, as calculated from a standard curve of bovine TNFα; data represent mean of two ELISA wells from each pooled supernatant.

9.2 Generation and characterisation of ovine macrophages from bone marrow stem cells

One hundred microliter aliquots representative of adherent and non-adherent cells were collected from each culture vessel at specific time points (day 0, 8 and 15) and cytospin slides prepared. Between the day of collection and day 15, the composition of cell

population changed at a variable rate depending on which culture system and which fraction (adherent or non-adherent cells) was considered. At the beginning of the period of culture *in vitro*, polymorphonuclear cells (PMNs; neutrophil and eosinophil-like cells, each with multi-lobed nuclei and eosin-stained cytoplasm for eosinophils) were predominant in each culture vessel used. Only a few lymphocyte-like cells were identified (1-2 %, small, medium sized cells with a round nucleus and small haematoxylin-stained cytoplasm); 10-12 % cells showed morphology consistent with monocyte/macrophage precursors and/or monocytes (large, round or kidney-shaped nucleus with surrounding medium to large hematoxylin stained cytoplasm). Progressing from day 8 towards day 15, the number of PMNs gradually decreased as the differentiated cells died and myeloid precursors of the PMN lineage did not further proliferate. At the same time, lymphocyte numbers remained unchanged or decreased and cells with monocyte- and macrophage-like appearance (large nucleus with chromatine granules and large cytoplasm rich in vacuoles, "foamy cell" appearance) became predominant around day 10 of culture. At this time, both the adherent and non-adherent fraction in each vessel showed predominant macrophage morphology (> 50 % cells), with the exception of non-adherent cells in the 6-well plate format. Adherent cells in the flask and 6-well plate reached the highest macrophage purity, with 95 % cells showing typical macrophage morphology (Table 9.1). At day 15 of culture, both adherent and non-adherent cells from all the culture vessels were harvested and counted. Viability was assessed by Trypan blue staining. Counts of viable macrophages are summarised in Table 9.2.

Table 9.1 The percentages of cell types in different sheep bone marrow cell *in vitro* cultures.

Culture vessel	Day 0				Day 8				Day 15			
	M/ MΦ	L	N	Eo	M/ MΦ	L	N	Eo	M/ MΦ	L	N	Eo
Bone marrow cells	11	3	38	48	n/a				n/a			
Teflon bag	n/a				40	2	39	19	77	0	17	6
Flask (adherent cells)					n/d	n/d	n/d	n/d	95	0	5	0
Flask (non-adherent cells)					32	2	51	16	62	1	7	30
6-well plate (adherent cells)					n/d	n/d	n/d	n/d	95	1	2	2
6-well plate (non-adherent cells)					31	0	41	28	45	0	26	29
24-well plate (adherent cells)					n/d	n/d	n/d	n/d	90	0	8	2
24-well plate (non-adherent cells)					27	2	42	29	33	0	22	45

Legend: M/MΦ, monocyte and/or macrophage-like cells; L, lymphocytes; N, mature neutrophils; Eo, eosinophils; n/a, not available; n/d, not determined.

Table 9.2 Ovine BM-MΦ cell counts after 15 days of *in vitro* culture in different vessels.

Culture vessel	Viable cell count at day 15 (x 10^6)	% of dead cells
Teflon bag	1.7	5
75 cm^2 Flask (adherent cells)	1.4	5
75 cm^2 Flask (non-adherent cells)	2.8	9
6-well plate (adherent cells)	0.95	10
6-well plate (non-adherent cells)	0.66	11
24-well plate (adherent cells)	0.62	9
24-well plate (non-adherent cells)	0.88	9

Taken together, these preliminary results indicate that in vitro culture of sheep BM-MΦ can be achieved by incubating cells for 15 days in the presence of 20 % FBS and 20 %

autologous serum. The optimal culture conditions which achieved the highest cell purity (95 %) combined with low cell mortality (5 %) were obtained by harvesting adherent cells from the 75 cm^2 polystyrene flask. The cells harvested from the polystyrene flask were used to gather preliminary data on the ability of these cells to produce cytokines following stimulation. The cells were stimulated with two types of LPS (O111:B4 *E. coli* LPS and *Rhodobacter sphaeroides* LPS). Although statistical analysis could not be carried out, the results in Figure 9.2 showed that these cells were able to produce detectable levels of TNFα above the unstimulated controls.

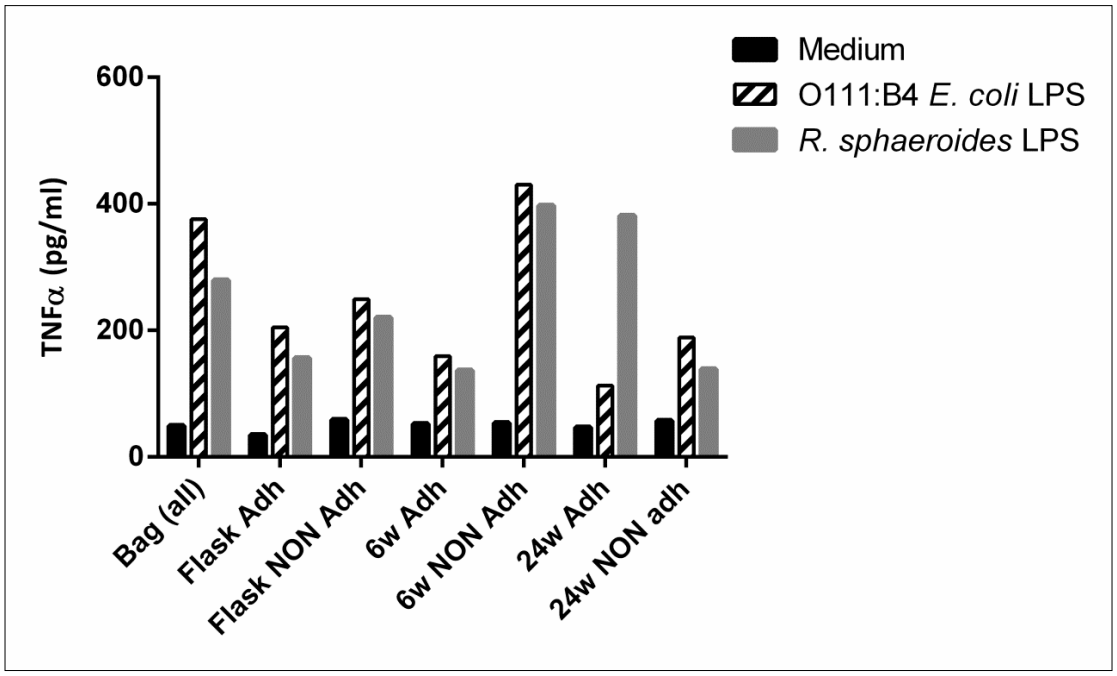

Figure 9.2 TNFα response in culture supernatant of ovine BM-MΦ generated in different vessels and stimulated with LPS.
Cells were stimulated with 10 µg/ml of either O111:B4 *E. coli* LPS or *R. sphaeroides* LPS. Supernatants were collected after 48 h of culture and levels of TNFα quantified by ELISA. BM-MΦ were generated in the culture vessels indicated under each set of bars [Bag (all): cells could not adhere therefore all cell types represented; Flask Adh or NON Adh: cells that adhered or did not adhere to the polystyrene flask; 6w Adh or NON Adh: cells that adhered or did not adhere to the 6-well culture plate; 24w Adh or NON Adh: cells that adhered or did not adhere to the 24-well culture plate]. All cells were harvested and stimulated in flat-bottomed 96-well plates. Data represent one experiment carried out in duplicate. Data shown are means of the two ELISA wells.

9.2.1 Intracellular non-specific esterase activity of BM-MΦ

Cytospin slides of bone marrow cells prepared immediately after collection showed a heterogeneous population of cells with highly variable non-specific esterase staining; all cells that showed monocyte, macrophage-like and monocyte precursor morphology stained with non-specific esterase, though the intensity of the stain was variable. Some cells showed an uniform black/grey cytoplasm of diffuse stain and others with only a few stained granules within a clear cytoplasm (black arrows in Figure 9.3, panel A). Cells with morphology consistent with PMNs did not stain. Bone marrow cells cultured *in vitro* for 15 days in polystyrene flasks had differentiated into BM-MΦ (population purity > 90 %) and also showed heterogeneous staining; all cells with macrophage-like morphology showed some degree of staining; some macrophage-like cells only stained well defined granules in their unstained cytoplasm (black arrow heads in Figure 9.3, panel B), whereas other macrophage-like cells showed a diffuse punctuate staining pattern and cells with morphology consistent with PMNs did not stain (red arrow heads, Figure 9.3, panel B).

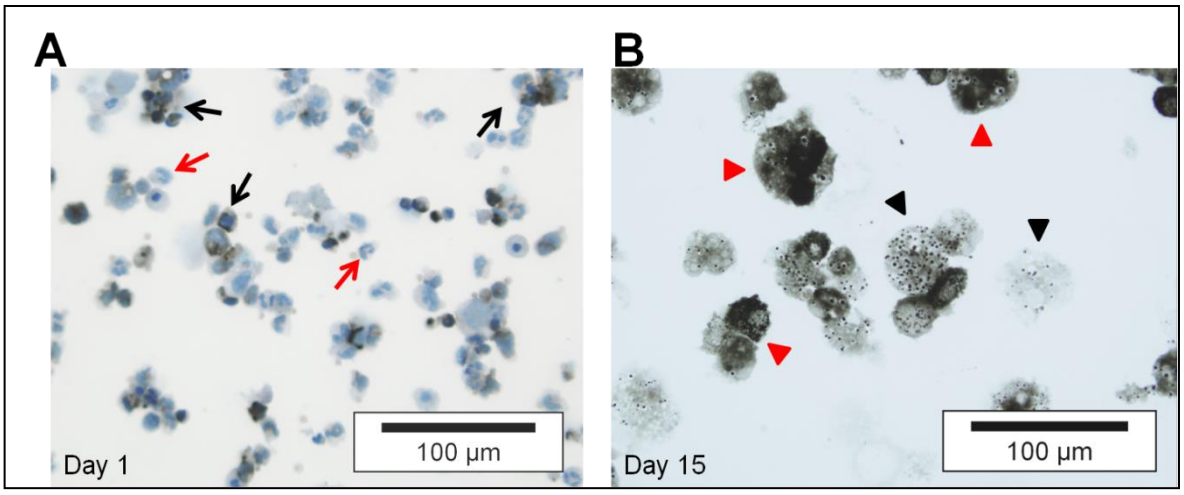

Figure 9.3 Staining for non-specific esterase in ovine bone marrow cells at day 0 of *in vitro* culture and BM-MΦ at day 15.
The bar shows the size of cells relative to 100 µm. Magnification of 200x was used to visualise stained cells. Panel (A): bone marrow cells were isolated from a sheep and stained for non- specific esterase. Black arrows: monocyte-like cells that stained more intensely; red arrows: PMN cells which did not stain. Panel (B): ovine BM-MΦ were harvested after 15 days in culture and stained for non-specific esterase. Black arrow heads: BM-MΦ with individual granule staining; red arrow heads: BM-MΦ with black granules filling the cytoplasm full. Note the increase in cell size from the monocyte-like cells indicated by black arrows in (A) compared to mature the macrophages in (B). The figures show representative cells derived from one of two sheep used in this experiment.

9.2.2 Expression of CD68

The expression of CD68 was investigated here in BM-MΦ cultures by immunocytochemistry (ICC). Bone marrow cells cultured *in vitro* for 15 day had differentiated into BM-MΦ (population purity > 90 %) and showed heterogeneous CD68 labelling; > 90 % cells with macrophage-like morphology showed some degree of labelling for CD68, though some possessed macrophage morphology without CD68 staining (Figure 9.4).

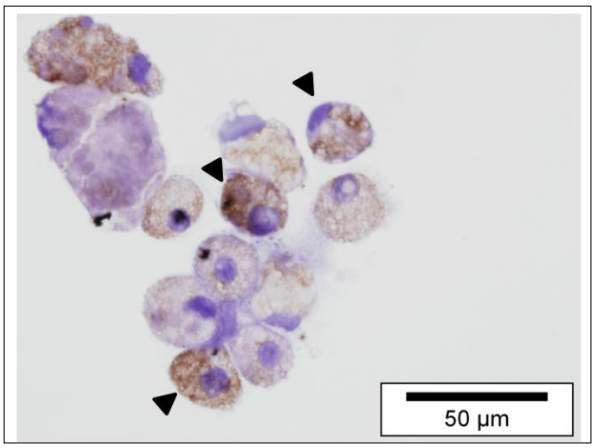

Figure 9.4 Immunocytochemical (ICC) labelling of CD68 in ovine BM-MΦ.
The bar shows the size of cells relative to 50 µm. Magnification of 400x was used to visualise positively labelled cells. BM-MΦ were cultured for 15 days and CD68 expression determined by ICC. Black arrow heads indicate the BM-MΦ cells that stained more intensely.

9.2.3 In vitro phagocytosis activity of ovine macrophages

The phagocytic activity of BM-MΦ was investigated by using fluorescent (FITC-labelled) *E. coli* cells in macrophage feeding assays. The results showed BM-MΦ were able to phagocytise the FITC-labelled *E. coli* cells. Dual fluorescent microscopy showed that co-localisation of green fluorescent particles (*E. coli*) occurred within the area of macrophage cytoplasm (BM-MΦ, Figure 9.5). BM-MΦ incubated without FITC-labelled *E. coli* cells did not show any fluorescent labelling (not shown).

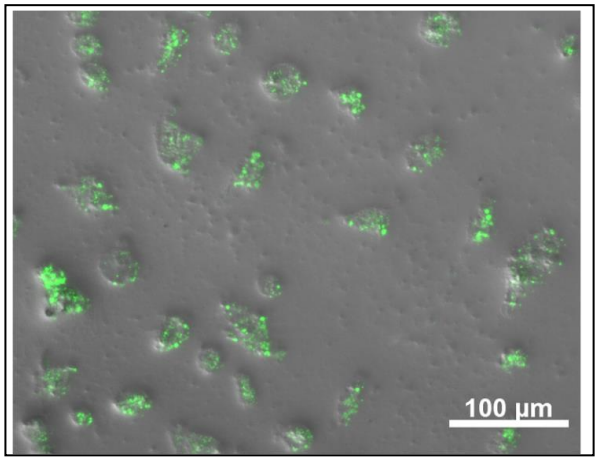

Figure 9.5 Fluorescent labelling of ovine BM-MΦ following phagocytosis of FITC-labelled *E. coli cells*.
Green fluorescent labelling within cells represent the phagocytosed FITC-labelled *E. coli* cells. Combined phase contrast microscopy and fluorescence microscopy demonstrating fluorescent labelling within BM-MΦ cells. Ratio of BM-MΦ to *E. coli* cells was 1:5. Magnification used was 200x; the bar shows the size of cells relative to 100µm. The figure is representative of two experiments.

9.2.4 Cytokine production of BM-MΦ upon stimulation

The production of IL-10 and TNFα was investigated, by ELISA, in *in vitro* BM-MΦ cultures. Cells were collected from the adherent cell fraction of the 15-18 day old *in vitro* cultures and stimulated for 48 h with LPS B (from *E. coli* O111:B4, Sigma), recombinant Tci-MIF-1 and recombinant ovine IL-4 (ov rIL-4). BM-MΦ were able to produce detectable levels of IL-10, however these were not found to be statistically significant between cells incubated without stimulation and cells that received LPS B, either recombinant Tci-MIF-1 version, or ov rIL-4. IL-10 production in cells stimulated with recombinant EcTci-MIF-1 was lower than in LPS-stimulated cells but this was not significantly different. The increase in IL-10 production observed in cells stimulated with LPS B compared to CeTci-MIF-1 was statistically significant ($p < 0.05$, Figure 9.6).

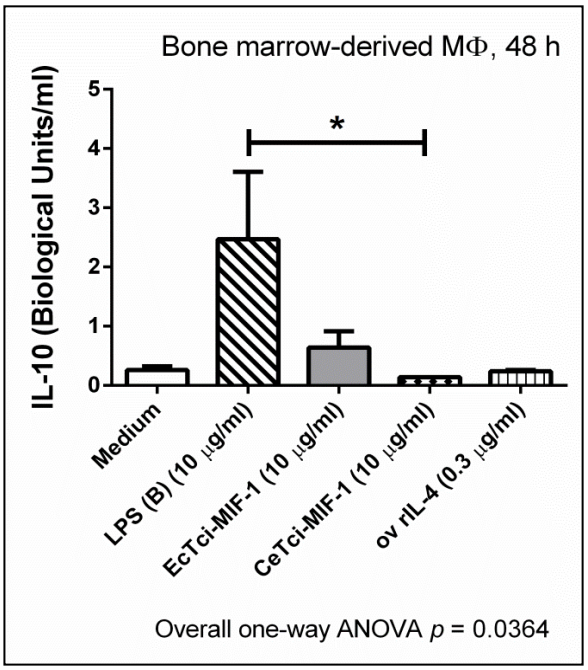

Figure 9.6 IL-10 detection in the cell supernatant of ovine BM-MΦ cultures stimulated with a range of molecules.
Cells were plated at 2 x 10^4/well and supernatants harvested after 48 h incubation with the molecules indicated below the graph (final concentrations), or with medium alone (unstimulated control). Data were transformed prior to carrying out statistical analysis to reduce skew using the formula y = 1/x, where x = IL-10 Biological Units/ml. Mean±SEM of untransformed data are shown [n=2 (CeTci-MIF-1 and IL-4 stimulation), n=4 (remaining BM-MΦ stimulation protocols). Overall one-way ANOVA p value is shown below the graph; * indicates 0.01 < p < 0.05.

The TNFα release of BM-MΦ cultures was also investigated. As previously shown for IL-10, LPS elicited the highest TNFα release in BM-MΦ supernatant, however this increase was not significant compared to unstimulated cells. Ovine recombinant IL-4 and both

recombinant Tci-MIF-1versions appeared to inhibit TNFα release in the BM-MΦ supernatant, but again these changes was not significantly different from unstimulated cell supernatant (Figure 9.7). Careful interpretation of ELISA data was required in both the IL-10 and TNFα experiments, as the results shown were based on small replicate numbers. This is likely to have resulted in highly variable standard errors.

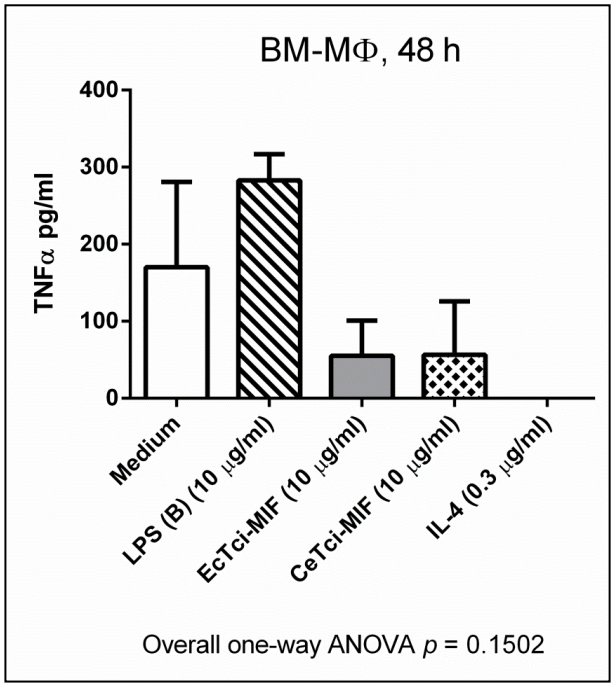

Figure 9.7 TNFα detection in the cell supernatant of ovine BM-MΦ cultures stimulated with a range of molecules.
Cells were plated at 2 x 10^4/well and supernatants harvested after 48 h co-incubation with the molecules indicated below the graph (final concentrations), or with medium alone (unstimulated control). Data represent mean±SEM [n=5 (medium and LPS (B)-stimulated cells), n=4 (EcTci-MIF-1), n=2 (CeTci-MIF-1 and IL-4 stimulation). Overall one-way ANOVA *p* value is shown below the graph; *p* > 0.05, not significant.

Overall, the results shown here confirm that functional sheep BM-MΦ can be generated *in vitro* and that these cells could provide a valuable insight in sheep basic immunology. However, further work is required to optimise the culture system and the stimulation protocols.

Appendix D - Reagents and Solutions

10.1 Tissue culture

10.1.1 Cell culture solutions

10.1.1.1 Sheep Wash Medium (SWM)

Hanks Balanced Salt Solution (HBSS) w/o Ca or Mg

2 % FBS

100 U/ml penicillin

0.1 mg/ml streptomycin

0.2 % gentamycin

10.1.1.2 ASC Culture Medium (ASC-CM)

Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Paisley, UK)

10 % FBS

2 mM glutamine

100 U/ml penicillin

0.1 mg/ml streptomycin

2.5×10^{-5} M β-Mercaptoethanol

10.1.1.3 Bone Marrow Culture Medium (BM-CM)

RPMI 1640 (Gibco)

2 mM L-glutamine

100 U/ml penicillin

100 µg/ml streptomycin

20 % FBS

20 % sheep autologous serum

Sodium Pyruvate (100 X, Sigma)

2.5×10^{-5} M β-Mercaptoethanol

2.5 % 1M HEPES (Sigma)

MEM non-essential amino acids (100 X, Sigma)

10.1.1.4 Bone marrow transport medium

Hanks Balanced Salt Solution (HBSS) w/o Ca or Mg

2 % FBS

100 U/ml penicillin

100 µg/ml streptomycin

7000 U/ml Heparin

0.2 % Gentamycin

70 U/ml nystatin

10.1.1.5 Standard Culture Medium (SCM)

RPMI 1640

10 % FBS

100 U/ml penicillin

0.1 mg/ml streptomycin

MEM non-essential amino acids (100 X, Sigma)

2 mM L-glutamine

2.5×10^{-5} M β-Mercaptoethanol

10.1.1.6 FACS medium

5 % FBS in 1X PBS with the addition of 0.2 % of sodium azide.

10.1.1.7 Cell fixating medium for FACS

20 % paraformaldehyde (PFA) was diluted to a final concentration of 1 % in 1X PBS.

10.1.1.8 MACS wash

0.5 % filter-sterilised bovine serum albumin (BSA) and 2 mM filter-sterilised EDTA in 1X PBS pH 7.2.

10.1.1.9 10X Red Blood Cells (RBC) Lysis Solution

1.5 M NH_4Cl	40.1 g
100 mM $NaHCO_3$	4.20 g
10 mM EDTA	1.85 g

Adjusted pH to 7.4 with 1 N HCl or 1 N NaOH, then adjusted volume to 500 ml. Stored less than 6 months at 4˚C. For use, dilute 10X solution 1:10 in dH_2O. The working solution was prepared fresh on the day of use. Kept working solution cold and discarded any unused portion. Pre warmed working solution at 37˚C before use.

10.1.1.10 Monocyte migration assay medium (MoMM)

RPMI 1640

100 U/ml penicillin

0.1 mg/ml streptomycin

Sodium Pyruvate (100 X, Sigma)

2.5 % 1 M HEPES

MEM non-essential amino acids (100 X, Sigma)

2 mM L-glutamine

2.5×10^{-5} M β-Mercaptoethanol

10.1.1.11 Quencher for monocyte migration assay (MoMQ)

As above (monocyte migration assay medium, 10.1.1.10), with addition of 5 % BSA.

10.1.1.12 Alveolar macrophages transport medium

Sterile PBS

70 U/ml nystatin

100 U/ml penicillin

0.1 mg/ml streptomycin

10.1.1.13 Alveolar macrophages culture medium

RPMI 1640

10 % FBS

2 mM L-glutamine

100 U/ml penicillin

0.1 mg/ml streptomycin

2.5 x 10^{-5} M β-Mercaptoethanol

10.2 Solutions for ELISA assays

10.2.1 Buffers and blocking solutions

10.2.1.1 Carbonate coating buffer

Na_2CO_3	0.795 g
$NaHCO_3$	1.465 g

Made up with the above in 400 ml dH_2O, adjusting the pH to 9.6 and making up to a final volume of 500 ml.

10.2.1.2 PBST

Phosphate Buffered Saline (PBS)

0.05 % Tween 20® (Sigma-Aldrich, Gillingham, UK).

10.2.1.3 PBST/Milk

PBST as above with the addition of 5 % non fat, dry milk (Marvel Dried Skimmed milk).

10.2.1.4 TBST

Tris	1.211 g
NaCl	29.22 g
Tween 20®	500 µl

Made up to 1L with dH_2O.

10.2.1.5 TBST/5% soya

As above with the addition of 5 % (w/v) of dried soya milk (Infasoy, Cow and Gate).

10.2.1.6 50mM Sodium Acetate Buffer

Dissolved 2.05 g sodium acetate (CH_3COONa; Sigma-Aldrich) in approximately 400 ml of dH_2O, adjusting the pH to 4.5 and making up to a final volume of 500 ml.

10.2.1.7 20mM Sodium periodate solution

Dissolved 42.8 mg of $NaIO_4$ (Sigma-Aldrich) in 10 ml 50 mM Sodium Acetate Buffer; adjusted pH to 4.5.

10.2.1.8 50mM Sodium Borohydride solution

Dissolved 18.9 mg of $NaBH_4$ (Sigma-Aldrich) in 10 ml TNTT. A 250 ml glass vessel must be used to prepare this solution, as it produces gas when $NaBH_4$ is mixed with the TNTT.

10.3 Reagents for cloning and protein expression

10.3.1 Buffers for bacterial and yeast growth

10.3.1.1 Luria-Bertani (LB) broth and agar

For 1 L solution:

1 % Bacto®-tryptone	10 g
0.5 % Bacto®-yeast extract	5 g
1 % NaCl	10 g
[15 % agar	15 g]

The volume was made up to 1 litre with distilled water and the pH was adjusted to 7.5 with NaOH. This solution was autoclaved to sterilise.

10.3.1.2 SOC medium

In 100 ml of distilled water:

Bacto®-tryptone	2 g
Bacto®-yeast extract	0.5 g
NaCl	0.05 g
250 mM KCl	1 ml

This solution was autoclaved for 15min. Once the liquid had cooled to below 60°C, the final two components were added:

0.5 ml filter-sterilised $MgCl_2$ (1.9 g $MgCl_2$ in 10 ml water)

2 ml filter-sterilised glucose (1.8 g glucose in 10 ml water)

Aliquots were taken and stored at -20 °C.

10.3.1.3 Low Salt LB broth and agar

For 1 L solution:

1 % Bacto®-tryptone	10 g

0.5 % Bacto®-yeast extract	5 g
0.5 % NaCl	5 g
[1.5 % agar	15 g]

Dissolved in 900 ml of dH_2O and pH was adjusted to 7.5 with 1 N NaOH, then made up to 1 L with dH_2O. The solution was autoclaved at 121°C for 15 min and stored at 4°C until used. When Zeocin® (25 µg/ml, Invitrogen, Paisley, UK) was added, the solution was stored at 4°C in the dark.

10.3.1.4 Yeast Peptone Dextrose medium (YPD), liquid and agar

For 1 L solution:

1 % Yeast extract	10 g
2 % peptone	20 g
[2 % agar	20 g]

Dissolved in 900 ml of dH_2O. The solution was autoclaved at 121°C for 15 min and stored at 4°C until use. Before use, 100 ml of a 20 % dextrose solution were added.

10.3.1.5 Yeast Peptone Dextrose Sorbitol medium (YPDS)

For 1 L solution:

1 % Yeast extract	10 g
2 % peptone	20 g
1 M Sorbitol	182.2 g
2 % agar	20 g

Dissolved in 900 ml of dH_2O. The solution was autoclaved at 121°C for 15 min and stored at 4°C until use. Before use, 100 ml of a 20 % dextrose solution were added. When Zeocin® (100 µg/ml, Invitrogen) was added, the solution was stored at 4°C in the dark.

10.3.1.6 Buffered Glycerol-complex Medium (BMGY)

For 1 L solution:

1 % yeast extract	10 g
2 % peptone	20 g

Dissolved in 700 ml of dH_2O. The solution was autoclaved at 121°C for 15 min and stored at 4°C until use. Before use, the following additions were made:

20 % dextrose solution	100 ml
1 M K Phosphate buffer, pH 6	100 ml

13.4 % Yeast Nitrogen Base (with NH_4, no amino acids)	100 ml
0.02 % biotin solution	2 ml
10 % Glycerol	100 ml

The complete medium was stored at 4°C until use.

10.3.1.7 Buffered Methanol-complex Medium (BMMY)

For 1 L solution:

1 % yeast extract	10 g
2 % peptone	20 g
1 M Sorbitol	182.2 g
2 % agar	20 g

Dissolved in 700 ml of dH_2O. The solution was autoclaved at 121°C for 15 min and stored at 4°C until use. Before use, the following additions were made:

20 % dextrose solution	100 ml
1 M K Phosphate buffer, pH 6	100 ml
13.4 % Yeast Nitrogen Base	100 ml
0.02 % biotin solution	2 ml
5 % Methanol	100 ml

The complete medium was stored at 4°C until use.

10.4 Solutions for handling of recombinant proteins

10.4.1 Protein purification

10.4.1.1 0.1M Sodium Phosphate Buffer (pH 7.4)

$NaH_2PO_4 \bullet H_2O$	3.1 g
NaH_2PO_4	10.9 g

Adjusted to pH 7.4, made up to 1 L with dH_2O and autoclaved. Stored at RT.

10.4.1.2 Binding Buffer A (Ni^{2+} column)

0.1 M Sodium Phosphate Buffer	20 ml
dH_2O	80 ml
NaCl (0.5 M)	2.92 g

Imidazole (20 mM)	0.136 g

Adjusted to pH 7.4 and stored at 4°C.

10.4.1.3 Elution buffers (1-4; Ni^{2+} column)

All elution buffers are based on:

0.1 M Na Phosphate buffer	20 ml
dH_2O	80 ml
NaCl (0.5 M)	2.92 g

Then for each elution buffer the following amounts of imidazole were added:

Elution buffer 1 (50 mM)	0.34 g
Elution buffer 2 (200 mM)	1.36 g
Elution buffer 3 (350 mm)	2.38 g
Elution buffer 4 (500 mM)	3.4 g

All buffers were adjusted to pH 7.4 and stored at 4°C.

10.4.1.4 Binding Buffer B (Co^{2+} column)

0.1 M Sodium Phosphate Buffer	20 ml
dH_2O	80 ml
NaCl (0.3 M)	1.94 g
Imidazole (20 mM)	0.136 g

Adjusted to pH 7.4 and stored at 4°C.

10.4.1.5 Wash buffer

0.1 M Sodium Phosphate Buffer	20 ml
dH_2O	80 ml
NaCl (0.3 M)	1.94 g
Imidazole (40 mM)	0.272 g

Adjusted to pH 7.4 and stored at 4°C.

10.4.1.6 Elution buffer (5; Co^{2+} column)

0.1 M Sodium Phosphate Buffer	20 ml
dH_2O	80 ml

NaCl (0.3 M)	1.94 g
Imidazole (165 mM)	1.122 g

Adjusted to pH 7.4 and stored at 4°C.

10.4.1.7 Dialysis Buffers

10.4.1.7.1 Dialysis Buffer 1

0.1 M Sodium Phosphate Buffer, 0.5 M NaCl pH 7.4.

10.4.1.7.2 Dialysis Buffer 2

0.1 M Sodium Phosphate Buffer, 0.3 M NaCl pH 7.4.

10.4.2 Solutions for use with D-galactose agarose gel

10.4.2.1 Binding buffer

0.1 M Sodium Phosphate Buffer

NaCl (0.15 M)	8.766 g

Adjusted to pH 7.2 and stored at 4°C.

10.4.2.2 Elution buffer

Binding Buffer (100 ml) with the addition of

D-galactose (0.1 M)	1.8 g

Stored at 4°C.

10.5 Solutions for use with *C. elegans*

10.5.1 Buffers and growth media

10.5.1.1 1M Potassium Phosphate Buffer

KH_2PO_4	108.3 g
K_2HPO_4	35.6 g

Salts were weighed and dH_2O added to 900 ml, stirred, pH adjusted to 6.0 with 85 % orthophosphoric acid or KOH and made up to 1 litre with dH_2O.

10.5.1.2 Nematode Growth medium (NGM)

For 500 ml of NGM:

NaCl	1.5 g

Agar	8.5 g
Peptone	1.25 g

The components were mixed in 486 ml of dH2O, stirred and autoclaved at 121°C for 15 min. The solid agar was stored at RT and when needed, melted in a microwave oven (full power until boiling) and left the settle for 15-30 min in water bath at 55°C. The following were added:

5 mg/ml cholesterol dissolved in ethanol (stored at -20°C)	0.5 ml
1 M KPO_4 Buffer pH 6.0	12.5 ml
1 M $MgSO_4$	0.5 ml
1 M $CaCl_2$	0.5 ml

The solution was stirred thoroughly and 20 ml aliquoted into new Petri dishes.

10.5.1.3 Peptone Rich Agar

For 500 ml of medium:

NaCl	1.2 g
Agar	25 g
Peptone	20 g

The components were mixed in 486 ml of dH2O, stirred and autoclaved at 121°C for 15 min. The solid agar was stored at RT and when needed, melted in a microwave oven (full power until boiling) and left the settle for 15-30 min in water bath at 55°C. The following were added:

5 mg/ml cholesterol dissolved in ethanol (stored at -20°C)	0.5 ml
1 M KPO_4 Buffer pH 6.0	12.5 ml
1 M $MgSO_4$	0.5 ml
1 M $CaCl_2$	0.5 ml

The solution was stirred thoroughly and 20 ml aliquoted into new Petri dishes.

10.5.1.4 M9 Buffer

Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	5 g
$MgSO_4 \cdot 7H_2O$	0.25 g

Salts were weighed and dH_2O added to 900 ml, stirred, then topped up to 1 L and autoclaved at 121°C for 15 min.

10.5.1.5 S Buffer

50 mM K_2HPO_4	12.9 ml
50 mM KH_2PO_4	87.1 ml
NaCl	0.585 g

The solution was autoclaved at 121°C for 15 min and stored at RT.

10.5.1.6 S Buffer for freezing worms

Solution prepared as above, with the addition of 30 % glycerin (v/v), then autoclaved.

10.5.1.7 Soft Agar Freezing Solution

NaCl	0.58 g
KH_2PO_4	0.68 g
Glycerol	30 g
1 M NaOH	0.56 ml
Agar	0.4 g

The solution was made up to 100 ml with dH_2O, autoclaved for 15 min and stored at RT.

10.6 Other solutions

10.6.1.1 50 X Tris-acetate/EDTA (TAE) Buffer

Trizma base	242 g
Glacial acetic acid	57.1 ml
50 mM EDTA	100 ml of 0.5 M solution

pH was adjusted to 7.7-8 using glacial acetic acid if required, made up to a volume of 1 L with dH_2O. The 1 X solutions were prepared adding 20 ml of the 50 X stock to 980 ml dH_2O.

10.6.1.2 6 X DNA loading Buffer

0.25 % Bromophenol blue

0.2 % Xylene cyanol FF

40 % (w/v) Sucrose in water

Stored at 4°C.

10.6.1.3 5 X TNTT

1 M Tris	50 ml
NaCl	146.1 g
Tween 20®	2.5 ml

The pH was adjusted to 7.4 using HCl and stored at 4°C. The 1 X solutions were made by mixing 200 ml of 5 X TNTT in 800 ml dH_2O.

10.6.1.4 0.1 M Citrate buffer (for use in fluorescence quencher solution)

0.1 M Citric acid	25.5 ml
0.1 M Na citrate dihydrate	24.5 ml

The solution was adjusted to pH 4.4 and made up to 100 ml with dH_2O.

10.6.1.5 Cysteine protease assay buffers

1) Cysteine was prepared as 0.5 M stock solution (100 X), aliquoted and stored at -20°C; DTT was prepared as 0.8 M stock solution (100 X), aliquoted and stored at -20°C.

2) Four reaction buffers stock at different pH were prepared as follows:

pH 3.5, 4.5 and 5.5 were 50 mM Sodium acetate buffer, 1 mM EDTA; 100 X cysteine and 100 X DTT were added fresh before use, to a final concentration of 5 mM cysteine and 8 mM DTT. The pH 7 buffer was 50 mM Tris-HCl buffer, 1 mM EDTA; 100 X cysteine and 100 X DTT were added fresh before use, to a final concentration of 5 mM cysteine and 8 mM DTT.

2) 7-Amino-4-methylcoumarin (AMC, Sigma-Aldrich) was dissolved in DMSO to a stock concentration of 10 mM and serial dilutions were prepared from that in DMSO.

3) The substrate Z-Phe-Arg-AMC (Enzo Life Sciences, Exeter, UK) was dissolved in DMSO to a stock concentration of 10 mM and serial dilutions were prepared from that in DMSO.

4) The cysteine protease inhibitor E-64 (Sigma-Aldrich) was dissolved in dH_2O to a stock concentration of 1 mM.

10.6.1.6 Dopachrome tautomerase assay buffers

1) Two hundred millilitres of reaction buffer 10 mM Na Phosphate Buffer, 1 mM EDTA was prepared by diluting 1:10 0.1M Na Phosphate buffer (this appendix, 8.1.4.1) and adding 200 µl of 1 M EDTA; pH 6.2.

2) 0.1 M 3,4-Dihydroxy-L-phenylalanine (L-DOPA methyl ester, Sigma-Aldrich) was prepared by dissolving 24.7 mg/ml in dH_2O.

3) 0.2 M sodium *m*-periodate (Sigma-Aldrich) was prepared by dissolving 42.8 mg/ml in dH_2O

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