Pathophysiology and immunomodulation associated with *Haemonchus contortus* infection

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In the Name of Allah the Most Gracious, the Most Merciful.

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ABSTRACT

The aim of this project was to investigate host-parasite interactions, which might lead to alternate strategies to control the sheep abomasal nematode *Haemonchus contortus*. The project focused on two aspects of host parasite interactions: the initiation of host pathology and suppression of host immune responses associated with the onset of infection.

Adult *H. contortus* ES products increased the permeability of Caco-2 cell monolayers and this increase could be blocked by single chain antibodies against ES products displayed on phage. Recombinant *H. contortus* enolase may be one of the active components of ES as it mimicked the action of ES products on Caco-2 cells.

This is the first study of immunomodulation by adult *H. contortus* ES products of the phenotypic and functional properties of human monocyte-derived dendritic cells (mdDCs). Incubation with ES products resulted in semi-maturation of mdDCs, with weak up-regulation of the co-stimulatory molecules CD40 and CD80 and increased surface expression of the tolerogenic markers CD32, CD305 and galectin-1. The highly variable responses of mdDCs of individual donors biased the group data, particularly in response to co-stimulation with ES products and LPS. This highlights genetic diversity in the immune system and possible difficulties in developing worm-based therapies.

The blastogenic activities of cells from lymph nodes collected from two groups of infected and vaccinated sheep were measured by ^{3}H-thymidine uptake after exposure to ConA or ES products. The Stimulation Index (SI) with ConA was 10-fold higher in cells from the older animals. Cells only from younger infected sheep had a reduced response to ConA and vaccinated groups with reduced parasite burdens had the highest SI. There was little response to ES products in older sheep, but in younger animals there was a trend for lymphocyte SI to be greater with 10% ES in sheep with the fewest parasites.

These experiments show that *H. contortus* ES products may facilitate the initiation of host pathology and the potential to modulate responses of dendritic and lymph node cells during parasitism. Further identification of the specific ES components responsible may allow disruption of their actions, resulting in resilient and immune sheep.

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List of Abbreviations

AAMs	alternatively activated macrophages
Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
ADJ	adjuvant
ADP	adenosine diphosphate
AEC	3-Amino-9-ethylcarbazole
AJ	adherens junctions
AK	arginine kinase
A. lumbricoides	*Ascaris lumbricoides*
AMcase	acidic mammalian chitinase
Amot	angiomotin
ANOVA	analysis of variance
APC	antigen presenting cell
aPKC	atypical protein kinase C
ASIP	aPKC isotype-specific interacting protein
ASP	*Ancylostoma* secreted proteins
B cells	B lymphocytes
BES	*Brugia malayi* ES
B. malayi	*Brugia malayi*
bp	base pair
BRSV	bovine respiratory syncytial virus
BSA	bovine serum albumin
CAR	coxsackievirus and adenovirus receptor
CarLA	Carbohydrate larval antigen
CCR7	chemokine (C-C motif) receptor 7
cDC	conventional dendritic cells
Cdc42	cell devision control protein 42 homolog
cDNA	complementary deoxyribonucleic acid
CDP	common DC progenitor

CHaFFs	chitinase and FIZZ family members CMP common myeloid progenitor
CIP4	Cdc42- interacting protein 4
CLA	cutaneous lymphocyte-associated antigen
CLR	C-type lectin receptors
ConA	concancavilin A
CPI	cysteine proteinase inhibitor (cystatin)
CTGF	connective tissue growth factor
Cy	cyanine
DC	dendritic cells
DC-SIGN	dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin
Der p 1	*Dermatophagoides pteronyssinus* allergen
DNA	deoxyribonucleic acid
DS	desmosome
EDN	eosinophil derived neurotoxin
ECL	enterochromaffin-like
E. coli	*Escherichia coli*
EGF	epidermal growth factor
E. granulosus	*Echinococcus granulosus*
ES	excretory secretory
ESGPs	eosinophil secondary granule proteins
ETP	early thymic progenitor
EU	endotoxin unit
FBS	foetal bovine serum
FEC	faecal egg count
F. hepatica	*Fasciola hepatica*
FITC	fluorescein isothiocyanate
FIZZ	found in inflammatory zone
FMOs	fluorescence minus one
g	gram
g	gravitational force
GAP	GTPase-activating protein

GAPDH	glyceraldehyde-3-phosphate
G. duodenalis	*Giardia duodenalis*
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
h	hour
*Hc*ES	*Haemonchus contortus* excretory secretory products
H. contortus	*Haemonchus contortus*
HES	*Heligmosomoides polygyrus* ES
HLA-DR	human leukocyte antigen-DR
H. polygyrus	*Heligmosomoides polygyrus*
H. pylori	*Helicobacter pylori*
HRP	horse radish peroxidase
HSC	hematopoietic stem cell
ICAM-1	intracellular adhesion molecule-1
iDC	inflammatory dendritic cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
INF	infected
IP-10	IFN-γ-inducible protein-10
IPSE	IL-4-inducing principle pf schistosome eggs
JAM	junctional adhesion molecules
JEAP	angiomotin-like-protein 1
kDa	kilodalton
KGF	keratinocyte growth factor
L	litre
L-NES	*N. brasiliensis* larval ES
LAIR-1	leucocyte-associated Ig-like receptor-1
LAP	latency-associated protein
LC	Langerhans cell
LDH	lysine dehydrogenase

LL	laminated layer
LPS	lipopolysaccharides
LSD	least significant difference
L1	first stage larva
L2	second stage larva
L3	third stage larva
L4	fourth stage larva
L5	fifth stage larva
M	molar
mAb	monoclonal antibody
MCP-1	monocyte chemo-attractant protein-1
mdDC	monocyte derived dendritic cell
MDP	macrophage DC progenitor
MFI	median fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
min	minute
MIP	macrophage inflammatory protein
ml	millilitre
mM	millimolar
M. marshalli	*Marshallagia marshalli*
MMPs	matrix metalloproteinase
MNC	mucous neck cells
mRNA	messenger ribonucleic acid
MUC5AC	mucin 5AC
MUC6	mucin 6
MUPP	multi-PDZ domain protein
Mv	microvilli
n	number
N. americanus	*Necator americanus*
N. brasiliensis	*Nippostrongylus brasiliensis*
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide

NI	non-infected
Ng	nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
NLR	NOD-like receptors
NOD-like	nucleotide-binding oligomerisation domain-like
NS	non-stimulated
O. ostertagi	*Ostertagia ostertagi*
O. volvulus	*Onchocerca volvulus*
OVA	ovalbumin
PAGE	polyacrelamide gel electrophrosis
PAMPs	pathogen-associated molecular patterns
PAR	partitioning defective protein
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PD-L	programme death ligand
PE	phycoerythrin
PerCP	peridinin chlorophyll
PFU	plaque forming units
pg	picogram
PHA	phytohaemagglutinin
p.i.	post-infection
PK	pyruvate kinase
pmol	picomole
PRRs	pathogen recognition receptors
Rac1	Ras-related C3 botulinum toxin substrate 1
RELM	resistin-like molecule
RGM1	rat gastric mucosal first
Rich1	Rho GAP interacting with CIP4 homologues
r*Hc*AK	recombinant *Haemonchus contortus* arginine kinase
r*Hc*ENO	recombinant *Haemonchus contortus* enolase

RhoA	Ras homolog gene family, member A
RIG-1	retinoic acid-inducible gene-1
RNA	ribonucleic acid
RO	reverse osmosis
scFvs	single chain antibody fragments
SDS	sodium duodecyl sulphate
S.E.M	standard error mean
SI	stimulation index
Sm	*Schistosoma mansoni*
S. mansoni	*Schistosoma mansoni*
S. venezuelensis	*Strongyloides venezuelensis*
SMC	surface mucous cells
SPN	serine proteinase inhibitor (serpin)
TAE	tris-acetate-EDTA
TBS	tris-buffered saline
T cells	T lymphocytes
T. circumcincta	*Teladorsagia circumcincta*
TCR	T cell receptors
T. crassiceps	*Taenia crassiceps*
T. cruzi	*Trypanosoma cruzi*
TEER	transepithelial electrical resistance
TES	*T. canis* ES
TGF	transforming growth factor
T_H0	naïve T cell
T_H1	type 1 immune response
T_H2	type 2 immune response
Tiam-1	T-lymphoma invasion and metastasis-inducing protein-1
TJ	tight junction
TLR	Toll-like receptors
TNF	tumour necrosis factor
T_{regs}	regulatory T cells
TSLP	thymic stromal lymphopoietin
T. spiralis	*Trichinella spiralis*

ZO	zonula occluding
ZAK	ZO-1 associated kinase
µCi	microcurie
µg	microgram
µl	microliter

INTRODUCTION

In pasture-based grazing systems, like those in New Zealand, gastrointestinal nematodes are major contributors to serious health and welfare issues and fiscal losses, because of low productivity and high treatment and control costs (Leathwick et al., 2001). Haemonchosis, caused by the blood-feeding abomasal nematode *Haemonchus contortus,* is one of the major constraints on small ruminant health and production in warmer areas worldwide and in some parts of New Zealand, because of its blood feeding (Rowe et al., 1988; Le Jambre, 1995). At present, control of gastrointestinal nematodes depends heavily on the use of chemical anthelmintics (Wolstenholme et al., 2004) and, where feasible, pasture management. Under intensive grazing conditions, however, clean pastures are not readily available and extensive use of anthelmintics has resulted in increasing resistance to these chemicals (Jackson, 1993; Waller et al., 1995; Borgsteede et al., 1997; Van Wyk et al., 1997). Moreover, there are concerns about drug residues in meat and the environment (Madsen et al., 1990; Lumaret, 1993).

Problems associated with chemical treatment of livestock can be addressed by developing immunologically-based methods for gastrointestinal nematode control, ideally vaccination (Newton and Munn, 1999). Vaccines would provide protection during the susceptible period between weaning and development of natural immunity and would most likely use antigens responsible for causing host pathophysiology or necessary for parasite survival. An additional target may be the parasite immunomodulators which suppress the host immune response (Maizels et al., 2004).

This project investigated two aspects of the host-parasite interaction which are hoped to provide targets for new therapies. The

first was how the parasite initiates host pathology, which appears to be chemically mediated by their excretory-secretory (ES) products. Worm products were tested on a Caco-2 cell model to investigate the possibility that *H. contortus* can modulate epithelial cell permeability to facilitate entry of worm chemicals into host tissues. The second objective was to investigate the ability of abomasal parasites to suppress host immunity, first by an *in vitro* study of the effects of *H. contortus* ES components on dendritic cells and secondly by examining the ability of lymphocytes collected from infected and vaccinated sheep to proliferate *in vitro* in response to mitogens.

Chapter 1

LITERATURE REVIEW

1.1. LIFE CYCLE OF ABOMASAL NEMATODES

The common trichostrongylid abomasal nematode parasites of sheep, *Haemonchus contortus*, *Teladorsagia circumcincta, Trichostrongylus axei* and *Marshallagia marshalli*, and *Ostertagia ostertagi* in cattle, have similar life cycles, which differ mainly in the length of the developmental stages (Sommerville, 1954; Silverman and Campbell, 1959). Fertilised eggs laid by adult female parasites are passed in the faeces of the host into the external environment. The first stage larvae (L_1) develop from the embryos inside the eggs and, after egg hatching in suitable environmental conditions of high humidity and temperature, L_1 moult to second (L_2), then to third stage larvae (L_3). The infective L_3 retain the L_2 cuticle as the sheath.

The infective L_3 are ingested by the host and exsheath in the rumen, stimulated by the bicarbonate buffer system (Sommerville, 1957; Rogers and Sommerville, 1960) and release of exsheathing fluid (Rogers and Sommerville, 1960) to become the parasitic third stage. The exsheathed L_3 move down to the abomasum, where they invade the gastric glands and pits of the fundic and pyloric regions. In the gastric glands, L_3 moult to L_4 and then to L_5, which become immature adult worms, the extent of development before emergence depending on the worm species and host factors. The larvae developing in glands, usually a single larva, cause localised swelling and nodules (Sommerville, 1953, 1954). Generally, *H. contortus* emerge into the abomasal lumen after only 2 - 4 days (Christie, 1970; Nicholls and Hayes, 1987), *T. circumcincta* after 5 - 6 days (Armour et al., 1966), *M. marshalli* after 18 days (Taylor et al., 1970) and *O. ostertagi* after 16 - 21 days (Fox et al., 1987), where they become adult worms closely associated with gastric mucus. Parasite development is affected by the infective dose (Dunsmore, 1960; Durham and Elliott, 1976), pre-existing adult worms (Michel, 1971) and the immune status of the host (Michel, 1971; Stear et al., 1995). The pre-patent period also varies with the species and in *H. contortus* ranges from 12 - 15 days (Nicholls and Hayes, 1987; Simpson et al., 1997) and is influenced by host resistance (Silverman and Patterson, 1960).

Hypobiosis, or arrested development of larvae in the host, occurs in most trichostrongylids when environmental conditions are unfavourable for larval development and survival or to synchronise the availability of infective larvae with the presence of susceptible young hosts (Michel, 1974; Gibbs, 1986). Whereas most species of arrested larvae are usually recovered from abomasal digests, 90% of arrested early L_4 *H. contortus* are associated with luminal mucus and only 10% were in tissue digests (Gatongi et al., 1998).

1.2. PATHOPHYSIOLOGY OF ABOMASAL PARASITISM

Abomasal nematode parasites often cause ill-thrift and production losses caused by loss of appetite, maldigestion, diarrhoea, leading to dehydration, and altered metabolism (Baker et al., 1965). Protein metabolism is particularly affected and may result in a negative nitrogen balance due to the anorexia, protein loss in mucus, mucosal cells and tissue fluid leakage and the cost of the immune response and tissue repair (Parkins and Holmes, 1989; Fox et al., 1993; Coop and Kyriazakis, 1999). Chronic infections can impair skeletal development (Coop and Kyriazakis, 1999). Anaemia often occurs in *H. contortus* infections, due to blood sucking by the parasite.

Effects on the infected abomasum include increased abomasal pH, stimulation of gastrin secretion, leading to increased serum gastrin concentrations, and greater leakage of pepsinogen into the circulation (Lawton et al., 1996). The structure of the infected tissues and cell composition changes, with reduced parietal cell numbers, enlarged pits and glands and expansion of the mucous neck cell population (Scott et al., 1998a). After a single larval infection, abomasal pathophysiology occurs at the time of parasite emergence from glands and within a day of transplantation of adult *H. contortus* or *T. circumcincta* (Lawton et al., 1996; Simpson et al., 1997). The minimal effect of developing larvae in glands and the rapid generalised response to luminal parasites suggest that chemicals released by parasites, their excretory/secretory (ES) products, are involved in initiating the pathophysiology.

1.2.1. Parietal cell loss and hypoacidity

Nematode infection of the abomasum inhibits acid secretion and causes widespread loss of parietal cells. Heavy worm burdens can raise abomasal pH to 5 and above (Lawton et al., 1996), but low adult worm burdens may raise pH only in their direct environment without affecting the pH of the abomasal contents. Less acidic conditions may be advantageous for worm survival, as only L_3 remained viable *in vitro*, whereas L_4 and adult *H. contortus* (Haag et al., 2005) and *T. circumcincta* (Lawton et al., 2002) were rapidly immobilised at pH 2.5 - 3.5. *In vivo*, parasites may be protected by the more neutral conditions in the mucus layer.

While larvae are developing in the glands, the pH of abomasal contents is unchanged and loss of parietal cells is confined to the local nodular lesions around the infected glands (Armour et al., 1966; Scott et al., 1998a). An abrupt rise in pH occurs at the time of emergence of parasites into the lumen, which varies from 2 - 4 days after a single infection of parasite-naïve sheep with *H. contortus* (Simpson et al., 1997) to 16 days in cattle infected with *O. ostertagi* (Jennings et al., 1966; Fox et al., 1987). This can be replicated within one day of transplantation of adult worms of several species (Anderson et al., 1985; McKellar et al., 1986, 1987; Lawton et al., 1996; Simpson et al., 1997; Scott et al., 2000). The duration of hypoacidity after larval infection is variable and abomasal pH may return almost to normal levels around patency, despite the continued presence of adult worms (Lawton et al., 1996). Individual animals probably differ in the inherent secretory capacity of gastric glands and the rate of regeneration of the functional parietal cell population (Scott et al., 1998a); these may overcome the inhibitory effects of the parasites. Immature parietal cells take about two days to become functional as they migrate, mainly downward, out of the progenitor zone in the glands (Coulton and Firth, 1983; Karam et al., 1997). This would be facilitated by hypergastrinaemia, which promotes parietal cell development and maturation (Coulton and Firth, 1983; Karam et al., 1997; Scott et al., 1998b).

A marked reduction in parietal cell numbers accompanies acid inhibition. In glands where larvae are present, mature parietal cells are replaced by undifferentiated or immature parietal cells and there are also fewer parietal cells in adjacent glands (Armour et al., 1966; Scott et al., 1998a). In non-nodular areas, marked parietal cell loss occurs about 5 days after parasites appear in the lumen (Scott et al., 2000). Transplantation of adult worms causes generalised parietal cell loss after only one day and the parietal cell population can decrease by 50% after 8 days (Scott et al., 1998a). As in other gastric pathologies (Ménétrier's disease and pernicious anaemia) in which parietal cells are inhibited or lost (Dempsey et al., 1992; Fox et al., 1996; Lacy et al., 1996), the pits enlarge and mucous neck cells (MNC) fail to mature to pepsinogen-secreting chief cells and remain as largely mucus-producing cells. These cellular changes occur because parietal cells control the architecture of the gastric mucosa through the release of members of the epidermal growth factor (EGF) peptide family and gastrin (reviewed by Simpson, 2000).

The identity of the inhibitors of acid secretion induced by parasitism is unknown, as is also their mode of action. It could be direct, by blocking the proton pump or disrupting the physiological regulation of the parietal cells through their ES products, or indirect through inflammatory mediators released by the presence of parasites in the abomasum. There is *in vitro* evidence that ES products could have direct effects on parietal cells (Merkelbach et al., 2002) or act indirectly on the histamine-secreting enterochromaffin-like (ECL) cells (Hertzberg et al., 1999), which play a key role in regulating acid secretion. The host inflammatory response may also contribute to the acid inhibition and increasing abomasal pH, as infiltration of inflammatory cells accompanies the hypoacidity and loss of parietal cells (Scott et al., 1998a, 2000). Granulocytes produce cytokines, including interleukin1-β (IL1-β) and tumour necrosis factor-α (TNF-α), which are capable of acid inhibition (Robert et al., 1991); IL1-β suppresses acid secretion by inhibiting histamine release from ECL (Prinz et al., 1997). In addition, tissue damage can be caused by other products of inflammation, such as reactive oxygen metabolites and cationic proteins (Levy and Kita, 1996).

Key questions are how can luminal parasites rapidly affect the parietal and chief cells located deep in the glands and how do they disrupt the mucosal defence system necessary for ES products to reach their target parietal cells. This effect could be caused by specific parasite molecules and may be reinforced by cytokines generated by inflammation. This was addressed in this project by examining whether ES products or their components increase the permeability of epithelial tissues, which could facilitate their actions on basolateral membranes of gastric gland cells. The lack of overt effects of worm chemicals on the epithelial cells in glands containing developing larvae is not unexpected in light of the unusual properties of the apical membranes of gland cells, which make them resistant to chemical absorption or damage (Waisbren et al., 1994).

1.2.2. Hypergastrinaemia

Increased circulating gastrin levels are typical of abomasal parasitism (Anderson et al., 1976; Fox et al., 1993). Gastrin is a key growth factor in maintaining fundic mucosal architecture (Wang et al., 2000) and is also vital for the development of the proton pump in parietal cells. In addition, gastrin is the prime acid secretogogue during a meal (Blair et al., 1987; Kovacs et al., 1989).

The main initial factor causing hypergastrinaemia is suggested to be removal of the inhibitory effects of acid feedback on the gastrin-producing G-cells and loss of parietal cells (Scott et al., 2000). Regulation of gastrin secretion by acid-feedback is well established (Becker et al., 1973). There was no evidence that ES products contribute directly to elevated plasma gastrin levels from *in vitro* experiments using sheep antral tissue pieces and ES products of either *T. circumcincta* (Lawton et al., 2002) or *H. contortus* (Haag et al., 2005). Elevated gastrin levels can be maintained when the abomasal pH decreases later in an infection (Lawton et al., 1996; Simpson et al., 1997) or even when abomasal pH did not rise (Scott et al., 2000), suggesting other gastrin stimulants are involved. These may be the inflammatory mediators histamine (Bado et al., 1994), TNF-α (Lehmann et al., 1996; Weigert et al., 1996) and IL1-β (Weigert et al., 1996), which are known to enhance gastrin release.

Unexpected drops in serum gastrin levels can be seen in some parasitised sheep despite markedly raised abomasal pH (Lawton et al., 1996; Simcock et al., 2006b). Although some samples of abomasal fluid taken from these sheep, contained *in vitro* inhibitors of gastrin secretion (Simcock et al., 2006a,b), there was no correlation *in vivo* between the presence of these inhibitors and reduced gastrin secretion. The source of these gastrin inhibitors appeared to be rumen bacteria which remained viable in the hypoacidic environment of the parasitised abomasum.

1.2.3. Hyperpepsinogenaemia

Pepsinogen, the inactive precursor of pepsin, is converted in an acid environment to the active aspartic protease which degrades protein into peptides. Pepsinogen is stored in granules in chief cells and MNC in the ovine fundic epithelium and also in surface mucous cells (SMC) in the bovine fundus (Yamada et al., 1988; Cybulski and Andren, 1990; Scott et al., 1999). Small amounts of pepsinogen appear in the circulation in unparasitised sheep (Lawton et al., 1996; Simpson et al., 1997), although most is released into the gastric contents. During infection with abomasal nematodes, serum pepsinogen levels increase, usually a little before the abomasal pH and serum gastrin increase (Anderson et al.,1985; McKellar, 1993; Hertzberg et al., 1995; Lawton et al., 1996).

Hyperpepsinogenaemia accompanies gastritis in humans with peptic ulcers or infected with *Helicobacter pylori* (Samloff et al., 1975; Hunter et al., 1993); the pepsinogen isoform responsible allows inflammation of the antrum or fundus to be distinguished in humans (Bodger et al., 2001). Leakage of pepsinogen could be from the basolateral membrane of the chief cells or more likely from the lumen through the surface mucosa of the stomach. In the parasitised abomasum, there is increased back-leakage of pepsinogen when parasites are present in the lumen, but not within glands, supporting diffusion occurring through a more permeable surface epithelium (Holmes and MacLean, 1971; McKellar, 1993). This could be facilitated by failure of pepsinogen conversion to pepsin in the hypoacidic abomasa (Jennings et al., 1966) and stimulation of the secretion of pepsinogen by the accompanying hypergastrinaemia (McKellar et al., 1986; Fox et al., 1989). ES products of adult *T. circumcincta* stimulated pepsinogen release *in vitro* from dispersed ovine gastric glands (McKellar et al., 1990) and from abomasal mucosal sheets collected from previously-infected, but not naïve, sheep (Scott and McKellar, 1998).

Also supporting back-leakage of pepsinogen through a more permeable surface mucosa are the ultrastructural observations of Murray (1969) and Murray et al. (1970), which showed loss of intercellular junctions, and enlarged intercellular spaces containing electron dense material, which they suggested was pepsinogen. Similarly, the increased turnover of plasma albumin and the calculated rate of leakage from the circulation support increased macromolecular diffusion (Holmes and MacLean, 1971), although another large molecule horseradish peroxidase did not appear to cross from the blood into abomasal contents (Stringfellow and Madden, 1979). The cause of the greater permeability has not been established and could be components of worm ES products, inflammation or a combination of the two.

1.3. GASTRIC MUCOSAL BARRIER

The gastric mucosa, where the parasite interacts with the host tissues, has a protective mucus gel layer which traps bicarbonate ions secreted by the SMC (reviewed by Allen et al., 1993). This creates a pH gradient between the neutral epithelial surface and the acidic lumen (Allen and Garner, 1980; Bahari et al., 1982), preventing damage from back diffusion of luminal H^+ and pepsin. The conditions in the mucus layer may also benefit the parasites, which are known to be sensitive to acid *in vitro* (Haag et al., 2005). The properties of the tight junctions of the epithelial tissues act as a cellular barrier to diffusion of chemicals both through the cells (transcellular) and through the intercellular spaces (paracellular) (Lichtenberger, 1999; Tsukita et al., 2001). Disrupting the integrity of gastric mucosal defence may be an important factor in the development of the pathophysiology associated with abomasal nematode infection. Büring (2009) has evidence from the effects of ES products on Caco-2 cells *in vitro* that parasites may increase epithelial cell permeability.

1.3.1. Mucus

The composition and physical properties of the mucus layer influence the protection it gives to the underlying mucosa and also the ability of pathogens to interact with the host. The fundic mucus gel contains alternating layers of MUC5AC, secreted by the SMC and pit cells, and MUC6, secreted by the MNC (Ota and Katsuyama, 1992). In the sheep pyloric region, there are no parietal cells and the secretion is alkaline and contains mucus (Harrison and Hill, 1962).

The major components of mucus are the high molecular weight mucins, which consist of a polypeptide core to which are attached mainly O-linked oligosaccharides (Forstner et al., 1982; Mukkur et al., 1985; Kobata, 1992).

The formation of disulphide bridges allows mucus to form a gel (Allen, 1981). The sol-gel transition of gastric mucins is pH dependent and influenced by the presence of proteins and glycolipids (Forstner et al., 1982). At around pH 4, gastric mucins change from the soft, visco-elastic gel present at acidic pH to the soluble proteins present at neutral pH (Cao et al., 1999; Celli et al., 2005). This is relevant to abomasal parasitism, as a loss of acidity is one of the features of infection with many parasite species and would be expected to reduce the protective function of the mucus overlying the mucosa. Mucus viscosity is increased by the interaction of calcium with sialic acids on the mucins (Tabak et al., 1982). The degree of sialylation and sulphation of the mucus sugars affect the gel forming properties and both modifications are known to alter in sheep with age and abomasal parasitism (Hoang et al., 2010a,b).

Very young and unweaned lambs have a thick layer of gastric mucins which are more highly sulphated and sialylated than in older animals (Hoang et al., 2010a,b) this may contribute to resistance to parasitism until after weaning (Zeng et al., 2001). Changes in monosaccharide composition occur during the first weeks of life, particularly reduced fucosylation and increased sialylation (Hoang et al., 2010a,b). The glycosylation of sheep abomasal mucins altered during infection with *H. contortus* or *T. circumcincta*: both parasites decreased fucosylation, sialylation and sulphation (Hoang et al., 2010a,b), but changes in the proportions of other monosaccharides differed during infection with the two parasites (Hoang et al., 2010b).

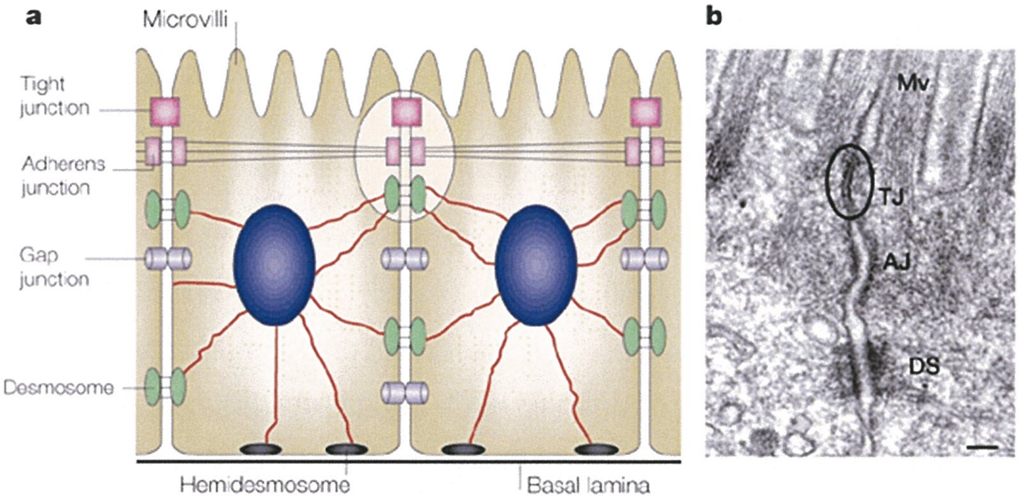

Fig. 1.1. Cell-cell junctions (Tsukita et al., 2001).

a: Schematic diagram, **b:** Elecctron micrograph in mouse intestinal epithelial cells. Scale bar, 200nm.

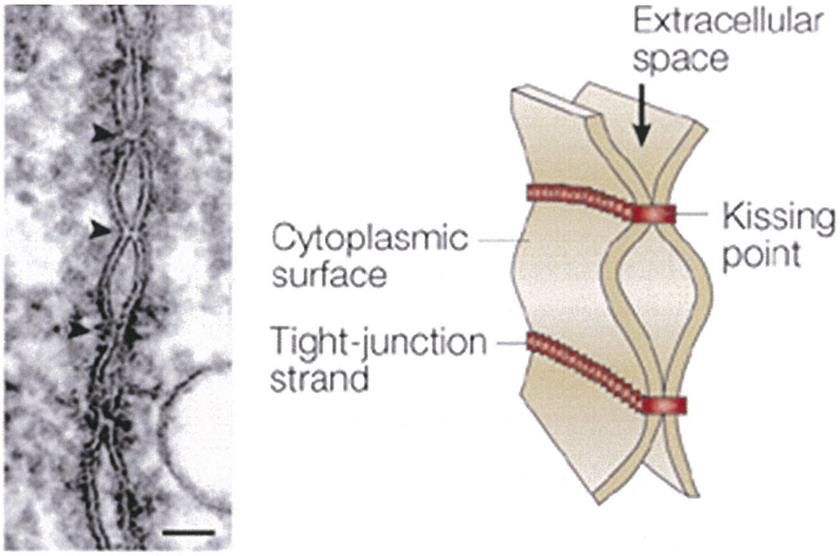

Fig. 1.2. Structure of tight junctions, showing kissing points. left: Electron micrograph, arrowheads showing kissing points (scale bar 20nm); right: Diagram of three dimensional structure of tight junctions (Tsukita et al., 2001).

The expression of mucin genes varies during the course of an *H. contortus* infection, MUC5AC being significantly decreased by Day 22, less in susceptible than in resistant sheep (Ingham et al., 2008; Rowe et al., 2009). Reduced production of mucin by the SMC would contribute to a lesser barrier to worm chemicals crossing the epithelium and reaching host tissues.

1.3.2. Epithelial cell integrity and permeability

The luminal surface of the stomach consists of a polarised layer of epithelial cells which regulates the movement of water and dissolved molecules across the gastric mucosa through cells (transcellular) and between cells (paracellular) (Mitic and Anderson, 1998). Maintenance of electrochemical and osmotic gradients depends on the membrane pumps and channels characteristic of each epithelium, aided by the paracellular pathway limiting back diffusion between the cells (Blasig et al., 2011).

Junctional complexes are present at the most apical part of the lateral membranes of epithelial cells; these are areas where the adjacent cells adhere to each other and also separate the apical from basolateral membranes, each with its own molecular structure. The junctional complexes consist of tight junctions, adherens junctions, desmosomes and gap junctions (Fig. 1.1); these connect to the cytoskeleton and signalling molecules at their cytoplasmic face (Tsukita et al., 2001).

1.3.3. Tight junctions

The tight junctions consist of a chain of “kissing points” where the external leaflets of the plasma membrane fuse to those of neighbouring cells (Fig. 1.2). The intercellular space is absent at

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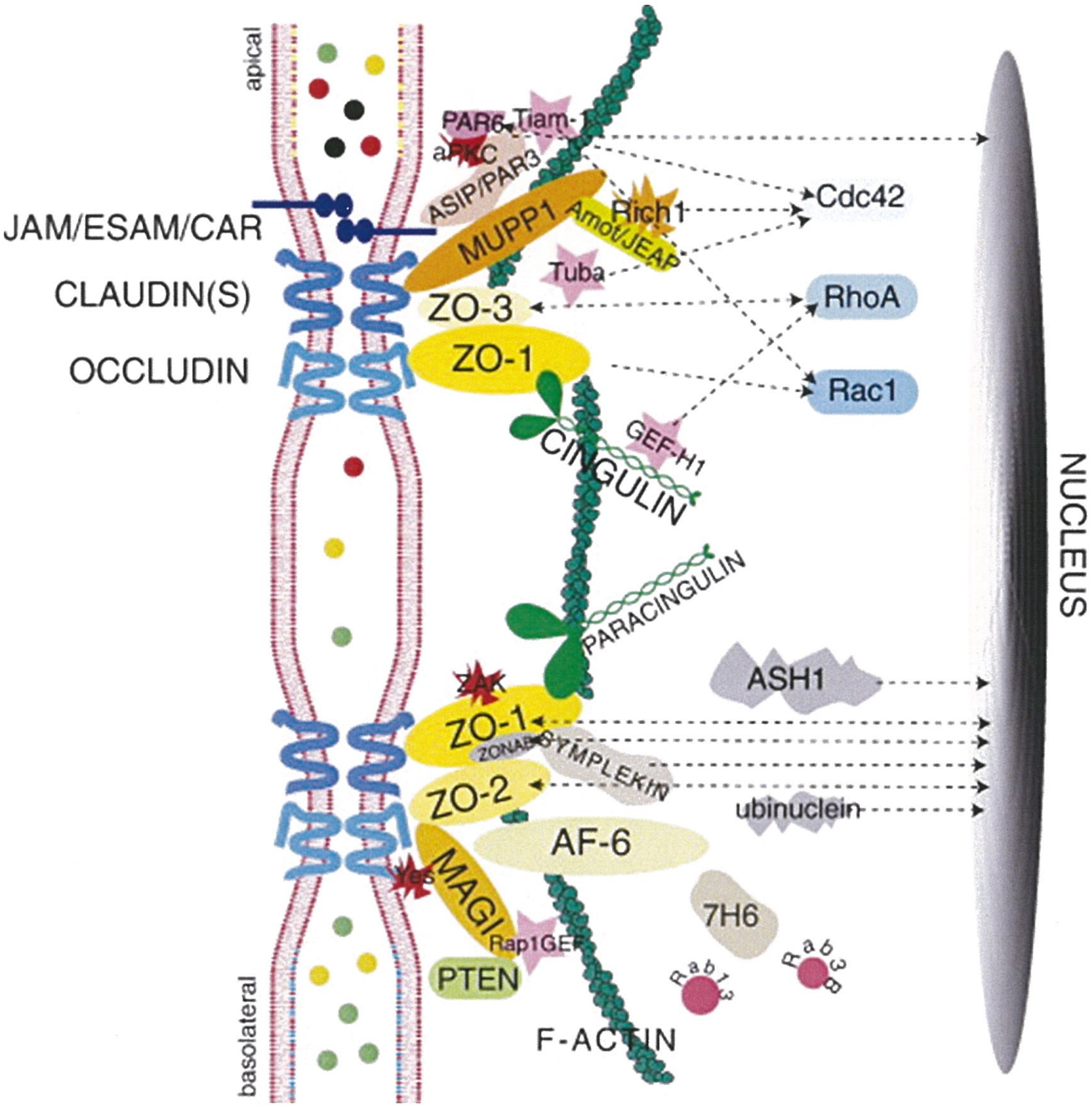

Fig. 1.4. Model of a tight junction, showing the transmembrane proteins occludin, claudins and JAM and the tight junction plaque proteins, which have scaffolding as well as regulatory functions. Kinases: red irregular shapes; guanine nucleotide exchange factors (GEF): pink stars; membrane traffic regulators: red circles; plaque protein-signalling interaction: dotted arrows; proteins with dual nuclear localization: arrows directed to nucleus (Guillemot et al., 2008).

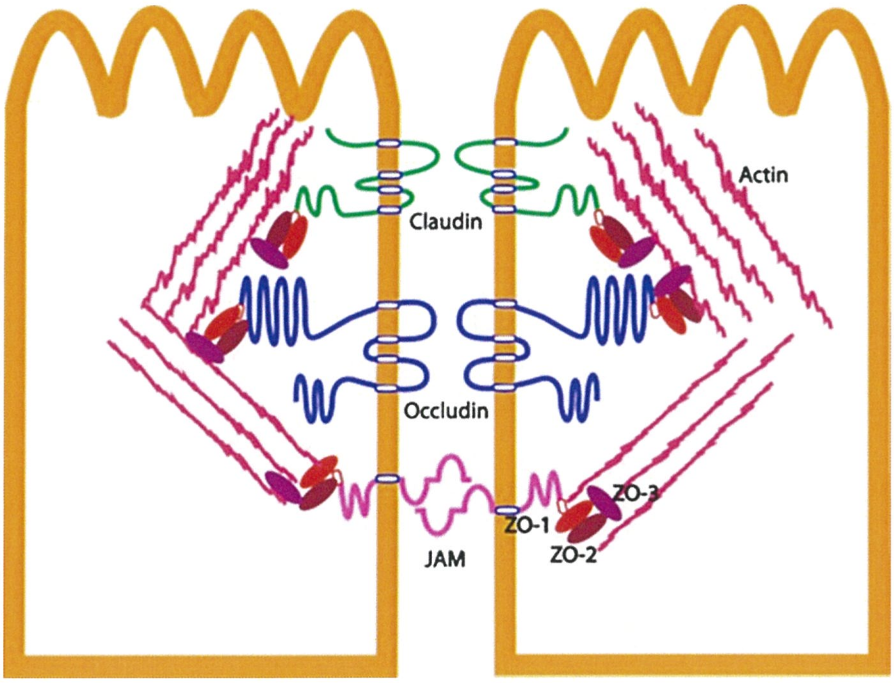

Fig. 1.3. The integral proteins claudins, occludins and junctional adhesion molecules (JAM) and the peripheral zonula occludin (ZO) proteins link the tight junction and actin cytoskeleton (Li et al., 2011).

these kissing points, whereas the opposing membranes of desmosomes and adherens junctions are 15 - 20nm apart. The tight junctions are considered to be responsible for intercellular sealing, impermeability and selective transport, which varies with the cell type and its physiological requirements (Furuse et al., 1998; Tsukita et al., 2001; Blasig and Haseloff, 2011). In the intestine, the tight junctions are considered "leaky", as large volumes of isotonic fluid are transported, in contrast to "tight" tight junctions in the stomach, as well as distal nephrons, where high gradients are set up (Ballard et al., 1995).

1.3.3.1. Molecular structure of tight junctions

Morphologically, tight junctions are complex networks of continuous fibrils within the cell membrane, although there is marked variation in the number and arrangement of the constituent filaments and associated proteins (Blasig and Haseloff, 2011). The main transmembrane proteins are occludin, claudin and junctional adhesion molecules (JAMs) (González-Mariscal et al., 2007; Myal et al., 2010). There are two isoforms of occludin (Muresan et al., 2000), at least 24 claudins have been identified in humans and mice, with more than two present in most cell types (Tsukita et al., 2001; Furuse, 2010) and at least 4 JAMs (Furuse, 2010).

Numerous peripheral proteins link the tight junction to the actin cytoskeleton and also are part of the intracellular signalling system (Fig. 1.3). The complexity of the arrangement of the peripheral/adaptor proteins associated with the integral proteins is illustrated in Fig. 1.4.

1.3.3.2. Permeability of tight junctions

Occludin is an important determinant of the permeability of tight junctions: transepithelial electrical resistance (TEER) decreases when the interactions of occludin with other molecules are disrupted (Balda and Matter, 1998) and permeability changes accompany overexpression or mutation of occludins (Tsukita and Furuse, 1999). Claudins and JAMs also influence the permeability of tight junctions (Saitou et al., 1998; Mitic et al., 2000; Tsukita et al., 2001). Claudins affect the conductivity and charge and size selection of the paracellular pathway (Van Itallie and Anderson, 2004). JAM-A antibodies disrupt intercellular connections and inhibit the restoration of TEER in epithelial cell monolayers (Liu et al., 2000), tricellulin depletion significantly reduced the ability to discriminate molecules by size and prevented the development of TEER (Ikenouchi et al., 2005). Over-expression of the crumb protein CRB3 disturbed the polarity of epithelial cells and delayed formation of tight junctions in epithelial monolayers (Roh et al., 2003). The peripheral proteins, the Zonula occludins ZO-1 and ZO-2, are essential for the polymerization of the protein constituents of tight junctions (Umeda et al., 2006) and calcium signalling (Furuse, 2010), while cingulin is involved in binding to actin in the cytoskeleton (Cordenonsi et al., 1999).

The permeability of tight junctions can be regulated by protein phosphorylation (Sawada et al., 2003; Gonzalez-Mariscal et al., 2008), peptides of the EGF family (Chen et al., 2001) or by structural remodelling (Ivanov et al., 2004, 2005; Shen et al., 2008). EGF appears to be involved in the increased resistance to apical acidification (Chen et al., 2001). There is continuous remodelling of tight junctions under physiological conditions, with about 70% of occludin and ZO-1 and 24% of claudin present in a mobile fraction (Shen et al., 2008). Occludin and claudin move within the plasma membrane and tight junction ZO-1 exchanges with an intracellular pool. Junctional proteins can be internalised and endocytosed (Ivanov et al., 2004, 2005; Shen et al., 2008), as well as the expression of proteins down-regulated.

1.3.3.3. Pathology associated with tight junctions

As part of the cell invasion process, many pathogens disrupt tight junctions and increase epithelial permeability by interacting with integral and peripheral proteins or the cytoskeleton, which attaches to tight junctional proteins. Cytokines, hypoxia, bacterial toxins and cytotoxic compounds also affect tight junction function (reviewed by Bonazzi and Cossart, 2011). Pathogens enter host cells, either through phagocytosis (Sansonetti et al., 1996) or by interacting with cell adhesion receptors on the surface of mucosal cells (Pizarro-Cerda′ and Cossart, 2006). Disruption of tight junctions often accompanies cancers (Turksen, 2010; Somi et al., 2012), autoimmune conditions (Clayburgh et al., 2004) and gastrointestinal infections. Depending on the epithelial tumours, there can be either decreased (Giepmans and van Ijzendoorn, 2009) or increased (Colegio et al., 2003) claudin expression.

There are numerous tight junction components targeted by viruses and bacteria, resulting in remodelling of the structure, degradation of proteins, down-regulation of expression of component proteins or reduced phosphorylation. Some act directly on tight junction proteins, e.g. retroviruses on JAM-A (Barton et al., 2001; Guglielmi et al., 2007), while other pathogens use signalling pathways or by modifying the cytoskeleton, such as coxsackievirus and adenovirus, which interact through their receptor CAR (Kerr, 1999; Walters et al., 2002). *Shigella* infections reduce ZO-1 and claudin-1 expression and alter the phosphorylation state of occludin, leading to a severe disruption of tight junctions (Sakaguchi et al., 2002). *Vibrio cholera* secretes a metalloprotease, which degrades the extracellular domain of occludin, thus dissociating ZO-1 from the tight junctions (Wu et al., 2000). Enteropathogenic and enterohemorrhagic *Escherichia coli* destabilise tight junctions by dephosphorylation of occludin (Dean and Kenny, 2004; Viswanathan et al., 2004).

Many pathogens alter epithelial permeability by a combination of tight junction remodelling with effects on protein function. Some *Salmonella* delocalise occludin, together with decreased ZO-1 expression (Boyle et al., 2006). Rotavirus causes delocalisation of claudin-3, ZO-1, and occludin (Dickman et al., 2000; Obert et al., 2000; Nava et al., 2004). The gastric pathogen *H. pylori* uses several virulence factors to disrupt the epithelial barrier and to invade these cells (reviewed by Wroblewski and Peek, 2011). Invasion involves moving ZO-1 and JAM-1 away from cell-cell contacts to form ectopic tight junctions at the bacterial-host interaction sites (Amieva et al., 2003; Saadat et al., 2007). *Clostridium difficile* and *Clostridium perfringens* indirectly disrupt tight junctions via the secretion of toxins which respectively dissociate ZO-1, ZO-2, and occludin from tight junctions (Nusrat et al., 2001) and bind and degrade occludin (Singh et al., 2000) and claudin-3 and -4 (Sonoda et al., 1999).

Many cytokines increase the permeability of tight junctions (reviewed by Bonazzi and Cossart, 2011): overproduction of TNF-α, interferon-γ (IFN-γ) and IL-1β increase epithelial permeability (Clayburgh et al., 2004; Al-Sadi et al., 2009). IFN-γ causes endocytosis of occludin, JAM-1 and claudin-1 and TNF-α down-regulates ZO-1 expression and redistributes ZO-1 away from the tight junction (Forster, 2008); IL-1β down-regulates occludin expression (Al-Sadi and Ma, 2007).

1.3.3.4. Parasitic infections

Parasitism is also associated with increased mucosal permeability, both *in vivo* and *in vitro*. Most detailed studies have been on protozoa, but metazoan parasites have also been reported to increase epithelial permeability. *In vivo*, elevated intestinal permeability and macromolecular uptake in giardiasis coincides with peak trophozoite colonisation and decreases to pre-infection levels when the parasites are cleared (Hardin et al., 1997). The hyperpermeability involves relocation of ZO-1, claudin-1, and F-actin (Scott et al., 2002) and is triggered by caspase 3 (Chin et al., 2002). *In vitro*, *Giardia* reduce the TEER of Caco-2 cells (Maia-Brigagão et al., 2012).

The house dust mite cysteine protease (Der p 1), which is excreted in faeces, increases the paracellular permeability of confluent lung epithelial cells *in vitro* (Wan et al., 1999), probably by cleavage of tight junction proteins. Colonic tissue collected from mice with a duodenal *Heligmosomoides polygyrus* infection showed a reduced TEER and altered expression of E-cadherin; this required adaptive immunity (Su et al., 2011). An inflammatory response was also implicated in the reduced TEER of *Hymenolepsis diminuta*-infected colonic tissues in Ussing chambers (Kosik-Bogacka et al., 2010) and immunohistochemical demonstration of relocation of occludin in the colon and ileum of infected rats from Days 8 - 40 post-infection (Kosik-Bogacka et al., 2011). A more direct demonstration of the effect of helminth chemicals on epithelial cells is the study of Büring (2009), which showed that *H. contortus* ES products caused cytoskeletal rearrangement in HeLa cells and displaced occludin and ZO-1 from Caco-2 cell tight junctions and reduced the TEER. Even in these *in vitro* studies, a role for inflammation is difficult to discount, as cultured epithelial cells also release cytokines when exposed to pathogens (Jung et al., 1995). This may compromise efforts to distinguish the direct effects of pathogens on tight junction proteins from secondary effects of cytokines released by their presence.

1.4. IMMUNE RESPONSE TO HELMINTHS

Unlike unicellular organisms, parasitic helminths cannot be readily phagocytosed by host immune cells, requiring different recognition systems and effector mechanisms to control them. Parasite ES products are believed to be responsible for eliciting both the innate and adaptive components of the host immune response. The protective immune response is a polarised type 2 immune (T_H2) response, but this is often actively suppressed by the parasites to prevent expulsion (reviewed by Harnett and Harnett, 2006; van Riet et al., 2009; McSorley et al., 2013; Grencis et al., 2014). In addition, there are physiological changes in mucus secretion and composition and gut motility, called “weep and sweep”, which in the intestine are suggested to contribute to worm expulsion (Anthony et al., 2007).

Immunity to gastrointestinal helminths is slow to develop, leaving lambs susceptible to repeated infection from weaning to about 6 months-of-age (Smith et al., 1985; Watson et al., 1994). The course of an infection in individual animals is influenced by the variation in the size of the worm burden, the ability to expel and reduce the fecundity of parasites and the resulting ill effects of infection (Barger, 1993; Mulcahy et al., 2004).

1.4.1. Host factors

Genetic differences between and within breeds affect worm burdens. Sheep can be classed as susceptible or resistant to gastrointestinal nematodes, depending on their ability to limit establishment and survival of parasites (Albers et al., 1987; Woolaston and Barker, 1996; McEwan et al., 1997). The cost of resistance can be reduced body growth: Riffkin and Dobson (1979) found an inverse relationship between growth rate and reactivity to

H. contortus antigens. Other animals, described as resilient, can maintain productivity during infection (Riffkin and Dobson, 1979; Albers et al., 1987; Bisset and Morris, 1996; Bisset et al., 2001).

Susceptibility to parasitism varies with age. Milk-fed lambs (Zeng et al., 2001) and calves (Satrija et al., 1991) usually have low gastrointestinal parasite burdens. While this may in part be due to maternal antibodies ingested in colostrum and milk (Andrews et al., 1995; Dempster et al., 1995), it may also result from the local environment created by mucins, which differ in composition from that in older animals (Hoang et al., 2010a,b). Ruminants younger than six months-of-age remain susceptible to helminth infection (Smith et al., 1985; Watson et al., 1994); thereafter most adult hosts are able to mount a protective response and expel worms from the gut (Mulcahy et al., 2004). Males are more susceptible than females (Barger, 1993).

Pregnancy (O'Sullivan and Donald, 1970; Coop et al., 1990) and nutritional status (van Houtert et al., 1995; Datta et al., 1998; Kahn et al., 2000; Sykes and Coop, 2001) affect susceptibility to nematodes. During the periparturient period, parasite immunity may be reduced in ewes, manifest as a higher faecal egg count (FEC) (O'Sullivan and Donald, 1970; Coop et al., 1990). Immunity of the ewes can be improved by increased dietary protein intake or by using their body protein reserves (Donaldson et al., 1998; Houdijk et al., 2001). Maintaining body growth requires extra protein (calculated to be 50 grams of protein per day) (Coop and Kyriazakis, 1999) to repair the parasitised gastrointestinal tract and mount the immune response (Sykes and Coop, 1976,1977). Parasitism can markedly reduce wool growth, which requires large amounts of sulphur amino acids (reviewed by Donald, 1979). The intake of protein has been determined to be more important than energy intake (reviewed by Black, 1987; Reis et al., 1992).

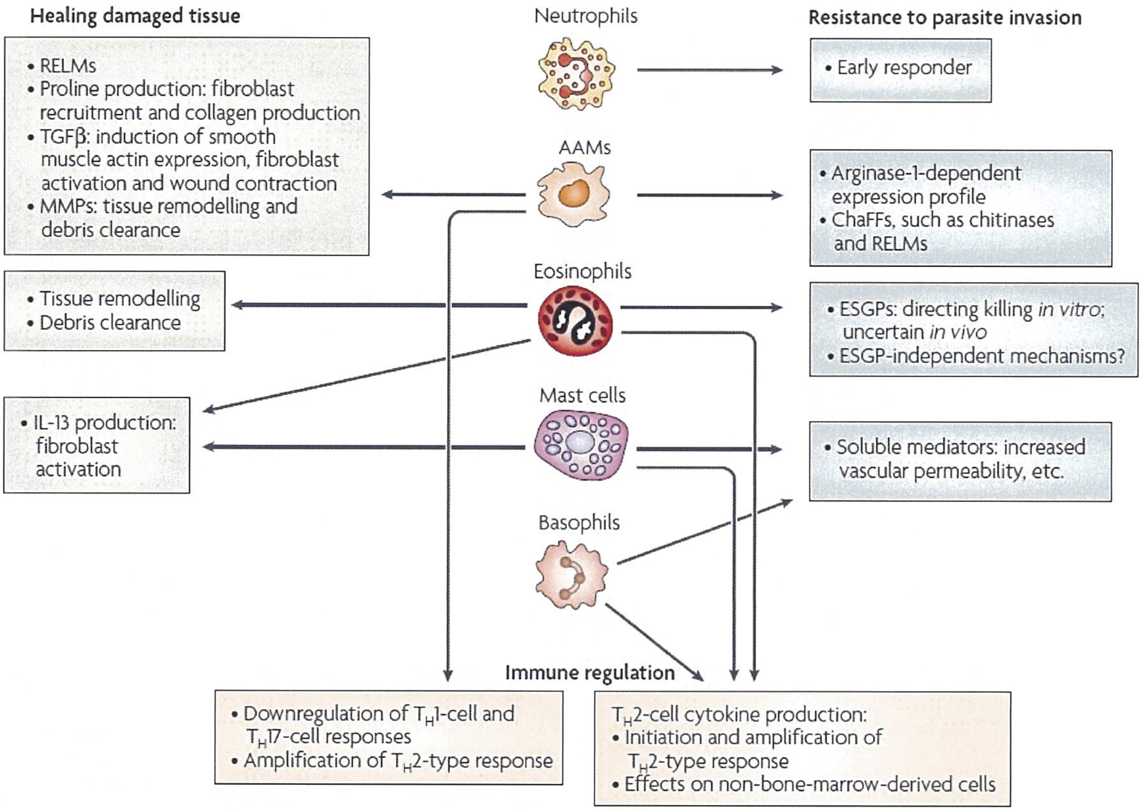

Fig. 1.5. An overview of functions of innate effector cells during the T_H2 immune response to helminths (Anthony et al., 2007).

1.4.2. T_H2 immune response

The protective $CD4^+$ lymphocyte response to gastrointestinal nematodes is a T_H2 response in sheep (Gill et al., 2000), but a more mixed T_H0 type in cattle (Gasbarre et al., 2001). Protection against *H. contortus* and other gastrointestinal helminths involves both innate and adaptive T_H2 components (McClure et al., 1996; Meeusen, 1999; Schallig, 2000; Meeusen et al., 2005; Nair et al., 2006; Alba-Hurtado and Muñoz-Guzmán, 2013). Fig. 1.5 gives an overview of T_H2 immunity, including the roles played by the cells recruited to parasitised tissues (neutrophils, alternatively activated macrophages (AAMs), eosinophils, mast cells and basophils) and the generation of the acquired antibody response (IgE, IgG and mucosal IgA) (reviewed by Smith, 1988; Meeusen, 1999; Balic et al., 2000b; Gasbarre et al., 2001; MacDonald et al., 2002). The complex interaction of different antigen presenting cells (APC), T cells and other innate effector cells and their chemicals and the production of antibody and associated cytokines makes it difficult to consider each of the components of the immune system in isolation.

1.4.2.1. Innate response

A primary infection with helminths, including abomasal nematodes, results in increased numbers of mucosal $CD4^+$ cells (T_H cells) (Kambara and McFarlane, 1996), lymphocytes, eosinophils and mucosal mast cells (Charleston, 1965; Armour et al., 1966; Salman and Duncan, 1984; Pfeffer et al., 1996). This response is enhanced in immune animal hosts, in which there is greater expansion of mast cells, the appearance of intraepithelial mast cells (globule leucocytes), eosinophilia, increased secretion of mucus containing anti-parasitic substances and increased gut motility (reviewed by Smith, 1988; Meeusen, 1999; Balic et al., 2000b; Gasbarre et al., 2001; MacDonald et al., 2002). The T_H2 response is also involved in promoting wound healing and is important during helminth infection, where epithelia are often damaged (Chen et al., 2012a).

Tissue and blood eosinophilia are features of gastrointestinal nematode infection (Schallig, 2000). Helminths activate the alternative complement pathway and bind opsonins on their surface, as well as generating the peptides C3a and C5, which are chemotactic for leucocytes, including eosinophils (Gasque, 2004; Giacomin et al., 2008b). Eosinophils accumulate in the abomasal tissue of *H. contortus*-infected sheep. It has been suggested that eosinophils inactivate L_3 through release of toxic chemicals, including eosinophil-specific peroxidase, major basic protein, lysosomal hydrolases, prostaglandins, leukotrienes and cytokines (reviewed by Meeusen, 1999; Balic et al., 2000a). These cells may not be essential for expulsion of all species of parasites in all hosts (Huntley et al., 1995; Schallig et al., 1997).

Mucosal mastocytosis is a marked features of gastrointestinal helminthiasis (Huntley et al., 1992), including infection with *H. contortus* (Schallig et al., 1997) or *T. circumcincta* and *Trichostrongylus vitrinus* (Huntley et al., 1995). Mast cells are essential for removal of parasites in many rodent infections, e.g. *Trichinella spiralis* (Urban et al., 2000), *Strongyloides ratti* (Abe and Nawa, 1988) and *Strongyloides venezuelensis* (Crowle and Reed, 1981; Lantz et al., 1998), but this was not the case for infection with *Nippostrongylus brasiliensis* (Crowle and Reed, 1981; Urban et al., 2000) or *Trichuris muris* in mice (Madden et al., 1991; Betts and Else, 1999; Koyama and Ito, 2000). The intraepithelial globule leucocytes are more closely correlated with nematode expulsion in sheep than are mast cells (Douch et al., 1986; Stear et al., 1995). Mast cells release pro-inflammatory cytokines, including TNF-α, proteinases, cytokines, histamine, leukotrienes and prostaglandins,

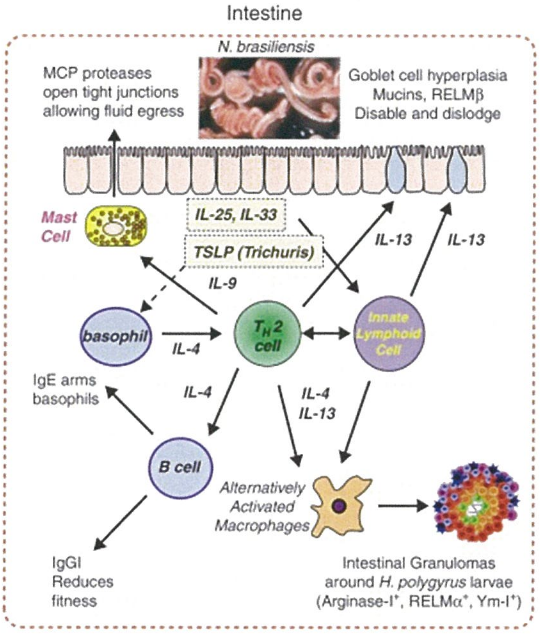

Fig 1.6 Cells and cytokines involved in immune responses in infection with *Nippostrongylus brasiliensis*. (Maizels et al., 2012b).

which may contribute to the inflammatory process and tissue damage (Galli et al., 1989).

Goblet cell hyperplasia, increased mucus production and hypermotility of the intestine have been implicated in worm expulsion from the intestine (Maizels et al., 2012a), but may be of lesser importance in the stomach. In the *H. contortus*-parasitised abomasum, expression of MUC5AC is reduced, not increased as might be expected; the reduction is greater in resistant sheep (Ingham et al., 2008; Rowe et al., 2009). Hypobiotic early L_4 *H. contortus* are found not in gastric tissues, but in the luminal mucus, and are able to maintain their position in spite of mucus turnover and gut motility (Gatongi et al., 1998). Even in the intestine, lumen dwelling worms are able to move up and down the intestine (Croll, 1976; Croll and Ma, 1977). The specific composition of the mucus, rather than the quantity, may be the key to worm expulsion. Ectopic expression of gastric MUC5AC in the caecum is associated with resistance of mice to *T. muris* (Hasnain et al., 2011). Sulphation of mucins may be an important feature in parasite resistance: milk-fed lambs have highly sulphated mucins (Hoang et al., 2010b); expulsion of adult *S. venezuelensis* from the intestine is increased in hamsters with highly sulphated glycans (Shi et al., 1994) and adult worms are unable to colonise sulphated mucus (Maruyama et al., 2002).

Cytokines released during the immune response to helminths result in polarisation of $CD4^+$ naïve T cells to a T_H2 phenotype. The complexity and interrelationships of the different cell types and cytokines involved are illustrated by the events provoked by *N. brasiliensis* (reviewed by Neill and McKenzie, 2011; Maizels et al., 2012b) (Fig 1.6). The response is initiated by parasites and their products interacting with APC, which include epithelial cells, B cells and dendritic cells (DCs), with subsequent activation of the other

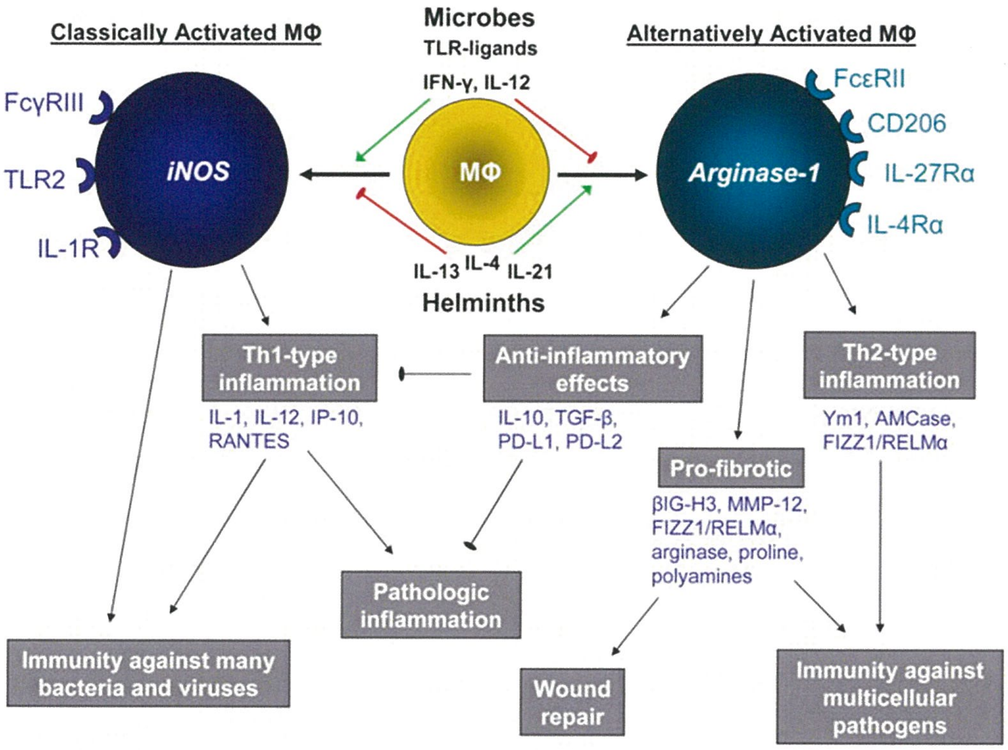

Fig 1.8 Comparison of generation and activation of classical macrophages (M1) and alternatively activated macrophages (M2). M1 are induced by microbes and M2 by helminths. (Kreider et al., 2007).

→ Induction ⊣ Blockage

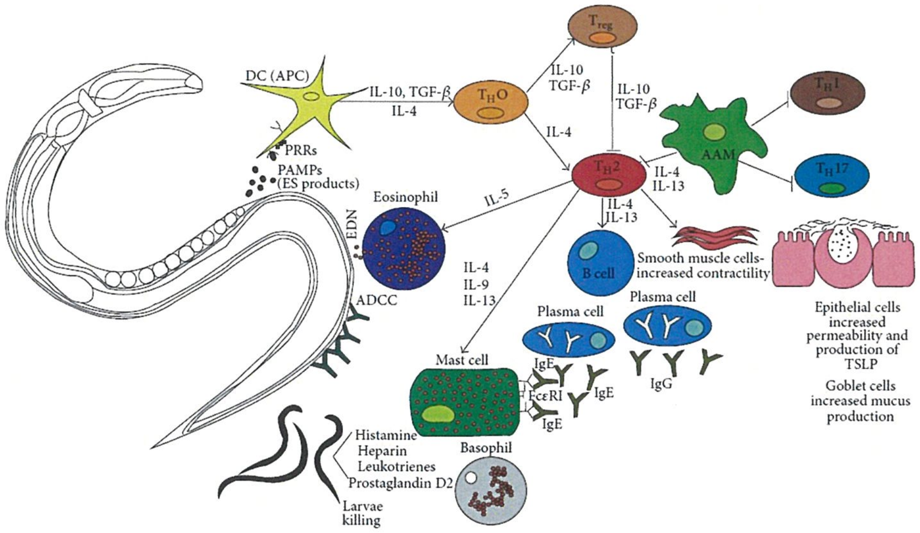

Fig. 1.7. Cytokines released by cells of the innate immune system after exposure of APCs to helminths. The downstream effects of these cytokines on other cells are also shown (Ditgen et al., 2014).

cells involved in generating resistance to the parasites (Fig. 1.7). The key cytokines which predominate in a T_H2 response, IL-4, IL-5 and IL-13, are upregulated in an *H. contortus* infection in sheep (Terefe et al., 2007). These are produced by a number of cell types.

Alternatively activated macrophages (AAMs) are another cell type induced during the T_H2 response to helminths. Macrophages originate from monocytes and in the tissues act as professional phagocytes (reviewed by Murray and Wynn, 2011). The subpopulations of macrophages are able to switch functions depending on the environment. AAMs (M2 macrophages) differentiate during helminth infections in a T_H2 cytokine environment, whereas classically activated macrophages (M1 macrophages) are induced by pro-inflammatory microbial antigens in a T_H1 cytokine environment (reviewed by Noël et al., 2004; Kreider et al., 2007). Fig 1.8 compares the stimuli required for their generation and the properties of these subsets of macrophages. AAMs are protective against helminths, mediate tissue repair and some are regulatory cells, expressing anti-inflammatory molecules, which include IL-10, resistin-like molecule (RELM)-α and arginase-1(Kreider et al., 2007).

In addition to the αß T cells, made up principally of $CD4^+$ T helper and $CD8^+$ cytotoxic cells, there is another major T cell population, the γδ T cells, which are particularly prominent in ruminants, especially young animals (Hein and Mackay, 1991). In sheep, as in other ruminant species, these lymphocytes are associated with and protect epithelial surfaces (Mackay, 1988; Hein and Dudler, 1997). Whereas αß T cells recognise peptides displayed on DCs and other APC, γδ T cells are stimulated by non-peptide antigens (reviewed Hayday, 2000; Wu et al., 2014). They are believed to be important in resistance to parasites and other infections: protection of mice against *Plasmodium berghei* (Inoue et

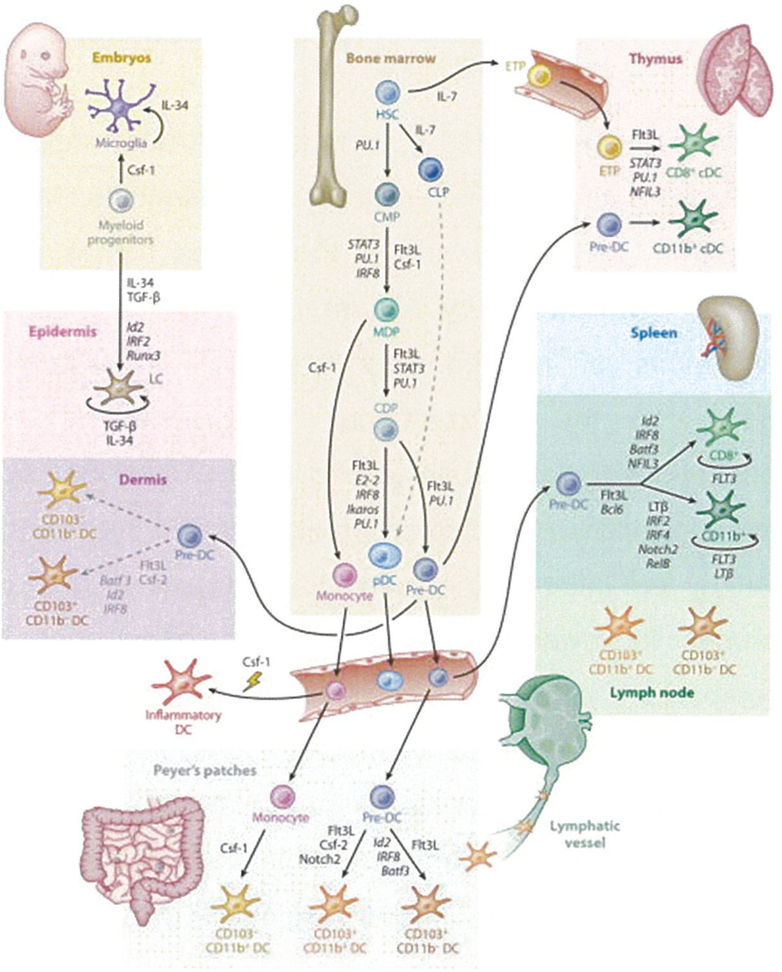

Fig. 1.10. Ontogeny of dendritic cell subsets (Merad et al., 2013).

Abbreviations: HSC, hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MDP, macrophage DC progenitor; CDP, common DC progenitor; ETP, early thymic progenitor; LC, Langerhans cell.

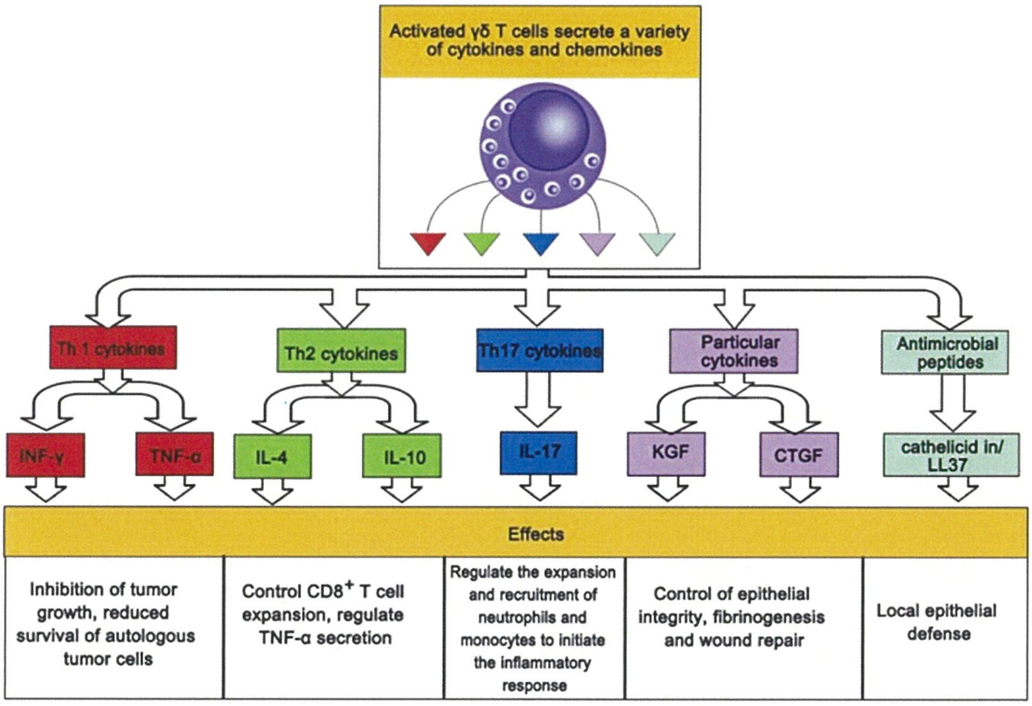

Fig. 1.9. Gamma delta T cell activation. Cytokines and chemokines generated by the 5 subsets and their effects on the other cells are shown (Wu et al., 2014).

al., 2012) and *N. brasiliensis* (Inagaki-Ohara et al., 2011) is γδ T cell-dependent and *Schistosoma japonicum* in the mouse liver are surrounded by γδ T cells (Xie et al., 2014). Different sub-sets of activated γδ T cells produce a range of cytokines and chemokines which interact with other components of the immune system (Wu et al., 2014), illustrated in Fig. 1.9.

1.4.2.2. Adaptive immunity

Parasite stimulation of a T_H2 response evokes adaptive immunity with the production of IgA, IgG and IgE (Smith, 1988; Schallig, 2000; Li et al., 2012). IgA and IgG antiparasite antibodies are associated with resistance to *H. contortus* infection (Schallig, 2000) and secretory IgA to *T. circumcincta* (Smith et al., 1985a; Strain et al., 2002). In particular IgA is implicated in lower faecal egg counts (FECs), reduced worm length and fecundity and delayed parasite development. Total IgE levels were increased 2 - 4 weeks after infection with *H. contortus* (Kooyman et al., 1997) and was more pronounced in *H. contortus* resistant sheep (Shakya et al., 2009). Increased IgE levels have also been reported in *O. ostertagi* infection in calves (Thatcher et al., 1989; Baker and Gershwin, 1993).

1.4.3. Dendritic cells

DCs are the only APCs which are able to initiate primary immune responses, as well as coordinate innate and adaptive immunity through antigen uptake and processing, release of cytokines and stimulation of T and B cells (reviewed by Banchereau et al., 2000; Merad et al., 2013; Schlitzer and Ginhoux, 2014). In the intestine, there are also tolerogenic DC of unknown ontogeny which secrete IL-10 and induce regulatory T cells (T_{regs}) (Fig. 1.10).

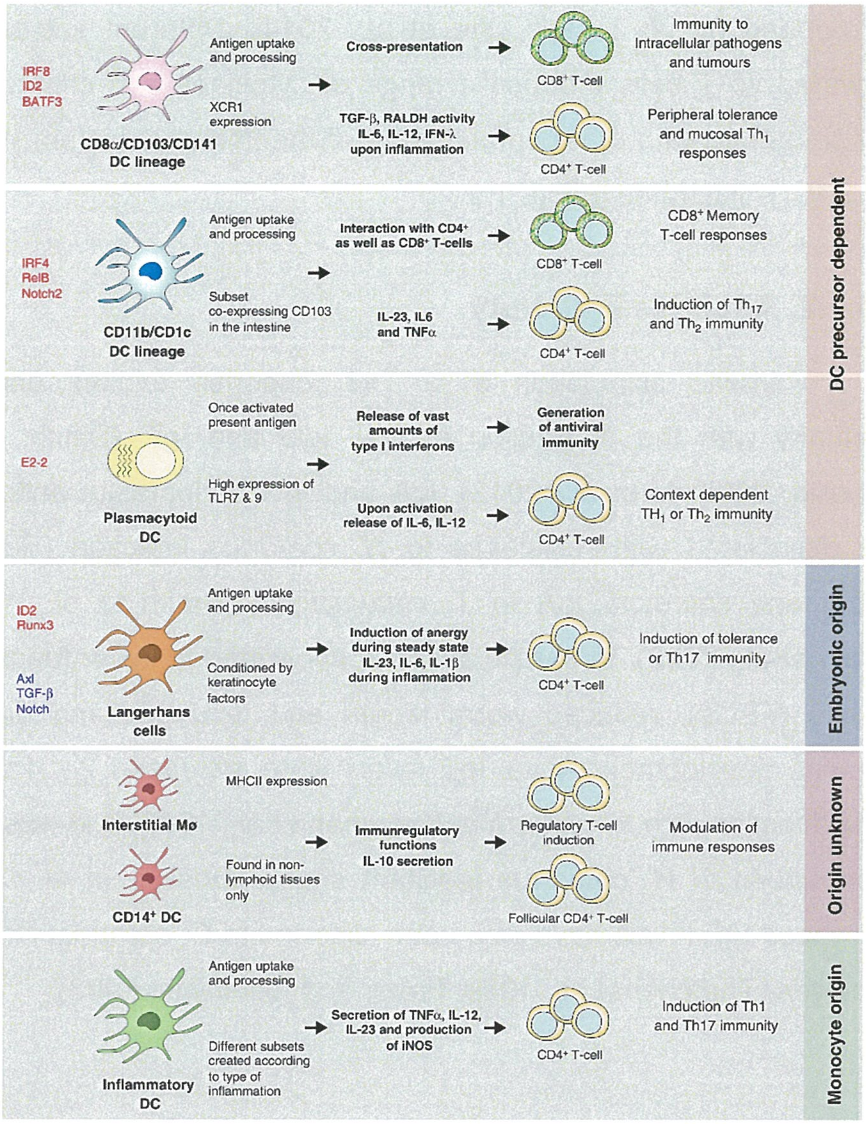

Fig. 1.11. Subsets of dendritic cells in mouse and human, showing transcription factor requirements and effects on T cells (Schlitzer and Ginhoux, 2014). (human: blue; mouse: red)

1.4.3.1. Origins and subsets

Human and mouse DCs have been studied most extensively; the subsets in the two species show many similarities, although cells are not identical in their gene expression and functions (reviewed by (Dutertre et al., 2014; Guilliams et al., 2014; Schlitzer and Ginhoux, 2014). There are three main lineages: conventional DC (cDC) derived from precursors in the bone marrow; the epidermal Langerhans cells derived from embryonic precursors, and monocyte-derived inflammatory DC (iDC) (Fig. 1.11). The stimuli needed to generate these cells from progenitors are summarised in Fig. 1.10. Differentiating subsets of DC based on expression of markers is difficult, as some markers are shared by cell types, there are species differences, and marker expression is different in activated and resting DCs. Gene expression is different in mice and human DCs from sheep DCs collected from lymph draining the either the skin or respiratory tract (Epardaud et al., 2004; McNeilly et al., 2006; Contreras et al., 2010; Crozat et al., 2010).

1.4.3.2. Antigen presentation and DC maturation

Immature cDCs, which are the progeny of myeloid DC, are either derived from blood and located exclusively in lymphoid tissue or are first resident in non-lymphoid tissue and migrate after maturation to lymph nodes (Randolph et al., 2008). Immature iDC are derived from monocytes. In tissues such as the gastrointestinal tract, immature cCD103$^+$ and iDC act as sentinel cells and upon exposure to pathogens and their products take up and process antigens and become mature DC. This maturation includes outgrowth of dendrites and decreased endocytotic capacity. Immature DCs have a basal level of secretion of chemokines and cytokines, which is changed by maturation.

Foreign antigens are recognised from their molecular patterns by pathogen recognition receptors (PRRs) (Wang et al., 2009) comprised of several molecular families including Toll-like receptors (TLR), cytoplasmic DNA sensors, C-type lectin, retinoic acid-inducible gene-1 (RIG-1) and nucleotide-binding oligomerisation domain-like (NOD-like) receptors (NLR) (Visintin et al., 2001; Janeway and Medzhitov, 2002). Antigen is internalised by DCs by endocytosis, micropinocytosis and phagocytosis, followed by proteolysis and translocation of peptide-major histocompatibility complex (MHC)II complexes to the surface for display (Banchereau et al., 2000). In mature DCs, these complexes are stable for several days on the cell surface, where they can be recognised by the T cell receptors (TCRs) on T cells.

In addition to the antigen presenting molecules MHC I and II, mature DCs have upregulated expression of co-stimulatory and immune regulatory molecules, such as CD80, CD86, CD83 and CD40. These direct naïve T cells to differentiate into T_H1 or T_H2 cells (Moser and Murphy, 2000; Tan and O'Neill, 2005) and to release pro-inflammatory cytokines and chemokines, including IL-6, IL-23, IL-12 and TNF (Kapsenberg, 2003). IL-12 released by TLR-activated DCs recruits macrophages to the site of infection and stimulates other cells to secrete IFN-γ and TNF-α, driving a T_H1 response and IgG production. Helminth activation of PRRs on DC is essential to induce a skewed T_H2 response: depletion of $CD11c^+$ cells *in vivo* inhibited expansion of T_H2 cells and release of T_H2 cytokines during a *Schistosoma mansoni* challenge (Phythian-Adams et al., 2010) and conversely an efficient T_H2 response against *N. brasiliensis* could be initiated when DCs pulsed with ES antigens were transferred into naïve recipient mice (Balic et al., 2004). T_H2 cells produce IL-4, IL-5 and IL-13 and induce production of IgE (Barton and Medzhitov, 2002). Parasites also down-regulate T_H1 responses by inhibiting IFN-γ (Uchikawa et al., 2000) and IL-12p70 production (Balic et al., 2004).

1.4.4. Antibody production in lymph nodes

DC migration to the draining lymph nodes is greatly increased during inflammation and is regulated by chemokine (C-C motif) receptor 7 (CCR7), a G protein-coupled receptor (GPCR) which initiates intracellular signalling cascades in the DCs (reviewed by (Merad et al., 2013). In addition, antigens <70Kd in size are taken up into lymph and carried to the regional nodes, where antigen can be directly taken up by the resident DCs. The iDC mature while migrating to the lymph node paracortical areas (Banchereau and Steinman, 1998; Dubois et al., 1999). The DC become the interdigitating DC, which are the only cells able to stimulate antigen-specific naïve T cells to proliferate, differentiate, secrete cytokines and express the CD40 ligand.

Lymphocytes continuously recirculate between the tissues and blood, which contains 2% of the total pool. The lymphocytes leave the tissues in draining lymph vessels, enter the lymph nodes and then leave in efferent ducts to re-enter the blood. DCs and lymphocytes interact in the lymph nodes, causing antigen-specific naïve T cells to be activated to effector or memory T cells (Battaglia et al., 2003). These T cells interact with $CD40^+$ B cells, inducing first short-lived plasmablasts that secrete IgM. With continuing stimulation the B cells differentiate into long-lived plasma cells or memory cells which produce other Ig subtypes. As the B cells mature, the amount of cytoplasm increases and very large amounts of antibody is produced per cell. In addition, B cells can act as APC and secrete cytokines, which differ for specific subsets of plasma cells (reviewed by Dang et al., 2014).

Switching of antibody subclass in mature plasma cells is caused by antigen stimulation and co-stimulatory signals (reviewed by Stavnezer et al., 2008). The basic mechanism is called class switch recombination, which involves creating breaks and recombination of DNA sequences in switch regions in the chromosomes of the mature B cell. IgG and IgA can be generated rapidly, but IgE production takes more cycles of recombination. IgE is the least abundant antibody subclass, but important in inflammation caused by helminth infection or allergies. The pool of B cells expressing IgE is kept small, there are few memory cells and the duration of the antibody response is shorter because of additional regulatory mechanisms (Yang et al., 2014).

1.5. HELMINTH IMMUNOMODULATION

Helminths produce a chronic, subclinical or asymptomatic infection in many animals, resulting in a host population in which there are only a few heavily infected individuals, but a large number with a low burden of parasites (Gaba et al., 2005; Hall et al., 2009). Evasion of host immunity allows time for parasites to mature and be transmitted to new host animals (Harnett and Harnett, 2006; Moreau and Chauvin, 2010). Active immunomodulation is also a feature of other types of chronic infection, such as leprosy, caused by *Mycobacterium leprae* (Kumar et al., 2013).

Immunomodulation in its widest sense could include induction of typical anti-parasite T_H2 responses and immunity through vaccination with *H. contortus* ES fractions (Schallig and Van Leeuwen, 1997), H-Gal or H-11 (Smith et al., 1994; Newton and Munn, 1999). The immune responses to vaccination may not mimic exactly natural immunity. Immunomodulation is more often

Product	**Helminth**	**Mechanism of action**	**References**
Tegumental antigen	*F. hepatica*	Suppresses IL-12p70, IL-6, IL-10 and TNF-α secretions from murine DCs. Supresses maturation of murine DCs	(Hamilton et al., 2009)
SEA (soluble egg antigens)	*S. mansoni*	Suppresses T_H1 response Suppresses TLR induced DC maturation Reduces IL-12, IL-6 and TNF-α secretions	(van Liempt et al., 2007)
Bm-CPI-2 (cysteine protease inhibitor)	*B. malayi*	Inhibits antigen processing via MHC II by interfering with asparaginyl endopeptidase	(Manoury et al., 2001)
rGco-gal-m/f (galectin)	*H. contortus*	Suppressor of inflammatory response Inhibition of cytokine production by modulating T cell development pathway, NF-$_{\kappa}$B pathway and free radical producing pathway.	(Wang et al., 2014b)
rHco-gal-m (galectin)	*H. contortus*	Supresses production of IL-6, IL-10 and TNF-α and increases IL-10 production in monocytes Restricts the expression of MHC II on monocytes Supresses T cell proliferation	(Wang et al., 2014a)
GAPDH	*H. contortus*	Inhibits complement function	(Sahoo et al., 2013)

Product	**Helminth**	**Mechanism of action**	**References**
Omega-1	*S. mansoni*	Reduces expression of co-stimulatory molecules and IL-12 by DCs	(Steinfelder et al., 2009)
Onchocystatin (cysteine protease inhibitor)	*Onchocerca volvulus*	Reduces CD86 and MHC II expressions on human monocytes Reduces antigen specific proliferation and induces IL-10 from stimulated peripheral blood monocytes	(Lustigman et al., 1992; Schönemeyer et al., 2001)
Glycans	*T. suis*	Supress pro-inflammatory phenotype of human DCs	(Klaver et al., 2013)
Sm16	*S. mansoni*	Supresses cutaneous inflammation	(Jenkins et al., 2005)
IFN-γ homologue	*T. muris*	Polarizes T_H1 immune response (bind the host IFN-γ receptors and alter the local cytokine milieu)	(Grencis and Entwistle, 1997)
Ts-MIF (MIF homologue)	*T. spiralis*	Similar to human MIF, inhibits recruitment of human peripheral blood monocytes	(Tan et al., 2001)
Antigen B	*E. granulosis*	Reduces expressions of co-stimulatory molecules on human DCs and induces T_H2	(Riganò et al., 2007)
PAS-1 (200 kilodalton (kDa) protein)	*A. suum*	Supresses pro-inflammatory cytokines and neutrophil influx after exposure to LPS	(Oshiro et al., 2005)
AvCystatin/Av17 (cysteine protease inhibitor)	*A. viteae*	Downregulates T cell responses. Interferes with macrophage MAP kinase signalling pathways to induce IL-10	(Hartmann et al., 1997; Klotz et al., 2011)
Bm-TGH-2 (TGF-β homologue)	*Brugia malayi*	Potentially influence T_{reg} differentiation by binding with mammalian TGF-β receptors	(Gomez-Escobar et al., 2000)

Table 1.1. Immunomodulators identified from helminth parasites of mammals.

Product	**Helminth**	**Mechanism of action**	**References**
Lysophosphatidyl-serine	*S. mansoni*	TLR2-dependent activation of DCs promotes T_{Reg} development	(van Riet et al., 2007)
Phosphatidylserine	*S. mansoni* *Ascaris lumbricoides*	TLR2-dependent activation of DCs promotes T_H2-type anti-inflammatory responses	(van Riet et al., 2009)
Lacto-*N*-fucopentose III	*S. mansoni*	Production of IL-10 and prostaglandin E2 by B-1 B cells	(Atochina and Harn, 2006; Harn et al., 2009)
ES62	*A. viteae*	Inhibition of B-2 B cell proliferation and induction of B-1 B cell-dependent IL-10 secretion TLR4 dependent inhibition of mast cell degranulation and inflammatory mediator production Reduces $CD4^+$ T cell proliferation and TLR4 dependent IL-2 production from macrophages after exposure to lipopolysaccharides (LPS) Induces an anti-inflammatory phenotype in DCs	Goodridge et al., 2005a,b; Harnett and Harnett, 2008)
Cathepsin (cysteine protease)	*S. mansoni*	Inhibits LPS induced IL-6, IL-12 and TNF production by macrophages by inhibition of TRIF signalling through endosomal degradation of TLR3	(Donnelly et al., 2010)
FheCL1 (cysteine protease)	*Fasciola hepatica*	Prevents release of macrophage inflammatory mediators by degrading TLR3	(Donnelly et al., 2010)
smCKBP (chemokine binding protein)	*S. mansoni*	Suppresses neutrophil migration and infiltration by blocking mammalian chemokine ligand CXCL8.	(Smith et al., 2005)

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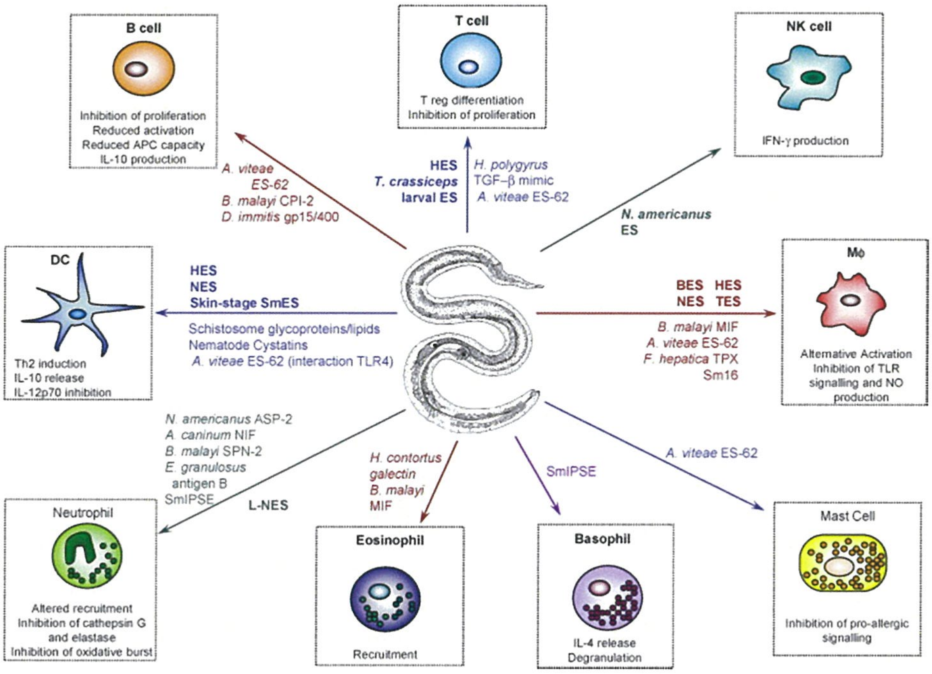

Fig. 1.12. Helminth induced immunomodulation by ES products (bold) and known molecules (Hewitson et al., 2009).

Abbreviations: ASP, *Ancylostoma* secreted proteins; BES, *B. malayi* ES; CPI, cysteine proteinase inhibitor (cystatin); HES, *H. polygyrus* ES; IPSE, IL-4-inducing principle of schistosome eggs; L-NES, *N. brasiliensis* larval ES; NES, *N. brasiliensis* adult ES; Sm, *Schistosoma mansoni*; SPN, serine proteinase inhibitor (serpin); TLR: toll-like receptor; TGF: transforming growth factor; TES: *T. canis* ES.

considered to be immunosuppression or skewed T_H responses deliberately produced by parasites to facilitate their survival. This is the definition used here. Immunomodulation has been extensively reviewed (Hewitson et al., 2009; Everts et al., 2010; Maizels et al., 2012b; McSorley et al., 2013; Grencis et al., 2014) with a principal focus on DCs, induction of T_{regs} and release of tolerogenic cytokines, particularly IL-10 and transforming growth factor (TGF)-ß. There is increasing clinical interest in exploiting immunosuppression by parasites of concurrent autoimmune and allergic diseases, such as ulcerative colitis, multiple sclerosis and asthma (Harnett and Harnett, 2010; McSorley and Maizels, 2012), not by live infections, but using the active parasite chemicals. (Maizels and Gause, 2014) have recently highlighted the complexity of such immunotherapy, as helminth infection and a T_H2 environment can allow latent viral infections to be reactivated.

Fig. 1.12 illustrates some of the many targets used by parasites for immunomodulation of the inflammatory cascade. In some cases, the identities of the active molecules are known, but in others crude parasite products, often fractions of ES products, have been used in experimental studies. The diversity of targets and types of molecules involved can be seen from some of the reported helminth immunomodulators (Table 1.1), which include host mimics, complement inhibitors, suppressors of host innate immune cells and DCs and inducers of T_{regs}, anti-inflammatory cytokines and suppressive AAMs.

1.5.1. Ruminant gastrointestinal parasites

Compared with parasites of humans and mice, less is known about ruminant gastrointestinal nematode immunomodulation (reviewed by McNeilly and Nisbet, 2014), although a similar range of mechanism seems to be employed.

1.5.1.1. Host mimics

Host TGF-ß is stimulated by exposure to helminth antigens and increases the T_{reg} population. A similar effect on T_{reg} prolifieration has been observed *in vitro* by TGF-ß mimics released by *Brugia malayi* (Gomez-Escobar et al., 2000) and *H. polygyrus* (Grainger et al., 2010). As genes encoding TGF-ß homologues are also present in *H. contortus* and *T. circumcincta*, (McSorley et al., 2010), these and other species may similarly induce T_{reg} expansion. A macrophage migration inhibitory factor (MIF) in larval *T. circumcincta* has been identified as a tautomerase (Nisbet et al., 2010). The gene for MIF-1 is also present in *H. contortus* and *O. ostertagi* (McNeilly and Nisbet, 2014).

1.5.1.2. Complement inhibition

The complement pathway is inhibited by binding of *H. contortus* glyceraldehyde-3-phosphate (GAPDH) to C3 (Sahoo et al., 2013) or calreticulin to C1q (Suchitra and Joshi, 2005). *H. contortus* can also inactivate C reactive protein (Suchitra et al., 2008). Hypodermin A (a serine protease) of the insect *Hypoderma lineatum* also binds C3 (Chen et al., 2014) and the calreticulin of *Necator americanus* binds C1q (Kasper et al., 2001). Calreticulin is a possible *H. contortus* vaccine candidate, as the *N. americanus* protein partially protected mice (Winter et al., 2005). As innate cellular recruitment is dependent on C3a and C5a (Volanakis, 2002), complement inactivation reduces neutrophil and eosinophil recruitment to parasites (Giacomin et al., 2005, 2008a,b; Rees-Roberts et al., 2010).

1.5.1.3. Lymphocyte proliferation

T cell hypo-responsiveness is a feature of chronic helminth infection (Maizels et al., 1993; Ricci et al., 2011; Mkhize-Kwitshana et al., 2014). T cell proliferation is reduced and apoptosis induced by ES products of *H. polygyrus* (Rzepecka et al., 2006), filaria (Allen and Macdonald, 1998), *Taenia crassiceps* (Spolski et al., 2000), *N. brasiliensis*, *Toxocara canis*, *Strongyloides stercoralis* (Werneck-Silva et al., 2006), *Onchocerca volvulus* (Hartmann et al., 2013), *F. hepatica* (Serradell et al., 2007) and *Metagonimus yokogawai* (Yu et al., 1997) and adult *B. malayi* (Miller et al., 1991). The mechanisms involved appear to be suppression of costimulatory molecules on activating cells or release of anti-inflammatory cytokines. The semi-maturity of DCs following *in vivo* parasite infection or *in vitro* ES product-stimulation promotes the expansion of Foxp3$^+$ T$_{regs}$ and is not usually adequate for full activation of T and B cell activation and proliferation and antibody secretion.

There are experiments which directly established the inhibitory activity of worm chemicals on proliferation of either blood or lymph node lymphocytes. Suppression of T cells by filarial cystatins (Hartmann and Lucius, 2003) and lacto-N-fucopentose III (Harn et al., 2009; Zhu et al., 2012) is accomplished by inducing IL-10 production. Cystatins also reduce T cell responses by decreasing the expression of costimulator molecules on the surface of macrophages, leading to an anti-inflammatory cytokine environment (Hartmann and Lucius, 2003).

CD4 T$_{regs}$ expressing Foxp3$^+$ have an important role in limiting tissue damage through their immunosuppressive role and are also exploited by numerous helminths as an immune evasion strategy (reviewed by van Riet et al., 2007; Hewitson et al., 2009; Geiger and Tauro, 2012; Maizels et al., 2012b; McSorley et al., 2013; Finlay et al., 2014). Evidence supporting their key role in immunosuppression includes correlation of T$_{reg}$ numbers with chronic infections and depletion of T$_{regs}$ causing parasite expulsion. Host TGF-ß is generated during helminth infections and is important in increasing the T$_{reg}$ population as are TGF-ß mimics released by *B. malayi*

(Gomez-Escobar et al., 2000) and *H. polygyrus* (Grainger et al., 2010). IL-10 secreted by T_{regs} has a role in immunosuppression, but does not appear to be essential in *H. polygyrus* infection (Maizels et al., 2012a), although there may be differences between host-parasite relationships, as for other aspects of protective immunity.

1.5.1.4. Dendritic cells

Helminths suppress immune responses and induce a tolerogenic environment by inhibiting DCs, so that they do not exhibit classical maturation, upregulation of co-receptors or release of pro-inflammatory cytokines (reviewed by White and Artavanis-Tsakonas, 2012). This has downstream effects on T and B cells, which are normally stimulated by activated DCs. In addition, DCs exposed to ES products of many helminths promote the expansion of Foxp3$^+$ T_{regs}. T_H1 responses are also reduced: *H. polygyrus* ES product stimulation of DCs reduced IL-12p70 responsiveness to TLR agonists like LPS (Segura et al., 2007). Helminth parasites of ruminants also suppress DCs. A high molecular weight surface glycolipid larval antigen (CarLA) on parasitic Trichostrongyles (Harrison et al., 2008) inhibits human monocyte-derived DCs (Pernthaner et al., 2012). Expression of immune genes was down-regulated in mixed cells, made up of 15% DC and 85% lymphocytes, which were collected from the intestinal afferent lymph of *T. colubriformis*-infected sheep (Knight et al., 2010).

Monocytes, the circulating precursors of DCs and macrophages, are also inhibited by worm products. A 66 kDa *H. contortus* glycoprotein in ES products inhibited the generation of hydrogen peroxide and nitric oxide by goat monocytes (Rathore et al., 2006). Goat monocytes incubated with *H. contortus* male galectin rHco-gal-m had decreased expression of MHCII, but not MHCI (Wang et al., 2014).

1.6. CONCLUSIONS

Parasitism has evolved to allow chronic infections despite the immune response of the host. Worm ES products are the major link between gastrointestinal parasites and their host and initiate both the immune response and the pathophysiology, but are also responsible for manipulation of the host to suppress inflammatory pathways leading to worm expulsion. To reach the host tissues, ES products cross the gut epithelial lining, a process which appears to be actively facilitated by these chemicals and important for both immunomodulation and the changes in host physiology which are likely to benefit parasite survival.

This project investigated the role of *H. contortus* ES products in the initiation of host pathology and in suppression of host immune responses. The experiments reported in Chapter 2 used a Caco-2 cell model to examine the permeabilisation of epithelial tissues, such as the abomasal epithelium, by adult *H. contortus* ES products, including the possibility that the immune sheep can block this property of ES products. In addition, enolase was investigated as a possible active component of the ES products. The second part of the study was down-regulation of host immune responses by adult worm chemicals. Experiments reported in Chapter 3, tested *in vitro* the modulation of the phenotypic and functional properties of human monocyte derived DCs (mdDCs). Chapter 4 describes an *in vitro* study of proliferation in response to ES products of lymphocytes collected from infected and vaccinated sheep.

Chapter 2

EFFECTS OF *H. CONTORTUS* ES PRODUCTS ON EPITHELIAL CELL PERMEABILITY

2.1. INTRODUCTION

2.1.1. Initiation of host pathology

Although the typical pathophysiology of increased abomasal pH and serum gastrin and pepsinogen concentrations and changes in gastric gland cell populations are well known (reviewed in Chapter 1, 1.2), how these are initiated by parasites has not been established. The onset of the pathophysiology and inflammatory responses coincides with the emergence of larvae or immature adult worms from the gastric glands and also rapidly follows transplantation of adult worms into naïve recipients (McKellar et al., 1986; Lawton et al., 1996; Simpson et al., 1997). This strongly suggests the involvement of parasite ES products in mediating the effects of luminal parasites, most likely through their action on the surface mucosa of the abomasum. To have an effect on the parietal and chief cells located deep in the glands, ES products probably cross the surface epithelium and act on the basolateral membranes of gastric gland cells. This entry of worm chemicals is likely to be facilitated by increased permeability of the mucosa.

2.1.2. Epithelial cell tight junctions

The polarised epithelium making up the gastric mucosa regulates the movement of water and dissolved molecules across the cells (transcellular) and between cells (paracellular) (Mitic and

Anderson, 1998). Adjacent cells adhere to each other at junctional complexes (Fig. 1.1), of which the tight junctions are considered to be responsible for intercellular sealing, impermeability and selective transport (Furuse et al., 1998; Tsukita et al., 2001; Blasig and Haseloff, 2011). The transmembrane protein occludin is an important determinant of the permeability of tight junctions and thus the TEER (reviewed in Chapter 1, 1.3.2), while the peripheral proteins, ZO-1 and ZO-2, are essential for the polymerisation of proteins of tight junctions (Umeda et al., 2006). One regulator of the permeability of tight junctions is structural remodelling (Ivanov et al., 2004, 2005; Shen et al., 2008). There is continuous remodelling of tight junctions under physiological conditions, with about 70% of occludin and ZO-1 and 24% of claudin in a mobile fraction (Shen et al., 2008). Occludin and claudin move within the plasma membrane, and tight junction ZO-1 exchanges with an intracellular pool. Junctional proteins can be internalised and endocytosed (Ivanov et al., 2004, 2005; Shen et al., 2008), and in addition protein expression is down-regulated.

2.1.3. Effect of pathogens on tight junctions

As part of the cell invasion process, many pathogens disrupt tight junctions and increase epithelial permeability by a combination of effects on protein function, tight junction remodelling or interacting with integral and peripheral proteins of the cytoskeleton, which attaches to tight junctional proteins (Chapter 1, 1.3.3.3). For abomasal nematodes to have an effect on the parietal and chief cells located deep in the glands, ES products probably cross the surface epithelium and act on the basolateral membranes of gastric gland cells. This entry of worm chemicals is likely to be facilitated by increased permeability of the mucosa, which may be caused by specific parasite molecules and may also be reinforced by cytokines generated by inflammation. Infection with many pathogens, including nematodes (reviewed in

1.3.3.3), has been reported to increase the permeability of the host gut (Scott et al., 2002; McDermott et al., 2003; Su et al., 2011). Increased abomasal permeability results in plasma protein and pepsinogen leakage into the abomasal lumen of infected animals (Holmes and MacLean, 1971; McLeay et al., 1973).

The increased mucosal permeability in the parasitised abomasum could be a direct effect of secreted chemicals on tight junctions of the epithelium or could indirect effects of inflammatory cytokines, which increase gut permeability (Ma et al., 2005; Al-Sadi and Ma, 2007) and *in vitro* reduce the TEER of Caco-2 cells (Van De Walle et al., 2010). Büring (2009) showed that *H. contortus* ES products caused cytoskeletal rearrangement in HeLa cells and reduced the TEER of Caco-2 cells at 6 and 24 hours. The specific molecules responsible were not investigated and could be any of the 100 - 200 proteins released *in vitro* by adult nematode parasites (Yatsuda et al., 2003; Craig et al., 2006; Wang et al., 2013), including proteases, structural components of the parasites, metabolic enzymes and many of unknown identity and function. The molecules in ES products responsible for the increased TEER may be able to be determined after recovery from 2-D gels and proteomic analysis. An alternative approach is to prepare pure recombinant candidate ES proteins by expression of these proteins in *E. coli* or other systems, using known gene sequences.

2.1.4. Host recognition of mediators

Further evidence that components of nematode ES products act *in vivo* as well as in *in vitro* model systems would be a demonstration that antibodies produced by immune sheep recognise and block the *in vitro* effects of ES products or fractions. Single chain antibody fragments (scFvs) libraries displayed on bacteriophage, created by coupling cloned light and heavy chain immunoglobulin-variable region-coding DNA, have proven to be a useful resource to identify antibodies to defined target antigens (Burton and Barbas, 1994; Winter et al., 1994). These libraries have been used to investigate antibody responses to immunogens in many vertebrates such as mice (Clackson et al., 1991), rabbits (Lang et al., 1996), sheep (Li et al., 2000), cattle (O'Brien et al., 1999) and chickens (Davies et al., 1995). Maass et al. (2007) developed a scFvs library to characterise larval surface antigens. This was derived from lymphocytes collected from sheep which were immune to nematodes because of field infection. Selective panning of this phage library provided antibodies for the present study to investigate host recognition of parasite chemicals involved in increased epithelial TEER.

2.1.5. Enolase

Candidate mediators of permeabilisation in ES products are likely to be those secreted by many pathogens, are immunologically recognised by the host and are known to have “moonlighting activities” involving interaction with host proteins. Glycolytic enzymes, such as enolase, have these properties, being secreted despite lacking a secretory sequence and are also found adhering to the surface of the organism. Enolase is present in the ES products of many helminths, including *T. circumcincta* (Craig et al., 2006), *H. contortus* (Yatsuda et al., 2003), *B. malayi* (Hewitson et al., 2008), *H. polygyrus* (Hewitson et al., 2011), *Schistosoma bovis* (Ramajo-Hernández et al., 2007) and *Clonorchis sinensis* (Wang et al., 2011). It is antigenic and recognised in helminth ES products by the serum of immune hosts (Jolodar et al., 2003; Pérez-Sánchez et al., 2006; Kiel et al., 2007; Marcilla et al., 2008). In addition to its role in glycolysis, enolase from different organisms has been reported to be a plasminogen receptor (Bernal et al., 2004; Ehinger et al., 2004), a lens crystallin (Tomarev and Piatigorsky, 1996), a heat shock protein (Aaronson et al., 1995), a transcription regulator which binds to nuclear RNA (Hernández-Pérez et al., 2011) and to bind the cell adhesin P-selectin (Díaz-Martín et al., 2013), salivary mucin (Ge et al., 2004) and actin (Arnold et al., 1971; Peng et al., 2014). It also evokes the production of specific IgE antibodies (Ishiguro et al., 1992), which is typical of parasitic infections.

Another enzyme in nematode ES products is arginine kinase (AK) (Craig et al., 2006; Kiel et al., 2007) which is also strongly antigenic (Kiel et al., 2007), but with no reported effects on host physiology. Comparison of the effects of enolase and AK on Caco-2 cell TEER may assist in distinguishing direct effects on permeability from indirect actions resulting from immune recognition.

Experiments reported in this Chapter examined the effects of ES products and its constituents on tight junction permeability using the intestinal tumour Caco-2 cell model. The possibility of using another epithelial cell model derived from gastric cells was investigated using rat gastric mucosal (RGM1) cells originally obtained from rat surface epithelial cells (Kobayashi et al., 1996). cDNA encoding *H. contortus* enolase and AK were cloned, expressed in *E. coli* and the recombinant proteins, purified and their enzymatic activities confirmed. The actions of the two recombinant enzymes were compared with that of adult worm ES products on Caco-2 monolayers. Phage displayed scFvs selected against ES products and recombinant enolase were used to investigate whether these specifically enriched scFvs are able to block the effects of ES products and recombinant enolase.

Fig. 2.1. Adult *H. contortus* on the mucosal surface of the parasitised sheep abomasum.

2.2. MATERIALS AND METHODS

2.2.1. Preparation of adult *H. contortus* ES Products

2.2.1.1. Maintenance of *H. contortus* cultures

The laboratory strain of *H. contortus* was originally obtained from the field and maintained by infection of nematode-free sheep and routine laboratory culture of L_3. Romney or Romney crossbred sheep were housed indoors and fed ad libitum with lucerne chaff harvested from paddocks not grazed by sheep (Denver Stock Feeds, Palmerston North New Zealand). Water was freely available. Animal experiments were carried out in accordance with the requirements of the Massey University Animal Ethics Committee (MUAEC 09/99) and AgResearch animal ethics standards (AE 13052 sustainable bio-control of nematode parasites).

All sheep were drenched (2ml/5kg) on arrival and again on the following day (1ml/5kg) with a broad spectrum anthelmintic (Matrix, Merial, New Zealand) and housed separately from uninfected sheep. Two weeks later, the absence of nematode infection was confirmed by faecal floatation (Appendix 1.1). Sheep were infected orally with approximately 10,000 L_3 and patency confirmed by faecal floatation on Day 17 post infection. L_3 were recovered from faeces (Appendix 1.3) and stored at 10°C in reverse osmosis (RO) water. L_3 viability was assessed microscopically prior to infection of sheep.

2.2.1.2. Collection of adult worms

Infected sheep were euthanased on Day 21 post infection and adult worms were collected. Fig. 2.1 shows adult *H. contortus* on the mucosal surface of the abomasum of a parasitised sheep. Clean worms were recovered from abomasal contents by setting in agar blocks, as described by Umair et al. (2013) (Appendix 1.4). Briefly, abomasal contents and washings were mixed 1:2 with warmed agar

Fig. 2.2. Formation of clumps of adult *H. contortus* worms during incubation in RP2 culture medium.

and after the agar blocks had set, they were placed in saline into which the adult worms migrated. Adult worms were collected with pipettes and placed in RP2 incubation medium.

2.2.1.3. Preparation of adult *H. contortus* ES products

Adult worms were incubated for a total of 4h in 37°C RP2 medium at a density of approximately 50mg/ml in T75 cell culture flasks (Fig. 2.2). Incubations were carried out in an atmosphere containing 5% CO_2 and 95% humidified air at 37°C. RP2 medium was also incubated under the same conditions to serve as a negative control. ES products were harvested after 1, 2 and 4h with replacement of the collected incubate with fresh medium at each time point. Worm viability was monitored during the incubation. Aliquots of incubates were filtered through 0.2µm filters (Minisart, Sartorius) into sterile tubes and stored either at 4°C for immediate use, or frozen at –80°C in small aliquots for single use only.

2.2.1.4. Estimation of lipo-polysacharide (LPS) content

The LPS content in each batch of ES products was measured using Pyrosat^R endotoxin kit (CAPE COD Inc, USA), which has a sensitivity of 0.25EU/ml. ES batches negative for LPS were used for further experiments.

2.2.2. Expression of adult *H. contortus* enolase in *E. coli*

2.2.2.1. Extraction of RNA from adult *H. contortus*

TRIzol (Invitrogen, USA) was used to extract total RNA. 1ml of TRIzol was added to 50 - 100µl of worms and frozen in liquid nitrogen. The mixture was ground with a mortar and pestle with extra liquid nitrogen being added as required, until a very fine white-pink powder was obtained.

Chloroform (0.2ml) per ml of TRIzol worm mixture was added and mixed well by vigorous shaking by hand for 15sec. The mixture was centrifuged at 12,000*g* for 15min at 4°C. The upper phase was removed to another tube, avoiding the interface; 0.5ml isopropanol was added, mixed and incubated for 10min at room temperature. The mixture was centrifuged at 12,000*g* for 10min at 4°C.

The supernatant was removed with care being taken to avoid the pellet; 1ml of 75% ethanol was added to the pellet and the mixture centrifuged at 7,500*g* for 5min at 4°C. The supernatant was removed, the tube centrifuged briefly and any remaining liquid was removed with a pipette. The pellet was air-dried for 5 - 10min at room temperature. The RNA obtained was resuspended in 50µl RNase-free water and quantified using a NanoDrop ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies, USA). The RNA was stored at –80°C until required.

2.2.2.2. cDNA synthesis

The extracted RNA was used as the template in the synthesis of first strand cDNA, using the iSCRIPT select cDNA synthesis kit (BIO-RAD, USA). Details of the cDNA synthesis method are given in Appendix 2.1. The cDNA product was quantified as above, diluted to 20ng/µl with sterilised deionised water and stored at –20°C until required.

2.2.2.3. Cloning of *H. contortus* enolase into the AY2.4 vector

2.2.2.3.1. Amplification of *H. contortus* enolase cDNA

H. contortus enolase cDNA was amplified from cDNA with the primers Hc enol-F (5'-ATGCCATATGATGCCTATCACGAAAATCCACG-3') and Hc enol-R (5'-TACGGCGGCCGCAACTGGATTGCGGAAGTTTTG-3'). The primers were based on the published *H. contortus* enolase sequence (Han et al., 2012) (Genbank Accession No. HM138086). The polymerase chain reaction (PCR) was carried out in a total volume of 20µl and contained 1µl cDNA (20ng/µl), 1µl dNTP mix (4mM of each dNTP), 1µl 50mM $MgCl_2$, 1µl of each primer (10µM), 2µl 10X reaction buffer, 0.1µl Platinum Taq DNA polymerase (5U/µl, Life Technologies, USA) and sterile deionised water to 20µl. The PCR was performed under following conditions: initial denaturation at 95°C for 10min, followed by 30 cycles each of 94°C for 30sec (denaturation), 60°C for 30sec (annealing) and 72°C for 1min (extension) and a final extension at 72°C for 10min. The *H. contortus* enolase cDNA PCR product was column purified using the DNA Clean and Concentrator-5 kit (Zymo Research, USA), eluted in 10µl sterile deionised water, quantified as in Section 2.2.2.1, and 1µl of cDNA PCR product were electrophoresed on a 2% agarose gel in tris-acetate-EDTA (TAE) buffer at 100 volts until the dye front was 80% of the way down the gel. DNA was visualised with SYBER Safe DNA gel stain (Life Technologies, USA). A PCR product of the expected size (1365 base pair) was obtained.

2.2.2.3.2. Isolation of the AY2.4 vector

E. coli containing the AY2.4 vector was grown from a glycerol stock in Luria Broth (LB) medium containing 100µg/ml ampicillin (LB-Amp). The recipe for LB medium is given in Appendix 2.2.1. Plasmid DNA was purified from a 10ml culture using the QIAprep spin miniprep kit (Qiagen, USA) following the manufacturer's instructions and quantified as in Section 2.2.2.1.

2.2.2.3.3. Restriction enzyme digestion of enolase cDNA PCR product and AY2.4 vector

The column purified PCR product was digested with the restriction enzymes NdeI and NotI in a reaction mixture containing 1µg column purified PCR product, 10µl 10x NEB buffer (New England

BioLab Inc, USA), 1µl 100x NEB BSA (New England BioLab Inc, USA), 1.5µl NdeI (20000U/ml, New England BioLab Inc, USA), 1.5µl NotI (10000U/ml, New England BioLab Inc, USA) and sterile deionised water to 100µl. The reaction mixture was incubated at 37°C for 4h, an additional 1.5µl of NdeI and NotI was added and the mixture incubated overnight at 37°C.

The AY2.4 vector was digested with the restriction enzymes NdeI, NotI and EcoRI in a reaction mixture containing 1µg AY2.4 purified plasmid DNA, 10µl 10x NEB EcoR1 buffer (New England BioLab Inc, USA), 1µl 100x NEB BSA, 1.5µl NdeI, 1.5µl NotI, 1.5µl EcoRI (20000U/ml, New England BioLab Inc, USA) and sterile deionised water to 100µl. The reaction mixture was incubated at 37°C for 2h, an additional 1.5µl of NdeI, NotI and EcoRI was added and incubated at 37°C for a further 2h. The digested vector was purified (Section 2.2.2.3.1) and quantified as described in Section 2.2.2.1.

2.2.2.3.4. Ligation of vector (AY2.4) and insert (*H. contortus* enolase)

The ligation was performed with a vector:insert ratio of 1:2 (pmol ends). The calculated pmol was 0.09pmol ends for 145ng of vector and 0.18pmol for 80ng of insert. The volume was made up to 5µl with sterile deionised water and an equal volume of Takara Mighty Ligation Mix (Clontech Laboratories Inc, USA) added prior to incubation at 16°C overnight, followed by 65°C for 20min.

2.2.2.4. Transformation of *E. coli* Top-10 competent cells with vector:insert

Ligated vector:insert (4µl) was transformed into chemically competent *E. coli* Top10 cells (Invitrogen, USA) according to the manufacturer's instruction. Ligation mixture (4µl) was added to a thawed vial of chemically competent *E. coli* Top-10 cells (Life Technologies, USA) and mixed gently, incubated on ice for 30min, heat shocked at 42°C for 30sec and returned immediately to ice. Room temperature super optimal broth with catabolite repression (S.O.C.) medium (Life Technologies, USA) (250µl) was added and the tubes incubated horizontally at 37°C and 200rpm for 1h.

2.2.2.5. Analysis of transformants

Transformed chemically competent *E.coli* Top-10 cells (~20µl) were plated onto LB-Amp agar plates and grown at 37°C overnight. Individual colonies were picked into 50µl sterile deionised water and the presence of insert confirmed by amplification with Hc enol-F and Hc enol-R primers under the conditions as described in Section 2.2.2.3.1. Transformants with the correct sized inserts were grown at 37°C overnight in 10ml LB-Amp. Plasmid DNA was extracted and sequenced by the University of Waikato DNA sequencing facility with the primers Hc enol-F, Hc enol-R, Ara-Hu (5'-ACTCTTTCTCCATACCC-3'), and Ara-rHu (5'-CATCCGCCAAAACAGCC-3').

The *H. contortus* enolase sequence was confirmed by alignment of the predicted protein sequence with that of Han et al. (2012). Alignments were performed using the Muscle alignment option in Geneious 5.6.5 (Biomatters Ltd, New Zealand) with the Blosum 62 similarity matrix used to determine 100% similarity.

2.2.2.6. Transformation of chemically-competent BL21 Star (DE3)

AY2.4 *H. contortus* enolase plasmid DNA (10ng in 1 - 5µl) was added to a thawed vial of chemically-competent BL21 Star (DE3) (Life Technologies, USA) and mixed gently, incubated on ice for 30min, heat shocked at 42°C for 30sec and returned immediately to ice. Room temperature S.O.C. medium was added (250µl) and the tubes incubated horizontally at 37°C and 200rpm for 30min.

2.2.2.7. Optimisation of expression of recombinant *H. contortus* enolase (r*Hc*ENO)

The entire transformation (250µl; plasmid DNA in BL21 Star (DE3)) was added to 10ml 2X yeast extract and tryptone (YT) medium (1.6% Bacto Tryptone, 1% Bacto Yeast Extract, 0.5% NaCl, pH 7.0) containing 100µg/ml ampicillin (2X YT-Amp) and incubated overnight at 37°C and 250rpm. Medium (25ml of 2X YT-Amp) was inoculated with 250µl of the overnight culture and incubated at 37°C and 250rpm until the OD_{600} was 0.5 - 0.8. The OD_{600} was monitored by removing 500µl aliquots every hour. L-arabinose was added to a final concentration of 0.1% and aliquots (500µl) were removed at 0, 2, 4, 6 and 16h and assessed for the level of recombinant protein expression and its solubility.

Bacteria were pelleted from the 500µl post-induction samples by centrifuging at 14000*g* for 5min. To obtain the soluble and insoluble fractions, 200µl B-Per (Pierce) reagent (Thermo Scientific, USA) was added to the pellets, which were vortexed for 1min and centrifuged at 14,000*g* for 5min. The supernatant (soluble) was removed and the pellet (insoluble) was resuspended in 200µl B-Per reagent.

2.2.2.8. Assessment of protein expression

Bacterial samples, from post-induction samples from Section 2.2.2.7, were analysed for the presence of enolase protein by electrophoresis on NuPAGE Novex 4 - 12% Bis-Tris mini gels (Life Technologies, USA). Briefly, bacterial pellet samples were thawed and resuspended in 100µl sterile deionised water. 2x SDS-Sample buffer (100µl) (Appendix 2.3.6) was added; the samples were heated to 100°C for 3min and briefly centrifuged. Samples (5µl) were loaded onto the mini gel and electrophoresed at 200 amps for 40min. Proteins were visualised with Coomassie Blue safe stain (Invitrogen, USA), as described by the manufacturer, or transferred to nitrocellulose. The remaining samples were stored at –20°C for reference and further use. Optimum expression of enolase protein was observed 4h post-induction.

The solubility of the expressed enolase protein was assessed by electrophoresis of the soluble and insoluble fractions from Section 2.2.2.7. 2x SDS-sample buffer (20µl) was added to equal amounts of both soluble and insoluble fractions, heated and centrifuged as above. Samples (10µl) were loaded onto mini gels, electrophoresed and visualised as above. The remaining samples were stored at –20°C for reference and further use. Maximum expression of soluble enolase protein was observed in the 4h post-induction sample.

2.2.2.9. Expression of r*Hc*ENO

The entire transformation from Section 2.2.2.6 was added to 10ml 2X YT-Amp medium and incubated overnight at 37°C and 250rpm. 2X YT-Amp medium (200ml) was inoculated with the 10ml overnight culture and incubated at 37°C and 250rpm until the OD_{600} was 0.6. The OD_{600} was monitored every hour as described in Section 2.2.2.7. L-arabinose was added to 0.1% and the culture grown for 4h at 37°C and 250rpm.

2.2.2.10. Preparation of bacterial cell lysate under native conditions

All work was performed on ice. After 4h of incubation, bacteria were harvested from the 200ml of culture medium by centrifugation for 5min at 5000rpm in a Sorvall SS-34 rotor. Bacterial cell pellets were stored overnight at –20°C and resuspended in 10ml of native binding buffer (10mM imidazole) (Appendix 2.3). Lysozyme (final concentration 1mg/ml) was added and the cell suspension was incubated on ice for 30min. The soluble extract was obtained by 3 rounds of freezing at –80°C and thawing at 37°C. This was followed by 6 sonications, each for 10sec, with 10sec cooling between. The cell suspension was passed through a 20 gauge needle then a 26 gauge needle to reduce viscosity and centrifuged at 10,000*g* and 4°C for 30min to pellet the cellular debris. The supernatant (soluble extract) was retained and 25µl removed for SDS-PAGE analysis.

2.2.2.11. Purification under native conditions

Nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, USA) was resuspended by inverting and gently tapping the bottle. Slurry resin (1ml) was washed 3 times with 5ml of native binding buffer containing 10mM imidazole.

The soluble extract was added to resin, and rotated for 120min at 4°C and loaded onto a column. The column was washed with 3x 2.5ml of native wash buffer containing 10mM imidazole followed by 3x 2.5ml of native wash buffer containing 40mM imidazole. Protein was eluted with 4x 1ml of native elution buffer containing 250mM imidazole (Appendix 2.3.4).

2.2.2.12. Dialysis

Eluted protein was dialysed against 2L of phosphate buffered saline (PBS) dialysis buffer (Appendix 2.3.5) at 4°C for 4h. The buffer was replaced with fresh buffer and dialysis continued overnight. Purified protein was stored at –20°C in 50% glycerol and dialysed again before use.

2.2.2.13. Protein estimation

Protein concentration was determined by the method of (Bradford, 1976) using bovine serum albumin (BSA) as standard.

2.2.2.14. Probing western blot for His-tagged proteins

Proteins were transferred to nitrocellulose using the iBlot Dry Blotting System (Invitrogen, USA). The nitrocellulose membrane was blocked for 3h in 4% skim milk powder (Anchor, New Zealand)/tris-buffered saline (TBS)/0.1% Tween-20 at room temperature with agitation prior to overnight incubation in anti-His HRP antibody (Sigma-Aldrich, USA) 1:2000/4% skim milk powder/TBS/0.1% Tween-20. The membrane was washed thrice with TBS/0.1% Tween-20 for 20min, followed by washing twice with TBS for 20min prior to detection of horse radish peroxidase (HRP). The washed nitrocellulose membrane was treated with 2ml of 3-amino-9-ethylcarbazole (AEC) stock in 50ml 50mM sodium acetate buffer containing 10μl hydrogen peroxide to detect HRP. Details of reagents used are given in Appendix 2.3.7.

2.2.2.15. r*Hc*ENO activity (E.C. 4.2.1.11)

The enzyme activity of r*Hc*ENO was measured at 25°C in a coupled assay with pyruvate kinase (PK) and lactic dehydrogenase (LDH) (Bergmeyer, 1974), in which 2-phosphoglycerate was converted successively to phospho(enol)pyruvate and pyruvate. The oxidation of NADH to NAD^+ was monitored spectrophotometrically at 340nm. The final reaction mixture (total volume 1ml, pH 7.4) contained 81mM triethanolamine, 1.9mM 2-phosphoglycerate, 0.12mM NADH, 5mM magnesium sulphate, 100mM potassium chloride, 1.3mM ADP, 7 units PK, 10 units LDH and 50μg enolase protein.

2.2.3. Expression of recombinant adult *H. contortus* arginine kinase (r*Hc*AK)

r*Hc*AK was expressed and enzyme activity (E.C. 2.7.3.3) estimated, according to (Umair et al., 2013). The enzyme and activity data were kindly provided by Dr S. Umair.

2.2.4. Production of negative control for recombinant enzymes

Untransformed *E. coli* strain BL21 (DE3) was grown in 200ml 2X YT without ampicillin to an OD_{600} 0.5 - 0.8. Bacteria were pelleted and the soluble extract obtained by freeze-thawing and sonication, as described in Section 2.2.2.10. The proteins were applied to a Ni-NTA column, according to the procedures detailed in Section 2.2.2.11.

2.2.5. Panning with *Hc*ES and r*Hc*ENO of an ovine single chain antibody library displayed on phage

The anti-*Hc*ES and anti-r*Hc*ENO single chain antibody fragments (scFvs) were obtained by panning an ovine scFvs library. The original library was constructed and the library was panned for this project by the Parasitology team at AgResearch Ltd, as described by Maass et al. (2007). Briefly, this library was prepared from B cell mRNA obtained from abomasal and mesenteric lymph nodes from two year-old Romney sheep naturally infected with poly-generic nematode challenge. The animals were found to be refractory to *T. colubriformis, T. circumcincta and H. contortus* infections.

Anti-*Hc*ES scFvs were selected by 3 rounds of panning with 4ml *Hc*ES (an equal mixture of 1h and 4h collections, Section 2.2.1.3) immobilised on immunotubes. This pre-selected ovine scFvs library was further selected for binding to 4ml r*Hc*ENO (10µg/ml in PBS).

2.2.6. RGM1 cell monolayers

The cell line, rat gastric mucosal cell first (RGM1) (Kobayashi et al., 1996), was maintained in culture, as described in Appendix 3.2.1. To establish monolayers, RGM1 cells were seeded on the apical

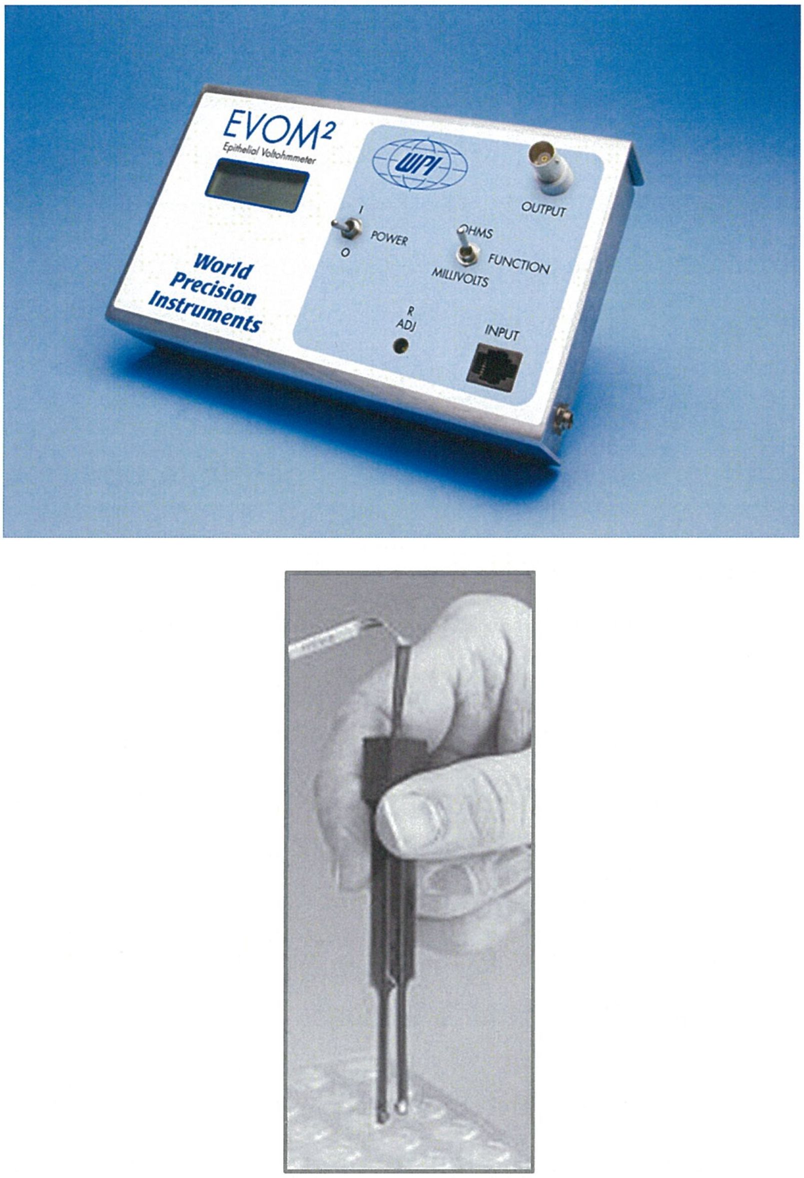

Fig. 2.4. Apparatus for measurement of transepithelial electrical resistance (TEER) of cultured cells. Top: epithelial volt-ohm meter (EVOM^{TM}; World Precision Instruments); bottom: StX2 "chopstick" electrodes.

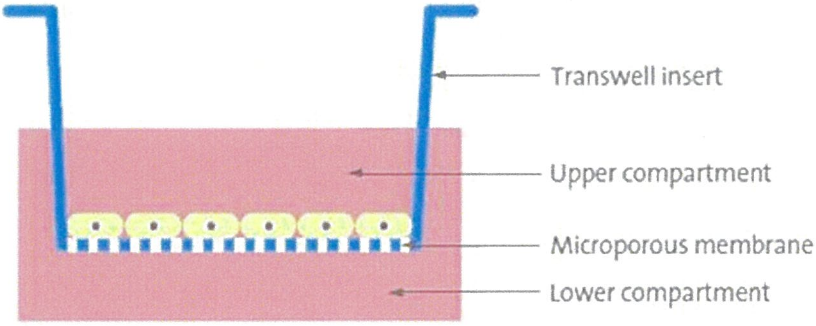

Fig. 2.3. Transwell® system. Top: a 12 well transwell plate with inserts; bottom: cross section of a typical transwell plate.

surface of polycarbonate and polyester tissue culture inserts (Corning 12-well Transwell plates; 12mm diameter, 0.4µm pore size) (Fig. 2.3) at 5×10^4 cells/ml, 5×10^5 cells/ml and 5×10^6 cells/ml in 500µl of cell culture medium; 1.5ml of cell culture medium was applied to the basolateral side. TEER was measured for 3 weeks with an epithelial volt-ohm meter and STX2 chopstick electrodes (EVOM^{TM}; World Precision Instruments, Inc., USA (Fig. 2.4), to determine whether a stable reading of at least 400 Ω/cm^2 could be obtained. The maximum TEER obtained was 165 Ω/cm^2.

2.2.7. Caco-2 cell monolayers

Human epithelial colorectal adenocarcinoma (Caco-2) cells were maintained as described in Appendix 3.2.2. To establish polarised monolayers, Caco-2 cells were seeded on the apical surface of polyester tissue culture inserts (Corning Clear 12-well Transwell plates; 12mm diameter, 0.4µm pore size (Fig. 2.3) at 5×10^5 cells/ml in 500µl of cell culture medium; 1.5ml of cell culture medium was applied to the basolateral side. Confluent cell monolayers used for experiments were formed in 7 - 14 days and had a stable TEER above 400 Ω/cm^2.

The effect of removal of cultures from the 5% CO_2 atmosphere in the incubator for TEER measurement at 2 hourly intervals was assessed; stable readings were obtained. The effects of changing the medium in the apical compartment were assessed: inadequate equilibration of the medium with CO_2 caused the pH to rise above pH 7.4 and reduced the TEER. Control incubates matched to the pH 7.2 of ES products did not change the TEER up to 8h, but increased the TEER by about 20% after 24h. For subsequent experiments, there was adequate equilibration of the medium with CO_2 to reach pH 7.4.

2.2.8. Incubation of Caco-2 monolayers with *H. contortus* products

Culture medium in the wells was discarded and replaced on the apical side with the test solution (500µl). Cells were incubated for 24h and TEER measured at 0, 2, 4, 6, 8 and 24h. The effect of worm products (*Hc*ES, r*Hc*ENO and r*Hc*AK) and the ability of scFvs to block the effects on TEER were assessed.

2.2.8.1. *H. contortus* ES products

Undiluted 2 - 4h *Hc*ES (*n* = 16) were added to the apical compartment after the time zero TEER reading. Medium incubated without worms and adjusted to the same pH as the ES products served as the control (*n* = 10).

2.2.8.2. Recombinant *H. contortus* enolase (r*Hc*ENO) and arginine kinase (r*Hc*AK)

The apical solution was replaced with 500µl culture medium containing 10µg in 50µl of either r*Hc*ENO (*n* = 10), r*Hc*AK (*n* = 4) or 50µl of the negative expression control (bacterial lysate) (*n* = 4). The solutions were incubated for 30min at 37°C in 5% CO_2 prior to applying to the monolayers.

2.2.8.3. Incubation of single chain antibodies displayed on bacteriophage with *H. contortus* ES products

The effect of replacing the apical solution with undiluted *Hc*ES was compared with *Hc*ES incubated for 30min at 37°C in 5% CO_2 with one of three scFvs displayed on phage. Anti-*Hc*ES phage in the same concentrations without ES products served as negative controls. Phage used with *Hc*ES were:

(1) unselected scFvs at final concentrations of 10^{12} and 10^{11} plaque-forming units (PFU)/ml (*n* = 2);

(2) anti-*Hc*ES scFvs at final concentrations of 10^{15}, 10^{14}, 10^{13} and 10^{12} PFU/ml ($n = 3$);

(3) anti-r*Hc*ENO scFvs at a final concentration of 10^{14} PFU/ml ($n = 4$).

2.2.8.4. Incubation of single chain antibodies displayed on bacteriophage with r*Hc*ENO

The effect of replacing the apical solution with r*Hc*ENO was compared with r*Hc*ENO incubated for 30min at 37°C in 5% CO_2 with one of three scFvs displayed on phage. Phage used with r*Hc*ENO were:

(1) anti-r*Hc*ENO scFvs at final concentrations of 10^{14}, 10^{13}, 10^{12} and 10^{11} PFU/ml ($n = 2$);

(2) anti-*Hc*ES scFvs at a final concentration of 10^{15} PFU/ml ($n = 4$);

(3) unselected scFvs at final concentrations of 10^{12} and 10^{11} PFU/ml ($n = 2$).

2.2.9. Immunochemical staining of tight junction proteins

The polyester membranes with Caco-2 monolayers were carefully removed from transwells and fixed in 4% paraformaldehyde (Appendix 4.1) for 30min at room temperature, then washed 3 times with PBS. Filter membranes were stored in 0.02% sodium azide in PBS at 4°C until stained.

Cells on filter membranes were exposed to acetone for 3min at –20°C, and then permeabilised with 0.2% Triton-X-100 in PBS for 15min at room temperature. Membranes were incubated with blocking buffer for 60min at 37°C. Antibody dilutions are given in Appendix 4.3. All antibodies were obtained from Life Technologies, USA. Two different batches of both ZO-1 and occludin primary antibodies were used to target ZO-1 and occludin.

Cells were stained with rabbit anti-ZO-1 antibody diluted in blocking buffer for 60min in a moist box at 37°C in the dark, followed by staining with Alexa Fluor 488 goat anti-rabbit IgG under the same conditions. For occludin, cells were stained with mouse anti-occludin antibody, diluted in blocking buffer (Appendix 4.2). Secondary staining for occludin was performed using either Alexa Fluor 647 goat anti-mouse IgG, or indirectly with biotin-conjugated goat anti-mouse IgG1, then a streptavidin Alexa Fluor 546 conjugate. Each step was carried out for 60min in a moist box at 37°C in the dark. Cells were washed 3 times with washing buffer after each staining step.

Membranes were mounted with vectashield mounting medium for fluorescence (Vector Laboratories, USA) and the slides observed under confocal microscopy (Leica SP5 DM6000B) at the Manawatu Microscopy and Imaging Centre (MMIC). Images were processed in ImageJ and Adobe Photoshop software.

2.2.10. Data analysis

TEER data for each well at successive time points were normalised to time zero (100%). Replicate data are presented as mean ± S.E.M. Graph Prism v5 was used to plot graphs and to analyse data using two-way ANOVA with Bonferri post-tests to compare data at successive time points.

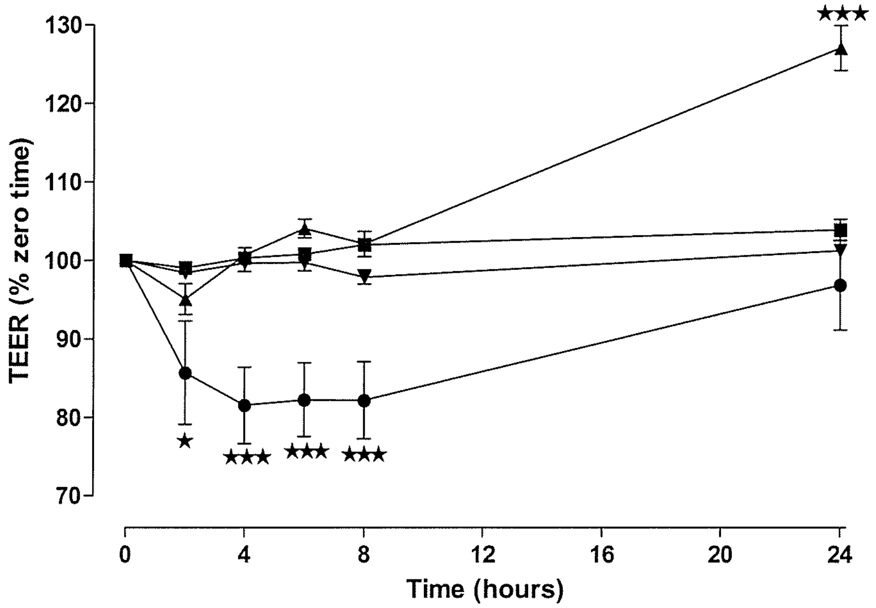

Fig. 2.7. Effects of exposure of Caco-2 cell monolayers to different culture media on the transepithelial resistance (TEER) (mean ± S.E.M) normalised to zero time. Symbols: (●) medium not equilibrated with 5% CO_2; (▲) pH adjusted medium; (▼) negative control medium equilibrated with 5% CO_2 (containing bacterial lysate). Also shown (■) are successive TEER readings without changing the equilibrated medium. Significant differences at each time point are shown: *: $p < 0.05$; ****: $p < 0.001$.

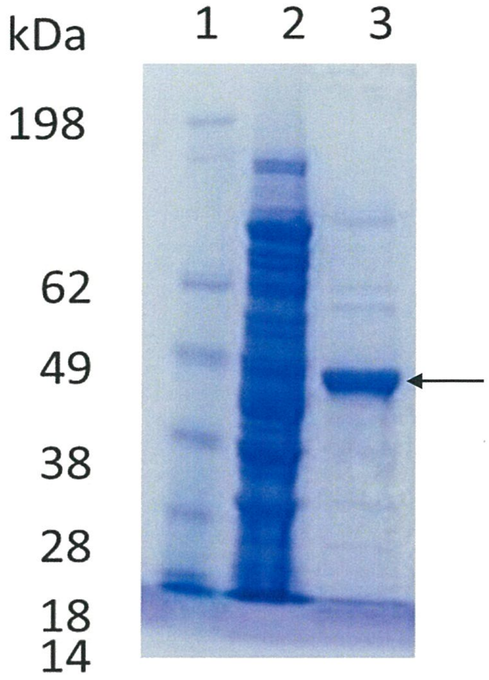

Fig. 2.6. SDS-PAGE of recombinant *H. contortus* enolase. Lane 1: standards; lane 2: non-purified enolase; lane 3: purified enolase (arrow).

Fig. 2.5. Alignment of *H. contortus* enolase #1 protein sequence (Genbank Accession No. KM502544) with *H. contortus* enolase #2 (ADK47524) and enolases from *Anisakis simplex* (Q8MU59), *Brugia malayi* (A8PFE3), *Onchocerca volvulus* (Q7YZX3), *Trichinella spiralis* (Q967U0), *Schistosoma bovis* (B2LXU1), *Schistosoma japonicum* (P33676), *Schistosoma mansoni* (Q27877), *Echinostoma caproni* (CAR47551), *Fasciola hepatica* (Q27655), *Echinococcus granulosus* (D0VLV3), *Taenia asiatica* (C9V487), and *Streptococcus pneumonia* (CAC8309). The percentage identity between the *H. contortus* #1 and the other sequences is indicated. Putative plasmin(ogen)-binding sites (red box; from Ehinger et al., 2004), residues involved in Mg^{2+} binding (shaded grey), substrate (2-phosphoglycerate) binding (shaded yellow) and the 'enolase-signature' (black box) are indicated (from de la Torre-Escudero et al., 2010). Differences between the *H. contortus* enolase #1 and #2 protein sequences occur at the C-terminus and are underlined in sequence #2.

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 Haemonchus contortus #1   -GK-ISIGMDVAASEFYK--QGKYDLDFKNPKSDPSKWLTGDQLAALYQTFIKEYPVVSI
 Haemonchus contortus #2   -GK-ISIGMDVAASEFYK--QGKYDLDFKNPKSDPSKWLTGDQLAALYQTFIKEYPVVSI
        Anisakis simplex   -GK-VSIAMDTAASEFYKADEKKYDLDFKNPNSDKSQWKTGDQLAEIYHSFVKEYPIVSI
           Brugia malayi   -GK-VAIAMDCAASEYYM----LYDLDFKNPTSDKAQWKTGDQMMEIYQSFIKEYPVVSI
     Onchocerca volvulus   -GK-VSIAMDCAASEYSKEADKLYDLKFKNPNSGKTQWKTGDQMMN-FQSFIKEYPVVSI
    Trichinella spiralis   -GK-VKIGMDIAASEFYKSKEKLYDLDFKNPESNAKDWKNGEQMLELYKDFVTNYPIVSI
       Schistosoma bovis   -GK-KEIGMDCAASEFHK--NGKYDLDFKNPHSAESAWLSPDAMTNVYKEMISKYPIVSI
   Schistosoma japonicum   -GK-VEIGMDCAASEYYK--KGKYDLDFKNPQSAESHWLSPDEMANVYKEMIQKYPIVSI
     Schistosoma mansoni   -GK-VEIGMDCAASEFHK--NGKYDLDFKNPHSAESTWLSPDAMANMYKQMISKFPIVSI
     Echinostoma caproni   -GK-VKIAMDSAASEFYK--EGKYDLDFKNPKSPASSWISSDAMADVYKKMMSTYPIVSI
       Fasciola hepatica   -GK-VXIAMDCAASEFYK--EGKYDLDFKNPKSQASSWITSDAMADVYKKMMSTYPIVSI
 Echinococcus granulosus   -GK-VKIGMDVAASEFYQ--DGNYNLDFKNPKAAASSIVSGSKLSDIYSEMISKYPIVSI
         Taenia asiatica   -GK-VKIGMDVAASEFYQ--NGKYNLDFKNPAAVASSIVPGSKLAEIYLEMLSKYPIVSI
Streptococcus pneumoniae   PGKDVFLGFDCASSEFYDKERKVY--DYTKFEGEGAAVRTSAEQIDYLEELVNKYPIITI
                            **   *  * *****       *  *                        *  ******

 Haemonchus contortus #1   EDAFDQDDWDNWGKLKASTN--IQLVGDDLTVTNPKRIRLAIDKKSCNCLLLKVNQIGSV
 Haemonchus contortus #2   EDAFDQDDWDNWGKLKASTN--IQLVGDDLTVTNPKRIRLAIDKKSCNCLLLKVNQIGSV
        Anisakis simplex   EDAFDQDDWENWTKFKSTLN--IQLVGDDLTVTNRERIQMAIDKKACDCLLLKVNQIGSV
           Brugia malayi   EDWFDQDDWENWTKALANTH--IQIVGDDLTVTNPKRIAMAAEKKACNCLLLKVNQIGSV
     Onchocerca volvulus   EDWFHEDDWHNWPKGLAKTN--IQIVGDDLTVPNPKRIALAAEKKACNCLLLKVNQIGSV
    Trichinella spiralis   EDGYDQDDWDNWAKMCSELK--IQIVGDDLTCTNPKRIQQAIDKKACNTLLLKVNQIGSV
       Schistosoma bovis   EDPVDQDDWETWPKLTASTN--IQIVGDDLTVTNPKRIKKAISSKACNCLLLKVNQIGSL
   Schistosoma japonicum   EDPFDQDDWDAWPKLTASTN--IQIVGDDLTVTNPKRIEKAIKVKACNCLLLKVNQIGSI
     Schistosoma mansoni   EDPFDQDDWETWPKLTSSTN--IQIVGDDLTVTNPKRIKQAIASKACNCLLLKVNQIGSL
     Echinostoma caproni   EDPFDQDDWPAWTKLTGECK--IQIVGDDLTVTNPLRVQKAIDQKACNCLLLKVNQIGSV
       Fasciola hepatica   EDPFDQDDWPAWTKLTGECK--IQIVGDDLTVTNPLRVQKAIDQKACNCLLLKVNQIGSV
 Echinococcus granulosus   EDPFDQDDWAAWTEFNAKAG--IQIVGDDLTVTNPERVQQAIDRKACNALLLKVNQIGSV
         Taenia asiatica   EDPFDQDDWAAWTAFNAKAG--IQIVGDDLTVTNPERVQQAIDKKACNALLLKVNQIGSV
Streptococcus pneumoniae   EDGMDENDWDGWKALTERLGKKVQLVGDDFFVTNTDYLARGIQEGAANSILIKVNQIGTL
                           **   ****             *******    *   *       * * ***********

 Haemonchus contortus #1   TESIEAAKLSRSNGWGVMVSHRSGETEDTFIADLVVGLATGQIKTGAPCRSERLAKYNQL
 Haemonchus contortus #2   TESIEAAKLSRSNGWGVMVSHRSGETEDTFIADLVVGLATGQIKTGAPCRSERLGQVQPA
        Anisakis simplex   TESIEAAKLARANGWGVMVSHRSGETEDTFIADLVVGLAVGQIKTGAPCRSERLAKYNQL
           Brugia malayi   TESIDAANLARKNGWGVMVSHRSGETEDTFIADLVVGLATGQIKTGAPCRSERLAKYNQI
     Onchocerca volvulus   TESIDAANLARKNGWGVMVSHRSGETEDTFIADLVVGLAAGQIKTGAPCRSERLAKYNQI
    Trichinella spiralis   TESIQACQLSRKNGWGVMVSHRSGETEDTFIADLVVGLATGQIKTGAPCRSERLAKYNQL
       Schistosoma bovis   TESIEACKLAQNAGWGVMVSHRSGETEDTFIADLVVGLCTGQIKAGAPCRSDRLAKYNQL
   Schistosoma japonicum   TESIEACKMAQKAGWGVMVSHRSGETEDNFIADLVVGLCTGQIKTGAPCRSERLAKYNQL
     Schistosoma mansoni   TESIEACKLAQDSGWGVMVSHRSGETEDTFIADLVVGLCTGQIKTGAPCRSDRLAKYNQL
     Echinostoma caproni   TESIQACKMAQSAGWGVMVSHRSGETEDNFIADLVVGLRTGQIKTGAPCRSERLAKYNQL
       Fasciola hepatica   SESIKACKMAQEAGWGVMVSHRSGETEDNFIADLVVGLRTGQIKTGAPCRSERLAKYNQL
 Echinococcus granulosus   TESIKACKMSRAAGWGVMVSHRSGETEDSTIADIVVGLRTGQIKTGAPCRSERLAKYNQL
         Taenia asiatica   TESIKACKMSRAAGWGVMVSHRSGETEDSTIADIVVGLRTGQIKTGAPCRSERLAKYNQL
Streptococcus pneumoniae   TETFEAIEMAKEAGYTAVVSHRSGETEDSTIADIAVATNAGQIKTGSLSRTDRIAKYNQL
                           ***  *  ***  **  ***********  **** *    ****  **  *****  *

                                                             Identity
 Haemonchus contortus #1   LRI-EEELG--KDAVYAGQN-FRNPV-
 Haemonchus contortus #2   ASYWRKNLG--KDAVYAGQN-FRNPV-         97%
        Anisakis simplex   LRI-EEELG--SAAVYAGEK-FRNPQA         85%
           Brugia malayi   LRI-EEELG--SAAIYAGQK-FRNPQA         81%
     Onchocerca volvulus   LRI-EEELG--SAAVYAGQK-FRNPQ-         80%
    Trichinella spiralis   LRI-EEELERQGIAKYAGEN-FHNPQ-         77%
       Schistosoma bovis   LRI-EEELG--AAAKYAGKN-FRHPKK         75%
   Schistosoma japonicum   LRI-EEELG--STAKYAGKH-FRHPQI         75%
     Schistosoma mansoni   LRI-EEELG--TAAKYAGKN-FRHPKV         75%
     Echinostoma caproni   LRI-EEDLG--SAAKYAGEN-FRRP--         74%
       Fasciola hepatica   LRI-EEDLG--GAAKYAGEN-FRRP--         73%
 Echinococcus granulosus   LRI-EEELG--PKAVYAGEH-FRNPL-         73%
         Taenia asiatica   LRI-EEELG--FKAVYAGEH-FRNPL-         73%
Streptococcus pneumoniae   LRI-EDQLG--EVAEYRGLKSFYNLKK         49%
                                  *     * * *   *
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```
                           1        10        20        30        40        50        60
                           |        |         |         |         |         |         |
 Haemonchus contortus #1   -MPITKIHARQIYDSRGNPTVEVDLYTEKGVF-RAAVPSGASTGVHEALELRDQDKKVHH
 Haemonchus contortus #2   -MPITKIHARQIYDSRGNPTVEVDLYTEKGVF-RAAVPSGASTGVHEALELRDQDKKVHH
        Anisakis simplex   -MPITRIHARQIYDSRGNPTVEVDLTTEKGVF-RAAVPSGASTGVHEALELRDGDKAVNH
           Brugia malayi   -MPITRVHARPIYDSRGNPTVEVDLTTDKGIF-RAAVPSGASTGVHEALELRDNDKAVNH
     Onchocerca volvulus   -MPITRVHARPIYDSRGNPTVEVDLTTEKGIF-RAAVPSGASTGIHEALELRDNDEAVNH
    Trichinella spiralis   -MSILDIHARQIFDSRGNPTVEVDLRTNRGIF-RAAVPSGASTGIHEALELRDGDKSQYM
       Schistosoma bovis   -MSIIAIHARQIFDSRGNPTVEVDLKTSKGLF-RAAVPSGASTGVHEALELRDTKSKAYM
   Schistosoma japonicum   -MAIIAIHARQIFDSRGNPTVEVDLKTAKGLF-RAAVPSGASTGVHEALELRDTNSKAYM
     Schistosoma mansoni   -MSILTIHARQIFDSRGNPTVEVDLKTSKGLF-RAAVPSGASTGVHEALELRDTNSKAYM
     Echinostoma caproni   -MAIKSIHARQIFDSRGNPTVGVDVTTAKGLF-RAAVPSGASTGVHEALELRDGPPG-YM
       Fasciola hepatica   -MAIKAIHARQIFDSRGNPTVEVDVTTAKGLF-RAAVPSGASTGVHEALELRDGPPG-YM
 Echinococcus granulosus   -MSILKIHARQIFDSRGNPTVEVDLTTSKGLF-RAAVPSGASTGVHEAVELRDADKNAYM
         Taenia asiatica   -MSIQKIHARQIFDSRGNPTVEVDLTTAKGMF-RAAVPSGASTGVHEAVELRDGDKNAYM
Streptococcus pneumoniae   MSIITDVYAREVLDSRGNPTLEVEVYTESGAFGRGMVPSGASTGEHEAVELRDGDKSRYG
                              *  **** * ******** *** *  * * *  ******** ********

 Haemonchus contortus #1   GKGVLKAVANINDKIAPALIAKNFCVTQQRDIDQFMLALDGTENKS-NLGANAILGVSLA
 Haemonchus contortus #2   GKGVLKAVANINDKIAPALIAKNFCVTQQRDIDQFMLALDGTENKS-NLGANAILGVSLA
        Anisakis simplex   GKSVLKAVANINDKIAPAIVAKNFCSTQQKEIDQFMLDMDGTANKA-NLGANAILGVSLA
           Brugia malayi   GKGVLKAVRNVNEHIGPALVAKNFCPTQQREIDHFMLELDGTENKGTKLGANAILGVSLA
     Onchocerca volvulus   GKGVLQAVGNVNEQIGPALVAKNFCPTQQREIDLFMLQLDGTENKA-KLGANAILGVSLA
    Trichinella spiralis   GKGVSKAVKNINEVIAPSLIAKNFEVTEQSLIDNFMLSLDGTENKS-VLGANAILGVSLA
       Schistosoma bovis   GKGVLTAVSNVNTIIAPALIQKNIPVTDQAAIDRFMIELDGTENKE-KLGANAILGVSLA
   Schistosoma japonicum   CKGVLTAVSNVNNIIAPALLKKQIPVTNQSEVDQFMIELDGKENKG-NLGANAILGVSLA
     Schistosoma mansoni   KKGVLTAVSNVNKIIAPALINKNIPVTNQAAIDKYMIDLDGTENKE-KLGANAILGVSLA
     Echinostoma caproni   GKGVLKAVSNVNNQIAPGLLKCGIPVTDQAGIDNFMLQLDGTPNKE-KLGANAILGVSLA
       Fasciola hepatica   GKGVLKAVANVNSQIAPNLIKSGINVTDQAAVDKFMLDLDGTPNKE-KLGANAILGVSLA
 Echinococcus granulosus   GKGVLNAVKNVNEVIAPALIKEKFVVTDQQRIDEFMIKLDGSPNKG-KLGANAILGVSLA
         Taenia asiatica   GKGVLNAVKNVNEVIAPALLKEKFIVTDQEKIDEFMIKLDGSPNKG-KLGANAILGVSLA
Streptococcus pneumoniae   GLGTQKAVDNVNNIIAEAII--GYDVRDQQAIDRAMIALDGTPNKG-KLGANAILGVSIA
                                 ** ***  *   **        *  **  ** ***  **   ************

 Haemonchus contortus #1   VAKAGAVHKGMPLYKYIAELAGVSKVILPVPAFNVINGGSHAGNKLAMQEFMILPVGATS
 Haemonchus contortus #2   VAKAGAVHKGMPLYKYIAELAGVSKVILPVPAFNVINGGSHAGNKLAMQEFMILPVGATS
        Anisakis simplex   VAKAGAVHKGMPLYKYIAELAGVKQVVMPVPSFNVINGGSHAGNKLAMQEFMIMPIGAKN
           Brugia malayi   VCKAGAVHKGMPLYKYIAELAGTKQIVLPVPAMNVINGGSHAGNKLAMQEFMIMPIGASS
     Onchocerca volvulus   VCKAGAVHKGMPLYKYIAELAGTRQIVLPVPAMNVINGGSHAGNKLAMQEFMIMPVGASS
    Trichinella spiralis   VCKAGAVHKGVSLYRYIADLAGVDKVIMPVPAFNVINGGSHAGNKLAMQEFMLLPIGASN
       Schistosoma bovis   VCKAGAAEAGLPLYRYIAKLAGHENVIMPVPAFNVINGGSHAGNKLAMQEFMILPTGASS
   Schistosoma japonicum   VCKAGAAELNLPLYRYIAKLAGHKDVIMPVPAFNVINGGSHAGNKLAMQEFMILPTGASS
     Schistosoma mansoni   VCKAGAAEAGLPLYRYIARLAGHEDVIMPVPAFNVINGGSHAGNKLAMQEFMILPTGASS
     Echinostoma caproni   VCKAGAAEKGLPLYKYIASLAGNNDVVMPVPSFNVINGGSHAGNKLAMQEFMIMPTGASS
       Fasciola hepatica   XCKAGAAEKGLPLYKYIATLAGNKEVIMPVPSFNVINGGSHAGNKLAMQEFMIMPTGASS
 Echinococcus granulosus   VCKAGAAEKGVPLYRHVADLAGNKDVVLPVPSFNVLNGGSHAGNKLAMQEFMILPTGAKS
         Taenia asiatica   VCKAGAAEKGVPLYRHIADLAGNKDVVLPVPSFNVLNGGSHAGNKLAMQEFMILPTGAKN
Streptococcus pneumoniae   VARAAADYLEIPLYSY---LGGFNTKALPTPMMNIINGGSHSDAPIAFQEFMILPVGAPT
                             ** *    * ** *   * *     ** *  *********   ** ******* **

 Haemonchus contortus #1   FHEAMRMGSEVYHHLKAEIKKRYGLDATAVGDEGGFAPNIQDNKEGLDLLKTAIDLAGYT
 Haemonchus contortus #2   FHEAMRMGSEVYHHLKAEIKKRYGLDATAVGDEGGFAPNIQDNKEGLDLLKTAIDLAGYT
        Anisakis simplex   FTEAMRMGSEIYHHLKAEIKKRYGLDATAVGDEGGFAPNIQDNREGLDLLNTAIALAGYT
           Brugia malayi   FSEAMRMGSEIYHYLKAEIKKRYGLDATAVGDEGGFAPNIQDNREGLDLLNTAIATAGYT
     Onchocerca volvulus   FSEAMRMGSEIYHYLKAEIEKRYGLDATAVGDEGGFAPNIQDNKEGLDLLNTAIATAGYT
    Trichinella spiralis   FKEAMRMGSEVYHHLKNCIKARYGLDATAVGDEGGFAPNIQDNKEALELLKEAIGKAGYE
       Schistosoma bovis   FTEAMKIGSEVYHNLKAVIKREYGLDACNVGDEGGFAPNIQDNMKGLQLLEEAIKIAGYT
   Schistosoma japonicum   FTEAMQMGSEVYHNLKAVIKREFGLDACNVGDEGGFAPNIQDNMKGLQLLEEAIKIAGYT
     Schistosoma mansoni   FTEAMQIGTEVYHNLKAVIKREYGLDACNVGDEGGFAPNIQDNMKGLQLLEEAIKIAGYT
     Echinostoma caproni   FAEAMKIGSEVYHHLKAVIKGKYGLDACNVGDEGGFAPNIQDNLEGLELLRTAIEKAGYT
       Fasciola hepatica   FTEAMKIGSEVYHNLRAVIKSKYGLDACNVGDEGGFAPSIQDNLEGLELLRTAIDKAGYT
 Echinococcus granulosus   FSEAMKMGTEVYHHLKSVIKGKYGLDACNVGDEGGFAPNIQDNMEGLELLKTAIDKAGYT
         Taenia asiatica   FTEAMKMGTEVYHHLKSVIKGKYGLDACNVGDEGGFAPNIQEHMEGLELLKTAIDRAGYT
Streptococcus pneumoniae   FKEALRYGAEIFHALKKILKSR-GLE-TAVGDEGGFAPRFEGTEDGVETILAAIEAAGYV
                           * **** * **** **  **   ***   *********  *     *  *  **  ***
```

2.3. RESULTS

2.3.1. r*Hc*ENO gene sequence

The protein sequence of the recombinant *H. contortus* enolase is shown in Fig. 2.5. The nucleotide and protein sequences have been deposited as Genbank Accession No. KM502544. This is identical to CDJ96217, but shares only 97% identity with the *H. contortus* enolase of Han et al. (2012) (HM138086 (nucleotide seq); ADK47524 (protein seq)). Differences occur at the C-terminus and are underlined in sequence #2 in Fig. 2.5. The percentage identity between this *H. contortus* and other helminth sequences is indicated and sites involved in binding of plasminogen, Mg^{2+} and substrate (2-phosphoglycerate) and the 'enolase-signature' are shown in Fig. 2.5.

2.3.2. Recombinant enzyme expression

The purified C-terminal His and E-tagged recombinant r*Hc*ENO was confirmed as a single band of about 49kDa on SDS-PAGE (Fig. 2.6). The presence of a His-tagged recombinant protein was confirmed by Western blotting. r*Hc*AK was a single band of 46kDa on SDS-PAGE (Dr S. Umair, pers. comm.). Both r*Hc*ENO and r*Hc*AK were shown to be functional enzymes, with activities of 580 ± 25 and 180 ± 10nmoles/min/mg protein respectively.

2.3.3. Effect of *H. contortus* ES products on Caco-2 cell monolayer TEER

Removing the culture plates from the incubator and making 2 hourly TEER measurements did not change the resistance (Fig. 2.7). The effect of pH of the apical solution is also shown in Fig. 2.7. Failure

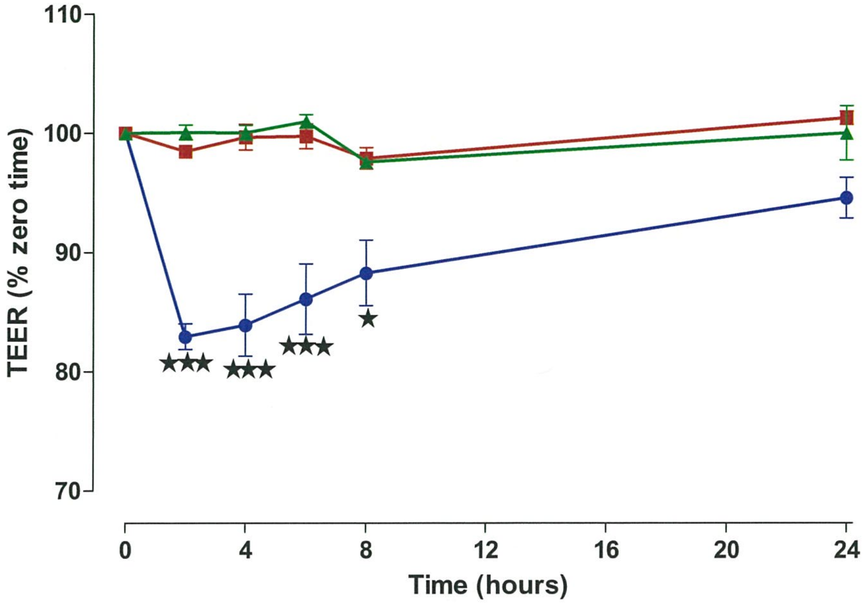

Fig. 2.13. Effects of exposure to recombinant *H. contortus* enzymes on the transepithelial resistance (TEER) (mean ± S.E.M) of Caco-2 monolayers normalised to zero time. Symbols: (●) *H. contortus* enolase (10µg) (n = 10); (▲) *H. contortus* arginine kinase (10µg) (n = 4) and (■) bacterial lysate (negative contrrol) (n = 4). Significant differences at each time point between enzymes and the negative control are shown: *: $p < 0.05$; ****: $p < 0.001$. PFU: plaque forming units.

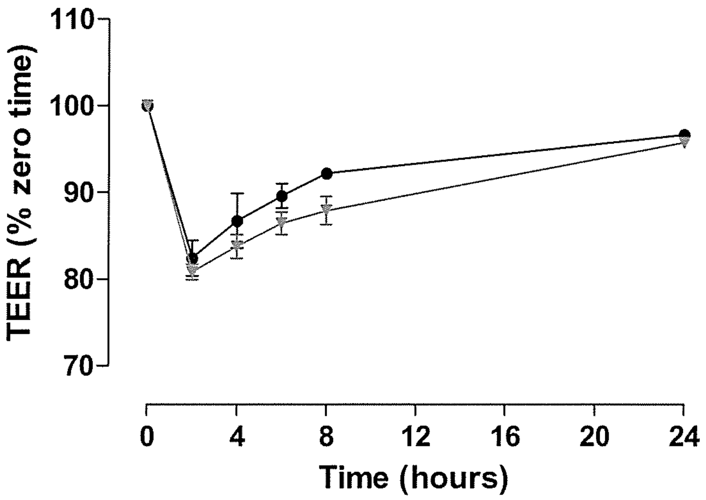

Fig. 2.12. Effects of phage displaying anti-r*Hc*ENO scFvs combined with *Hc*ES on the transepithelial resistance (TEER) of Caco-2 monolayers. Values are shown as mean ± S.E.M, normalised to zero time. Symbols: (●) undiluted *Hc*ES (*n* = 4) and (▼) *Hc*ES incubated with anti-r*Hc*ENO 10^{14} PFU/ml (*n* = 4). PFU: plaque forming units.

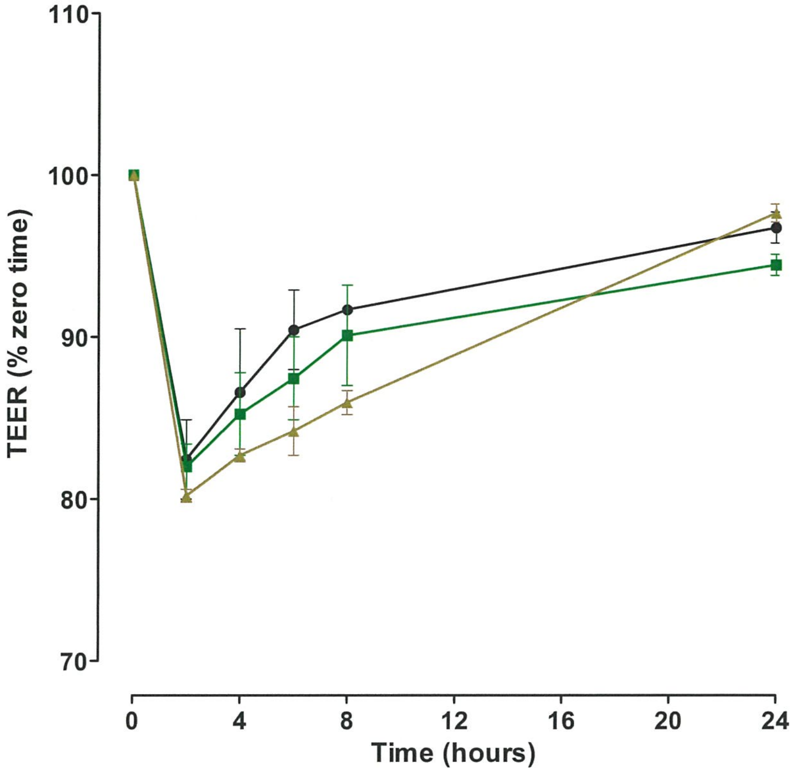

Fig. 2.11. Effects of phage displaying unselected scFvs combined with *Hc*ES on the transepithelial resistance (TEER) of Caco-2 monolayers. Values are expressed as mean ± S.E.M, normalised to time zero. Symbols: (●) undiluted *Hc*ES (*n* = 4); *Hc*ES incubated with unselected scFvs: (■) 10^{12} PFU/ml (*n* = 4) and (▲) 10^{11} PFU/ml (*n* = 4). PFU: plaque forming units.

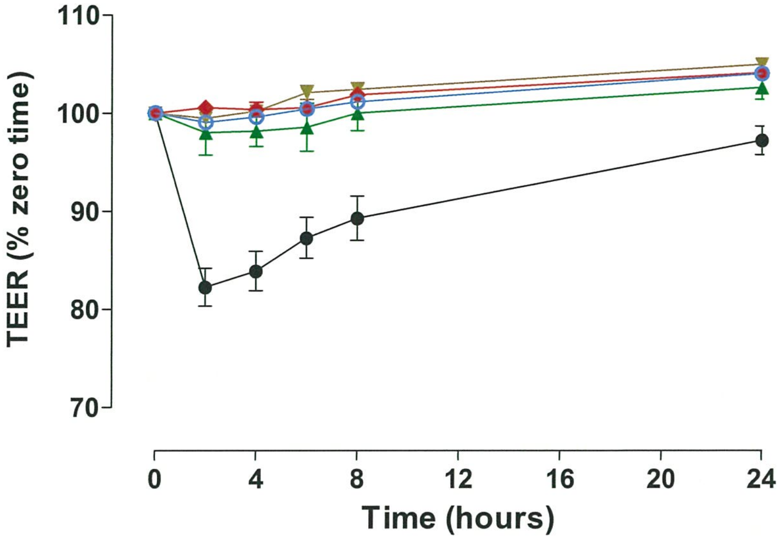

Fig. 2.10. Effects of phage displaying anti-*Hc*ES scFvs on the transepithelial resistance (TEER) of Caco-2 monolayers. Values are expressed as mean ± S.E.M, normalised to time zero. Symbols: culture medium containing anti-*Hc*ES scFvs: (▲) 10^{15} PFU/ml (*n* = 3); (▼) 10^{14} PFU/ml (*n* = 3); (♦) 10^{13} PFU/ml (*n* = 3) and (o) 10^{12} PFU/ml (*n* = 3). For reference, the effect of undiluted *Hc*ES (*n* = 3) is shown (●). PFU: plaque forming units.

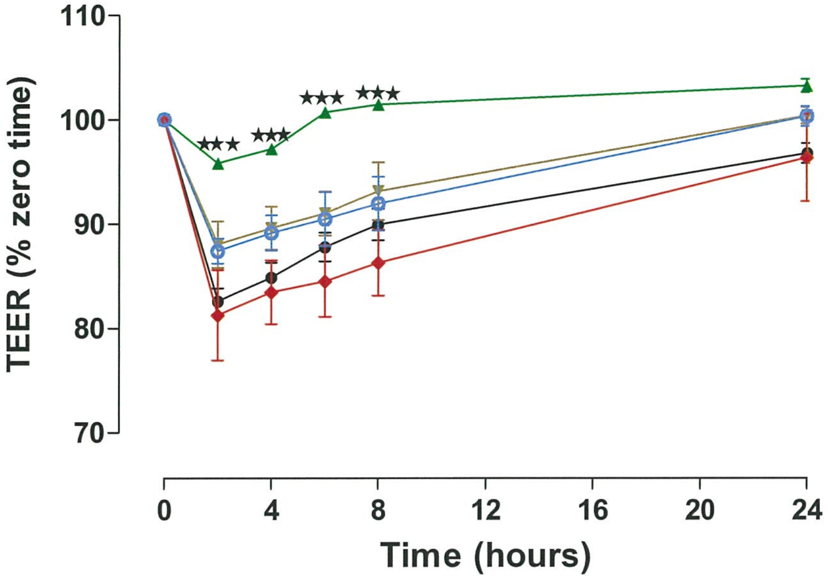

Fig. 2.9. Inhibition with phage displaying anti-*Hc*ES single chain antibodies of the effects of *Hc*ES on the transepithelial resistance (TEER) of Caco-2 monolayers. Values are expressed as mean ± S.E.M, normalised to time zero. Symbols: (●) undiluted *Hc*ES ($n = 3$); *Hc*ES incubated with anti-*Hc*ES scFvs: (▲) 10^{15} PFU/ml ($n = 3$); (▼) 10^{14} PFU/ml ($n = 3$); (♦) 10^{13} PFU/ml ($n = 3$) and (o) 10^{12} PFU/ml ($n = 3$). Significant differences at each time point are shown: ***: $p < 0.001$. PFU: plaque forming units.

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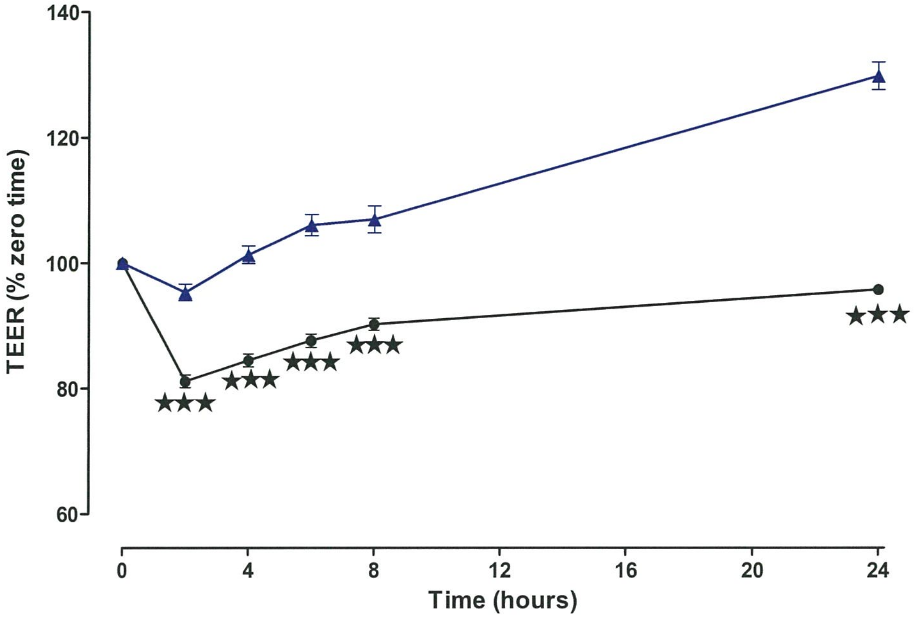


Fig. 2.8. Effects of exposure to *H. contortus* ES products (●) and control medium (▲) on the transepithelial resistance (TEER) (mean ± S.E.M, *n* = 16), normalised to zero time, of Caco-2 cell monolayers. Significant differences at each time point are shown: ****: $p < 0.001$.

to allow equilibration of the bicarbonate buffer with CO_2 raised the pH and significantly reduced TEER, whereas 30min pre-incubation with 5% CO_2, as shown with the negative control for recombinant enzymes, produced a solution with pH 7.4 which did not alter TEER. Medium at pH 7.2 (ES product control incubate) increased TEER only after exposure for 24h.

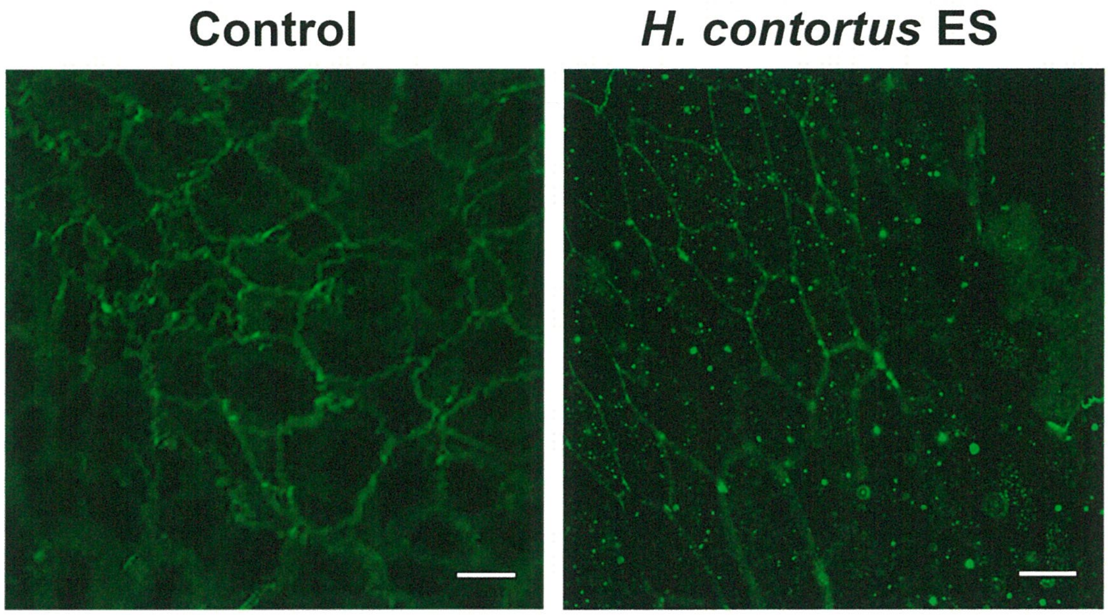
Apical exposure to *Hc*ES products significantly reduced the TEER ($p < 0.001$) at all time points from 2 - 24h (Fig. 2.8). The greatest mean reduction was 19% at 2h.

2.3.4. Interaction of single chain antibodies displayed on bacteriophage with *H. contortus* ES products

Phage displaying anti-*Hc*ES scFvs at a concentration of 10^{15} PFU/ml, but not at lower concentrations, significantly blocked the effect of *Hc*ES at 2 - 8h ($p < 0.001$) (Fig. 2.9). No concentration of phage alone had any effect on TEER (Fig. 2.10). Neither unselected scFvs (10^{12} PFU/ml)(Fig. 2.11) nor anti-r*Hc*ENO scFvs (10^{14} PFU/ml) (Fig. 2.12) blocked the effect of *Hc*ES.

2.3.5. Effects of r*Hc*ENO and r*Hc*AK on Caco-2 cell monolayer TEER

Exposure to r*Hc*ENO significantly reduced the TEER ($p < 0.001$) compared with the negative control (expression vector minus the plasmid) from 2 - 8h (Fig. 2.13). The time course and magnitude of the changes in TEER were very similar to those produced by *Hc*ES. r*Hc*AK did not reduce TEER significantly (Fig. 2.13).



Control

H. contortus ES

Fig. 2.17. Effect of exposure to *H. contortus* ES products (right) or control medium (left) on the location of ZO-1 in the tight junctions of Caco-2 cell monolayers. Scale bar: 10µm.

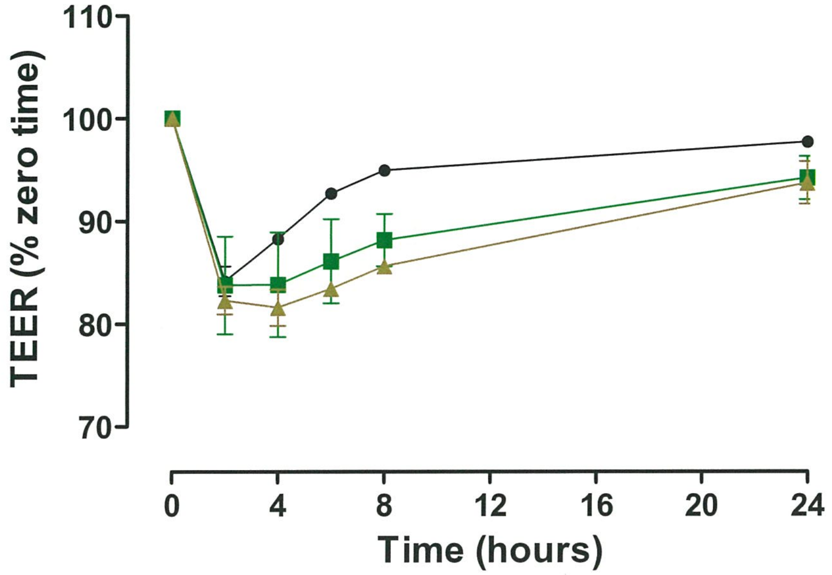


Fig. 2.16. Effects of phage displaying anti-*Hc*ES scFvs combined with r*Hc*ENO on the transepithelial resistance (TEER) of Caco-2 monolayers. Values are shown as mean ± S.E.M, normalised to zero time. Symbols: (●) r*Hc*ENO (*n* = 4); r*Hc*ENO incubated with anti-*Hc*ES scFvs: (▲) 10^{15} PFU/ml (*n* = 4) and (■) 10^{14} PFU/ml (*n* = 4). PFU: plaque forming units.

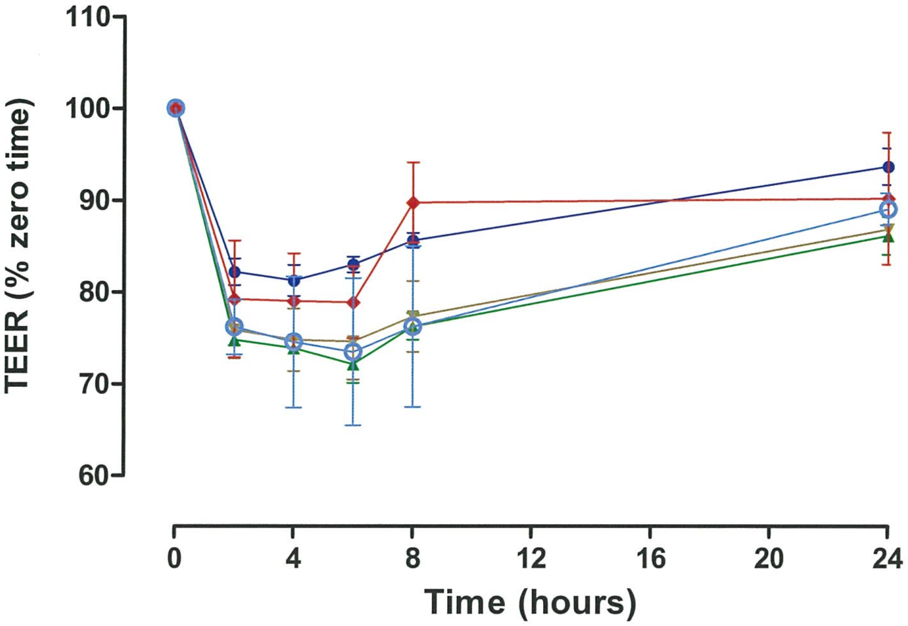


Fig. 2.15. Effects of phage displaying anti-r*Hc*ENO scFvs combined with r*Hc*ENO on the transepithelial resistance (TEER) of Caco-2 monolayers. Values are shown as mean ± S.E.M, normalised to zero time. Symbols: (●) r*Hc*ENO (*n* = 2); r*Hc*ENO incubated with anti-r*Hc*ENO scFvs: (▲) 10^{14} PFU/ml (*n* = 2); (▼) 10^{13} PFU/ml (*n* = 2); (♦) 10^{12} PFU/ml (*n* = 2) and (O) 10^{11} PFU/ml (*n* = 2). PFU: plaque forming units.

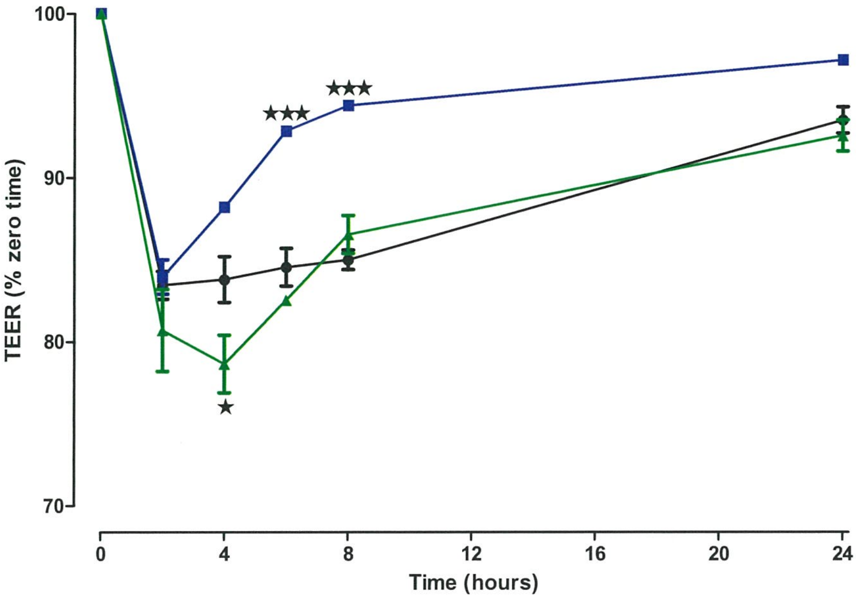


Fig. 2.14. Inhibition of phage displaying unselected single chain scFvs of the effects of r*Hc*ENO on the transepithelial resistance (TEER) of Caco-2 monolayers. Values are expressed as mean ± S.E.M, normalised to time zero. Symbols: (●) r*Hc*ENO (n = 4); r*Hc*ENO incubated with unselected scFvs: (■) 10^{12} PFU/ml (n = 4) and (▲) 10^{11} PFU/ml. Significant differences from r*Hc*ENO at each time point are shown: *: $p < 0.05$ or ***: $p < 0.001$. PFU: plaque forming units.

2.3.6. Interaction of single chain antibodies displayed on bacteriophage with recombinant *H. contortus* enolase

Unselected scFvs at a concentration of 10^{12} PFU/ml reduced the effect of enolase on TEER at 6 and 8h ($p < 0.001$) (Fig. 2.14) and at a concentration of 10^{11}/ml increased the effect of enolase at 4h ($p < 0.05$). Neither anti-r*Hc*ENO scFvs (10^{14} PFU/ml) (Fig. 2.15) nor anti-*Hc*ES scFvs (10^{15} PFU/ml) blocked the effect of r*Hc*ENO (Fig. 2.16).

2.3.7. Effect of *H. contortus* ES products on ZO-1 and occludin

ZO-1 staining was weak, but did show internalisation of ZO-1 after exposure to ES products (Fig. 2.17). Occludin signals were too weak to be interpreted. Separate staining with ZO-1 and occludin antibodies or combining the two primary antibodies for double staining did not improve signal strength.

2.4. DISCUSSION

These experiments extended the preliminary observations of Büring (2009) that adult *H. contortus* ES products can increase epithelial tissue tight junction permeability of monolayers of Caco-2 cells. Antibodies displayed on phage derived from lymphocytes of field-immune sheep blocked the effect of ES products. Recombinant *H. contortus* enolase, but not arginine kinase, also reduced the TEER of Caco-2 monolayers, suggesting enolase may be one of the active components of ES products in increasing the permeability of the gut during parasitism. Phage-displayed scFvs selected sequentially for binding to *Hc*ES, followed by selection for binding to r*Hc*ENO, did not block enolase activity in the Caco-2 cell model.

2.4.1. Epithelial cell models

RGM1 cells, originally obtained from rat gastric surface epithelial cells (Kobayashi et al., 1996), were cultured in an attempt to produce a gastric epithelial cell model which formed adequate tight junctions for the study of ES products. These cells are used for experiments on wound healing and barrier function using histological methods (Suzuki et al., 2008), but there are few studies of RGM1 cell permeability using TEER measurements, because of the low TEER they develop. Although the cells grew well in flasks, on transwell inserts, the TEER readings were inconsistent and the maximum resistance achieved was 165 Ω/cm^2, probably too low for reliable studies of permeabilising agents. Similarly low TEER were recorded for cultured human gastric cells (HGE-20) which were used to study the effects of *H. pylori* on epithelial cells (Marcus et al., 2013). As failure to form adequate tight junctions is not unusual for cultured gastric cells (Basque et al., 2001), the classical Caco-2 model, derived from a human colon cancer is often used for such studies.

In the present study, Caco-2 cells gave repeatable results, provided care was taken with experimental conditions, particularly the pH of the medium. Removing the culture plates from the incubator and making 2 hourly TEER measurements did not change the resistance (Fig. 2.7). Prolonged exposure of the medium to air allowed the HCO_3:CO_2 ratio to change and the pH to rise, which could be observed visually from the change in indicator colour. Exposure of the Caco-2 cells to this medium with low CO_2 reduced TEER (Fig. 2.7), whereas pre-incubation in a CO_2 atmosphere restored the medium to pH 7.4 and did not significantly alter TEER. This pre-incubation procedure was carried out for all studies, except those involving ES products, which had a pH of about pH 7.2; control incubates without worms were therefore adjusted to match that pH. Apical acidification of epithelial cells increases TEER (Lindén et al., 2007), but these effects were observed at lower pH (pH 5) than in the present study and only after incubation for 48 hours, not during short-term exposure. The ES products and adjusted control solutions used here had a pH much closer to pH 7.4 than in their study, but mild acidity could explain the 20% increase in TEER at 24 hours with the control solution (Fig. 2.7).

2.4.2. *H. contortus* ES increased permeability

Increased permeability of the gut epithelium accompanies parasitism by nematodes (Holmes and MacLean, 1971; Su et al., 2011) and infection with unicellular pathogens (Scott et al., 2002). This could result from direct effects of the organism on the epithelial cells or be secondary to host immune responses. Cultured epithelial cells also develop increased permeability upon exposure to bacteria and protozoa (Maia-Brigagão et al., 2012). There are many different mechanisms involved in tight junction dysfunction, including structural changes in tight junction proteins caused by direct interactions with a pathogen chemical or the pathogen itself.

A direct role for worm chemicals in opening tight junctions is suggested by the rapid fall in TEER of Caco-2 cell monolayers on exposure to adult *H. contortus* ES products, which reduced the TEER of Caco-2 cell monolayers by approximately 25%. This confirms the observations of Büring (2009), which were made after 6 and 24 hours of exposure of Caco-2 cells to ES products. The effect was rapid, being greatest after 2h, after which the TEER slowly recovered over 24h (Fig. 2.8). Staining of tight junction proteins was disappointing, with only ZO-1 showing internalisation (Fig 2.17) and occludin signals being very weak. Büring (2009), however, clearly showed internalisation of both junctional proteins after exposure to ES products of both adult *H. contortus* and *T. circumcincta*. Adult *T. circumcincta* ES products reduced the TEER of Caco-2 cell monolayers in a very similar manner to *H. contortus* ES products (Deng and Umair, pers. comm.), indicating that closely related abomasal nematodes appear to employ similar strategies to facilitate access of their chemicals to host tissues.

2.4.3. Host recognition of component(s) of ES products

Pools of single chain antibody fragments (scFvs) were made by panning an ovine scFvs library originally constructed from B cell mRNA from abomasal and mesenteric lymph nodes from naturally infected immune sheep (Maass et al., 2007). These two year-old sheep were refractory to *T. colubriformis, T. circumcincta and H. contortus* infections. The antibody pools were (a) scFvs from the original library (b) anti-*Hc*ES scFvs, selected by three rounds of panning with *Hc*ES and (c) anti-r*Hc*ENO, selected from preparation (b) by one round of panning for binding to r*Hc*ENO.

Selected phage displaying anti-*Hc*Es scFvs blocked the *in vitro* effects of *Hc*ES on epithelial permeability, suggesting the immune host can neutralise the active chemicals produced by the parasite. Anti-*Hc*ES scFvs (10^{15} PFU/ml) significantly blocked the effect of *Hc*ES from 2 - 8h ($p < 0.001$) (Fig. 2.9). Phage alone had no effect on the TEER (Fig. 2.10). Neither unselected scFvs (10^{12} PFU/ml) (Fig. 2.11) nor anti-r*Hc*ENO scFvs (10^{14} PFU/ml) (Fig. 2.12) blocked the effect of *Hc*ES. These phage concentrations were the maximum achievable during the assay, as adding a higher volume would compromise the TEER. The final concentration of phage may have been too low in the unpanned library and the antibodies in the pool of anti-r*Hc*ENO scFvs may have also been too low to block either r*Hc*ENO or *Hc*ES. Panning against *Hc*ES and r*Hc*ENO were carried out sequentially; more specific enrichment of neutralising antibodies may have been obtained had the pannings been done independently.

2.4.4. Permeabilising component of ES products

The component(s) of the ES products active on tight junctions have not been identified, but could be any of the 200 proteins or other chemicals known to be present (Yatsuda et al., 2003). One approach could be to attempt chemical separation of ES products; the one chosen here was to test candidates selected from ES components known to interact with mammalian proteins. While it is possible that excreted ammonia contributes to opening of tight junctions, adult worm ES products usually contain only around 100μM ammonia after 4 hours of incubation (Simpson et al., 2009). This is much less than the 50mM ammonia used to produce a 20% decrease in TEER of cultured kidney MDCK cells (Vastag et al., 2005) or by 10 - 15mM ammonia in Caco-2 cells exposed to *H. pylori* (Lytton et al., 2005).

Enolase is one of the most extensively studied of the many “moonlighting enzymes” with functions apart from their metabolic roles. The best known of these is the ability of enolase to bind extracellular matrix proteins, particularly plasminogen. It was selected for investigation because of its reported properties and consistent identification as an immunogenic protein in helminth ES products (Jolodar et al., 2003; Kiel et al., 2007; Marcilla et al., 2008). Enolase lacks a secretory signal, which earlier led to its being discounted as a true nematode ES product (Vercauteren et al., 2003), however, it is now known to be secreted *in vitro* and also *in vivo* (Morphew et al., 2007; Liu et al., 2012; Lorenzatto et al., 2012). It is both secreted and present on the surface of unicellular organisms, mammalian cells (Pancholi, 2001) and helminths (Ramajo-Hernández et al., 2007; Wang et al., 2011; Liu et al., 2012; Lorenzatto et al., 2012) and has been implicated in bacterial, fungal and autoimmune diseases and in cancer (Pancholi, 2001). After the binding of *F. hepatica*, *E. caproni* and *O. volvulus* enolase to human plasminogen *in vitro*, host activators convert plasminogen to the protease plasmin, suggesting a role in tissue invasion by promoting the degradation of the extracellular matrix or attachment of organisms to the mucosa (Jolodar et al., 2003; Marcilla et al., 2007).

2.4.4.1. Production of recombinant *H. contortus* enolase (r*Hc*ENO)

The sequences for enolase in the laboratory strain of *H. contortus* (Genbank Accession No. KM502544) which were expressed in this experiment were identical to those of the Moredun Haco strain (CDJ96217), but shared only 97% amino acid identity with the *H. contortus* enolase of Han et al. (2012). Enolase catalyses the dehydration of 2-phospho-D-gycerate to phosphoenol pyruvate during glycolysis and the reverse reaction during gluconeogenesis (Brewer and Ellis, 1983). r*Hc*ENO was shown to be a functional glycolytic enzyme and had identifiable substrate and plasminogen binding sites (Fig. 2.5).

2.4.4.2. r*Hc*ENO reduced Caco-2 cell monolayer TEER

Recombinant enolase mimicked the action of ES products in reducing the TEER of Caco-2 cell monolayers, suggesting it is one of the active ES molecules. The time course and magnitude of the changes in TEER were very similar for r*Hc*ENO and *Hc*ES (Figs. 2.8 and 2.13), whereas the negative control (bacterial lysate) was without effect on the TEER. In contrast, r*Hc*AK, another antigenic enzyme secreted *in vitro* by many helminths (Kiel et al., 2007), did not alter the

TEER of Caco-2 cell monolayers (Fig. 2.13). The response to enolase, but not AK, suggests that the effects of worm chemicals are less likely to be caused by immune responses and cytokine release from Caco-2 cells than by a specific effect on the cells, either at the tight junctions or the attached cytoskeleton. Inflammatory mediators cannot be ruled out, as cultured epithelial cells both release endogenous cytokines when exposed to pathogens (Jung et al., 1995) and are responsive to exogenous cytokines (Van De Walle et al., 2010).

Bacterial enolase binds to Caco-2 cells (Glenting et al., 2013) and enolase of many pathogens bind to host extracellular proteins to facilitate invasion and pathogenicity (Pancholi, 2001). These proteins include fibronectin (Donofrio et al., 2009), plasminogen (Bernal et al., 2004; Ehinger et al., 2004), salivary mucin (Ge et al., 2004) and actin (Arnold et al., 1971; Peng et al., 2014). Büring (2009) observed cytoskeletal disruption in HeLa cells exposed to *H. contortus* ES products, suggesting this may also occur in Caco-2 cells. Therefore, its ability to bind to actin and associate with the cytoskeleton (Ottlinger and Lin, 1988; Pal Bhowmick et al., 2009; Lorenzatto et al., 2012) may be the most relevant properties to explain the effect of enolase on increasing the permeability of Caco-2 cell monolayers.

2.4.5. Host recognition of r*Hc*ENO

Host antibodies prevented r*Hc*ENO reducing the TEER of the monolayers, but unlike *Hc*ES, which was blocked by phage displaying anti-*Hc*ES scFvs, the phage effective for enolase was from the unpanned library. Unselected scFvs at a concentration of 10^{12}/ml reduced the effect of r*Hc*ENO on TEER at 6 and 8h ($p < 0.001$) (Fig. 2.14). In contrast, the same phage at a concentration of 10^{11}/ml increased the effect of enolase at 4h ($p < 0.05$). This latter observation is difficult to explain and may be due to experimental conditions or a chance observation, as the significance was at the 5% level and false positives are likely to occur occasionally. The failure of either of the panned libraries to block the activity of the recombinant enzyme may be due to selection of too few phage displaying antibodies specific for the part of the enolase molecule which interacts with tight junctions of Caco-2 cells.

2.4.6. Conclusions

These experiments confirmed the ability of adult *H. contortus* ES products to disrupt tight junctions of cultured epithelial cells and expanded the time course studied in the preliminary experiments of Büring (2009). The reduction in TEER was greatest after exposure for 2 hours, the first time point studied and slowly increased again over 24 hours. It was also shown that the immune host recognises and produces blocking antibodies against the adult *H. contortus* ES products responsible for permeabilisation of epithelia. Production of these antibodies indicates that there is exposure *in vivo* to key epitopes on the active ES components or uptake of intact molecules.

Recombinant enolase, but not AK, mimicked the effect of ES products in opening tight junctions. This suggests that enolase could be one of the active components of ES products increasing the permeability of epithelial cells in culture and probably of abomasal tissues *in vivo*. Initiation of the pathophysiology rapidly follows exposure of the gastric mucosa to chemicals released by luminal worms, either after emergence from gastric glands or by transplantation of adult worms into an uninfected host. As native enzymes of abomasal nematodes are recognised by the immune host (Yatsuda et al., 2003; Kiel et al., 2007) and the recombinant enzymes are recognised by immune serum (pers. comm. Dr S Umair), a direct effect of enolase is more likely than a response dependent of inflammation. Inflammation and down-regulation of a protective host response also accompany the pathophysiology *in vivo* and the two are probably inseparable. In Chapter 3, the effects of adult *H. contortus* ES products on dendritic cells are reported and the proliferative response of lymphocytes collected from infected and vaccinated sheep exposed to adult *H. contortus* ES products is described in Chapter 4.

Chapter 3

IMMUNOMODULATION OF HUMAN MONOCYTE DERIVED DENDRITIC CELLS BY *H. CONTORTUS* ES PRODUCTS

3.1. INTRODUCTION

Parasites, like micro-organisms which cause chronic infections (Kumar et al., 2013), modulate the host response to enhance their survival and transmission to new hosts (Harnett and Harnett, 2006a; van Riet et al., 2007; Moreau and Chauvin, 2010). Helminth immunomodulation, defined as active immunosuppression (Hewitson et al., 2009; Everts et al., 2010; Maizels et al., 2012b; McSorley et al., 2013; Grencis et al., 2014), is reviewed in Chapter 1, 1.5.

3.1.1. Immune response to helminths

H. contortus and other gastro-intestinal helminths induce a protective innate and adaptive T_H2 response (McClure et al., 1996; Meeusen, 1999; Schallig, 2000; Meeusen et al., 2005; Nair et al., 2006; Alba-Hurtado and Muñoz-Guzmán, 2013), summarised in Fig. 1.5. There is also a tolerogenic component produced by parasite immunomodulatory chemicals, which target multiple points in the immune cascade. Many helminth immunomodulators have been identified only as ES products or in crude fractions, although in a few cases the molecular structure has been determined (Table 1.1). These helminth chemicals include host mimics, complement inhibitors, suppressors of host innate immune cells and inducers of T_{regs}, anti-inflammatory cytokines and suppressive AAMs.

Much less is known about immunomodulation by parasites of the ruminant gut than for parasites of humans and mice (reviewed by McNeilly and Nisbet, 2014). As well as the complement system (Suchitra and Joshi, 2005; Suchitra et al., 2008; Sahoo et al., 2013), dendritic cells, lymphocytes, monocytes, macrophages and natural killer cells have been identified as targets of *H. contortus* immunomodulators (Chapter 1, Section 1.5).

3.1.2. Immunomodulation of dendritic cells

DCs are the only professional APCs which are able to initiate primary immune responses, as well as coordinate innate and adaptive immunity through antigen uptake and processing, release of cytokines and stimulation of T and B cells (reviewed by Banchereau et al.,2000; Merad et al., 2013; Schlitzer and Ginhoux, 2014). The type of stimulation of the DCs determines the type of inflammatory response, such as the polarised T_H2 response to parasites (Balic et al., 2004; Phythian-Adams et al., 2010). During helminth infections, DCs do not show classical maturation, up-regulation of co-receptors or release of pro-inflammatory cytokines. Their ES products induce and then down-modulate the T_H2 response and stimulate DCs to induce both tolerance and inflammation (reviewed by Maizels et al., 2004; White and Artavanis-Tsakonas, 2012). This has negative downstream effects on T and B cells, which are normally stimulated by activated DCs, and promotes the expansion of Foxp3$^+$ T_{regs}.

In vitro experiments have shown that many helminths and their products suppress DCs maturation and modulate their response to stimulants, such as lipopolysaccharides (LPS), inducing a tolerogenic-like phenotype. This has been observed for ES products of *H. polygyrus* (Segura et al., 2007), adult *N. brasiliensis* (Balic et al., 2004), larval *T. spiralis* (Gruden-Movsesijan et al., 2011) and *T. crassiceps*

(Terrazas et al., 2011), *Ascaris lumbricoides* pseudocoelomic fluid (Dowling et al., 2011), *F. hepatica* tegumental antigens (Hamilton et al., 2009) and the laminated layer (LL) of larval *Echinococcus granulosus* (Casaravilla et al., 2014).

Helminth parasites of ruminants also suppress DCs. A high molecular weight surface glycolipid larval antigen (CarLA) on parasitic Trichostrongyles (Harrison et al., 2008) inhibits human monocyte-derived DCs (Pernthaner et al., 2012). Expression of immune genes was down-regulated in mixed cells, made up of 15% DC and 85% lymphocytes, which were collected from the intestinal afferent lymph of *T. colubriformis*-infected sheep (Knight et al., 2010).

3.1.3. *In vitro* dendritic cell model

Experiments described in this Chapter examined the response of human mdDCs to ES products of adult *H. contortus*. An attempt was also made to develop a reliable sheep model of mdDCs, however, this was not entirely successful. Experiments on human mdDCs included studying the effects of ES products and CarLA and of co-stimulation with ES products and LPS on the expression of surface molecules and release of cytokine and chemokines into the incubation medium.

3.1.3.1. Surface expressed molecules

The surface molecules monitored are involved in antigen presentation (MHCII), co-stimulation of lymphocytes (CD83, CD40, CD80 and CD86) and regulation of immune responses (galectin-1, CD32 and CD305). A total of 18 cytokines and chemokines with a range of activities in immunity were assayed in the incubation medium. Some of these, such as intercellular adhesion molecule-1 (ICAM-1), are present on the cell surface and released as soluble molecules, although the former may be more important for DC function.

3.1.3.1.1. Antigen presentation

MHCII (HLA-DR). Parasite antigens are recognised by PRRs, including TLR, cytoplasmic DNA sensors, C-type lectin, dendritic cell specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), RIG-1 and NLR (Visintin et al., 2001; Janeway and Medzhitov, 2002). Typically, nematode protein antigens are recognised by PRRs on DCs (Wang et al., 2009), then internalised, followed by proteolysis, complexing with MHCII and the peptide-MHCII complexes translocated to the surface for display (Banchereau et al., 2000). Together with expression of co-stimulatory molecules, the peptide-MHCII complexes activate T cells and B cells leading to antibody production.

3.1.3.1.2. Co-stimulatory molecules

CD83 is a glycoprotein member of the Ig superfamily which is expressed on DCs and other cells, including activated T (Wolenski et al., 2003) and B lymphocytes (Hock et al., 2001). CD83 expression on DCs activates the DCs and provides a co-stimulatory signal for naïve and memory T cells (Aerts-Toegaert et al., 2007; Breloer and Fleischer, 2008). It is released as a soluble form, which inhibits monocyte differentiation into DCs *in vitro* (Lin et al., 2014).

CD40 activates T and B cells, which express the CD40 receptor (Ma and Clark, 2009). Ligation of CD40 on DCs up-regulates expression of CD80, CD83 and CD86 (Caux et al., 1994) and secretion of pro-inflammatory cytokines (Cella et al., 1996).

CD80 and **CD86** are co-stimulatory molecules on DCs which regulate T cell activation. CD80 and CD86 are both ligands of CD28 and CD152, which have opposing stimulating and inhibitory effects on T cells according to differential affinities and rates of association and dissociation (Sansom et al., 2003; de la Fuente et al., 2012). The

CD80/CD86/CD28/CD152 system has an important role in the regulation of T cell activation by DCs, as well as induction of tolerance.

3.1.3.1.3. Regulatory molecules

Galectin-1. Fifteen galectins are known to bind carbohydrates containing N-acetyllactosamine (reviewed by Rabinovich et al., 2002; Camby et al., 2006; Rabinovich and Toscano, 2009; Cedeno-Laurent and Dimitroff, 2012). Galectins are present both within the cell and extracellularly, where they bind to surface receptors (Rabinovich and Toscano, 2009). Intracellular galectin-1 is a monomer, but the extracellular form is a homodimer, which has several immunomodulatory actions as well as promoting cell-cell adhesion. It causes apoptosis of pro-inflammatory T cells (Deák et al., 2015) and stimulates T_{reg} differentiation and IL-10 secretion (Camby et al., 2006; Cedeno-Laurent and Dimitroff, 2012).

CD32. The affinity and specificity of binding of the Fc portion of four classes of Ab to receptors on human cells determines the resulting signalling pathways which are activated (Bruhns et al., 2009). Whereas non-primates express only the inhibitory IgG FcγII receptor CD32B, human mdDCs express both CD32A and CD32B, two isoforms which are activating and inhibitory respectively (Boruchov et al., 2005). CD32A is the most abundantly expressed human Fcγ receptor and is present on most inflammatory cells (Tan Sardjono et al., 2003). Activation of CD32A by binding of immune complexes is inhibited by binding of complexes to CD32B, which acts as a regulator of the resulting inflammation (Van Montfoort et al., 2012). Although antibody-antigen complexes induce maturation of mdDCs and activation of T cells, the release of IL-10, rather than IL-12, suggests a more tolerogenic phenotype (Bánki et al., 2003).

CD305 (also called leucocyte-associated Ig-like receptor-1 (LAIR-1)) is a transmembrane inhibitory receptor (Meyaard, 2008, 2010) which is expressed on almost all immune cells. It down-regulates the activity of these cells (Lebbink and Meyaard, 2007; Meyaard, 2008) and regulates the differentiation of blood monocytes to mdDCs by granulocyte-macrophage colony-stimulating factor (GM-CSF) reporter-mediated activation signalling (Poggi et al., 1998). Collagen is a strong ligand for CD305 and, in collagen-rich tissues, binding of collagen to CD305 regulates immune responses (Omiya et al., 2009).

3.1.3.2. Cytokines and chemokines

Of the 20 cytokines/chemokines assayed in the incubation medium, IL-4 and GM-CSF were not considered, as they were included in the incubation medium to stimulate monocyte conversion to immature DCs.

LAP (latency-associated protein) is expressed on the surface of immature DCs, where it binds TGF-ß; this prevents T_H1 cell activation and maintains differentiation of T_{regs} and tolerance (Gandhi et al., 2007).

IL-1α and IL-1β are distinct mediators of innate immunity and inflammation that share a common receptor (Dinarello, 2010, 2011; Garlanda et al., 2013). The precursor of IL-1α is constitutively produced in epithelial tissues and is released by cell necrosis and initiates sterile immunity. An activated form is present on monocytes. IL-1β is produced by monocytes, DCs and other haemopoetic cells, requires activation after release and has numerous inflammatory, metabolic and physiological effects.

IL-17A is a pro-inflammatory cytokine, which is secreted by activated memory T cells and drives T cell responses through ICAM-1 and enhances secretion of chemokines, including IL-8, monocyte chemo-attractant protein-1 (MCP-1) and GM-CSF (Aggarwal and Gurney, 2002; Moseley et al., 2003; Gaffen, 2008, 2011). There do not appear to be reports of IL-17A being secreted by DCs.

IL-13 is an immunoregulatory cytokine secreted principally by activated T_H2 cells (Hershey, 2003). It is important in the effector phase of allergic inflammation. IL-13 and IL-4 are recognised by a subunit on the IL-4 receptor, thus sharing many actions. Both cytokines have an anti-inflammatory role, causing macrophages and monocytes to undergo alternative activation (Bhattacharjee et al., 2013), although the affinities for the receptor leads to IL-13 enhancing DC suppression of IFN-γ secretion by T cells and IL-4 enhancing secretion of T_H2 cytokines (Webb et al., 2007).

IL12p70. IL-12 is a pro-inflammatory cytokine, which is produced by DCs, macrophages and B cells in response to microbial stimulation. There are many roles for IL-12, including the positive feedback loop between APC IL-12 and T cell IFN-γ, which is important for driving a T_H1 response (Heystek et al., 2003; Vignali and Kuchroo, 2012). The IL-12 family share different combinations of chains, some of which can be secreted as active monomers. IL-12 is composed of two subunits, IL-12p35 (MW 35 kDa) and IL-12p40 (MW 40 kDa), making up the entire cytokine IL-12p70.

TNF-α. During acute inflammation, TNF-α (cachectin) an inflammatory cytokine produced by macrophages, monocytes and DCs, confers immunity and is responsible for cell necrosis or apoptosis (Idriss and Naismith, 2000). As TNF receptors are present on almost all cells, TNF-α acts on many tissues and has effects beyond inflammation, such as organogenesis and sleep regulation (Vilček and Lee, 1991; Locksley et al., 2001).

IL-10. During infection with viruses, bacteria, fungi, protozoa, and helminths, IL-10 is a key immunoregulator which inhibits the activity of macrophages, NK cells and $T_H1$cells. As these cells are required for pathogen clearance, this may be delayed in its absence (Couper et al., 2008). IL-10 inhibits MHC class II and costimulatory molecule expression on monocytes and macrophages and limits the production of proinflammatory cytokines, including IL-1α and β, IL-6, IL-12 and TNF-α, and the chemokines MCP1, IL-8, IP-10, and macrophage inflammatory protein (MIP)-ß (reviewed by (Moore et al., 2001).

IP-10 (IFN-γ-inducible protein-10) is a chemo-attractant for activated T cells, has a role in effector T cell responses and stimulation of monocytes and NK cells and maturation of bone marrow progenitor cells (Dufour et al., 2002; Shanmugam et al., 2006). Regulatory DCs down-regulate T cell responses by production of IP-10 and inhibition of T_H1 cell proliferation (Qian et al., 2007).

IL-6 is an activator of acute phase responses and a stimulator of lymphocytes (Jones, 2005). IL-6 secretion by DCs limits the extent of T_H2 immunity (Mayer et al., 2014), alters the T_{reg} phenotype to one with less Foxp3 expression and promotes host susceptibility to helminth infection (Smith and Maizels, 2014). *S. mansoni* induces IL-6 synthesis in the lung blood vessels to reduce eosinophilia and escape the inflammatory response (Angeli et al., 2001).

IL-8 is a long lasting inflammatory mediator produced by many cell types, not only immune cells, which attracts and activates particularly neutrophils, as well as basophils and T cells (Seitz et al., 1991; Remick, 2005).

IFN-γ is the only type II interferon. It is released by activated T_H1 and NK cells and stimulates macrophages and DCs to increase antigen presentation, attracts leucocytes and regulates Ag production and class switching by B cells. There is also release of IFN-γ by IL-12- stimulated

APC, where it acts locally to self-activate and also activate nearby cells (Frucht et al., 2001; Schroder et al., 2004).

IFN-α is structurally different from IFN-γ and binds to different receptors. IFN-α interferes with viral replication and its secretion is classically stimulated by viral infection; it is a general stimulant of the immune system (Rönnblom, 2011; Porritt and Hertzog, 2015).

MCP-1 is one of the key chemokines that regulate the migration of monocytes, macrophages, memory T cells and DCs and their infiltration into sites of inflammation (Carr et al., 1994; Xu et al., 1996; Deshmane et al., 2009). It also stimulates the secretion of IL-4 by T cells (Karpus et al., 1997) and is associated with polarised T_H2 responses (Chensue et al., 1995; Handel and Domaille, 1996).

MIP-1α and MIP-1β are chemo-attractants for monocytes, macrophages, DCs and T cells; MIP-1α preferentially attracts $CD8^+$ T cells and MIP-1β $CD4^+$ T cells (Maurer and von Stebut, 2004).

ICAM-1 (CD54) is an adhesion molecule which, together with secreted chemokines, is used by immature DCs to stimulate $CD4^+$ cells in the absence of exogenous antigen (Real et al., 2004). ICAM-1 on DCs is rapidly internalised and associated with MHCII and recycled to the immunological synapse, where priming of T cells occurs (Jo et al., 2010). The soluble form was measured in the culture supernatant, not surface ICAM-1, as many cytokines and their receptors are shed into the extracellular fluid and subsequently excreted in urine (Novick et al., 1989). Changes in the soluble form may not necessarily reflect changes in the surface molecules.

E-selectin. Activated DCs migrate to the regional lymph nodes and are replaced by precursors and immature DCs which migrate from the blood into inflamed tissues. This migration involves immature DCs binding to both P- and E-selectin using its E-selectin ligand CLA (cutaneous lymphocyte-associated antigen) (Pendl et al., 2002), which is also expressed on T cells, neutrophils and monocytes (Kieffer et al., 2001). On endothelial cells, E-selectin serves as a chemotactic agent for phagocytes (Lo et al., 1991; Kumar et al., 2001) and stimulates angiogenesis (Koch et al., 1995). A truncated, 94 kDa soluble form of E-selectin is released from endothelial cells (Leeuwenberg et al., 1992), probably similar to that measured in the incubation medium of the DCs.

3.2. MATERIALS AND METHODS

3.2.1. Generation of human monocyte derived dendritic cells (mdDCs)

Human monocyte derived dendritic cell experiments were performed under AgResearch ethical approval "The recognition and processing of molecules from livestock nematode parasites by human dendritic cells, reference # CEN/10/12/060".

DCs were generated from monocytes isolated from the blood of healthy volunteers, according to the procedure described by Pernthaner et al. (2012). Monocytes were isolated using an antiCD14 monoclonal antibody (mAb) directly conjugated to magnetic beads (Miltenyi Biotec, Germany), using a standard magnetic cell separation protocol (AutoMacs, Miltenyi Biotec). Typically, isolated $CD14^+$ monocytes had a purity of at least 95%, as assessed by flow cytometry, using either a standard indirect staining procedure for the CD14 mAb used for cell separation or a different CD14 antibody clone conjugated with phycoerythrin (PE).

Monocytes were cultured in six well tissue culture plates (Nunc, Denmark) at an approximate density of $1.5x10^6$ cells/ml in RPMI 1640 cell culture medium (Sigma-Aldrich, USA) (pH 7.4), supplemented with 10% foetal calf serum (FCS), 100ng/ml GM-CSF (PeproTech Inc., USA) and 50ng/ml IL-4 (BioLegend, USA), at 37°C in 5% CO_2 in air. Half of the cell culture medium was replaced with fresh medium on Days 3 and 5. The development of monocytes into immature mdDCs was verified microscopically on Day 7 by the development of dendrites and typical loose attachment to the plate surface. This development of immature mdDCs from monocytes was later confirmed by flow cytometry on non-stimulated (NS) mdDCs by observing the expression of CD14 and CD11c.

3.2.2. Generation of sheep mdDCs

$CD14^+$ monocytes were isolated from sheep blood (*n* = 5) by magnetic separation, as described in Section 3.2.1, using human CD14 mAb-conjugated beads, which are known to cross react with sheep cells. Purity of $CD14^+$ monocytes was assessed either by anti-human CD14 mAb directly conjugated with PE, or by an indirect staining procedure using an anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Dead cells were recognized through staining with 7AAD (BioLegend, USA).

The human anti-CD14-PE mAb did not recognise sheep CD14+ cells, therefore an indirect staining procedure with an anti-mouse FITC-conjugated antibody was used to evaluate the purity of sheep $CD14^+$ cell populations. Magnetic separation resulted in 60 - 80% enrichment of $CD14^+$ cells, of which 40 - 50% were dead. As the magnetic separation procedure did not result in sufficient numbers of live $CD14^+$ cells required for the assays and co-selected for dead cells, generation of DCs from sheep monocytes was abandoned.

3.2.3. Experimental overview

Two experiments (#1 and #2) were each carried out independently on 2 days. The aim principally was to determine the effects on human mdDCs of ES products alone and of co-stimulation with LPS and ES products on the expression of surface markers (both experiments) and release of cytokines and chemokines (Expt #2). In addition, in Expt #1, the effects of CarLA and ES products were compared and ovalbumin (OVA) was tested as an alternative stimulant to LPS. In a separate experiment, uptake of CarLA by mdDCs from 8 donors was determined.

Donors of mdDCs. For Expt #1, the Day 1 donors were 1 - 4 and on Day 2 the donors were 3 - 6. For Expt #2, the donors were 1, 2, 4 and 7 on each of the 2 days. For the CarLA uptake experiment, the donors were 1, 2, 4, 7, 8, 9, 10 and 11. Donors were 2 females and 9 males aged 30-50; 3 South Asian and the rest Caucasians.

ES products. The ES products used were the same 2 - 4h incubate in RP2 medium used on Caco-2 cells (Chapter 2, Section 2.2.1). For Expt #1, the final concentrations tested were 0, 0.4%, 2% and 10%, whereas for Expt #2, the concentrations were 0, 0.5%, 1.25%, 2.5%, 5% and 10%.

3.2.4. Expression of mdDC surface markers and secreted cytokines/chemokines

The incubations of mdDCs with test substances were carried out in 96 well tissue culture plates (Nunc, Denmark). The expressions of HLA-DR (MHCII), CD40, CD86, CD80, CD32 and CD305 were determined in both Expts, plus CD83 in Expt #2. Surface galectin-1 was determined in Expt #1; for Expt #2, cellular (surface and intracellular) galectin-1 were measured either on the cells after staining other surface molecules in the panel or in separate wells.

MdDCs from the original 6 well tissue culture plates were transferred to 96 well plates on Day 7 of culture. In Expt #2, all wells were set up in duplicate. There were 2 wells containing control NS cells with no test substances in each plate; these were used to normalise values from each plate and donor. The plates were incubated for 24h at 37°C in 5% CO_2 in air.

The supernatant was aspirated and discarded in Expt #1. In Expt #2, supernatant samples were stored –80°C until assayed for cytokines and chemokines (Section 3.2.9).

3.2.4.1. Test substances

Each test well contained 200µl of mdDCs in complete culture medium, to which 30µl of the test substance(s) or control medium was added to make a total volume of 230µl.

ES products. The final concentrations of ES products were 0.4%, 2% and 10% in Expt #1 and 0.5%, 1.25%, 2.5%, 5% and 10% in Expt #2.

CarLA. CarLA from *T. colubriformis* was prepared, as described by Pernthaner et al. (2012), and provided by the Parasitology team at AgResearch Ltd. In Expt #1, CarLA was tested in a final concentration of 10µg/ml.

OVA. In Expt #1, OVA (Sigma-Aldrich, USA) was tested in a final concentration of 10µg/ml.

Lipopolysaccharide (LPS). LPS from E. coli OIII:B4 (Sigma-Aldrich, USA) was tested in a final concentration of 100ng/ml in Expt #1 and at 10ng/ml and 30ng/ml in Expt #2.

Table 3.1. Antibodies to surface antigens on mdDCs in panels A and B, showing fluorochromes, clones and final concentrations.

Antibody	Fluorochrome	Clone	Final concentration (µg/µl)	Panel
CD14	PE	HCD14	0.01	A,B
CD11c	Pacific Blue	3.9	0.02	A,B
HLA-DR	PerCP/Cy5.5	L243	0.01	A
CD86	Alexa Fluor 647	IT2.2	0.02	A
CD40	PE/Cy7	5C3	0.002	A
CD32	FITC	FUN-2	0.02	B
CD305 (LAIR-1)	PerCP/Cy5.5	NKTA255	0.005	B
CD80	PE/Cy7	2D10	0.01	B
CD83	Alexa Fluor 647	HB15e	0.02	B

Abbreviations: PE, phycoerythrin; PerCP, peridinin chlorophyll; Cy, cyanine; FITC, fluorescein isothiocyanate.

Co-stimulation with ES products and LPS. In Expt #2, final concentrations of 10ng/ml and 30ng/ml LPS were tested in combination with 0.5%, 1.25%, 2.5%, 5% and 10% ES products.

3.2.5. Staining for surface antigens

To quantify surface antigens, combinations of mAbs were used for 6 - 8 colour flow cytometry. Two antibody panels (A and B) were used, which recognise respectively HLA-DR (MHCII), CD40 and CD86 (A) and CD32, CD305, CD80 and CD83 (B); CD14 and CD11c were included in both panels (BioLegend, USA). Fluorochrome and clones of these mAbs are described in Table 3.1. The fixable viability dye eFluor® 780 (eBioscience, USA) was used to identify live cells. Human TruStain FcX (BioLegend, San Diego, USA) was also added to the cocktail to prevent non-specific binding of mAbs to Fc receptors on mdDCs. Optimised final concentrations for each antibody (Table 3.1) in the cocktails for panel A and B were established prior to experiments.

MdDCs were stained with 50µl cocktails of optimal concentrations of mAbs in PBS. Stained cells were washed with PBS and fixed in Fluorofix fixation buffer (BioLegend, San Diego, USA) and finally re-suspended in PBS. Single cell stains and fluorescence minus one (FMOs) samples for each antibody were also prepared for each experiment. The detailed procedure for staining is described in Appendix 5.1.

3.2.6. Staining for total cellular galectin-1

Cellular galectin-1 staining was performed either on cells after staining for surface antigens or on separate cells. The detailed procedure is described in Appendix 5.2. Briefly, cells were fixed in Fluorofix fixation buffer (BioLegend, USA) and re-suspended in permeabilisation wash buffer (BioLegend, USA). Rabbit anti-galectin-1 mAb (Abcam, Cambridge, UK) was used as the primary antibody and FITC-goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, USA) was used as a secondary antibody; both were diluted in permeabilisation wash buffer.

3.2.7. Flow cytometry for surface antigens and total galectin-1

Flow cytometry files were acquired using a three laser, ten parameter FACSVerse (Becton Dickinson, San Jose, USA), running BD FACSuite software. Prior to each experimental session, the FACSVerse was performance verified using the in-built quality control steps in the FACSuite software, in conjunction with BD FACSuite CS&T research beads (Becton Dickinson, USA). Samples were acquired using the plate loader feature, making use of the precise volumetric control on the FACSVerse to maximise data acquisition (maximum of 100,000 events acquired/sample for 70 sec). Single stain populations for each antibody-fluor combination were used for generation of a compensation matrix for each panel of antibodies in each experiment. FMO controls were used to determine the background fluorescence in each detector.

Data were analysed using FlowJo (Tree Star, Inc. Ashland, USA) using a hierarchical gating strategy. To identify precisely the cells of interest, the following gating strategy was adopted: (a) time gate (to identify and exclude any artefacts/bubbles in the fluid stream); (b) DC scatter gate (selected by forward and side scatter); (c) dead cell exclusion gate (dead cells excluded by selecting eFluor® 780 bright cells); (d) single cell gate (populations selected using forward scatter area versus forward scatter height to exclude doublets) and (e) immature DC gate (selected by CD11c positive expression). Levels of expression of antigens within a cell population were measured as median fluorescence intensity (MFI).

3.2.8. Cytokine assay

Supernatants collected from test wells (Section 3.2.5) were assayed for cytokines and chemokines. Cell culture medium was also processed as the negative control. ES products in cell culture medium, at the same concentrations used in the experiments with mdDCs, were also processed to determine whether cytokines were present.

A FlowCytomix 20plex multiple analyte kit (eBioscience Bender MedSystems, Austria) was used to detect 18 cytokines and chemokines (E-selectin, ICAM-1, IFN-α, IFN-γ, IL-1α, IL-1β, IL-6, IL-8, IL-10, IL12p70, IL-13, IL-17A, IP-10, LAP, MCP-1, MIP-1α, MIP-1β, TNF-α). GM-CSF and IL-4 secretion levels were excluded from analyses as both were included in the culture medium for DCs. Standard procedures recommended by the manufacturer were used, as described in Appendix 5.3. Briefly, samples and standards were stained with bead mixture, biotin conjugate and finally with streptavidin-PE in appropriate buffers provided with the kit. Acquisition was performed on FACSVerse for 30sec and the event count was set at 10,000/sec. Data were analysed using FlowCytomix Pro (eBioscience Bender MedSystems, Austria).

3.2.9. CarLA uptake assay

In a separate experiment, purified CarLA (Section 3.2.5.1) at concentrations of 0, 10 and 100µg/ml was incubated with human mdDCs for 24h in a 96 well plate. MdDCs derived from monocytes of 8 donors were harvested, washed with PBS and fixed with 1x Fluorofix (fixation buffer) (BioLegend, San Diego, USA) for 20min and washed twice with Perm buffer (BioLegend, San Diego, USA) (800*g* for 2min). The cells were stained with biotinylated mouse anti-CarLA mAb (PAB1) for 30min, followed by staining with streptavidin-PE for 30min. After each staining, cells were washed 3 times with 200µl permeabilising buffer by centrifugation at 800*g* for 2min. Data were acquired on FACSVerse and the MFI of the PAB1 signal was calculated in FlowJo.

3.2.10. Statistical analysis

GraphPad Prism v5 (Graph Pad Software Inc., USA) was used to graph data and for analyses unless stated otherwise. For group analyses, data were normalised to NS values, taken as 100%. In Expt #1, the effects of OVA and LPS were compared by non-parametric one-way ANOVA, with Dunn's post-test. The effects of two concentrations of LPS (Expt #2) were analysed by non-parametric one-way ANOVA, with Dunn's post-test. The effects of CarLA were compared with NS cells by paired t-tests. The effects of ES products on surface and cellular galectin-1 were determined by two-way ANOVA, with Dunn's post-tests; a non-linear curve of best fit (one phase decay) was fitted to the data for normalised total cellular galectin-1.

The effects of ES products were analysed by one-way ANOVA, using least significant difference (LSD) comparisons. Co-

Table 3.2 Expressions of surface markers (median fluorescence intensity (MFI)) in non-stimulated mdDCs of Donors 1-6 (Expt #1)

Expt Days	Donors Markers	1	2	3	4	5	6
1	CD32	398	1366	926	885		
2				626	386	1024	566
1	CD80	676	3054	2754	1313		
2				973	569	1435	632
1	CD86	349	1110	523	301		
2				3840	902	2461	8255
1	HLA-DR	656	1123	807	515		
2				1335	785	484	1265
1	CD305	115	675	134	104		
2				128	185	445	91
1	CD40	1506	2541	2328	1619		
2				2716	1897	1913	2947

Table 3.3 Expressions of surface markers (median fluorescence intensity (MFI)) in non-stimulated mdDCs of Donors 1, 2, 4 and 7 (Expt #2).

Expt Days	Donors Markers	1	2	4	7
1	CD32	923	7561	9331	918
2		2121	15697	11068	2265
1	CD80	2067	2755	3522	1734
2		1065	2382	1730	1098
1	CD86	182	238	353	527
2		427	980	409	591
1	HLA-DR	5164	6217	9355	4263
2		12017	28815	13807	8225
1	CD305	664	921	881	458
2		352	1241	675	287
1	CD40	3598	2241	3086	2239
2		3609	6354	4082	4030
1	CD83	70	323	219	476
2		10	10	10	10

stimulation of ES products and LPS were analysed by two-way ANOVA and restricted maximum likelihood (REML) analysis, kindly performed by Dr J. Koolaard, AgResearch Ltd (summarised in Appendices 7.1 – 7.3). Data are presented as means; significant differences were determined from calculated LSD for significance at $p < 0.05$, $p < 0.10$ and $p < 0.15$.

3.3. RESULTS

All experiments were conducted on human mdDCs. Although 60 - 80% enrichment of sheep CD14+ cells was achieved, 40 - 50% of them were dead.

3.3.1. Effect of ES products on expression of cell surface molecules

Two experiments were carried out: for Expt #1, the Day 1 donors were 1 - 4 and on Day 2 the donors were 3 - 6. For Expt #2, the donors were 1, 2, 4 and 7 on each of the 2 days.

3.3.1.1. Non-stimulated mdDCs

All NS mdDCs expressed all cell surface molecules, although CD83 expression was generally low, barely detectable in some cells. The considerable variation in absolute values for mdDCs from different donors, even from the same donor on different days, is apparent from Tables 3.2 and 3.3 for Expts #1 and #2 respectively. In Expt #2, mdDCs from Donors 2 and 4 had higher expressions than cells from Donors 1 and 7 of CD32, CD305, MHCII and CD80. Expression of CD86 was quite variable. For subsequent group analyses, data were normalised to respective NS values because of the individual donor variation.

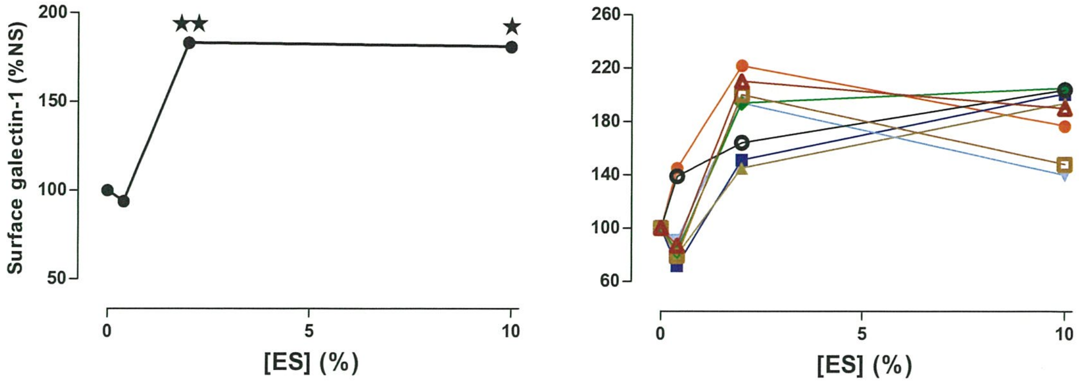


Fig. 3.5. Normalised expressions of surface galectin-1 of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (*n* = 8). Right panel: individual donor cells; symbols: (●) donor 1, (■) donor 2, (▲) donor 3, (♦) donor 4, (▼) donor 3a, (**O**) donor 4a, (□) donor 5 and (Δ) donor 6. *: $p < 0.05$; **: $p < 0.01$.

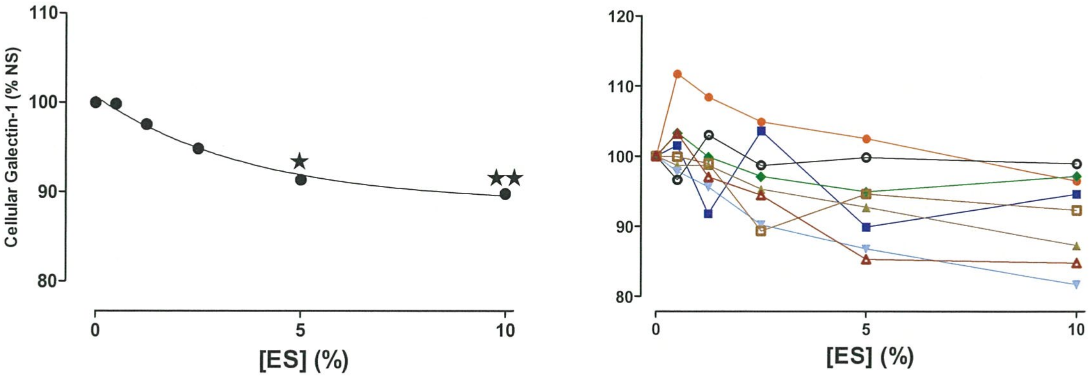


Fig. 3.6. Normalised expressions of cellular (Surface and intracellular) galectin-1 of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (*n* = 8); the curve of best fit is shown using one phase decay equation. Right panel: individual donor cells; symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. *: $p < 0.05$; **: $p < 0.01$.

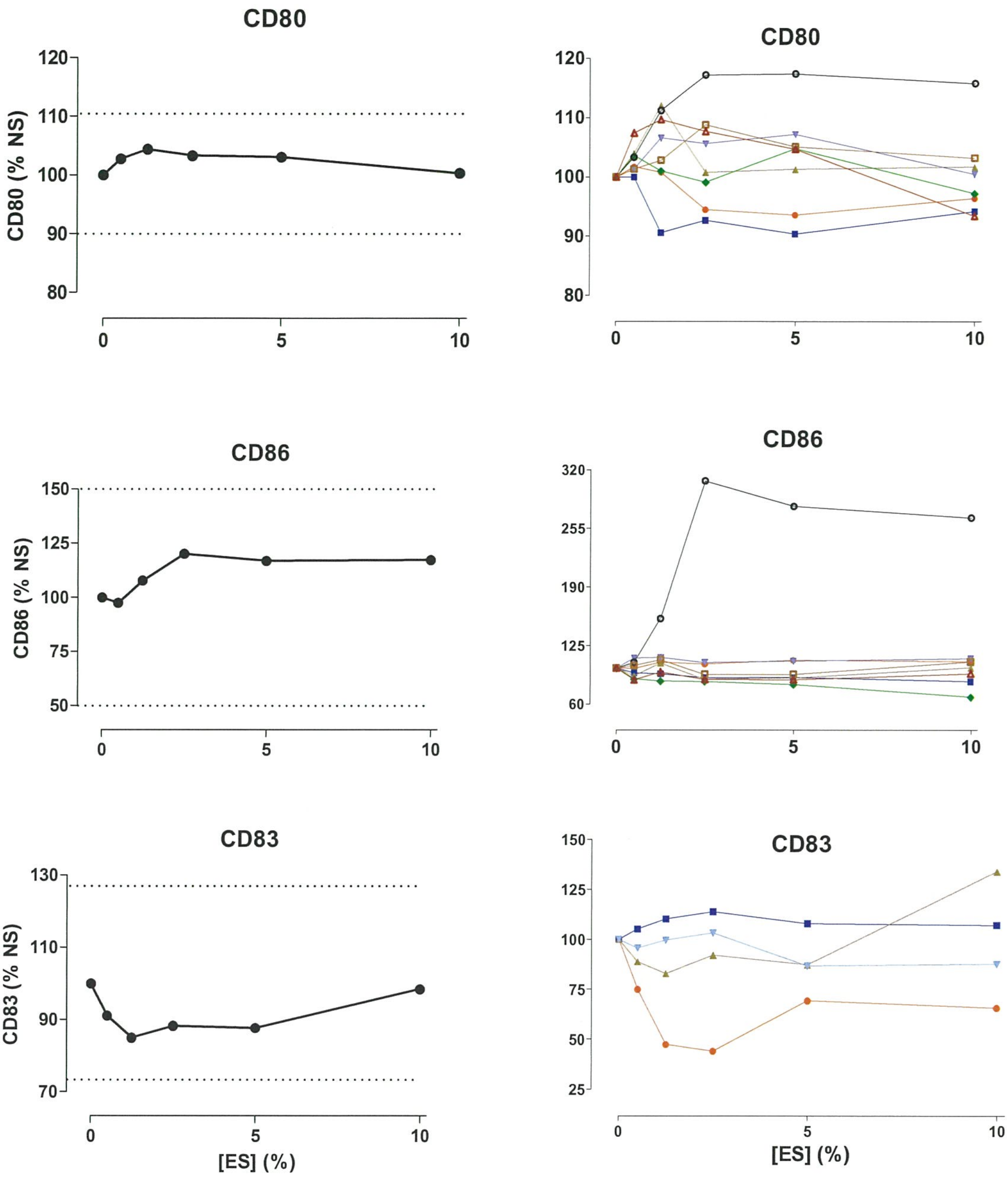


Fig. 3.4. Normalised expressions of surface CD80, CD86 and CD83 of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (*n* = 8); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Right panel: individual donor cells; Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a.

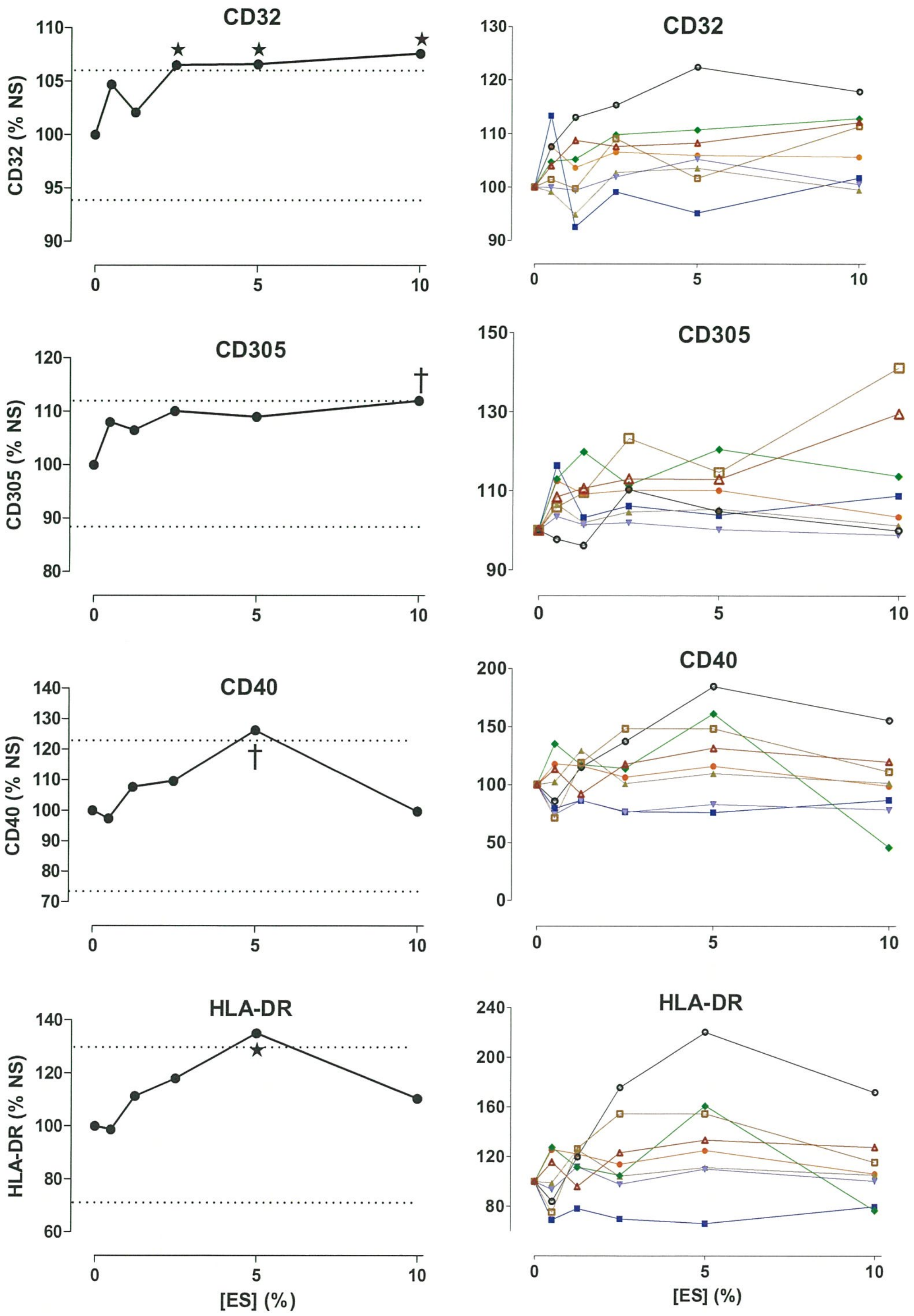


Fig. 3.3. Normalised expressions of surface CD32, CD305, CD40 and HLA-DR of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (n = 8); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Right panel: individual donor cells; Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. $*$: $p < 0.05$, †: $p < 0.1$.

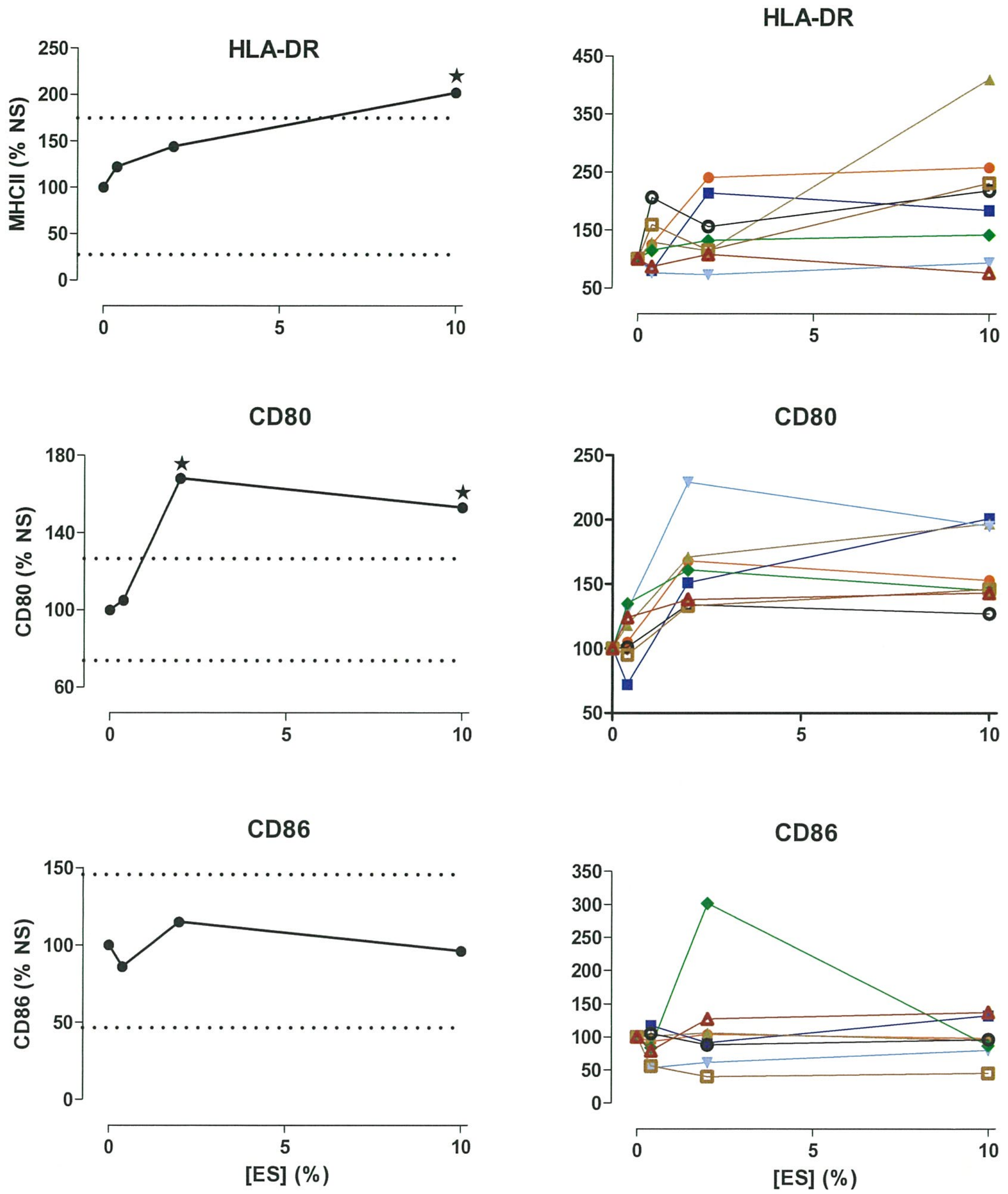


Fig. 3.2. Normalised expressions of surface HLA-DR, CD80 and CD86 of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (*n* = 8); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Right panel: individual donor cells; symbols: (●) donor 1, (■) donor 2, (▲) donor 3, (♦) donor 4, (▼) donor 3a, (**O**) donor 4a, (□) donor 5 and (Δ) donor 6. *: $p < 0.05$.

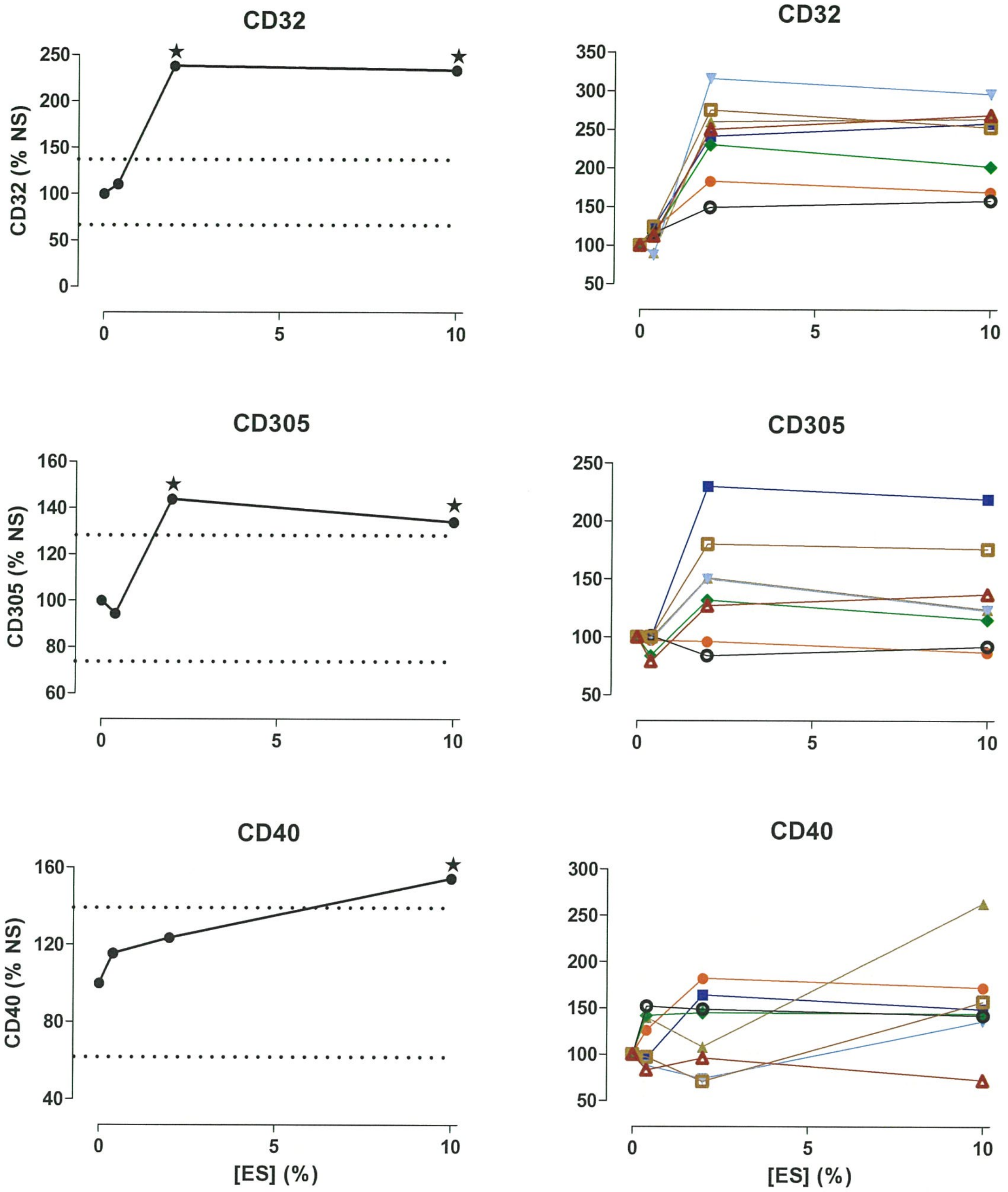


Fig. 3.1. Normalised expressions of surface CD32, CD305 and CD40 of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (*n* = 8); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Right panel: individual donor cells; symbols: (●) donor 1, (■) donor 2, (▲) donor 3, (♦) donor 4, (▼) donor 3a, (**O**) donor 4a, (□) donor 5 and (Δ) donor 6. *: *p* < 0.05.

3.3.1.2. ES-stimulated mdDCs

The principal changes in cell surface molecule expression were similar in the two experiments (Figs 3.1 - 3.4), although there were more significant group differences in Expt #1. Very large responses for individual cells, but not others, and greater variation between donor cells on particular days are likely to influence the group data. Overall, CD32, CD305, CD40 and HLA-DR expressions were increased by ES products. CD80 was increased for all donor cells in Expt #1 and tended to increase in Expt #2. CD86 and CD83 were unchanged. Cells from Donor 2 often differed most from other cells, but the responses on the three days were not consistent.

In Expt #1, expressions of CD32, CD305 and CD80 were increased by 2% and 10% ES products, HLA-DR and CD40 increased by 10% ES products only and CD86 was unchanged (Figs 3.1 - 3.2). Only CD32 and CD80 increased for all donor cells and CD305 and HLA-DR increased in all but two. In Expt #2, there were significant increases in CD32 and HLA-DR expressions ($p < 0.05$), CD305 and CD40 tended to increase ($p < 0.10$) and expressions of CD86, CD80 and CD83 were unchanged (Figs 3.3 - 3.4). The maximum response was not always at the highest concentration of ES products. There was only one set of data for each of the four donors for CD83 expression,as data were lost during the experimental procedure.

3.3.1.3. Galectin-1 expression by ES-stimulated mdDCs

Surface galectin-1 was measured in Expt #1, along with other surface molecules, whereas in Expt #2, the much greater cellular (surface and intracellular expression) was separately assayed. Whereas surface galectin-1 expression was increased by all cells and as a group (Fig. 3.5), cellular galectin-1 was decreased in a concentration-dependent manner (Fig. 3.6). The data significantly fitted a one phase decay model ($p < 0.05$). Cellular expression of

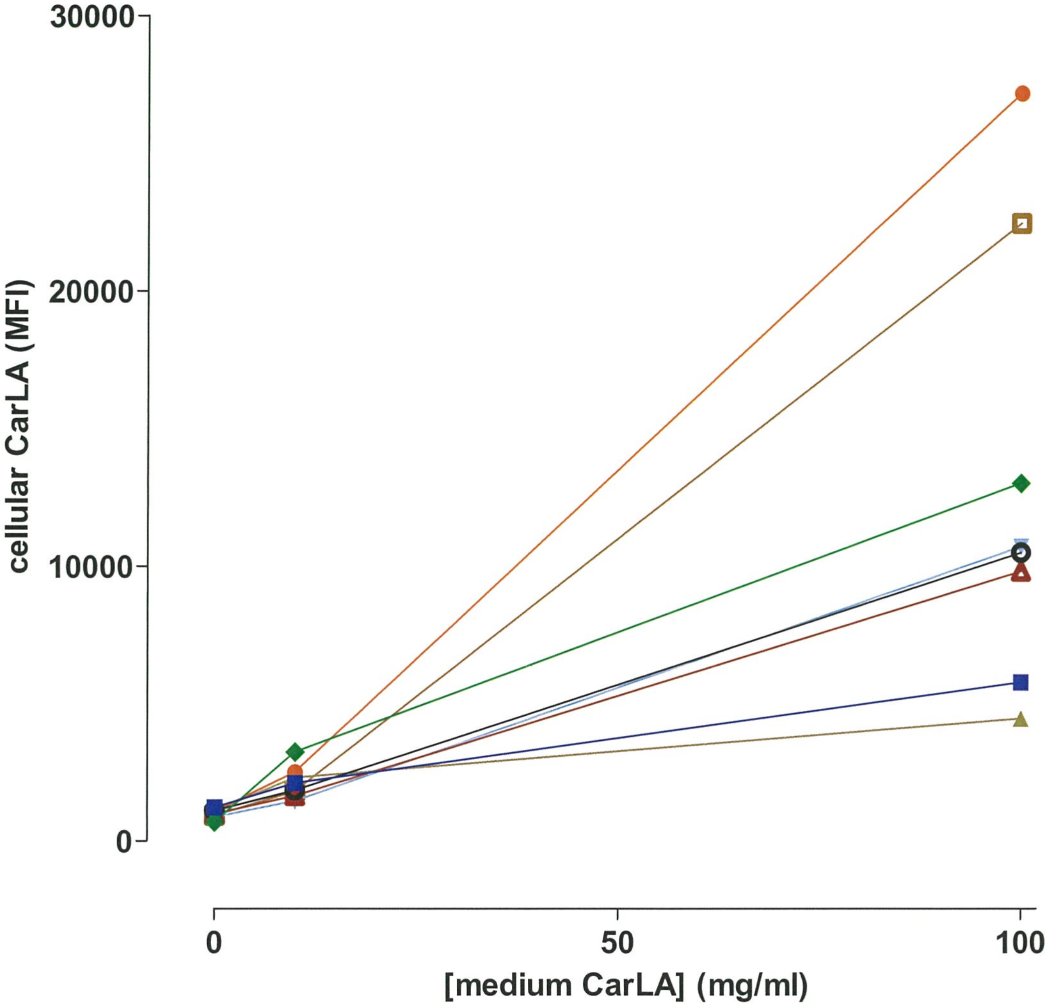


Fig. 3.8. CarLA uptake (median fluorescence intensity (MFI)) by mdDCs of individual donors from medium containing 0 - 100µg/ml of *T. colubriformis* CarLA. Symbols: (●) donor 1, (■) donor 2, (♦) donor 4, (▼) donor 7, (▲) donor 8, (□) donor 9, (Δ) donor 10 and (**O**) donor 11.

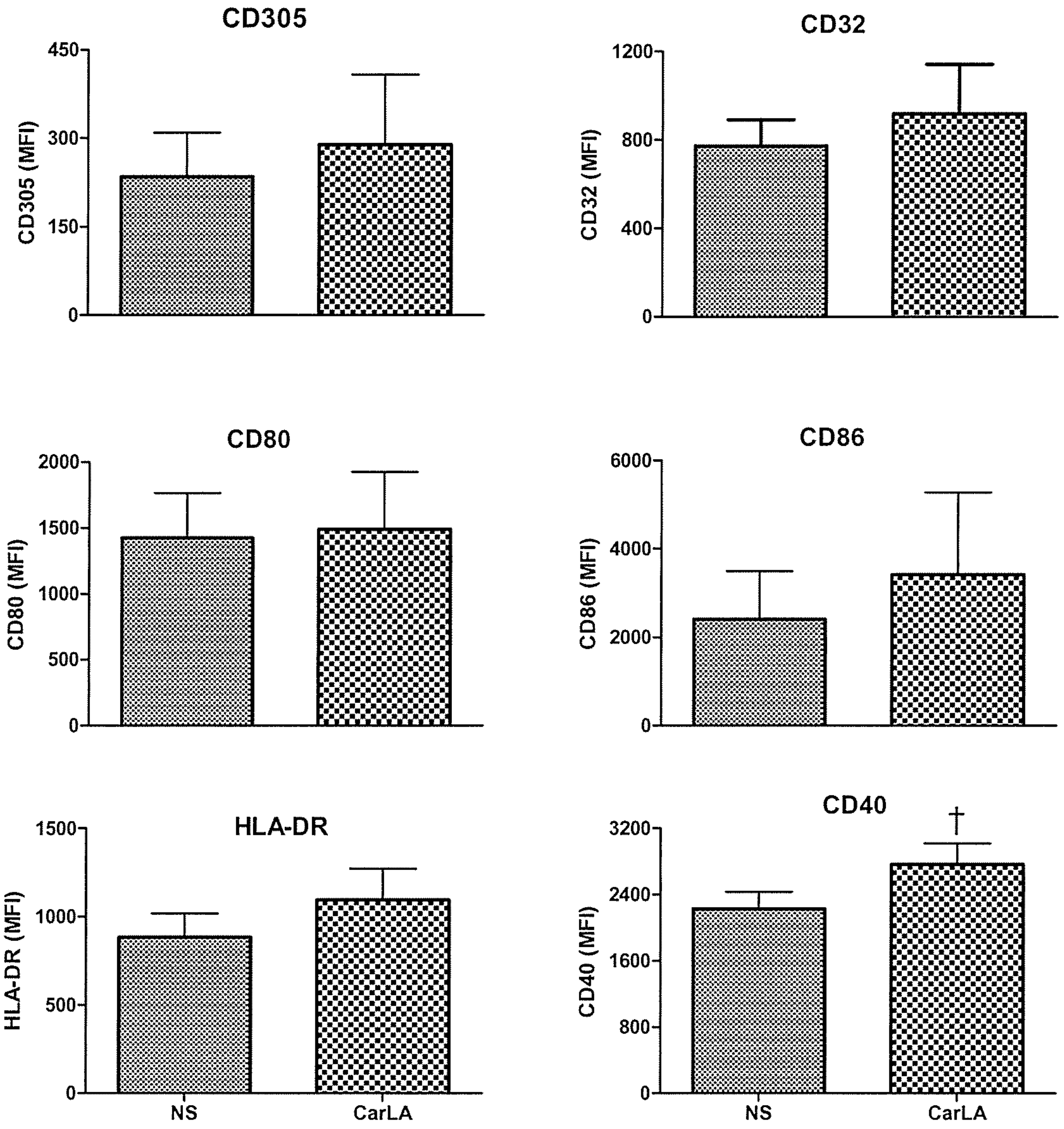


Fig. 3.7. Expressions (median fluorescence intensity (MFI)) (mean ± SEM; $n = 8$) of surface CD305, CD32, CD80, CD86, HLA-DR and CD40 of mdDCs either non-stimulated (NS) or exposed to 10µg/ml of *T. colubriformis* CarLA. †: $p < 0.1$.

Table 3.4. Effect of CarLA on expressions of surface markers (mean fluorescence intensity (MFI)) by mdDCs of Donors 1-6. UD: undetectable.

Days	Donor	1		2		3		4		5		6	
	Marker	NS	CarLA	NS	CarLA	NS	CarLA	NS	CarLA	NS	CarLA	NS	CarLA
1	CD32	398	464	1366	2091	926	1057	885	559				
2						626	682	386	429	1024	1639	566	434
1	CD80	676	588	3054	1640	2754	4350	1313	1549				
2						973	1442	569	497	1435	1014	632	856
1	CD86	349	178	1110	1433	523	553	301	456				
2						3840	2444	902	UD	2461	14007	8255	4813
1	HLA-DR	656	711	1123	1207	807	853	515	611				
2						1335	1659	785	UD	484	1787	1265	836
1	CD305	115	81	675	982	134	147	104	84				
2						128	134	185	145	445	648	91	89
1	CD40	1506	1702	2541	2435	2328	2822	1619	2711				
2						2716	3619	1897	UD	1913	3563	2947	2500

galectin-1 by mdDCs of Donors 2 and 4 was higher in NS cells than from Donors 1 and 7 and exposure to ES products either caused a lesser decrease or even an increase in total galectin-1.

3.3.2. Effect of CarLA on expression of cell surface molecules

The effect on the expression of cell surface molecules of exposure to 10µg/ml CarLA was examined in Expt #1. Generally, there was a trend for CarLA and ES products to produce similar qualitative changes in surface marker expression. Data for mdDCs of individual donors are presented in Table 3.4 and group data in Fig. 3.7. There was no significant change in expression for any surface molecule, although CD40 approached a significant increase ($p < 0.10$). Except for CD80, the group means for cells exposed to CarLA were numerically greater than for NS cells, although this was not the case for all individual cells. MdDCs of Donors 4 and 6 differed in being stimulated less by CarLA than were cells of other donors (Table 3.4).

3.3.3. CarLA uptake

CarLA uptake was high from incubation medium with a concentration of 100µg/ml CarLA, but very low when the concentration was 10µg/ml (Fig. 3.8). CarLA uptake varied greatly between mdDCs of different donors: very low for Donors 2 and 8, moderate for Donors 7, 10, 11 and 4 and high for Donors 1 and 9.

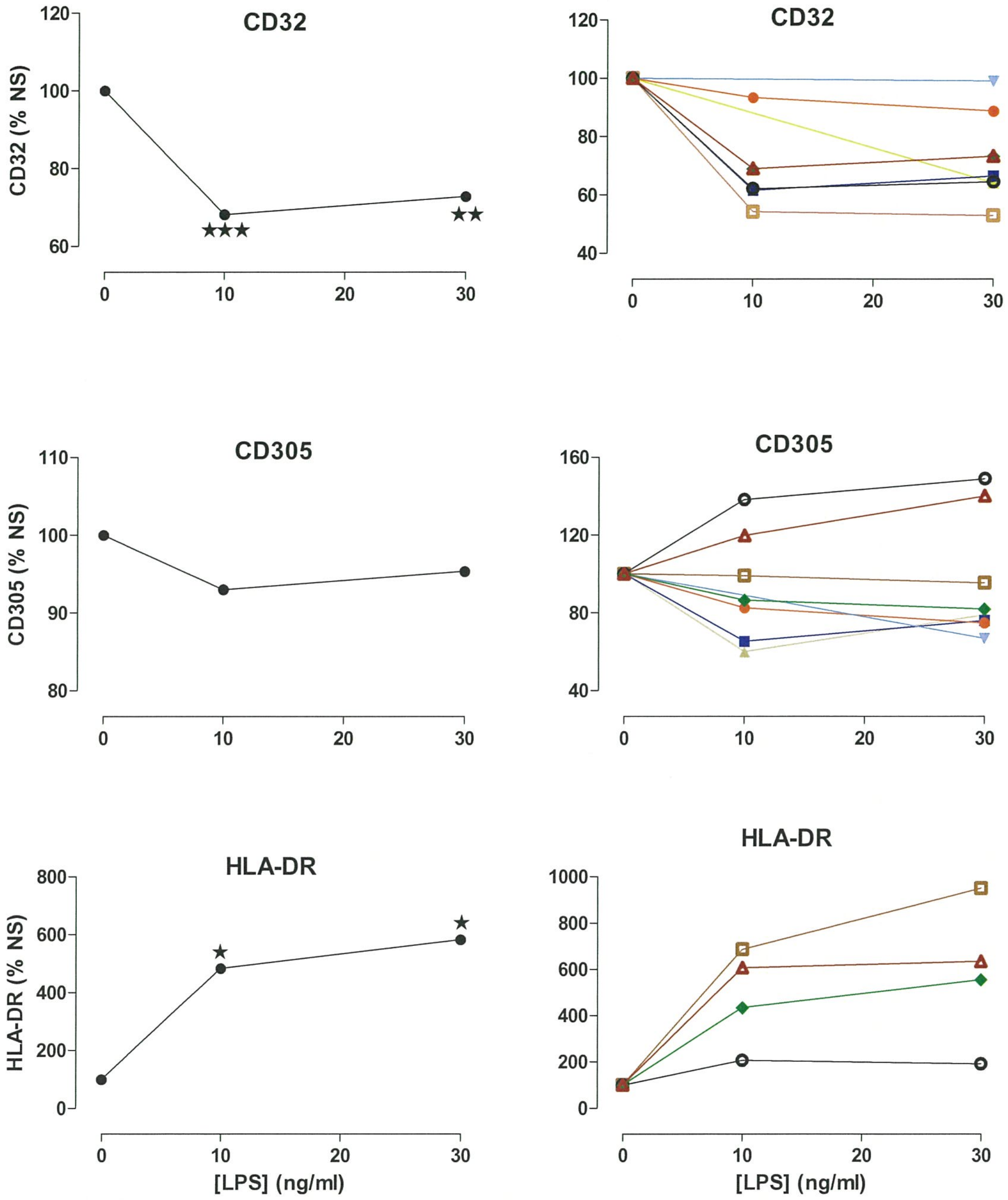


Fig. 3.12. Normalised expressions of surface CD32, CD305 and HLA-DR of mdDCs exposed to 0 - 30ng/ml of LPS. Left panel: group means (*n* = 8). Right panel: individual donor cells; Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (**Δ**) donor 7a. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

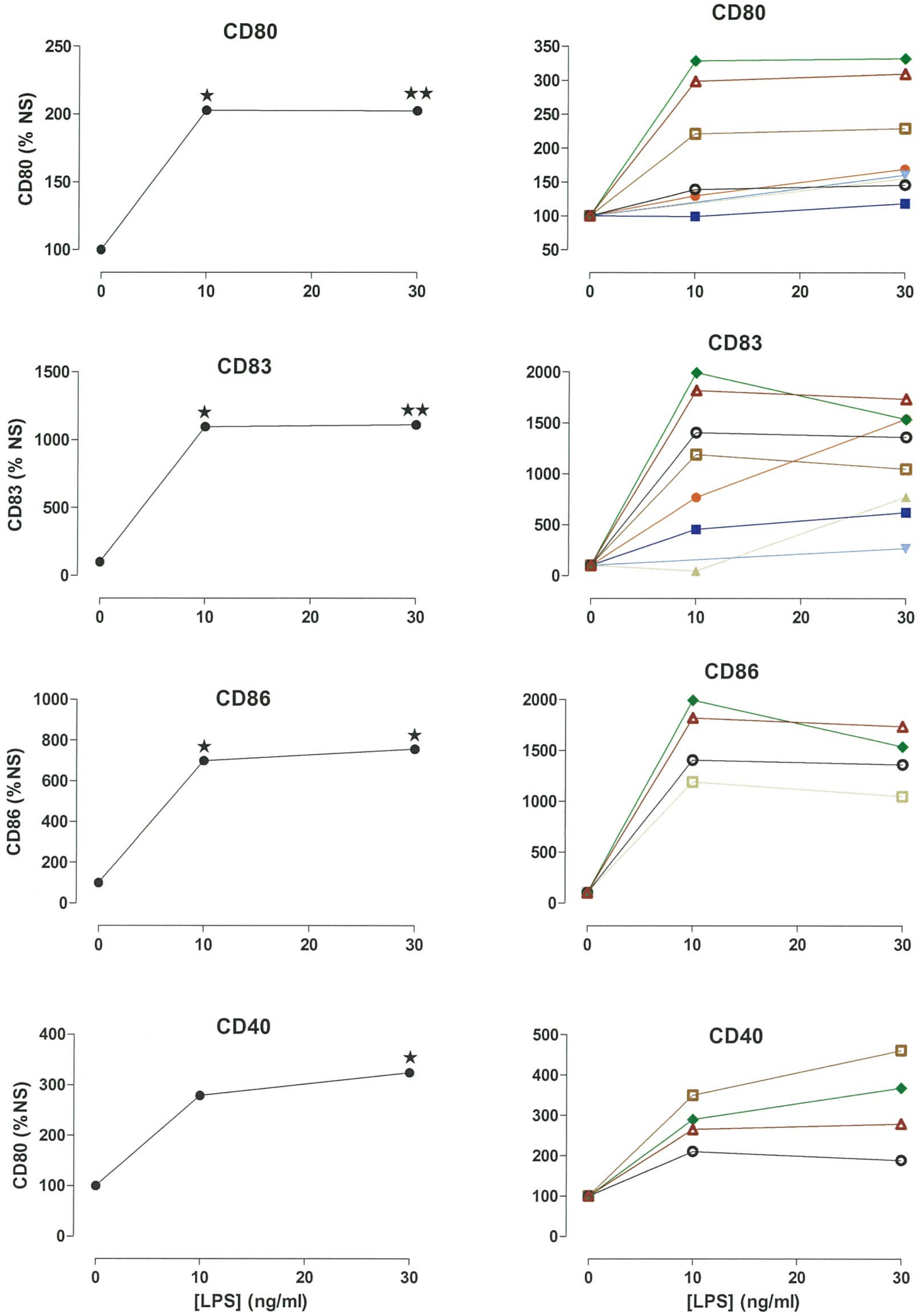


Fig. 3.11. Normalised expressions of surface CD80, CD83, CD86 and CD40 of mdDCs exposed to 0 - 30ng/ml of LPS. Left panel: group means (*n* = 8). Right panel: individual donor cells; Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (**Δ**) donor 7a. *: $p < 0.05$; **: $p < 0.01$.

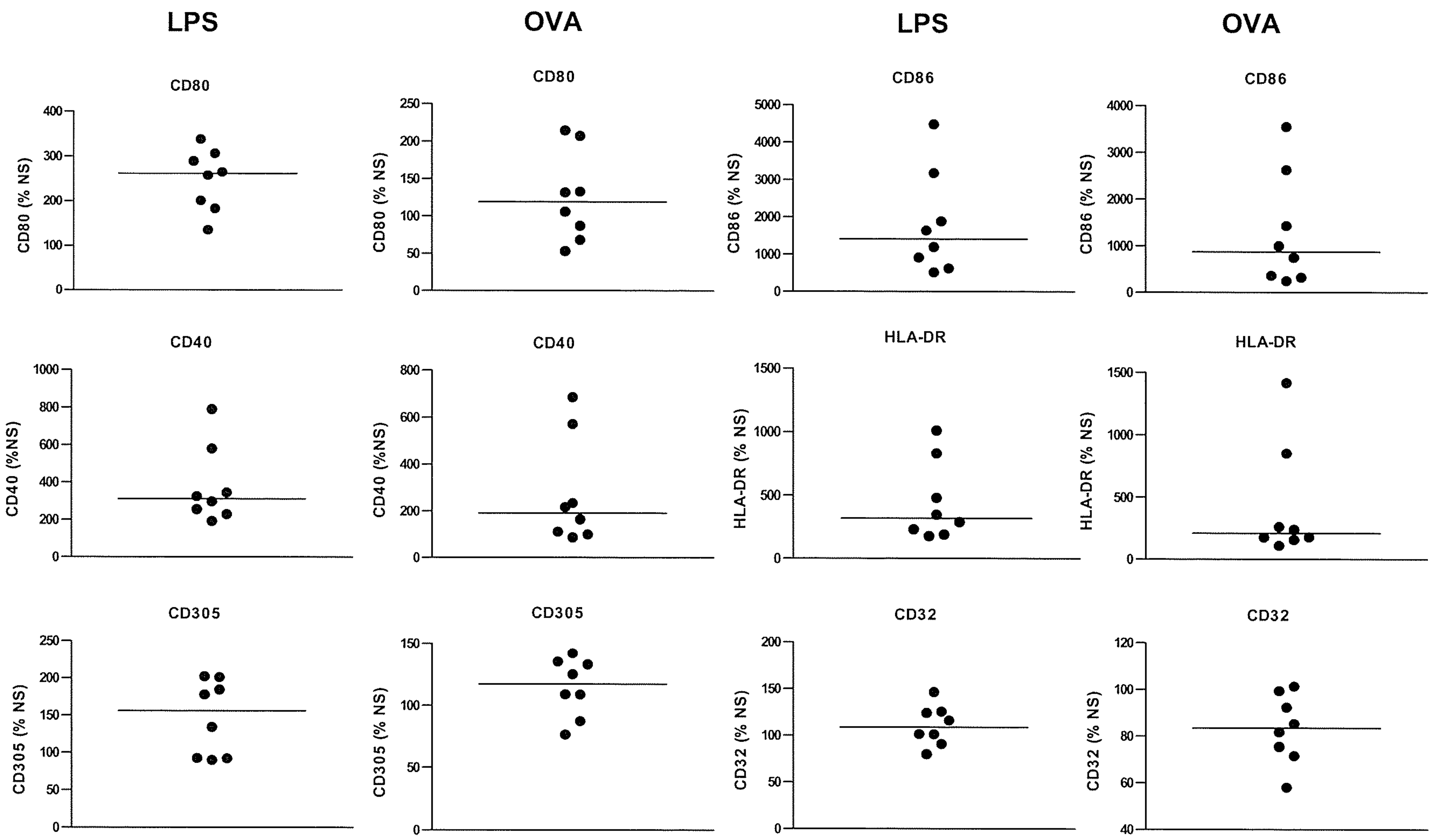


Fig. 3.10. Normalised expressions of surface CD80, CD86, CD40, HLA-DR, CD305 and CD32 by individual donor mdDCs exposed to 100ng/ml LPS or 10μg/ml ovalbumin (OVA). Lines show median for the group.

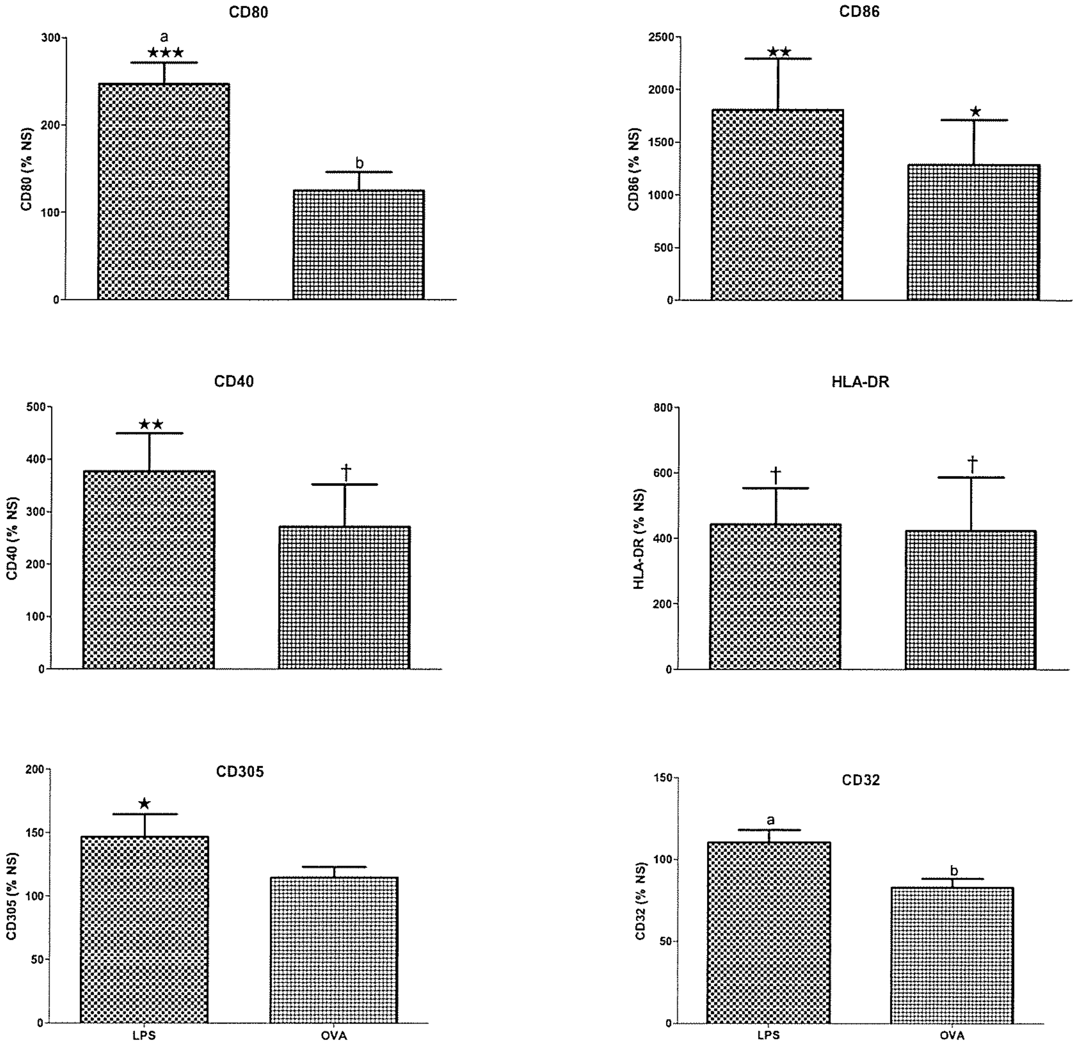


Fig. 3.9. Normalised expressions (mean ± SEM; $n = 8$) of surface CD80, CD86, CD40, HLA-DR, CD305 and CD32 by mdDCs exposed to 100ng/ml LPS or 10µg/ml ovalbumin (OVA). Significant differences from non-stimulated cells are shown: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; †: $p < 0.1$ and between LPS and OVA ($p < 0.05$) by different superscripts.

3.3.4. Effect of LPS or OVA on expression of cell surface molecules

Over both experiments, LPS was strongly stimulatory for surface expression of CD80, CD86, CD40, HLA-DR and CD83, but had less consistent effects on CD305 and CD32. In Expt #1, the concentration of LPS was 100ng/ml (Figs 3.9 - 3.10) and 10 and 30ng/ml in Expt #2 (Figs 3.11 - 3.12). Quantitative variation in expression of surface markers by mdDCs of different donors was prominent, with cells of Donor 2 generally less responsive to LPS than mdDCs of other donors.

Co-stimulatory molecule and MHCII expression was increased overall. In both Expts #1 and #2, the expressions of CD80, CD86 and CD40 were significantly increased. HLA-DR increased in Expt #2 and was approaching significance in Expt #1 ($p < 0.1$). CD83 was monitored only in Expt #2, in which it significantly increased. Effects on the regulatory molecules were more variable. Expression of CD305 was unchanged by the lower LPS concentrations in Expt #2, but the median significantly increased by about 50% with 100ng/ml LPS in Expt #1 (Fig. 3.10). Individual donor variation was prominent, several cell populations showing reduced or unchanged CD305 expression. CD32 expression decreased significantly with both 10 and 30ng/ml LPS (Fig. 3.12), but was unchanged with 100ng/ml LPS (Fig. 3.9).

OVA had generally similar, but lesser, effects than LPS in Expt #1, in which the effects of 10µg/ml OVA and 100ng/ml LPS were compared with expression of surface molecules by NS cells (Figs 3.9 - 3.10). OVA significantly increased expression only of CD86 ($p < 0.05$), whilst the increases in HLA-DR and CD40 were approaching significance ($p < 0.10$). CD32 expression was below NS

Table 3.5 Upper and lower detectable limits of cytokines and chemokines. Values in pg/ml, except for ICAM-1, LAP and E-selectin which are in ng/ml.

Cytokine/chemokine	Lower limit	Upper limit
IL-12p70	20	20000
LAP	1.9	1000
IFN-γ	26	20000
MIP-1ß	3.2	3000
MCP-1	40	5500
IL-10	25	20000
MIP-1α	12	9000
IL-8	12	9000
ICAM-1	5.5	2200
IL-6	20	18000
IL-1α	1.4	1000
IFN-α	27	20000
IL-13	24	20000
IP-10	26	12500
IL-17A	12	10000
IL-1ß	20	20000
TNF-α	25	20000
E-selectin	4	2500

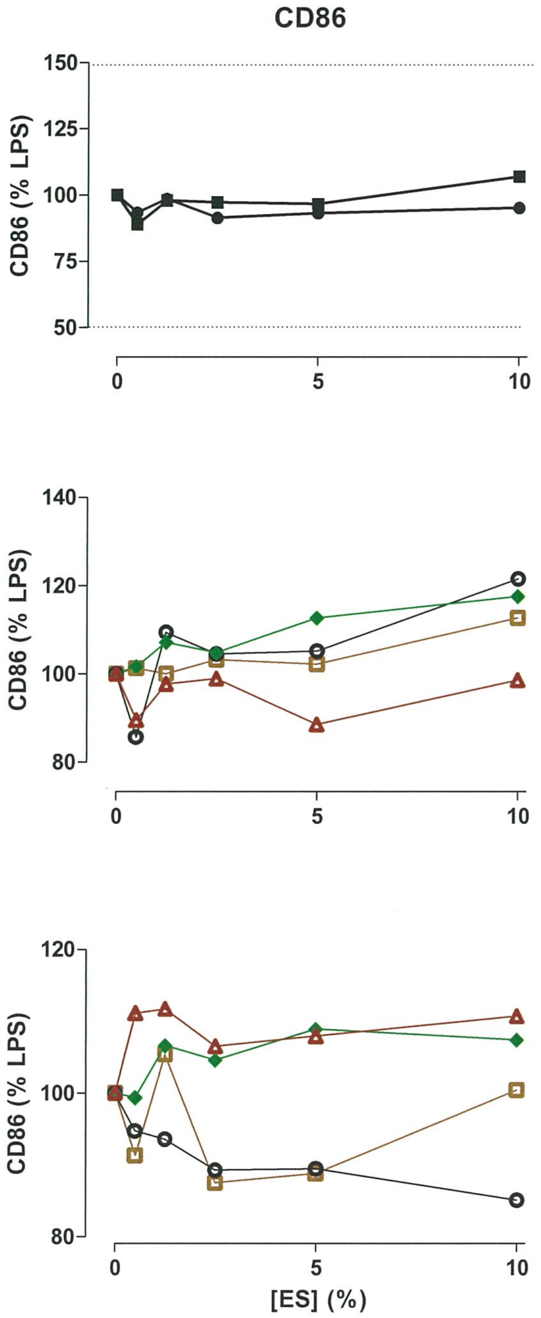


Fig. 3.15. Normalised expressions of surface CD86 of mdDCs co-exposed to 0-10% *Hc*ES products and 0-30ng/ml of LPS. Top row: group means (*n* = 8); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis; symbols: (●) LPS 30 ng/ml, (■) LPS 10ng/ml. Middle row: individual donor mdDCs co-exposed to 0-10% *Hc*ES products and 10ng/ml of LPS. Bottom row: individual donor mdDCs co-exposed to 0-10% *Hc*ES products and 30ng/ml of LPS; symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a.

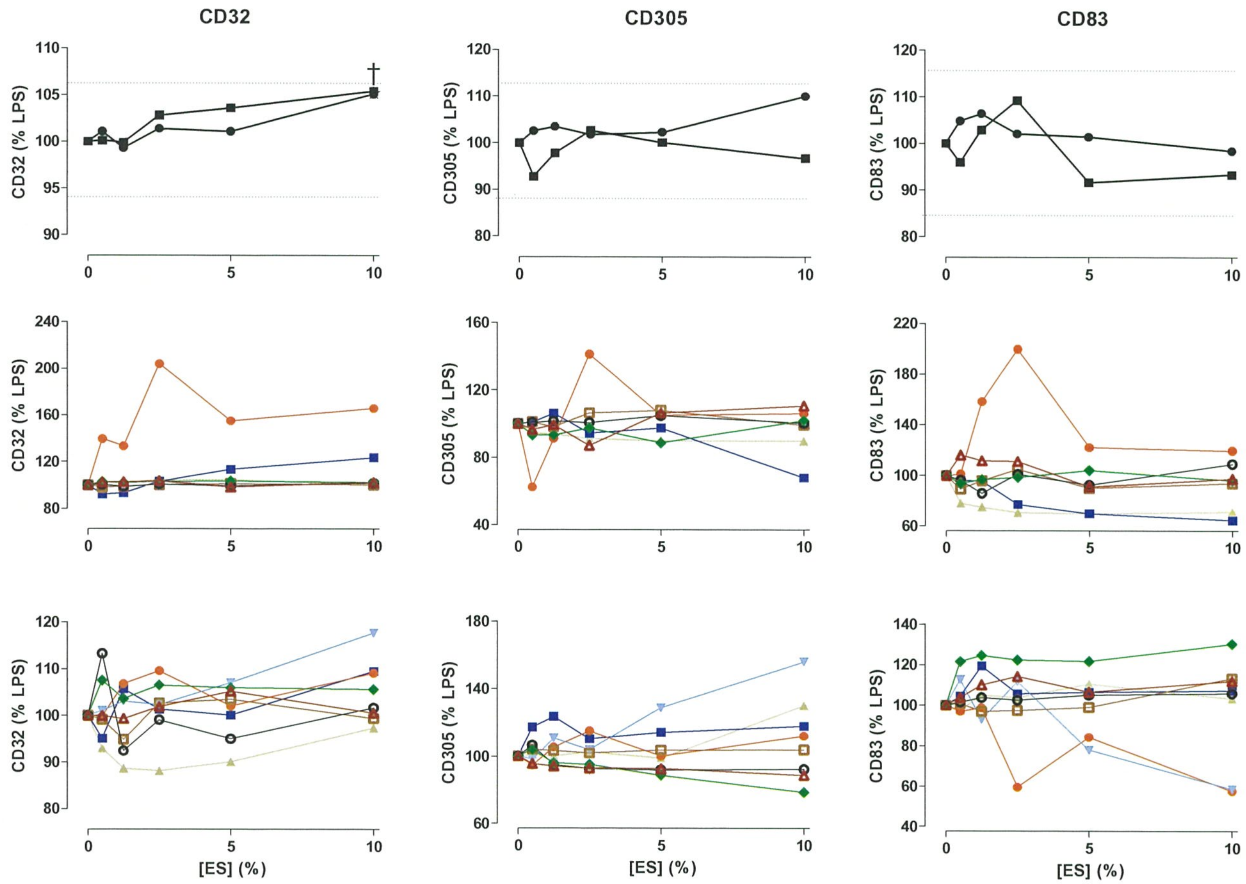


Fig. 3.14. Normalised expressions of surface CD32, CD305 and CD83 of mdDCs co-exposed to 0 - 10% *Hc*ES products and 0 - 30ng/ml of LPS. Top row: group means (n = 8); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Symbols: (●) LPS 30 ng/ml, (■) LPS 10ng/ml. Middle row: individual donor mdDCs co-exposed to 0 - 10% *Hc*ES products and 10ng/ml of LPS. Bottom row: individual donor mdDCs co-exposed to 0 - 10% *Hc*ES products and 30ng/ml of LPS. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. †: $p < 0.1$.

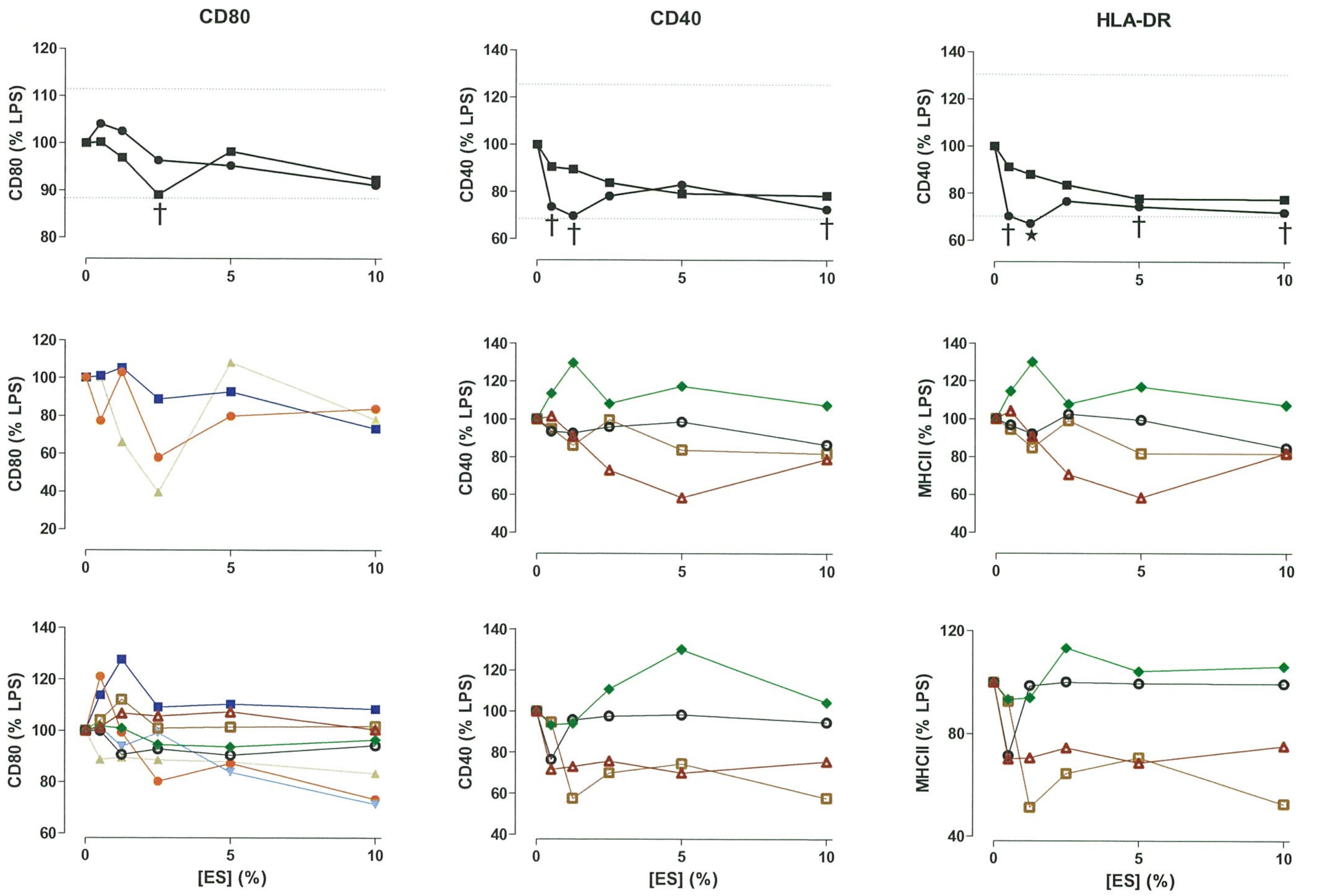


Fig. 3.13. Normalised expressions of surface CD80, CD40 and HLA-DR of mdDCs co-exposed to 0 - 10% *Hc*ES products and 0 - 30ng/ml of LPS. Top row: group means ($n = 8$); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Symbols: (●) LPS 30 ng/ml, (■) LPS 10ng/ml. Middle row: individual donor mdDCs co-exposed to 0 - 10% *Hc*ES products and 10ng/ml of LPS. Bottom row: individual donor mdDCs co-exposed to 0 - 10% *Hc*ES products and 30ng/ml of LPS. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (◆) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. *: $p < 0.05$, †: $p < 0.1$.

for the group and for 7 of the 8 individual mdDCs. There were significant differences between OVA and LPS for CD80 and CD32 (Fig. 3.9).

3.3.5. Effect of LPS and ES product co-stimulation on expression of cell surface molecules

There were no significant group effects of ES products and LPS co-stimulation compared with LPS alone (Figs 3.13 - 3.15). Individual responses were very variable, and group means were often strongly influenced by large changes in mdDCs of particular donors, as well as by opposite effects on individual cells, particularly for LPS 30ng/ml. Donor 1 mdDCs often responded differently from other cells, in many cases being strongly stimulated compared with others, or failing to be inhibited when other decreased markedly. Donor 4 and 7 cells most often showed the greatest inhibitions.

3.3.6. Effect of ES products on secretion of chemokines and cytokines

The lower and upper limits of the assays for the cytokines and chemokines are given in Table 3.5. Of the 20 cytokines/chemokines assayed, only 18 are reported, as IL-4 and GM-CSF were constituents of the incubation medium. LAP, IL-1α and IL-1β were not detected under any experimental conditions. The ES products added to the cell culture medium did not contain detectable cytokines or chemokines when assayed at the final concentrations in the medium.

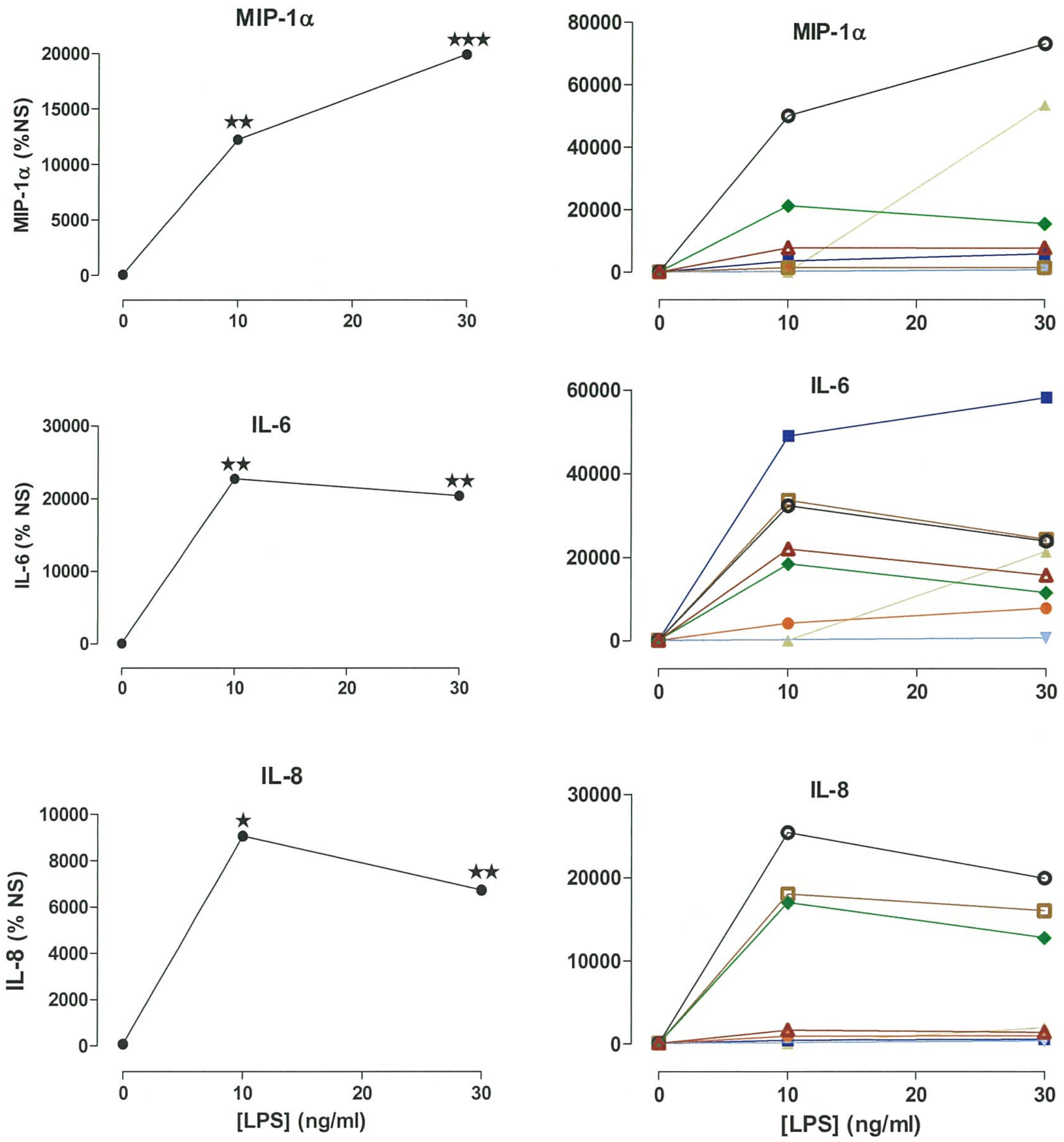


Fig. 3.20. Normalised secretion of MIP-1α, IL-6 and IL-8 by by mdDCs exposed to 0 - 30ng/ml of LPS. Left panel: group means (*n* = 8). Right panel: individual donor cells. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (**Δ**) donor 7a. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

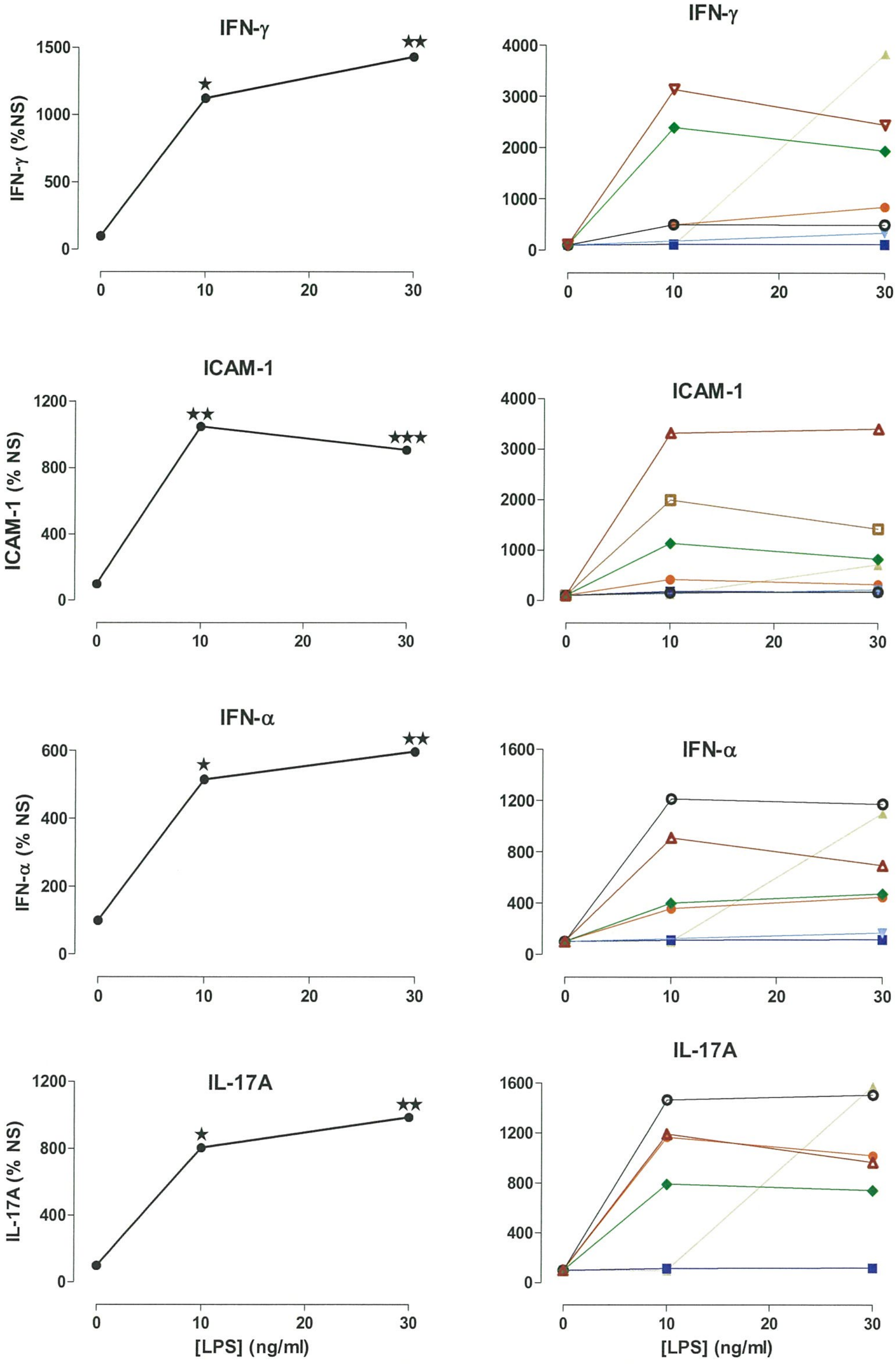


Fig. 3.19. Normalised secretion of IFN-γ, ICAM-1, IFN-α and IL-17A by mdDCs exposed to 0 - 30ng/ml of LPS. Left panel: group means (*n* = 8). Right panel: individual donor cells. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

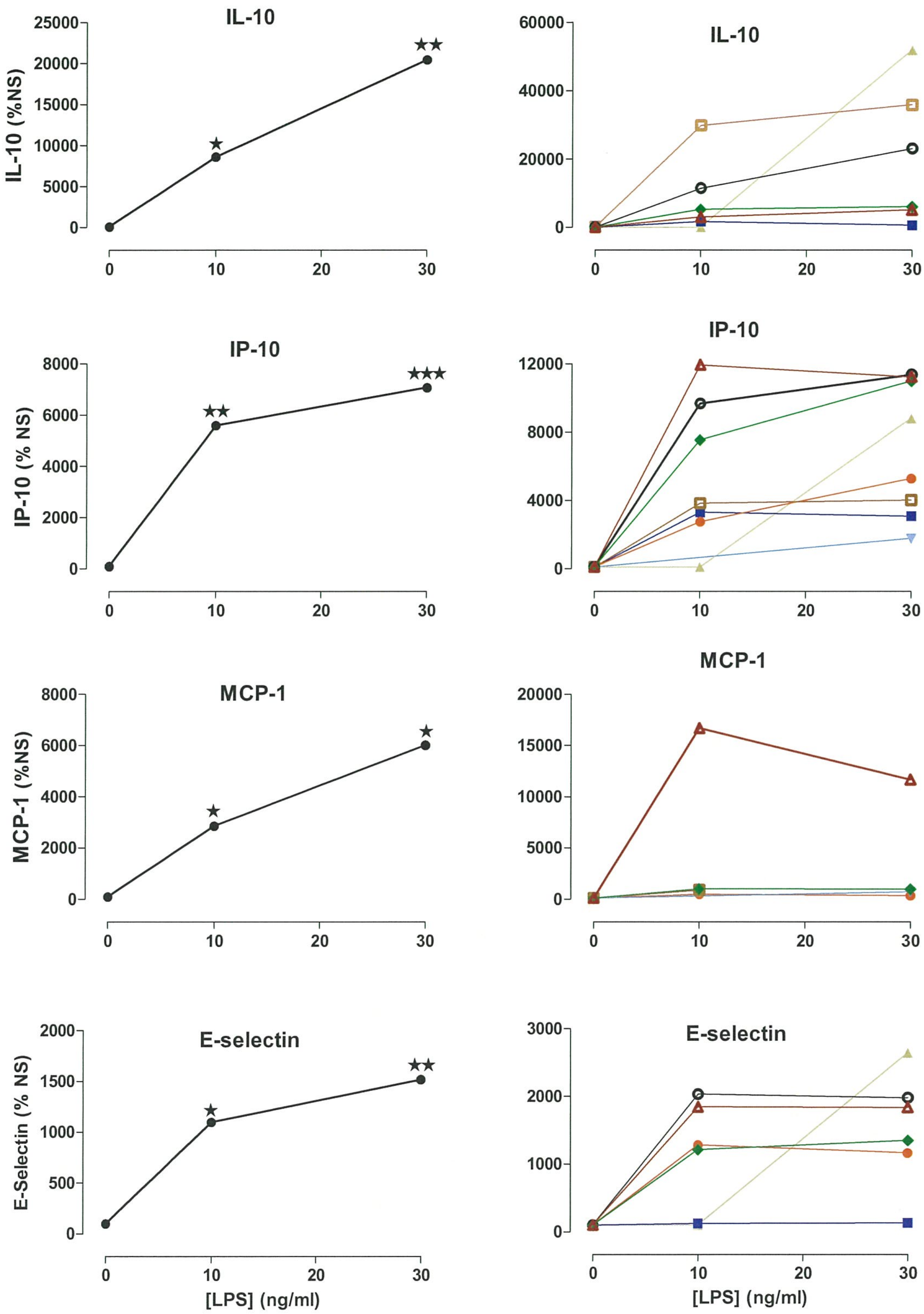


Fig. 3.18. Normalised secretion IL-10, IP-10, MCP-1 and E-selectin by mdDCs exposed to 0 - 30ng/ml of LPS. Left panel: group means (*n* = 8). Right panel: individual donor cells. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

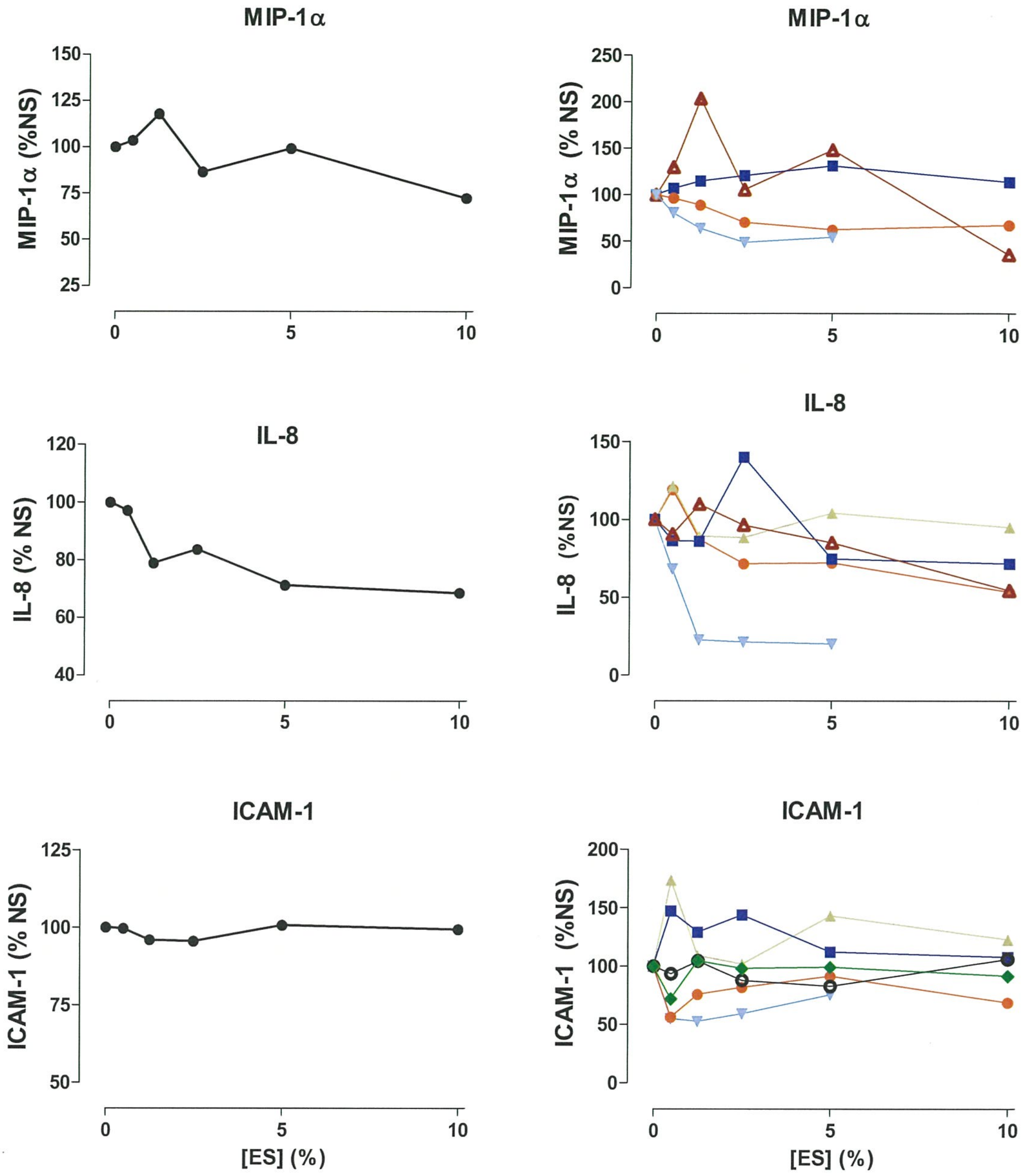


Fig. 3.17. Normalised secretion of MIP-1α, IL-8 and ICAM-1 of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (*n* = 8). Right panel: individual donor cells. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a.

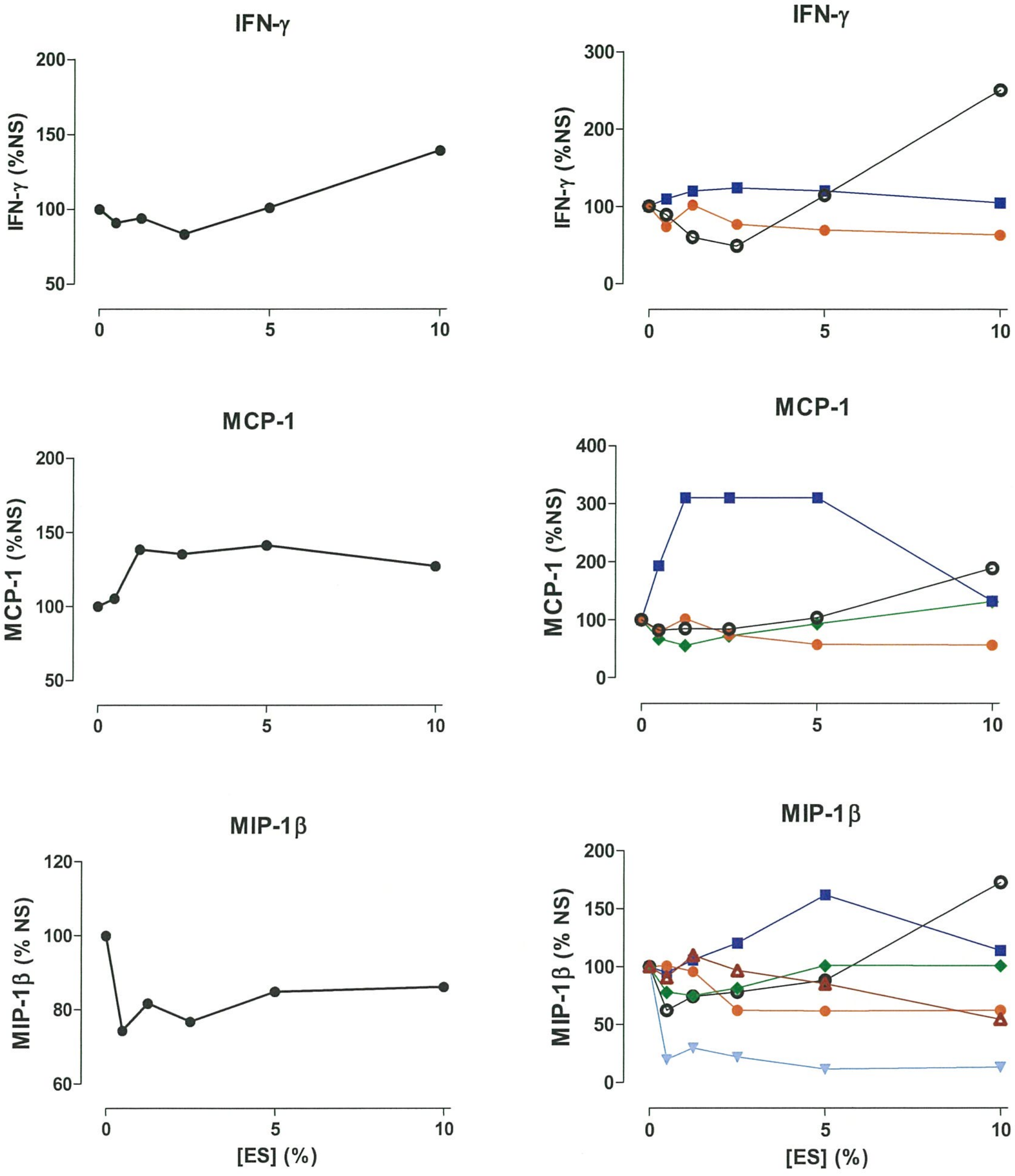


Fig. 3.16. Normalised secretion of IFN-γ, MCP-1 and MIP-1β of mdDCs exposed to 0 - 10% *Hc*ES products. Left panel: group means (*n* = 8). Right panel: individual donor cells. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a.

Table 3.6 Secretion of cytokines and chemokines by non-stimulated mdDCs of Donors 1, 2, 4 and 7. Values are in pg/ml except for ICAM-1, LAP and E-selectin, which are in ng/ml. UD: undetectable.

Day	Cytokine/ chemokine	Donor			
		1	2	4	7
1	IFN-γ	46	425	UD	44
2		UD	113	UD	UD
1	MCP-1	200	>5500	UD	235
2		157	716	UD	UD
1	MIP-1α	31	78	UD	24
2		UD	UD	UD	17
1	MIP-1β	77	72	5	82
2		90	58	UD	151
1	IL-8	506	246	38	1298
2		UD	UD	UD	67
1	ICAM-1	18	37	7	16
2		7	40	UD	UD
1	IFN-α	24	124	UD	UD
2		UD	UD	UD	UD
1	IL-13	UD	60	UD	UD
2		UD	UD	UD	UD
1	IL-17A	UD	75	UD	UD
2		UD	UD	UD	UD
1	E-selectin	UD	30	UD	UD
2		UD	UD	UD	UD
1	IP-10	UD	UD	UD	UD
2		UD	UD	UD	UD
1	TNF-α	UD	UD	UD	UD
2		UD	UD	UD	UD
1	IL-6	UD	UD	UD	UD
2		UD	UD	UD	UD
1	IL-10	UD	UD	UD	UD
2		UD	UD	UD	UD
1	IL12-p70	UD	UD	UD	UD
2		UD	UD	UD	UD

3.3.6.1. Non-stimulated mdDCs

The concentrations of cytokines and chemokines secreted by NS mdDCs are given in Table 3.6. There were marked differences between the cells of the 4 donors, particularly cells of Donor 4, which produced almost no cytokines/chemokines and Donor 2, which secreted the highest concentrations. Only IFN-γ, MCP-1, MIP-1α, MIP-1β, IL-8 and ICAM-1 were secreted in detectable levels by mdDCs of 3 or more donors. On one day, the maximum assayable concentration of MCP-1 was measured in the supernatant for cells of Donor 2. Also on one day, IFN-α, IL-13, IL-17A and E-selectin were detected from Donor 2 cells and IFN-α from Donor 1 mdDCs. IL-6, IP-10 and TNF-α were not detected in any supernatant of NS cells.

3.3.6.2. ES-stimulated mdDCs

Exposure to ES did not cause cytokines/chemokines to be released unless the NS cells also secreted measurable levels. The responses of individual donor cells and the group means for IFN-γ, MCP-1, MIP-1α, MIP-1β, IL-8 and ICAM-1 are shown in Figs 3.16 - 3.17. The very variable individual responses were consistent with the lack of significant effects of ES products overall. Donor 2 cells more often increased secretion, whereas cells of other donors either showed no response or decreased secretion. Changes in group means were often dominated by one donor, such as the decreases in MIP-1α, MIP-1β, IL-8 and ICAM-1 recorded from Donor 7 cells on Day 1.

3.3.7. Effect of LPS on secretion of chemokines and cytokines

LPS greatly increased secretion of 11 cytokines/chemokines (Figs 3.18 - 3.20). There was no measurable release of IL12p70 or IL-13. For most cytokine/chemokines, the differences in response to

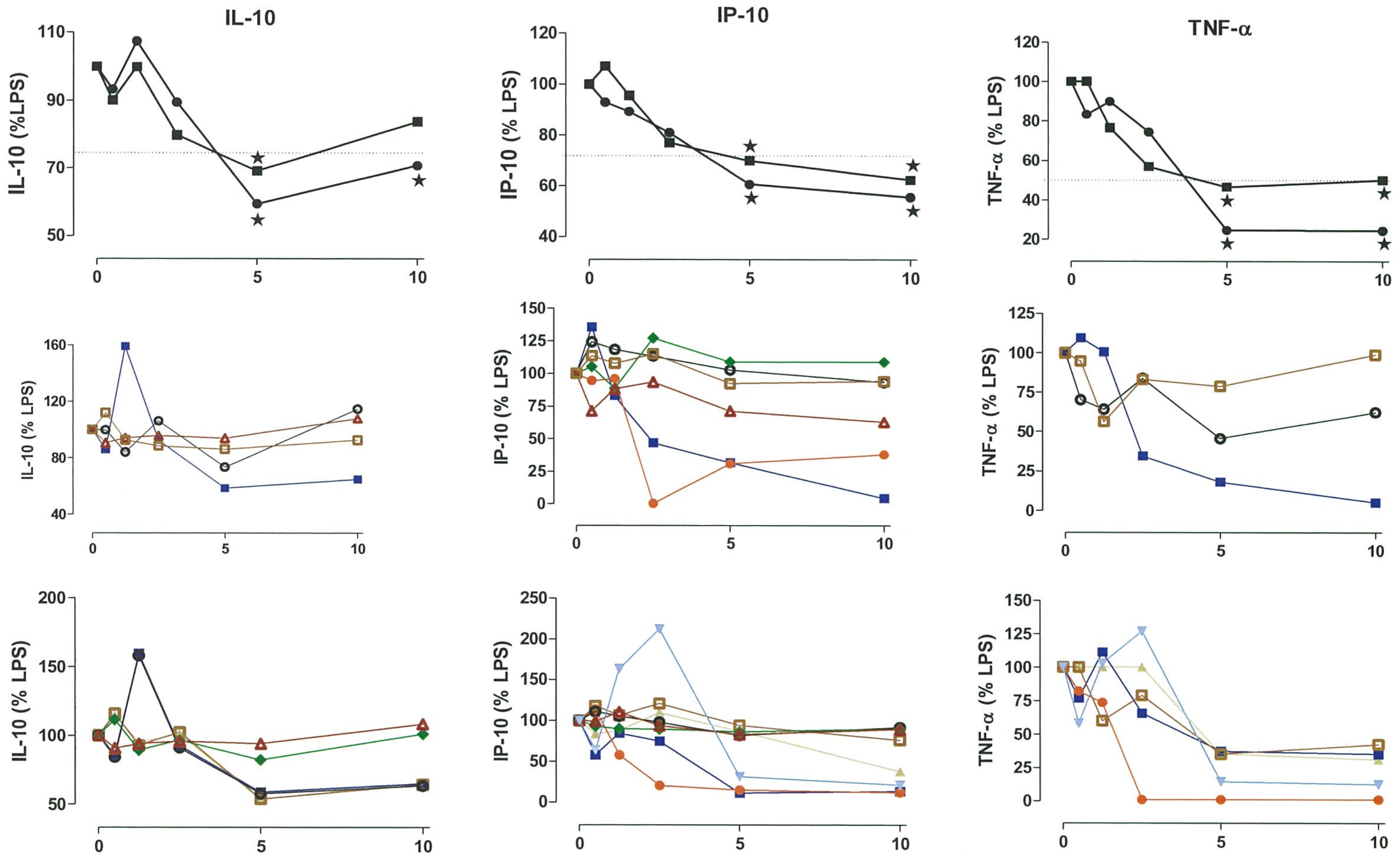


Fig. 3.21. Normalised secretion of IL-10, IP-10 and TNF-α by mdDCs exposed to 0 - 10% *Hc*ES products and 0 - 30ng/ml of LPS. Top row: group means ($n = 8$); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Symbols: (●) LPS 30 ng/ml, (■) LPS 10ng/ml; Middle row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 10ng/ml of LPS; Bottom row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 30ng/ml of LPS. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. *: $p < 0.05$.

the two LPS concentrations were small, the main exception being IL-10. MCP-1 concentrations were too high to be assayed for cells of Donor 2 and on one other day (Donor 4); these were not included in the graphs or analyses, however, there was a significant group increase with LPS for other donor cells (Fig. 3.18).

The effects of LPS on TNF-α and MIP-1ß could not be assessed accurately, as the concentration in the supernatant exceeded the maximum assayable level on many days, thus underestimating the percentage increase. These were not included in the Figures. MIP-1ß was released in low concentrations by most NS cells and on 2 days remained low with LPS. In the presence of 30ng/ml LPS, MIP-1ß exceeded the maximum on 6 of the 8 days and with both concentrations of LPS on 5 days.

TNF-α was not released by NS cells, nor by either concentration of LPS on 2 days, but was too high to be assayed with both LPS concentrations on 3 days and with the higher LPS on another 2 days. Although TNF-α was greatly increased by 30ng/ml, 10ng/ml LPS had little effect on many cells.

3.3.8. Effect of LPS and ES products co-stimulation on secretion of chemokines and cytokines

Results are presented in groups of cytokines/chemokines which were similarly affected by co-stimulation with ES products and LPS.

3.3.8.1. ES products reduced the response to LPS

ES products overall significantly reduced the effects of LPS on IL-10, IP-10 and TNF-α, although cells from individual donors varied, particularly for IL-10 and IP-10 (Fig. 3. 21). These data did not

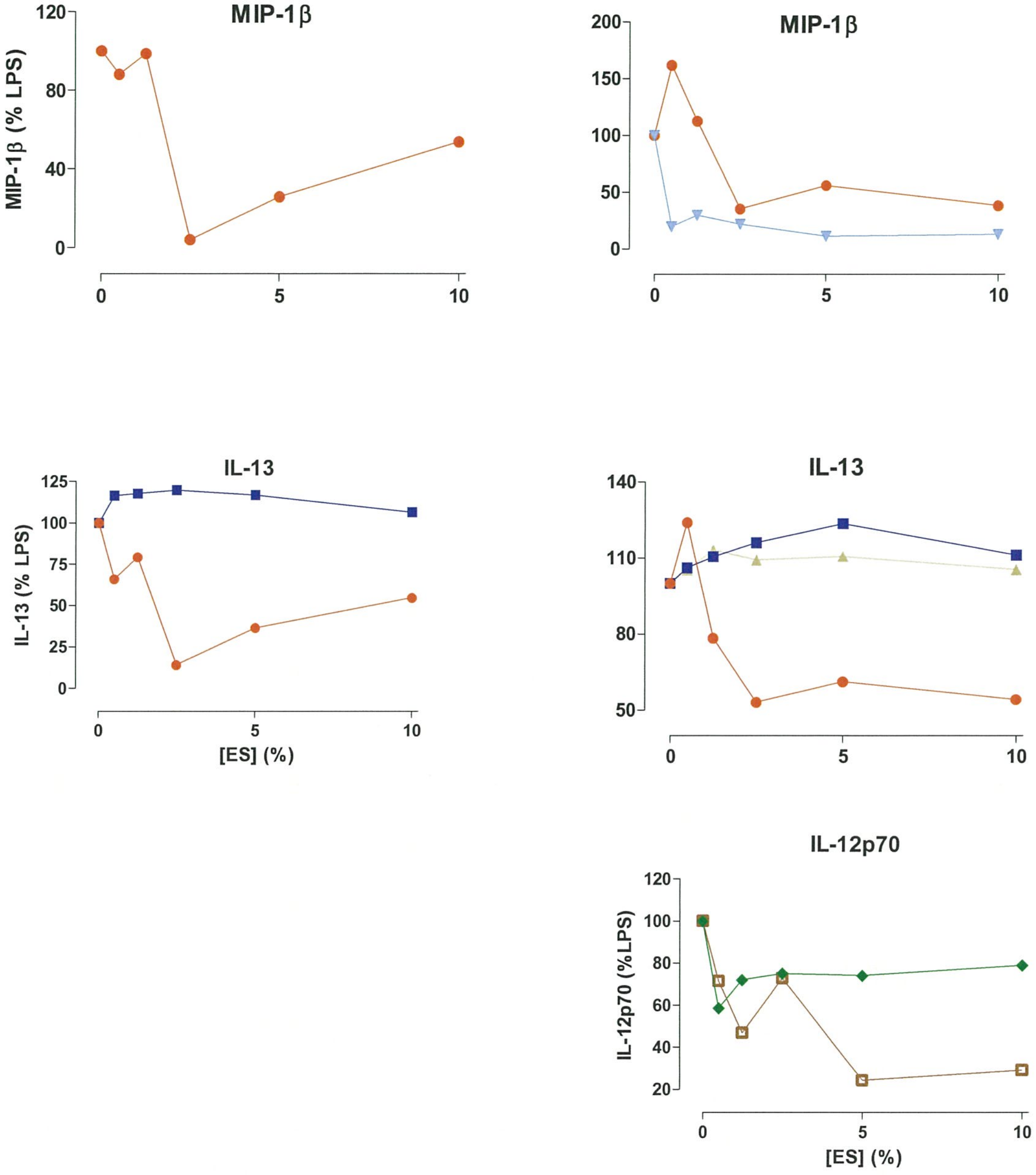


Fig. 3.25. Normalised secretion of MIP-1β, IL-13 and IL-12p70 by mdDCs cexposed to 0 - 10% *Hc*ES products and 0 - 30ng/ml of LPS. Left panel: exposed to 0 - 10% *Hc*ES products and 10ng/ml of LPS. Right panel: exposed to 0 - 10% *Hc*ES products and 30ng/ml of LPS. IL-12p70 levels were detectable only with 30ng/ml of LPS. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a and (□) donor 4a.

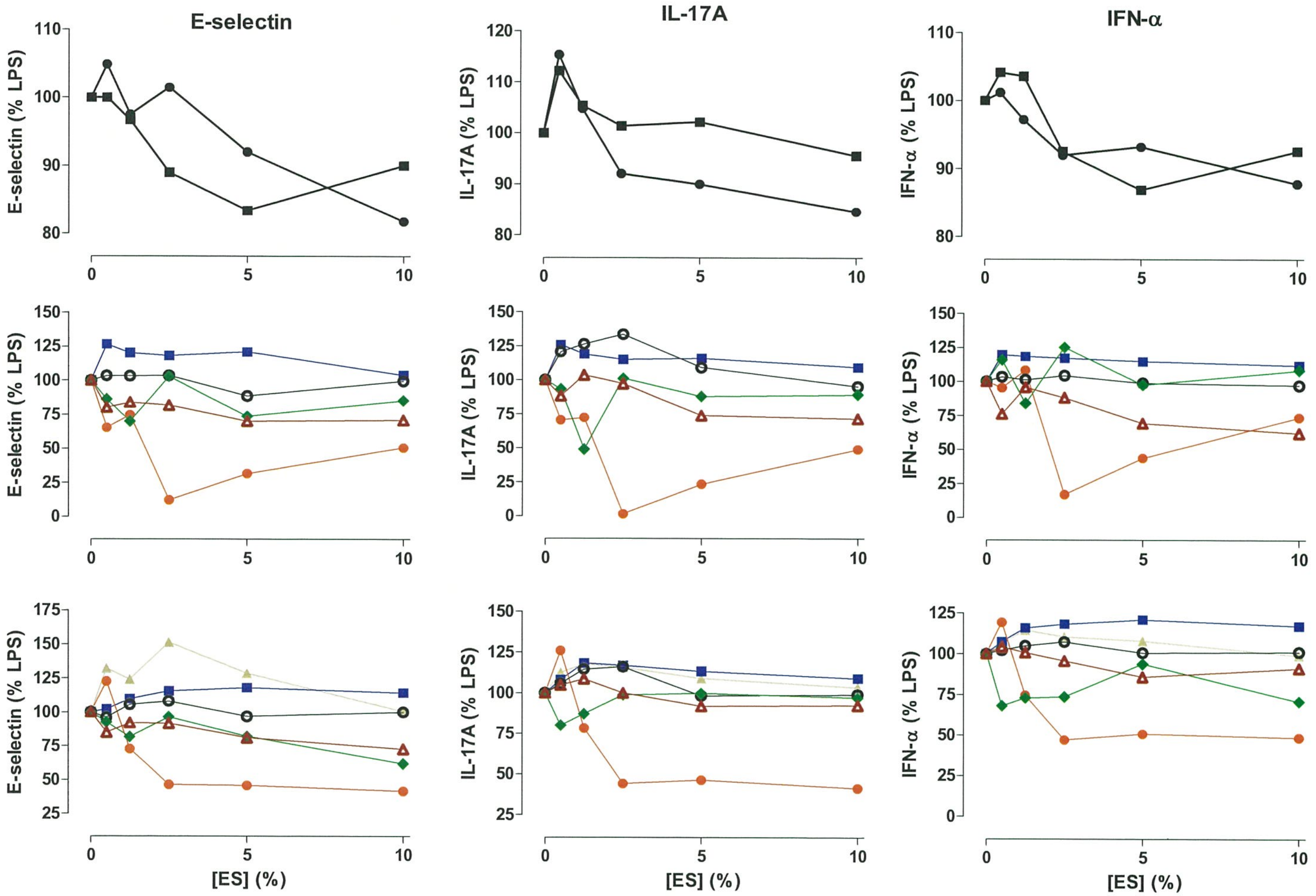


Fig. 3.24. Normalised secretion of E-selectin, IL-17A and IFN-α by mdDCs exposed to 0 - 10% *Hc*ES products and 0 - 30ng/ml of LPS. Top row: group means (*n* = 8); symbols: (●) LPS 30 ng/ml, (■) LPS 10ng/ml. Middle row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 10ng/ml of LPS. Bottom row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 30ng/ml of LPS. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (◆) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a.

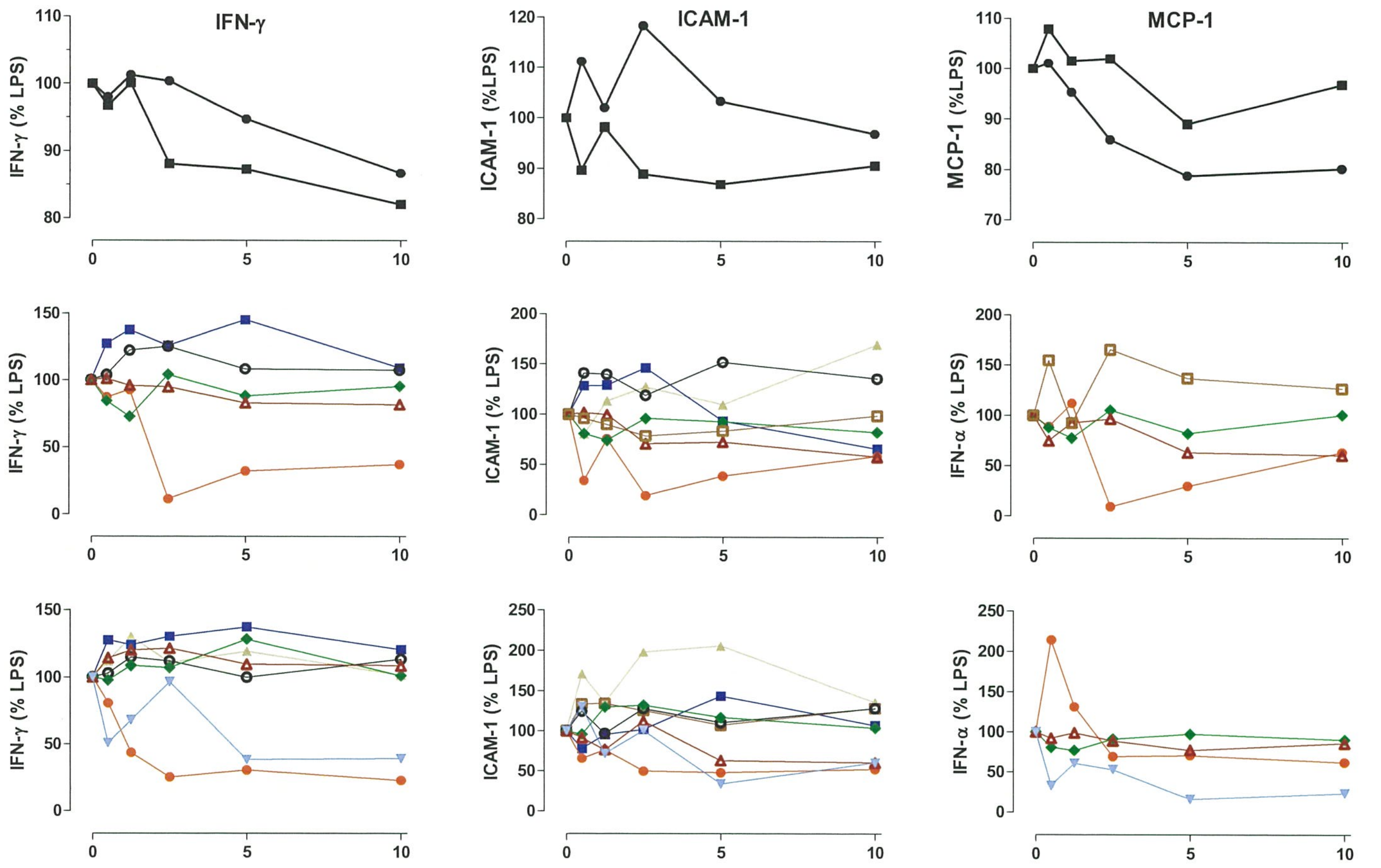


Fig. 3.23. Normalised secretion of IFN-γ, ICAM-1 and MCP-1 by mdDCs exposed to 0 - 10% *Hc*ES products and 0 - 30ng/ml of LPS. Top row: group means ($n = 8$); symbols: (●) LPS 30 ng/ml, (■) LPS 10ng/ml. Middle row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 10ng/ml of LPS. Bottom row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 30ng/ml of LPS. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a.

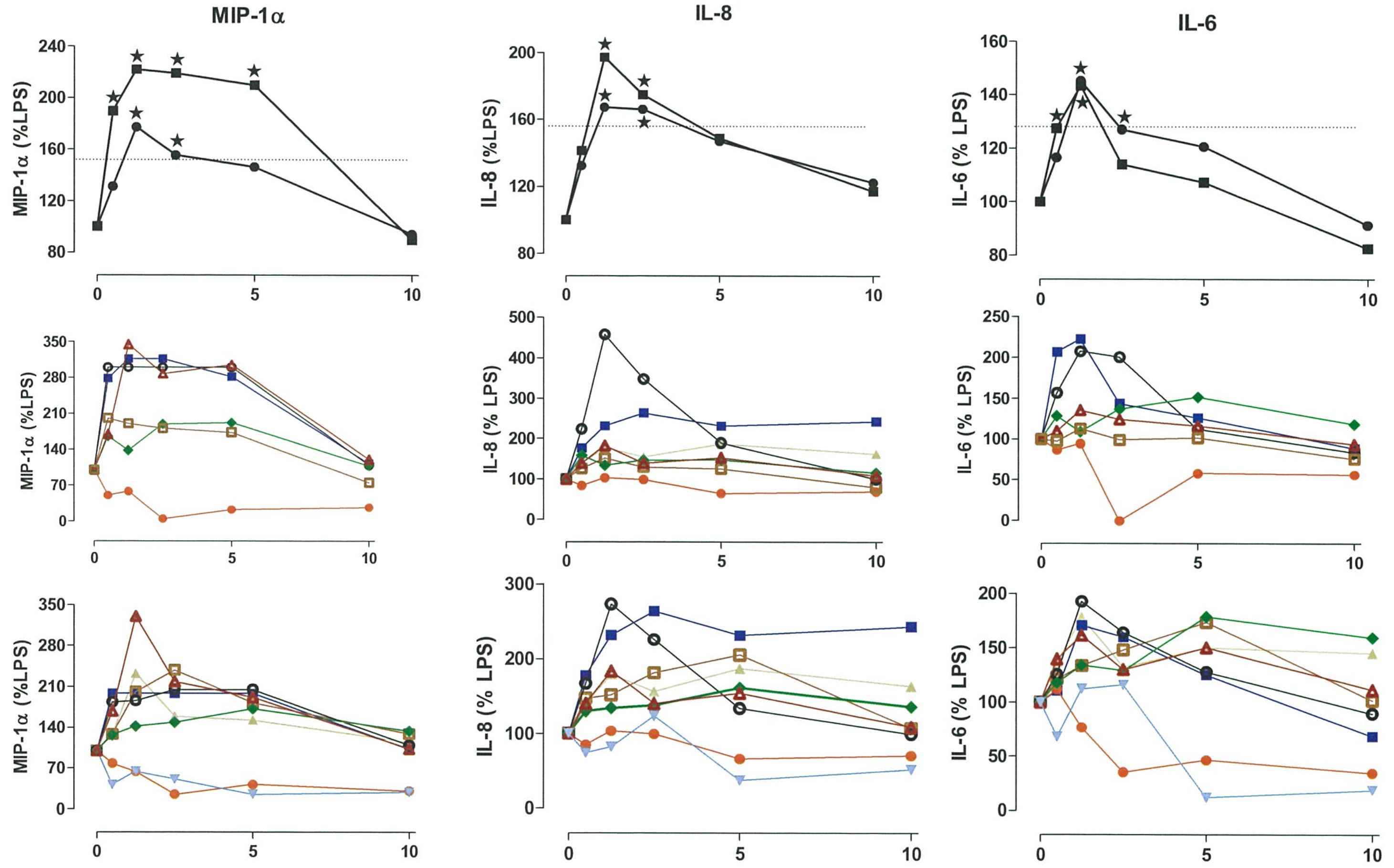


Fig. 3.22. Normalised secretion of MIP-1α, IL-8 and IL-6 by mdDCs exposed to 0 - 10% *Hc*ES products and 0 - 30ng/ml of LPS. Top row: group means (*n* = 8); dotted lines show the 95% confidence intervals calculated by restricted maximum likelihood (REML) analysis. Symbols: (●) LPS 30 ng/ml, (■) LPS 10ng/ml; Middle row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 10ng/ml of LPS; Bottom row: individual donor mdDCs exposed to 0 - 10% *Hc*ES products and 30ng/ml of LPS. Symbols: (●) donor 1, (■) donor 2, (▲) donor 4, (▼) donor 7, (♦) donor 1a, (**O**) donor 2a, (□) donor 4a and (Δ) donor 7a. *: $p < 0.05$.

include 5 data sets in which all TNF-α concentrations were above the maximum assayable level.

3.3.8.2. ES products increased the response to LPS

ES products overall significantly increased the effects of LPS on MIP-1α, IL-8 and IL-6 at lower, but not the highest ES product concentrations (Fig. 3.22). Cells from Donor 1 were different from those of other donors, generally responding to ES products by inhibition of secretion. The largest increases were seen with cells from Donor 2.

3.3.8.3. ES products did not change the response to LPS

ES products did not change the response to LPS for IFN-γ, ICAM-1, MCP-1, E-selectin, and IFN-α (Figs 3.23 - 3.24). MIP-1ß was released in assayable levels for only 3 data sets and could not be analysed. IL-1α was barely measurable in only 2 cases and was considered undetectable, as in all other experiments. MIP-1ß, IL-13 and IL-12p70 were detectable only for a few cells and although were analysed probably did not generate meaningful data for the experiment as a whole (Fig. 3.25).

3.4. DISCUSSION

These experiments have shown that adult *H. contortus* ES products have immunomodulary actions on the DC, as has been observed for many other helminths. Parasites evoke a polarised T_H2 response in the host, combined with the generation of T_{reg} cells and inhibition of T_H1 and T_H17 responses (reviewed in Chapter 1, Section 1.4.2). DCs are required for this process (Balic et al., 2004), augmented by antigen presentation and T cell activation by basophils (Wynn, 2009), although these latter cells do not appear to be essential (Siracusa et al., 2011). In the present study, *H. contortus* chemicals results only in partial maturation of human mdDCs and reduced some aspects of the strong T_H1/T_H2 response evoked by bacterial LPS.

3.4.1. Dendritic cell model

All reported experiments used a classical *in vitro* system consisting of DCs generated from blood monocytes (Bender et al., 1996; Romani et al., 1996; Pernthaner et al., 2012). An unsuccessful attempt was made to develop a similar sheep mdDC model, as this species is a natural host for *H. contortus* and it would have been interesting to compare the responses of sheep and human DCs to worm products. A limited experiment could have been conducted, although fewer ovine cell surface and cytokine assay antibodies are available than for human or mouse cells. In addition, the costs of specific ovine growth factors (GM-CSF and IL-4) and mAb are high.

Human $CD14^+$ monocytes, isolated by magnetic separation using an anti-CD14 mAb directly conjugated to magnetic beads, had a purity of at least 95% (Pernthaner et al., 2012). The same procedure was used to isolate $CD14^+$ monocytes from sheep blood using human CD14 mAb-conjugated beads, which are known to cross react with sheep cells. This resulted in 60 - 80% enrichment of $CD14^+$ cells, of which 40 - 50% were found to be dead. The purity of sheep $CD14^+$ cell populations had to be assessed by indirect staining with an anti-mouse FITC-conjugated antibody, as the human anti-CD14-PE mAb was found not to recognise sheep $CD14^+$ cells. As there were insufficient live $CD14^+$ cells and co-selection for dead cells, generation of DCs from sheep monocytes was abandoned at this stage and only the human monocytes were transformed into DCs by culture with 100ng/ml human GM-CSF and 50ng/ml IL-4 (Pernthaner et al., 2012). Immature DCs were initially verified by the development of dendrites and later confirmed by the expression of CD11c.

Whilst it was disappointing that a sheep DC model could not be used to study immunomodulation by *H. contortus* chemicals, there is interest in the response of human cells to parasite immunomodulators. Clinicians wish to use parasite-derived chemicals, rather than live infections, to treat autoimmune and allergic diseases, such as ulcerative colitis, multiple sclerosis and asthma (Harnett and Harnett, 2010; McSorley and Maizels, 2012).

3.4.2. Effect of ES products on DC surface molecules and cytokine secretion

This appears to be the first report of *H. contortus* ES products modulating the phenotypic characteristics of human mdDCs and is consistent with numerous studies in other helminths. All NS mdDCs expressed all cell surface molecules examined, although CD83 expression was generally low, barely detectable in some cells. Human mdDCs were not fully activated by exposure to *H. contortus* ES products, similar to the reports of “semi-mature” DCs exposed to chemicals of other helminths, including ES products of adult *N. brasiliensis* (Balic et al., 2004), larval *T. spiralis* (Gruden-Movsesijan et al., 2011) and *T. crassiceps* (Terrazas et al., 2011), *A. lumbricoides* pseudocoelomic fluid (Dowling et al., 2011), *F. hepatica* tegumental antigens (Hamilton et al., 2009) and the laminated layer (LL) of larval *E. granulosus* (Casaravilla et al., 2014).

The principal changes in cell surface molecule expression were similar in the two experiments: CD32, CD305, CD40, CD80, HLA-DR and galectin-1 expressions were increased by ES products, whereas CD86 and CD83 were unchanged. Very large responses for individual donor cells and variation between donor cells on different days are likely to influence the group data. The effects of ES products on surface molecule expression by DCs (Figs 3.3 - 3.4) were quantitatively small compared with the effects of LPS (Figs 3.11 - 3.12). Exposure to ES products did not cause additional cytokines/chemokines to be released above those secreted by NS mdDCs, so that only IFN-γ, MCP-1, MIP-1α, MIP-1β, IL-8 and ICAM-1 were consistent detected (Figs 3.16 - 3.17). The very variable individual responses was consistent with the lack of significant effects of ES products overall.

Generally, CarLA prepared from *T. colubriformis* produced similar qualitative changes in surface marker expression to those seen with ES products (Table 3.4, Fig. 3.7). There was no significant change in expression for any surface molecule, although CD40 was approaching a significant increase ($p < 0.10$). Pernthaner et al. (2012) reported that human mdDCs recognise and internalise both the purified and the native form of CarLA, however, this did not result in classical maturation of DC. There was only transient or minor up-regulation of CD86, CD83, HLA-DR and CD40. Exposure of mdDCs to purified CarLA resulted in the increased production of the pro-inflammatory cytokines IL-6 and to a lesser extent of IL-8 and TNF-α, and a reduced production of the anti-inflammatory cytokine IL-1RA. CarLA therefore has little ability to mature and functionally alter mdDC function.

3.4.2.1. Antigen presentation

Overall, the expression of MHCII antigen-presenting molecule HLA-DR was moderately increased, but not all donor cells responded to ES products (Figs 3.1 and 3.3). Increased HLA-DR expression is consistent with translocation of peptide-MHCII complexes to the surface for display, following recognition of ES product antigens by PRRs on DCs (Wang et al., 2009), internalisation and processing (Banchereau et al., 2000). These changes were small compared with the increases produced by LPS (Fig. 3.12). Other helminth products similarly failed to upregulate surface MHCII display to a level sufficient for T cell activation. Adult worm homogenate derived from *H. polygyrus* did not induce MHCII (Segura et al., 2007) and DCs isolated from patients infected with *N. americanus* had reduced expression of HLA-ABC and HLA-DR (Fujiwara et al., 2009).

3.4.2.2. Co-stimulatory molecules

The weak up-regulation of co-stimulatory molecules is consistent with only a semi-mature status. Semi-mature DCs lacking high expression of co-stimulatory molecules are tolerance inducing DCs inducing T_{regs}, unlike the fully mature DCs which activate T and B cells (Lutz and Schuler, 2002; Frick et al., 2010). Human CD83, CD40, CD80, CD86 and HLA class I and II molecules are markers for maturation of mdDCs generated in the presence of GM-CSF and IL-4 (Banchereau et al., 2000) into mature DCs, which direct naïve T cells to differentiate into T_H1 or T_H2 cells (Moser and Murphy, 2000; Tan and O'Neill, 2005; Hu et al., 2009).

CD83 expression on DCs activates the DCs and provides a co-stimulatory signal for naïve and memory T cells (Aerts-Toegaert et al., 2007; Breloer and Fleischer, 2008). CD80 and CD86 are co-stimulatory molecules on DCs which regulate T cell activation. Ligation of CD40 upregulates expression of CD80, CD83 and CD86 (Caux et al., 1994) and secretion of pro-inflammatory cytokines (Cella et al., 1996). The CD80/CD86/CD28/CD152 system has an important role in the regulation of T cell activation by DCs, as well as induction of tolerance.

H. contortus ES products did not increase surface expressions of all of the co-stimulatory molecules. Overall, CD40 (and HLA-DR) were significantly increased by ES products (Figs 3.2 and 3.3). CD80 was increased for all donor cells in Expt #1 (Fig. 3.1), but only tended to increase in Expt #2 (Fig. 3.4). CD86 and CD83 were unchanged.

Many helminth products also do not induce full maturation of DCs, with little or no increase in expression of the co-stimulatory molecules, although differences exist in the specific responses, not only between species, but also in response to similar ES products. In the present experiment, this was also the case for mdDCs cultured from the blood of different donors.

Expressions of CD40, CD80 CD86 were unchanged in the presence of ES products or adult worm homogenate of *H. polygyrus* (Segura et al., 2007). CD86 and HLA-DR expressions were reduced in DCs isolated from humans infected with *N. americanus* (Fujiwara et al., 2009). ES products of *N. brasiliensis* induced increased CD86 expression but little change in CD80 and MHCII in mouse DCs (Balic et al., 2004). Unicellular parasites have similar effects: bovine mdDCs stimulated with *Giardia duodenalis* showed little or no up-regulation of CD40 and CD80, although there was increased expression of MHCII (Grit et al., 2014).

3.4.2.3. Regulatory molecules

The immune response is determined by the balance of positive and negative signals, thus inhibitory receptors play an important role in limiting inflammation, inducing tolerance and preventing autoimmunity (reviewed by de la Fuente et al., 2012). Cell surface receptors, cytokines and other soluble proteins and signalling molecules interact to regulate immune responses. These mechanisms include the CD80/CD86/CD28/CD152 system described above for regulation of T cell activation by DCs, as well as induction of tolerance via galectin-1, CD305 and CD32 expressions.

Surface galectin-1 expression was increased by *H. contortus* ES products (Fig. 3.5), whereas cellular galectin-1 (surface and intracellular) was reduced (Fig. 3.6), due to either or both reduced gene expression and increased relocation to the extracellular compartment. Galectins are expressed both within the cell and extracellularly, where they bind to surface receptors (Rabinovich and Toscano, 2009). The extracellular form has several immunomodulatory actions as well as promoting cell-cell adhesion. It causes apoptosis of pro-inflammatory T cells (Deák et al., 2015) and stimulates T_{reg} differentiation and IL-10 secretion (Camby et al., 2006; Cedeno-Laurent and Dimitroff, 2012).

Surface CD32 was increased by *H. contortus* ES products (Figs 3.1 and 3.3). Human mdDCs express both CD32A and CD32B, two isoforms which are activating and inhibitory respectively (Boruchov et al., 2005). As the antibody used in the present experiments does not distinguish between CD32A and CD32B, it was not possible to determine which isoform was up-regulated. As other surface markers indicated that these mdDCs were semi-mature, not fully activated, CD32B is possibly the isoform involved. Activation of CD32A by binding of immune complexes is inhibited by binding of complexes to CD32B, which acts as a regulator of the resulting inflammation (Van Montfoort et al., 2012).

Increased expression of surface CD305 (Figs 3.1 and 3.3) is consistent with development of more tolerogenic DCs in the presence of worm chemicals. CD305 down-regulates the activity of these cells (Lebbink and Meyaard, 2007; Meyaard, 2008) and also regulates the differentiation of blood monocytes to mdDCs by GM-CSF (Poggi et al., 1998).

3.4.2.4. Cytokines and chemokines

Immature NS mdDCs of at least 3 of the 4 donors expressed only IFN-γ, MCP-1, MIP-1α, MIP-1β, IL-8 and ICAM-1 and subsequent exposure to ES products did not cause any additional cytokines/chemokines to be released. There were marked differences between the cells of the 4 donors, particularly cells of Donor 4, which produced almost no cytokines/chemokines. Donor 2 mdDCs secreted the highest concentrations, including the maximum assayable concentration of MCP-1, and more often increased secretion on exposure to ES products. The very variable individual responses were consistent with the lack of significant group effects of ES products overall. In most cases, there was little change (or a tendency to decrease) in pro-inflammatory cytokine/chemokine release for individual mdDCs after exposure of mdDCs to *H. contortus* ES products, suggesting lack of activation. In addition, the pro-inflammatory cytokines IL-6, IP-10 and TNF-α were not detected, nor was there secretion of IL-12p70, which is released by mature TLR-activated DCs and drives a T_H1 response (Kapsenberg, 2003; Lichtenegger et al., 2012).

Cells of Donor 2 were generally activated by parasite chemicals, unlike the other donor cells. Unfortunately, the cytokine responses to ES products and CarLA cannot be compared for this donor, as no cytokines were measured in the experiment where CarLA was tested. Interestingly, Donor 2 cells were one of two populations which took up very little CarLA from medium, unlike cells from other donors (Fig. 3.8). Pernthaner et al. (2012) observed that purified CarLA increased production of the pro-inflammatory cytokines IL-6 and to a lesser extent IL-8 and TNF-α, and a reduced production of the anti-inflammatory IL-1RA from immature mdDCs.

Reductions or lack of change for three of the donor cells are consistent with failure of DCs to be fully activated. Parasites and their products generally down-regulate T_H1 responses by inhibiting IFN-γ (Uchikawa et al., 2000) and IL-12p70 production (Balic et al., 2004; Maizels et al., 2004) and inducing tolerogenic responses (reviewed by van Riet et al., 2007; White and Artavanis-Tsakonas, 2012). Similar to the effects of *H. contortus* ES products, adult worm homogenate and ES products of *H. polygyrus* did not induce DC maturation or cytokine production, with no change in expressions of CD40, CD54, CD80, CD86 and MHCII and production of IL-12p40, IL-12p70, IL-10 and TNF-α (Segura et al., 2007). Protozoa also induce semi-mature DCs: bovine mdDCs stimulated with *G. duodenalis* showed increase expression of MHCII, but no or little upregulation of the maturation markers CD40 and CD80, although these cells induced proliferation of T cells secreting TNF-α, TGF-β, IL-4 and IL-10 (Grit et al., 2014).

3.4.3. Immunomodulation by ES products of the response to LPS

3.4.3.1. Exposure to LPS

LPS was strongly stimulatory for surface molecule expression and release of many cytokines/chemokines. Quantitative variation in expression of surface markers by mdDCs of different donors was prominent, with cells of Donor 2 generally less responsive to LPS than mdDCs of other donors. For most cytokine/chemokines, the differences in response to 10 and 30ng/ml LPS were small, the main exception being IL-10. Gram-negative bacterial LPS ligates CD14 on the surface of DCs, initiating pro-inflammatory processes through TLR-4 signalling; co-stimulatory molecules are up-regulated and high levels of the cytokines TNF-α, Il-6, Il-8 and IL-12 are secreted (Verhasselt et al., 1997; Banchereau et al., 2000).

The effects on DCs in the present experiment generally were in agreement with reported studies. There were large increases in surface expression of MHCII and co-stimulatory molecules (CD80, CD86, CD40, HLA-DR and CD83), but there were less consistent effects on the regulatory molecules CD305 and CD32. Individual donor variation was prominent, several cell populations showing reduced or unchanged CD305 expression. CD32 expression decreased significantly with both 10 and 30ng/ml LPS (Fig. 3.12), but was unchanged with 100ng/ml LPS (Fig. 3.9). LPS greatly increased secretion of 11 cytokines/chemokines (Figs 3.18 - 3.20) and induced the secretion of many not secreted by NS cells or mdDCs exposed to ES products. Release of TNF-α, MCP-1 and MIP-1ß was very high in most cases, however, there was no measurable release of IL-12p70 or IFN-γ.

OVA was a weaker stimulant than LPS on surface molecules, significantly increasing only the expression of CD86 ($p < 0.05$), while the increases in HLA-DR and CD40 were approaching significance ($p < 0.10$). CD32 expression was below NS for the group and for 7 of the 8 individual mdDCs. As this batch of OVA was later found to contain LPS (Dr A. Pernthaner, pers. comm.), the response to OVA may have been due to contaminating LPS.

3.4.3.2. Co-stimulation with ES products and LPS

H. contortus ES products down-regulated cytokine/chemokine release by LPS-stimulated mdDCs, but had no significant group effects on the expression of surface molecules (Figs 3.7 - 3.9). The low ES concentrations further increased MIP-1α, IL-8 and IL-6 secretion produced by LPS alone, whereas this was reversed as the ES concentration increased to 10% (Fig. 3. 22). Other cytokine release was significantly reduced (Fig. 3.21) or unchanged (Fig. 3.23 - 3.24). The highly individual responses, large in some cases, biased the group data and there were even opposite effects on individual cells, particularly for LPS 30ng/ml. Cells from Donor 1 were different from those of other donors, generally responding to co-stimulation by inhibition of secretion, whereas the largest increases were seen with cells from Donor 2.

There are many reports of helminth products modulating immune responses of DCs to LPS and inducing a less inflammatory and more tolerogenic environment, although the particular surface molecules and cytokines affected vary between studies. ES products of *H. polygyrus* inhibited TLR-ligand induced DC activation, seen as reductions in CD40, CD86 and MHC II expressions and secretion of IL-12p70, IL-10 and TNF-α (Segura et al., 2007). *T. suis* soluble products suppressed LPS-induced secretion by human mdDCs of pro-inflammatory cytokines/chemokines, including IL-12, TNF-α and IL-6 (Klaver et al., 2013). Larval *E. granulosus* LL potentiated the up-regulation by 10ng/ml *E. coli* LPS of surface CD86 and CD80 and secretion of IL-10 by bone marrow-derived DCs (BMDCs), while inhibiting up-regulation of CD40 and IL-12 secretion; MHCII expression was not altered (Casaravilla et al., 2014). Protozoa similarly induced semi-maturation of DCs, e.g. *T. cruzi* trypomastigotes reduced IL-12, TNF-α, and IL-6 secretion as well as the expression of the surface markers HLA-DR and CD40 by LPS-activated DCs (Van Overtvelt et al., 1999).

3.4.4. Individual donor responses

The most marked feature of these experiments was the highly individual responses to parasite chemicals of mdDCs cultured from monocytes of different human donors and on different days. Variability in surface molecule expression and cytokine release was already apparent in immature NS mdDCs and responses were not uniform following exposure to parasite products and LPS. MdDCs of the four donors (1, 2, 4 and 7) studied at least twice each, showed unusual features, however, no consistent relationships between NS expression and responses to ES products or co-stimulation with LPS were apparent.

Each culture of mdDCs had individual characteristics and sometimes two cultures from the same donor did not respond similarly to stimulants. In general, there were smaller differences in responses by cells from the same donor than between cells from different donors. Before stimulation, immature Donor 7 mdDCs released almost no cytokines in contrast to Donor 2 cells, which had high expression of surface molecules, cellular galectin-1 and secretion of cytokines. Donor 1 NS cells had low expression of some surface molecules, but average cytokine release. CarLA uptake was very high for Donor 1 cells, but CarLA did not stimulate increased expression of surface molecules, in contrast to Donor 2 cells, which showed very low CarLA uptake, but had high responsiveness of expression of surface molecules and cytokine secretion to ES products. LPS alone and co-stimulation with LPS plus ES products had lesser stimulatory effects on surface molecules of Donor 2 cells than others, but induced large increases in many cytokines. Donor 4 NS cells had average expression of surface molecules and almost no cytokines/chemokines were secreted by NS or ES-stimulated cells.

Whilst this variation between individual cultures made it difficult to obtain statistically significant group effects and reach generalised conclusions about the effects of treatments, studying human cells from donors of different ethnic backgrounds has highlighted the differences between experiments performed on inbred mice and on volunteer human subjects. Whereas studies on closely genetically related animals may show clear effects of specific treatments, these may be applicable only to a restricted population.

Variation in responses of DCs from different donors is not surprising, as individual cells in an apparently uniform culture differ in mRNA and transcript levels in response to identical stimulation (Shalek et al., 2013, 2014). In the former experiment, 18 single mouse BMDCs cells in a culture were collected after 4 hours of exposure to LPS, whereas in the latter experiment, 1700 single cells were analysed at different time points during exposure to a variety of stimuli. In part, heterogeneity reflected the degree of maturity of the cell and partly differences in regulatory circuits activated. There is likely to be a genetic component to DC variability, perhaps related to expression of surface molecules, receptors and secreted cytokines and chemokines. Conti et al. (2008) found that peripheral blood monocytes from different donors varied in the density of GM-CSF receptors, and although this did not affect differentiation into mdDCs driven by IL-4 and GM-CSF, it did in the absence of IL-4. There could also be differences in the cultures prepared on different days, either due to small variations in culture conditions or according to the exposure of the monocytes to stimuli from infectious agents or inflammatory conditions before their collection, culture and differentiation into DCs.

The remarkable variation in response of human mdDCs to parasite chemicals in this study suggests that clinical treatments based on helminth immunomodulators may need careful evaluation. Maizels and Gause (2014) have recently highlighted the complexity of such immunotherapy, as helminth infection and a T_H2 environment can allow latent viral infections to be reactivated. Some patients may not respond as predicted to worm therapy and either adverse reactions or failures in the treatment could occur. Whilst no clear pattern emerged from the four cell donor in the main part of the study, it was clear that one donor was more responsive to *H. contortus* ES products and less so to LPS than the other three cell populations, particularly on one of the two days. This particular donor is usually resident in a South Asian country and has experienced infections with helminths and malaria, which may be related to his response to ES products. No distinctive features of the other donors were apparent, apart from Donor 1 having a rare blood group (AB negative). If similar variations in response to worm chemicals occur in sheep, this may prove in the future to have a bearing on susceptibility or resistance to helminths.

These experiments have shown that *H. contortus* ES products alone only partly activated mdDCs and that co-stimulation of mdDCs with ES products and LPS resulted in an attenuated LPS response. The semi-mature state of DCs following *in vivo* parasite infection or *in vitro* ES product-stimulation is not usually adequate for full activation of the adaptive immune system, with T and B cell activation and proliferation and antibody secretion. The experiment reported in Chapter 4 investigated whether cells cultured from the lymph nodes of sheep with a range of parasite burdens had reduced proliferative capacity. This would be evidence of immunomodulation *in vivo* by *H. contortus*.

Chapter 4

IN VITRO PROLIFERATION OF LYMPH NODE CELLS FROM SHEEP INFECTED WITH *H. CONTORTUS*

4.1. INTRODUCTION

H. contortus ES products only partly activated human mdDCs *in vitro* and reduced LPS-stimulation of mdDCs (Chapter 3), consistent with the failure of helminth infections to induce classical maturation of DCs, up-regulation of their co-receptors and release of pro-inflammatory cytokines. Helminths and their ES products instead provoke and then down-modulate the T_H2 response, resulting in DCs which induce both tolerance and inflammation (reviewed by Maizels et al., 2004; White and Artavanis-Tsakonas, 2012). This has down-stream effects on the adaptive immune system, proliferation of lymphocytes and antibody production in lymph nodes.

4.1.1. Immune response to helminths

Haemonchosis, like other helminth infections causes an initial T_H1, followed by a T_H2-polarised immune response (Gill et al., 2000; Kabagambe et al., 2000; Terefe et al., 2007) and T cell hypo-responsiveness (Maizels et al., 1993; Ricci et al., 2011; Mkhize-Kwitshana et al., 2014). DCs recognise parasite antigens, internalise and metabolise them, and display peptide-MHCII complexes on their surface (Banchereau et al., 2000) (reviewed in Chapter 1, 1.4.3). Mature DCs also have upregulated expression of co-stimulatory and immune regulatory molecules, which direct naïve T cells to

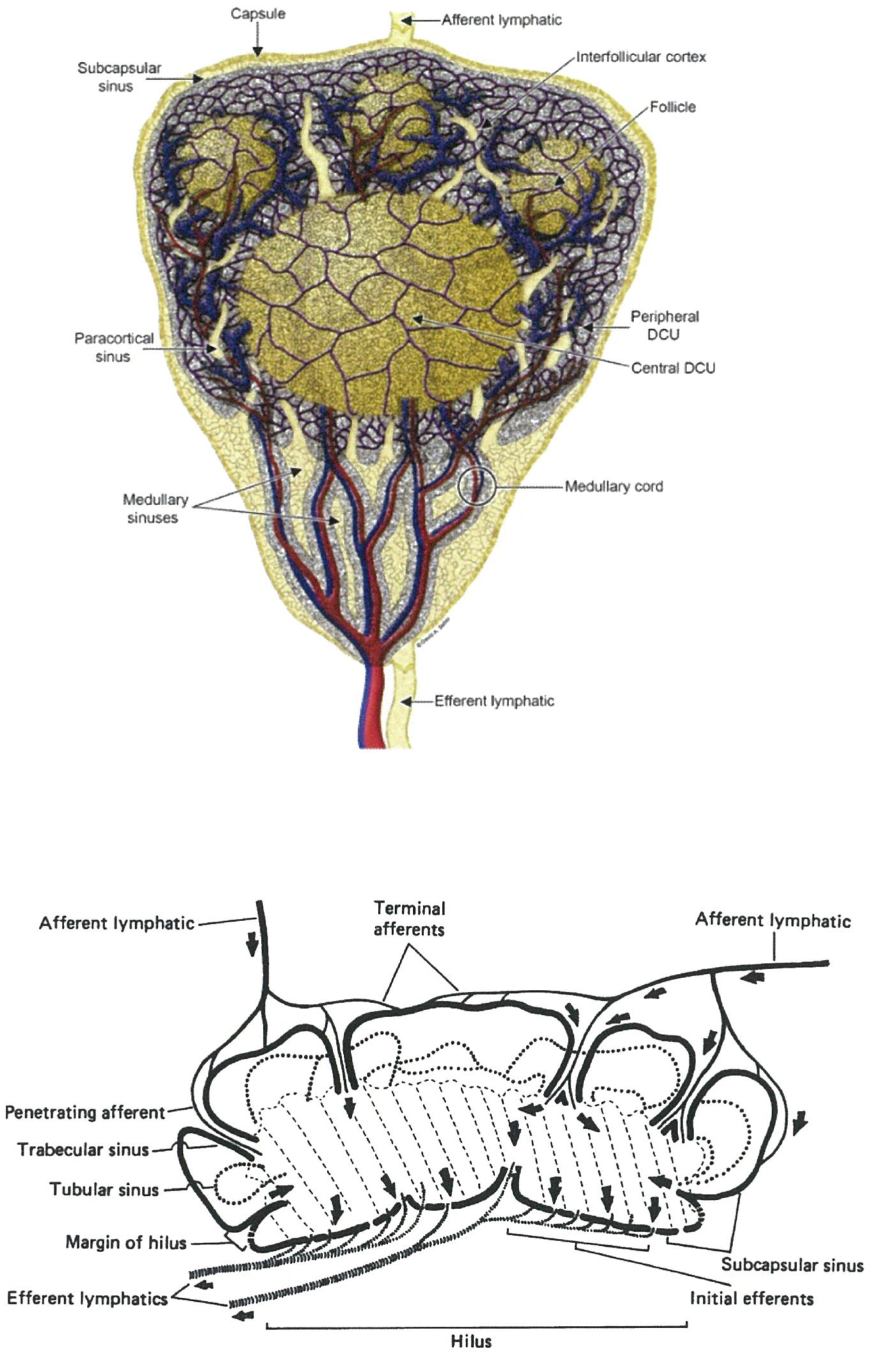


Fig. 4.1. Lymph node structure. Top: generalised mammalian node containing a single lymphatic nodule (Willard-Mack, 2006). DCU: deep cortical unit. Bottom: Sheep ileofemoral lymph node, showing species differences in lymphatic vessel pathways (Heath and Spalding, 1987).

differentiate into T_H2, not T_H1 cells, and release the appropriate cytokines and chemokines. Helminth activation of PRRs on DCs is essential to down-regulate T_H1 responses and induce $CD4^+$ T_H2 cells which are required to control (Vignali et al., 1989) or clear (Katona et al., 1988) parasite infections.

4.1.2. Lymph nodes

Lymph nodes, where APC and naïve lymphocytes interact to produce antibodies, are made up of one or more lobules, surrounded by a capsule (Willard-Mack, 2006). Fig. 4.1 shows the generalised structure and differences in afferent and efferent vessel pathways in sheep nodes. Lymph containing Ags, DCs and cytokines/chemokine from infected tissues enters in afferent lymphatics, flows through the sinuses around the lobules and leaves in the single efferent lymphatic duct. As each lobule drains a different drainage field and could be exposed to different Ags, APCs and chemicals, they can have different immunological activities, sizes and appearances.

The lobules and sinuses are filled with a network of fibroblastic reticular cells forming channels through which the lymphocytes migrate. Clusters of macrophages (sinus histiocytes) adhere to the reticular network in the sinuses, where they remove cell debris, red cells, bacteria and particles. The macrophage population increases with a greater need to remove particulate matter. Some mast cells also migrate in from tissues. Cells, lymph and particulate matter cannot passively move into the lobules through the surrounding membrane, but actively migrate into the lobules.

Lymphocytes enter the lymph node in the afferent lymphatic duct, fill the lobules and constantly migrate into the lobule through high endothelial venules. They leave via the paracortical sinuses, then exit the lymph node in the efferent lymphatic to recirculate to other nodes and organs. The iDCs in the tissues take up antigen, migrate to the lymph node as they mature and lose the ability to take up antigen, and present it to T cells in the in the paracortex of the lobule (Chapter 1, 1.4.3.2). There are also follicular DCs resident in the lymphatic follicles which present antigen to the B cells, which synthesise antibodies.

4.1.3. Antibody production in lymph nodes

The T_H2 response evokes production of IgA, IgG and IgE (Smith, 1988; Schallig, 2000; Li et al., 2012). IgA and IgG anti-parasite antibodies are associated with resistance to *H. contortus* (Schallig, 2000) and secretory IgA to *T. circumcincta* infection (Smith et al., 1985; Strain et al., 2002). DC migration to the draining lymph nodes is greatly increased during inflammation (reviewed by Merad et al., 2013) (Chapter 1, 1.4.4). In addition, antigens <70Kd in size are taken up into lymph and carried to the regional nodes, where antigen can be directly taken up by the DCs residing in the node.

iDCs mature while migrating to the lymph node paracortical areas, where they activate naïve and antigen-specific T and B cells (Banchereau and Steinman, 1998; Dubois et al., 1999). A subset of DCs becomes the interdigitating DC, which stimulate antigen-specific naïve T cells to proliferate, differentiate, secrete cytokines and express the CD40 ligand. Memory T cells allow a rapid response to the presence of a previously encountered antigen.

Activated T cells interact with $CD40^+$ B cells, first inducing short-lived plasmablasts that secrete IgM. With continuing activation, there is class-switching, so that longer-lived plasma cells secrete other sub-classes of antibodies (reviewed by Nutt et al., 2015). The first interaction is extra-follicular. The more potent and specific antibody production results from activated B cells re-entering the B cell follicular area, interacting with the T follicular helper cells (T_{HM}) and proliferating extensively to form germinal centres which produce high-affinity antibodies and also memory B cells (De Silva and Klein, 2015; Kurosaki et al., 2015).

4.1.4. Immunosuppression of lymphocytes

T cell hypo-responsiveness is a feature of chronic helminth infection (Maizels et al., 1993; Ricci et al., 2011; Mkhize-Kwitshana et al., 2014). The semi-maturity of DCs following *in vivo* parasite infection or *in vitro* ES product-stimulation promotes the expansion of $Foxp3^+$ T_{regs} and is not usually adequate for full T and B cell activation, proliferation and antibody secretion. CD4 T_{regs} expressing $Foxp3^+$ limit tissue damage and are exploited by numerous helminths as an immune evasion strategy (reviewed by (van Riet et al., 2007; Hewitson et al., 2009; Geiger and Tauro, 2012; McSorley and Maizels, 2012; Finlay et al., 2014) (Maizels et al., 2012) (Chapter 1, 1.5.1.3). Reduced T cell activation has often been indirectly deduced from studies of changes in DC or macrophage phenotype, in which there is suppression of co-stimulatory molecules or release of anti-inflammatory cytokines. However, there are experiments which directly established the inhibitory activity of worm chemicals on proliferation of either blood or lymph node lymphocytes *in vitro*.

4.1.5. *In vitro* lymphocyte proliferation assays

A mouse or guinea pig local lymph node assay (LLNA) was developed as a screening test for contact hypersensitivity reactions (Kashima et al., 1996; Basketter et al., 2007). This test used lymph nodes draining distinct skin areas sensitised to chemicals and has been extended to studies of immune responses to such compounds (van den Berg et al., 2005). It has been shown that a skin sensitiser can also have systemic effects, such as inducing proliferation in the spleen, as well as the local lymph nodes (Chipinda et al., 2009).

The proliferative ability of immune cells has been frequently studied in cultures in the presence of a mitogen, such as concancavilin A (ConA), phytohaemagglutinin (PHA), LPS or a specific antigen. The measure of proliferation is the Stimulation Index (SI), derived from the rate of incorporation of ^{3}H-thymidine by dividing cells. The cells of choice are most often blood lymphocytes (Zimmerman et al., 1983; Turner et al., 1985; Woldehiwet, 1987; Haig et al., 1989), although similar methods have been used for cells collected from the spleen (Barriga, 1980), mammary glands (Kensinger et al., 1990) or lymph nodes (usually considered to be lymphocytes) (Ellis and DeMartini, 1985; Pernthaner et al., 2005a,b).

4.1.6. Mitogen-induced lymphocyte proliferation

The proliferative responses to mitogens are generally depressed in lymphocytes collected from animals infected with viruses, bacteria and parasites, although the mechanisms involved may differ for intracellular and extracellular organisms. The peripheral blood mononuclear cells (PBMC) of sheep infected with the Rickettsia *Ehrlichia phagocytophila* had reduced proliferative responses to mitogens, including ConA, PHA and LPS, although they were able to proliferate in the presence of live or heat-inactivated organisms (SI of 10 - 12) (Gokce and Woldehiwet, 1999). The lymphocytes of lambs infected with bovine respiratory syncytial virus (BRSV) had reduced proliferative responses to PHA (Sharma and Woldehiwet, 1991) and live or inactivated BRSV inhibited PHA-induced proliferation of healthy ovine lymphocytes *in vitro* (Keles et al., 1998); this was not due to release of prostaglandins or inhibitors of IL-1 or IL-2. Similarly, respiratory syncytial virus inhibited the PHA-induced blastogenic response of human cord blood lymphocytes, which released a soluble inhibitor into the medium (Preston et al., 1992).

Many helminths are also capable of inhibiting *in vitro* proliferation of lymphocytes collected either from blood or lymph nodes. Two such studies are one in *H. polygyrus*-infected mice, in which an IgE-reactive fraction of both ES products and worm extract of *H. polygyrus* was inhibitory to antigen-stimulated lymph node proliferation (Rzepecka et al., 2006), and another in *F. hepatica*-infected lambs, in which the responses of PBMC to ConA were suppressed (Zimmerman et al., 1983). *H. contortus* antigens are similarly capable of suppressing T cell proliferation: ConA-induced T cell proliferation was decreased by *H. contortus* male worm galectin Hco-gal-m (Wang et al., 2014a), by a 66 kDa adult *H. cont*ortus ES antigen (Rathore et al., 2006) and by *H. contortus* larval antigens (Gill et al., 1991).

The blastogenic activity of lymphocytes appears to be influenced by the susceptibility of the host to parasitism. Unstimulated lymphocytes from resistant sheep had greater blastogenic activity than those from susceptible sheep at the time of maximal activity three weeks after infection with *T. colubriformis*, however, lymphocytes from the susceptible sheep responded more strongly to ConA (Pernthaner et al., 1995), as did PBMC from resistant compared with random-bred lambs stimulated with larval or adult *H. contortus* antigen (Gill, 1994). Vaccination-induced immunity may also influence lymphocyte proliferative ability. Torgerson and Lloyd (1993b) observed a greater proliferative response to *H. contortus* antigen of PBMC from vaccinated and naïve sheep than from infected sheep.

In the present experiments, there was evidence of immunomodulation *in vivo* in the lymph nodes collected from sheep in two trials given different levels of protection by vaccination with four and six different combinations of *H. contortus* antigens respectively. *In vitro* proliferation of cells cultured from the lymph nodes in response to ConA or adult *H. contortus* ES products showed lymphocyte suppression was greatest in unprotected sheep and that blastogenic activity tended to recover in vaccinated sheep with the lowest worm burdens.

4.2. MATERIALS AND METHODS

4.2.1. Experimental overview

Abomasal lymph nodes were collected from sheep in two vaccination trials against *H. contortus*, performed by the Parasitology team at AgResearch Ltd, Palmerston North. Animal experiments in this trial were carried out in accordance with the requirements of AgResearch animal ethics standards (Grasslands Animal Ethics Committee approvals #12944 and #13262). Parasitological data were provided by AgResearch Ltd.

Cells harvested from the lymph nodes were cultured in 96 well plates and stimulated with 0 - 10% adult *H. contortus* ES products (*Hc*ES) or 2.5µg/ml ConA. The lymph node cell SI was calculated and secretion of IFN-γ and IL-4 into the incubation medium were measured.

4.2.2. Vaccination Trial #1

Groups of 9 sheep were vaccinated with 4 different combinations of recombinant *H. contortus* proteins (Groups A1 - A4); group A4 was reduced to $n = 8$ during the trial. The control groups were: non-infected (NI), infected (INF) and infected and injected with adjuvant (ADJ).

Romney cross sheep 3 months-of-age were drenched with 1ml/5kg of Matrix (Merial, New Zealand) and grazed on pasture that had not been grazed by sheep for more than 3 years. The sheep were vaccinated twice (on Weeks 1 and 4) with the appropriate antigen combination. The sheep were drenched on Week 6, because of low levels of natural infection with the cattle parasite *Cooperia* sp. On Week 7, all groups except group NI were challenged with a total of 8000 *H. contortus* L_3 given in equal doses on each of 3 successive days.

The levels of protection against *H. contortus* were assessed on Weeks 2 - 8 p.i. by FEC, using a modified McMaster technique (Waghorn et al., 2014) and worm counts were performed at necropsy.

4.2.3. Vaccination Trial #2

Groups of 8 sheep were vaccinated with 6 different combinations of recombinant *H. contortus* proteins (Groups B1 - B6), but group B5 was reduced to $n = 7$ during the trial. The control groups were: non-infected (NI) ($n = 4$) and infected and injected with adjuvant (ADJ).

Romney cross sheep 8 months-of-age were drenched before the trial, vaccinated and challenged on Week 7, as in Trial #1. The levels of protection against *H. contortus* were assessed on Weeks 2 - 11 p.i. by FEC and worm counts were performed at necropsy.

4.2.4. Culture of lymph node cells

Animals were humanely killed and exsanguinated. The abomasal lymph nodes were excised immediately after death and put into 5ml ice-cold PBS. Any attached tissue was removed carefully from each lymph node and a 1mm thick cross sectional slice was collected and dissected into small pieces in PBS. Single cell suspensions were prepared by repeated pipetting followed by passing the cell suspensions through a cell strainer. The suspensions were washed twice in PBS-EDTA (Appendix 6.2.4) by centrifugation at 400*g* for 4min. Cells from each lymph node were counted and appropriate volumes of the cell suspension were added to ensure equal densities of cells (approximately 1×10^6 per ml) for each node preparation.

4.2.5. Stimulation with ES products or ConA

200µl aliquots of cells were seeded into 96 well culture plates at a concentration of 1×10^6 per ml in RPMI cell culture medium (Sigma-Aldrich) enriched with 5% FBS (Life Technologies), 1% antibiotic solution (Pen Strep, Life Technologies, USA), 1% sodium pyruvate and 1% glutamax (Life Technologies). Separate plates were similarly set up for the collection of supernatants for measuring secreted IFN-γ and IL-4. For the cell proliferation assay, all treatments were performed in triplicate, but a single well for the cytokine assay plates. Each assay contained control wells of NS cells. Cells were cultured at 37°C in 5% CO_2 in air for 6 days, either in the presence of 2.5µg/ml ConA (Sigma-Aldrich) or *Hc*ES to make final concentrations of 1.25%, 2.5%, 5% and 10% in a final volume of 30µl cell culture medium. ES products were collected, as described in Chapter 2, 1.2.1.3. The 2 - 4h *Hc*ES preparation was used for cells from Trial #1 and the 1 - 2h *Hc*ES collection for Trial #2.

For the cell proliferation assay plates, on Day 5, 0.5µCi of methyl-^{3}H thymidine (Perkin Elmer, USA) was added to each well and the plates incubated overnight at 37°C. The plates were then frozen at –80°C until cell harvesting. For the cytokine assay plates, on Day 6, 100µl of each supernatant was collected and frozen at –80°C until assayed.

4.2.6. Stimulation index

Frozen cells in the proliferation assay plates were thawed at 37°C and harvested on Filtermat B (PerkinElmer, USA), using Harvester96 Mach III (Tomtec, USA) as described in Appendix 6.1. Radioactivity was detected with a Microbeta Trilux 1450 (PerkinElmer, USA) and recorded as counts per minute (CPM). The SI was calculated as the mean CPM of stimulated cells, divided by the mean CPM of non-stimulated cells.

4.2.7. IFN-γ and IL-4 assays

Similar sandwich ELISA assays were used to measure the two cytokines in the incubation media. An ELISA-based bovine IFN-γ specific ELISA assay kit (MCA5638KZZ, AbD Serotec, UK) and IL-4 reagents (AbD Serotec, UK) were used according to the manufacturer's recommendations. Nunc-immuno Microwell 96 well flat bottom plates (Nunc, Denmark) were used for the assays. Plates were sealed with adhesive plate sealers during all incubations.

4.2.7.1. IFN-γ

Coating antibody was diluted 1:200 in ELISA buffer (Appendix 6.2.1) and 50µl was pipetted into the wells. The plates were incubated at 4°C overnight and then washed 3 times with wash buffer (0.2M NaCl, 0.05% Tween-20 in distilled water). Blocking buffer (4% BSA in PBS) 100µl per well was added and incubated for 1h at room temperature. After incubation, the plates were washed 3 times with wash buffer.

Lyophilised IFN-γ standard was reconstituted by adding 6245µl distilled water and standards from 0.025 to 50ng/ml were prepared. Stored supernatants were thawed at room temperature. 50µl of samples and standards were placed in the designated wells, sealed and incubated for 1h at room temperature, followed by 3 washings with wash buffer.

50µl of detection antibody, diluted 1:500 in wash buffer, was pipetted into the wells and incubated at room temperature for 1h, followed by 3 washings with wash buffer.

100µl of Pierce High Sensitivity Streptavidin-HRP conjugate (Thermo scientific, USA), diluted 1:10000 in ELISA buffer, was added to each well and incubated for 1h at room temperature and then washed with washing buffer 3 times. 100µl TMB substrate (Appendix 6.2.3) was added to each well and incubated for 30min while agitating at room temperature. After the incubation, 50µl stop solution (0.2M sulphuric acid) was added to each well. Plates were read on a Wallac VICTOR^2 1420 multi-label counter (PerkinElmer, USA) at 450nm.

4.2.7.2. IL-4 assay

The protocol was the same as for IFN-γ assay. The coating antibody (mouse anti-bovine IL-4) and detection antibody (mouse anti-bovine IL-4-biotin) were used at concentrations of 2.5µg/ml and 5µg/ml respectively. Recombinant bovine IL-4 was used as the IL-4 standard.

4.2.8. Statistical analysis

Data were graphed using Graph Prism v5. Statistical analyses were performed with by Graph Prism v5 unless indicated otherwise. The parasitological data were analysed by non-parametric one-way ANOVA (Kruskal-Wallis) with Dunn's post-test. The proliferative data for ALN were analysed by one-way ANOVA on logarithmically

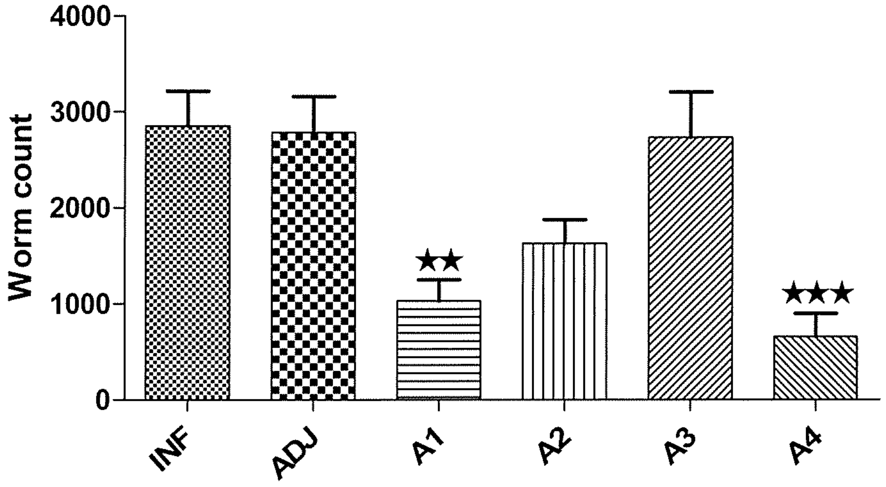


Fig. 4.4. Worm counts (mean ± SEM) of sheep in vaccination trial #1. Groups INF, ADJ and A1 - A3: $n = 9$; group A4: $n = 8$. Significant differences from the infected unvaccinated group (I) are shown: **: $p < 0.01$; ***: $p < 0.001$.

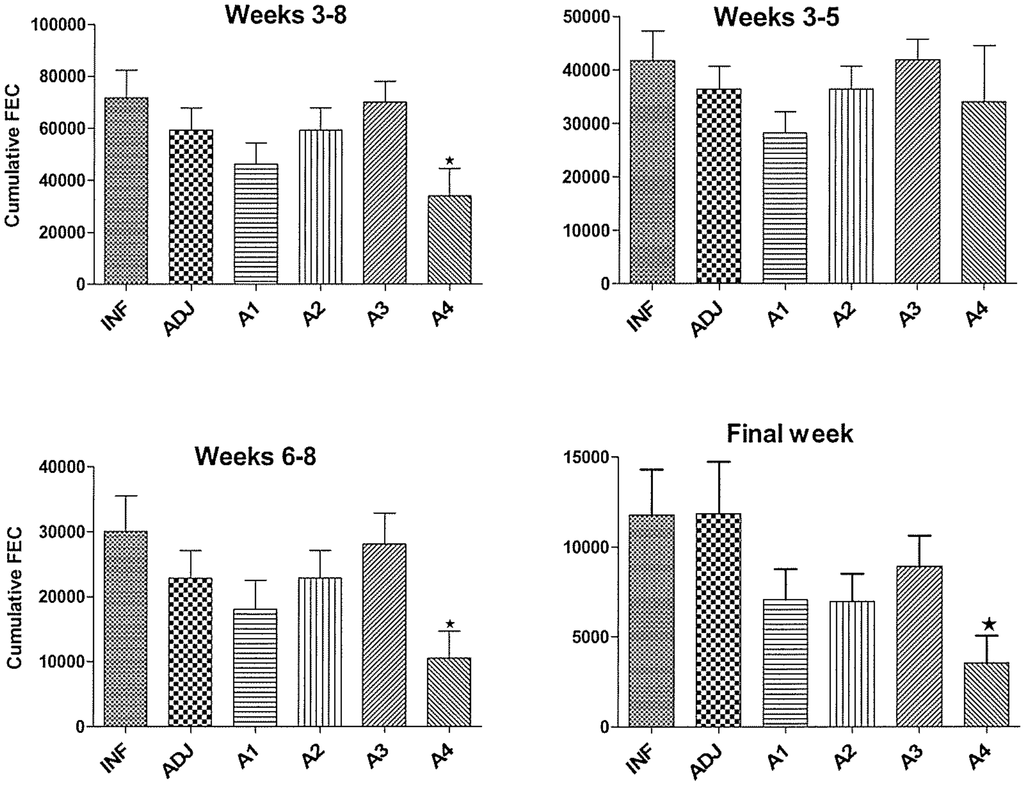


Fig. 4.5. Cumulative FEC (mean ± SEM) for Weeks 3 - 8, 3 - 5, 6 - 8 and the final week of sheep in vaccination trial #1. Groups INF, ADJ and A1 - A3: $n = 9$; group A4: $n = 8$. Significant differences from the infected unvaccinated group (I) are shown: * : $p < 0.05$.

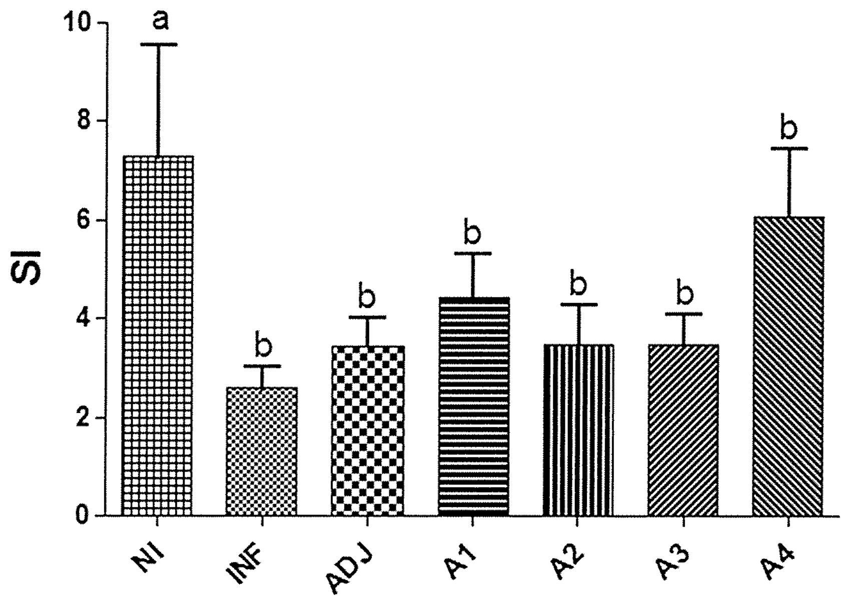


Fig. 4.2. Effect of ConA on the Stimulation Index (SI) (mean ± SEM) of cells from abomasal lymph nodes of sheep in vaccination trial #1. Groups NI: n = 5; INF and A1-A3: n = 9; group A4: n = 8. Means with different superscripts are significantly different, $p < 0.05$.

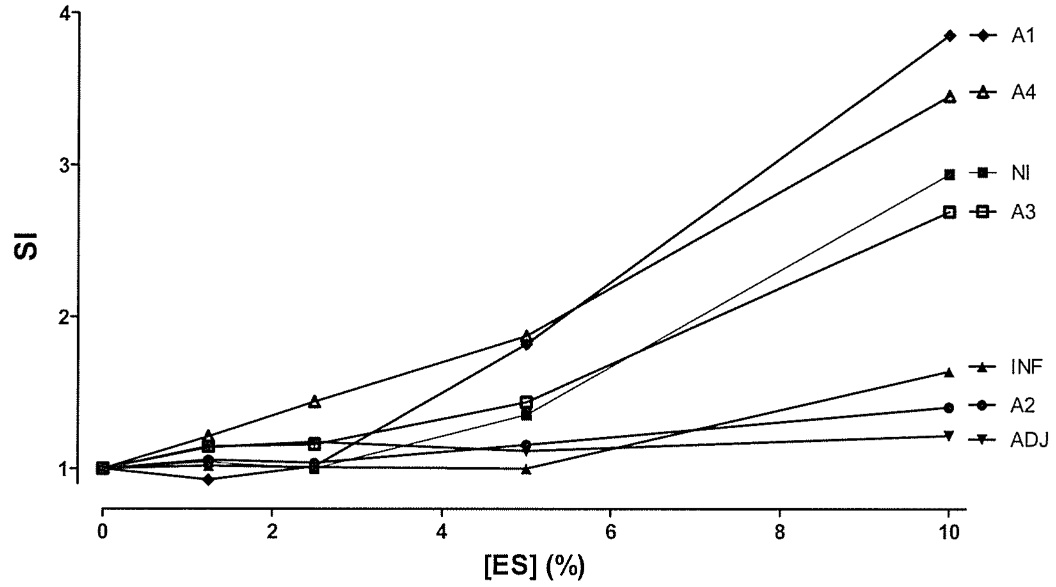


Fig. 4.3. Effect of 0 - 10% *H. contortus* ES products on the Stimulation Index (SI) (mean ± SEM) of cells from abomasal lymph nodes of sheep in vaccination trial #1. Groups NI: n = 5; INF, ADJ and A1– A3: n = 9; group A4: n = 8. There were no significant differences.

transformed data using Tukey's test for LSDs, kindly carried out by Dr J. Koolaard, AgResearch Ltd (summarised in Appendix 7.4).

4.3. RESULTS

4.3.1. Vaccination Trial #1

4.3.1.1. Cell proliferation

ConA stimulation had a greater effect on lymph node cells from NI sheep than from infected groups, whether vaccinated or not. All SI group means were above 1. The NI group had a significantly higher ($p < 0.05$) SI than any other group, whose means were not different from each other (Fig. 4.2). Group A4 had the highest numerical SI group mean, followed by group A1.

Exposure to *H. contortus* ES products during culture had variable effects on cells from different sheep within the groups, so that there were no significant group differences in SI (Fig. 4.3). Means for each group were numerically greater for 10% *Hc*ES products than for lower concentrations for each treatment group. The greatest means were for the vaccinated groups A1 and A4, next for NI and A3, whereas the SI for infected, ADJ-treated and infected and vaccination group A2 were very low.

4.3.1.2. Parasitology

Adult worm counts were significantly lower in vaccinated groups A4 ($p < 0.001$) and A1 ($p < 0.01$) than in the infected non-vaccinated sheep (Fig. 4.4). Vaccination group A2 had intermediate worm counts, but these were not statistically significantly different from those in the infected group.

Cumulative FEC were calculated for the whole period when eggs appeared in the faeces until necropsy (Weeks 3 - 8 p.i.), as well as for Weeks 3 - 5 and 6 - 8 p.i. and the final week (Fig. 4.5). No

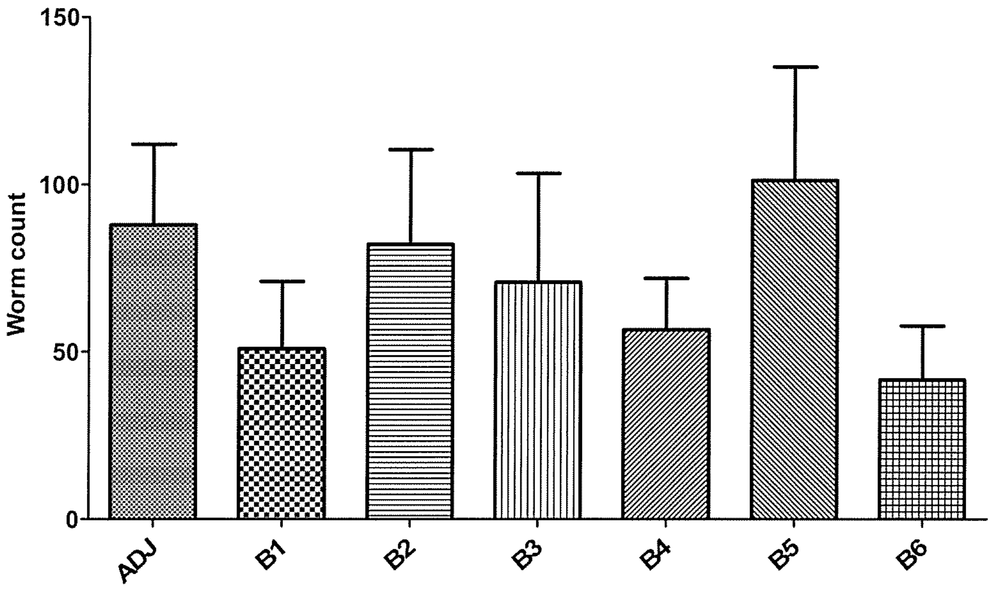


Fig. 4.8. Worm counts (mean ± SEM) of sheep in vaccination trial #2. All groups: *n* = 8, except group B3: *n* = 7. There were no significant differences.

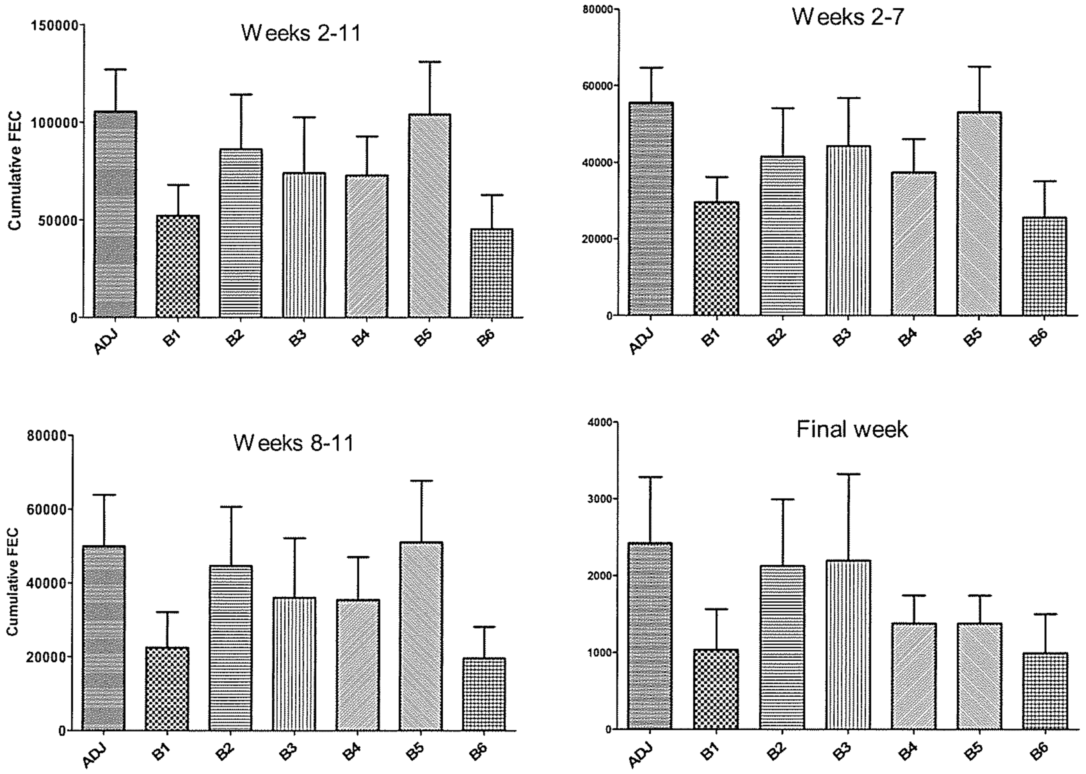


Fig. 4.9. Cumulative FEC (mean ± SEM) for Weeks 2 - 11, 2 - 7, 8 - 11 and the final week of sheep in vaccination trial #2. All groups: *n* = 8, except group B3: *n* = 7. There were no significant differences.

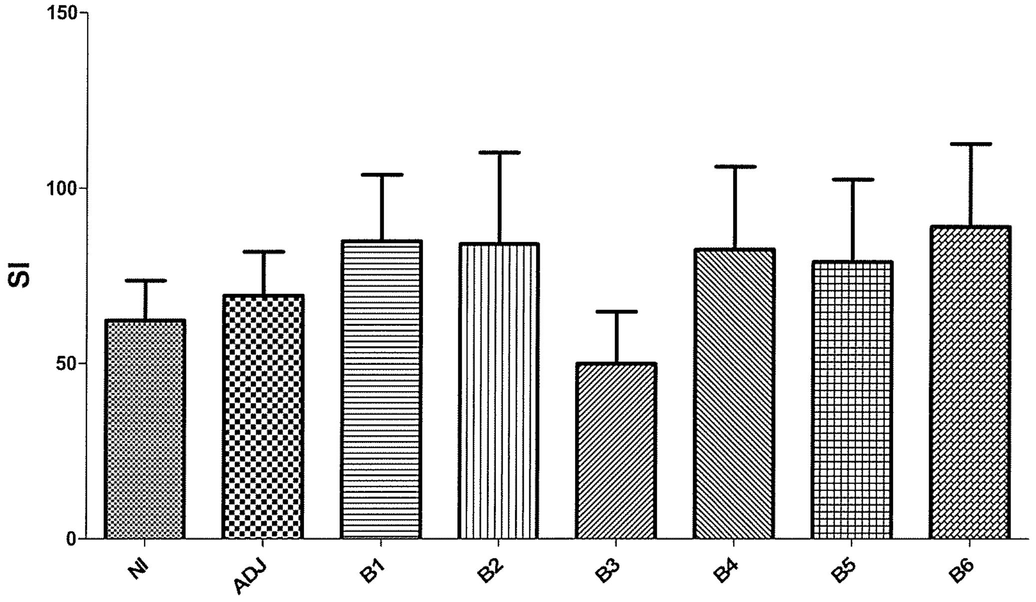


Fig. 4.6. Effect of ConA on the Stimulation Index (SI) (mean ± SEM) of cells from abomasal lymph nodes of sheep in vaccination trial #2. Groups NI: *n* = 4; ADJ and B1 - B6: *n* = 8, except group B3: *n* = 7. There were no significant differences.

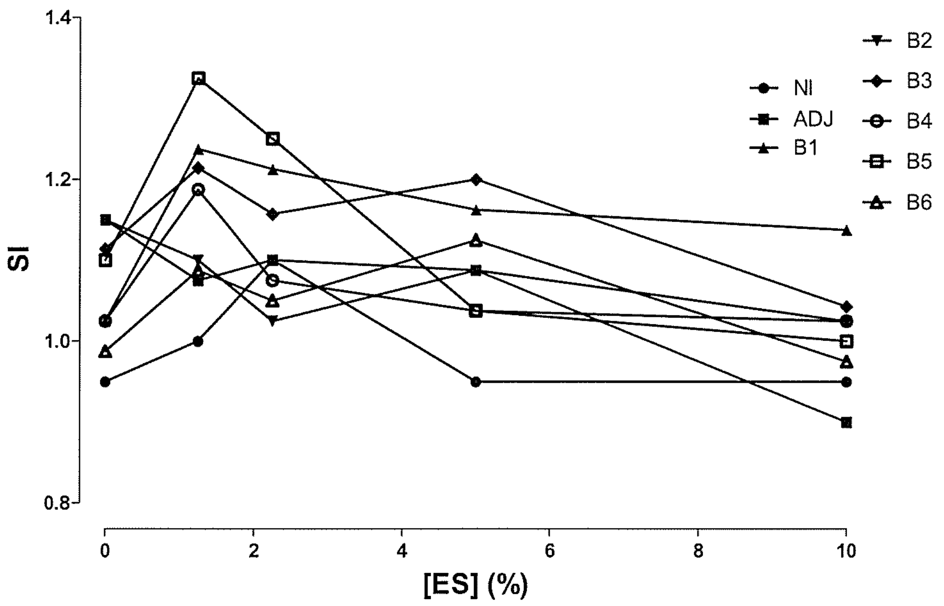


Fig. 4.7. Effect of 0 - 10% *H. contortus* ES products on the Stimulation Index (SI) (mean ± SEM) of cells from abomasal lymph nodes of sheep in vaccination trial #2. Groups NI: *n* = 4; ADJ and B1 - B6: *n* = 8, except group B3: *n* = 7. There were no significant differences.

group FEC differed during the first 3 weeks of collection, however, significantly lower cumulative FEC were present in vaccinated group A4 for Weeks 3 - 8, 6 - 8 p.i. and the final week ($p < 0.05$). Group A1 had the next lowest FEC for these periods.

4.3.2. Vaccination Trial #2

4.3.2.1. Cell proliferation

ConA stimulation had no significantly different effects on the SI lymph node cells from all groups (Fig. 4.6). Group B3 had numerically the lowest SI group mean and all other vaccinated groups had numerically greater means than the non-vaccinated group (ADJ), however, there was large variation within the groups.

Exposure to *H. contortus* ES products during culture had no significantly different effects on the SI lymph node cells from all groups (Fig. 4.7). The SI were all very low, the greatest SI being approximately 1.3. For each group, the highest numerical values were for low concentrations of ES products.

4.3.2.2. Parasitology

Adult worm counts were not significantly different between groups (Fig. 4.8). Vaccination group B6 had the numerically lowest worm counts and group B1 nearly as low.

Cumulative FECs were not significantly different between groups for any of the collection periods (Fig. 4.9). The rankings of the group means for each cumulative FEC were similar to the ranking for respective worm counts (Figs 4.8 - 4.9). Vaccination groups B1 and B6 had the numerically lowest cumulative FEC and worm counts, with group B4 the next lowest.

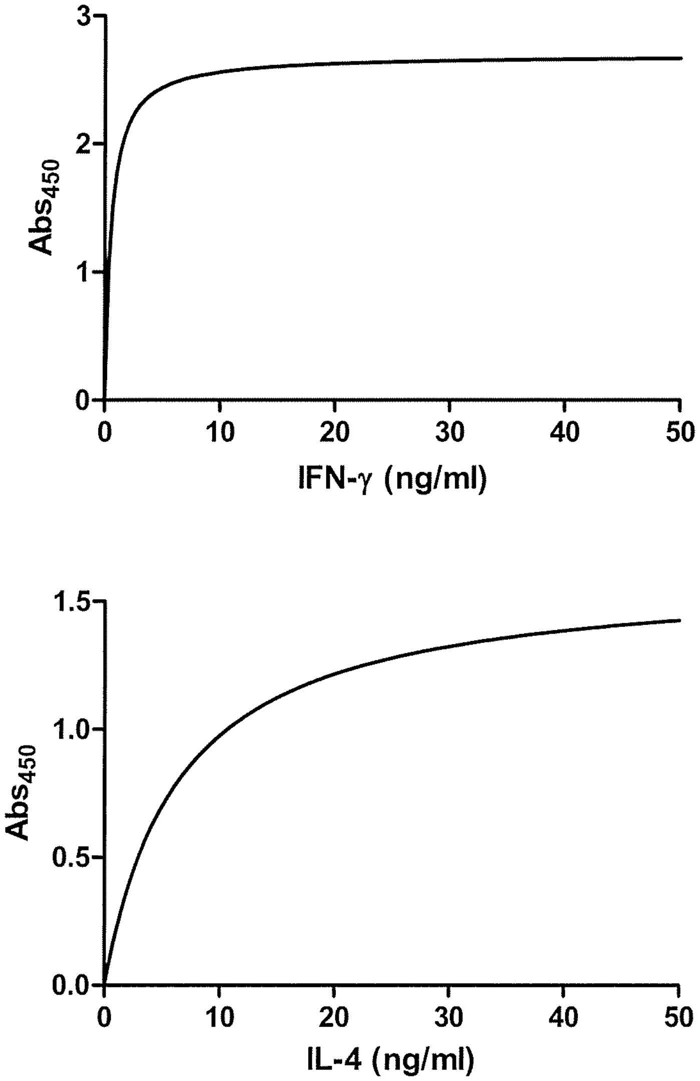


Fig. 4.10. Standard curves for the cytokine assays. Top: IFN-γ; bottom: IL-4.

4.3.3. IFN-γ and IL-4

The standard curves for the cytokine assays are shown in Fig. 4.10. There were no detectable concentrations of either IFN-γ or IL-4 in the incubation media.

4.4. DISCUSSSION

Parasites, like micro-organisms which cause chronic infections (Kumar et al., 2013), modulate the host response to enhance their survival and transmission to new hosts (Harnett and Harnett, 2006; (van Riet et al., 2007; Moreau and Chauvin, 2010) (reviewed in Chapter 1, 1.5.). *In vitro* experiments (Chapter 3) have shown that DCs are a target for immunomodulators in *H. contortus* ES products. The proliferation in response to ConA and ES products of mixed abomasal lymph node cells collected from infected and partially-protected vaccinated sheep has now shown that lymphocytes are also a target of these chemicals. Compared with those from uninfected sheep, lymphocytes of heavily parasitised sheep had poor proliferative responses to both ConA and ES products. There was a trend for suppressed proliferation to be reversed in those vaccinated groups with lower worm burdens, suggesting the level of parasitism determined the degree of immunosuppression.

4.4.1. Lymphocyte pool

Naïve lymphocytes are generated in the thymus and released into the circulation at a decreasing rate with age (Bains et al., 2009); some naïve $CD4^+$ cells continue to divide slowly after release. In the thymus, developing and maturing T cells express either the αß TCR or, less frequently, the γδ TCR (reviewed by

Kannan et al., 2012). Rearrangement of gene segments in the TCR receptor results in 2.5 x 10^8 unique human αß TCRs. γδ T cells are particularly prominent in ruminants, especially young animals (Hein and Mackay, 1991) and are believed to be important in resistance to parasites (Chapter 1, 1.4.2.1).

Lymphocytes continuously recirculate between the blood (2% of the pool) and tissues. The lymphocytes leave the tissues in draining lymph vessels, enter the lymph nodes and leave in efferent ducts and re-enter the blood. APC and lymphocytes interact in the lymph nodes, causing antigen-specific naïve T cells to be activated to effector or memory T cells, creating differences in the populations of lymphocytes in peripheral blood and lymph nodes (Battaglia et al., 2003). Analysis of the lymphocyte populations from the two sources showed higher B:T cell and $CD4^+$:$CD8^+$ T cell ratios in lymph nodes than in blood, however, the notable differences were in the phenotypes of the different T cells, rather than the percentages of each cell type.

The lymphocyte population of abomasal lymph nodes is likely to differ according to the time of collection after infection with nematodes, as the cellular profiles of the abomasal mucosa and draining lymph nodes change during the course of a primary infection with *H. contortus* (Balic et al., 2000a). At 3 days p.i., the percentage of $CD4^+$ cells in the lymph node increased, but decreased to that of control sheep by 5 days p.i., due to the doubling of the weight of the node, although the absolute numbers were increased. The percentage of T cells expressing MHCII increased at 5 days p.i. There was little increase in γδ T cells in the lymph nodes, unlike the abomasal tissues. Adult worms were present at 27 - 36 days p.i., when more activated B cells were present, but there were lower percentages of $CD4^+$, $CD8^+$ and γδ T cells in the lymph nodes.

4.4.2. T cell receptor

The structure, ligand-binding properties and signalling pathways associated with T cell receptors are now well defined (reviewed by Wucherpfennig et al., 2010). The αßTCR is non-covalently associated with three CD3 signalling molecules to form the TCR-CD3 complex. The best characterised interaction with ligand is the binding to the αßTCR of peptide-MHC complexes, but the binding of lipid Ag-CD1 complexes and non-classical binding of MHCI by γδ TCRs are well known.

In conventional T cells, the same TCR receptor is used for signalling antigen recognition during the life-cycle of the T cell, from development in the thymus, response of naïve T cells to generate effector (T_H1, T_H2, T_H17 or T_{reg}) and memory T cells and the response of memory T cells to re-exposure to the antigen, however, different intracellular signalling pathways are activated in each case (reviewed by Kannan et al., 2012 and Guy et al., 2013).

4.4.3. Stimulation of lymphocyte proliferation by ConA

The binding of particular mitogenic lectins to the surface glycans of cells of the immune system induces cell proliferation, activation of effector cells and release of cytokines (reviewed by Singh and Walia 2014). These mitogens cause non-specific effects on all cells carrying the appropriate glycans, in contrast to specific antigen, which may activate only about 0.01% of lymphocytes. Other anti-mitogenic lectins bind to co-stimulatory molecules and can antagonise mitogens in co-culture. ConA and PHA are two lectins which selectively bind to T cells through the CD3 component of the T cell receptor complex (TCR-CD3) (Janossy and Greaves, 1972;

Chilson and Kelly-Chilson, 1989; Pani et al., 2000; Singh and Walia, 2014).

H. contortus infection depressed the ability of T cells to proliferate when the TCR was stimulated by ConA. In the younger lambs in Trial #1, the SI was higher for T cells from NI animals than for cells from infected lambs, whether vaccinated or not ($p < 0.05$) (Fig 4.2). In the older animals in Trial #2, all SI were about 10-fold higher than the proliferation seen in cells from the lambs in Trial #1, although there were no group differences and individual animal variation was very large (Fig. 4.6). Vaccination with some combinations of *H. contortus* antigens both reduced parasite burdens and partially restored the blastogenic activity of T cells. In the younger animals, groups A4 and A1 had significantly lower worm counts (and cumulative FEC for A4) and the SI in both groups were numerically greater than in the INF group, although not statistically significantly different. The pattern of group means for SI were the inverse of those for worm counts and cumulative FEC for the latter part of the infection, suggesting these trends may become significant if larger numbers of animals were studied. In Trial #2, some animals in all groups are likely to have naturally developed immunity from their prior field exposure, as well as the challenge infection, making it difficult to detect any group differences. Vaccination groups B1 and B6 had the least mean worm counts and FEC and the highest SI in response to ConA (Figs 4.6 and 4.8 - 4.9).

No cytokines were detected in any of the culture supernatants. The standard curves for IL-4 and IFN-γ indicated that the assay components were active and not the reason for the negative result. These anti-bovine antibodies are stated by the supplier to recognise sheep cytokines. No cytokines may have been released or they may have been inactivated either during the 6 day incubation or subsequent storage at –80°C. The long incubation period is more likely to be responsible, since cytokine release may occur rapidly and maintenance at 37°C may be too long for stability. Six days is close to the maximum time recommended by the suppliers for storage of reconstituted cytokine solutions at 4°C. In the future, cytokines should be included in incubation experiments as positive controls and the supernatant collected after a shorter time, probably 1 - 2 days.

4.4.4. Proliferative response to *H. contortus* ES products

Exposure to *H. contortus* ES products during culture had little effect on the proliferation of lymph node cells in the older sheep (Fig. 4.7), the greatest SI being approximately 1.3. The response was greater in the younger animals (Fig. 4.3), but there were no significant group differences in SI. Means for each group were numerically greater for 10% *Hc*ES than for lower concentrations for each treatment group. There was some evidence that partial protection was associated with greater cells proliferation, as groups A1 and A4, which also responded more strongly to ConA, had the highest mean SI. The cells from infected and ADJ-treated infected groups showed almost no proliferative response. The ES products in the two trials were collected from 2 - 4 and 1 - 2 h of worm incubation respectively, however, this is unlikely to be important, as both incubates were equally active in reducing tight junctional permeability in CaCo-2 cells.

H. contortus ES products generally had modest effects (up to 5-fold SI) on lymphocyte proliferation, of similar magnitude to those reported for *F. hepatica* ES products (Moreau et al., 2002) and *H. contortus* larval antigen (Haig et al., 1989; Gill et al., 1991; Torgerson and Lloyd, 1993a,b). Resistance to nematodes is associated with lymphocytes with greater proliferative capacity: lymphocytes from resistant sheep had greater responses to larval and adult worm extracts than random-bred sheep, particularly 7 days p.i. (Gill, 1994) and sheep able to expel challenge infections of *T. colubriformis* had higher blastogenic activity than cells from unchallenged sheep (Pernthaner et al., 2005a). This was not the case for the older sheep in Trial #2, whose cells showed almost no response to parasite ES products, but might have been expected to have greater levels of protective immunity to parasite infection than the younger lambs.

A large number of studies of many helminths have shown ES products, their fractions or purified antigens either directly inhibit mitogen-stimulated proliferation of lymphocytes or act indirectly by reducing co-stimulatory expression on activating cells or release anti-inflammatory cytokines (Hartmann and Lucius, 2003). Cervi and Masih (1997) used catalase to reverse the inhibition by *F. hepatica* ES antigen of ConA-induced proliferation of spleen mononuclear cells, suggesting the involvement of H_2O_2 and O_2 in the immunosuppression. There are several candidates for the *H. contortus* lymphocyte immunosuppressors, including male worm galectin Hco-gal-m (Wang et al., 2014a) and a 66 kDa adult worm antigen (Rathore et al., 2006).

Results in the present experiment, which appears to be the only direct study of lymph nodes from vaccinated sheep, are consistent with *in vivo* inhibition of the blastogenic activity of lymphocytes during a parasite infection. The trend towards restoration of the proliferative capacity to ConA in the partially protected groups could be due to exposure to a lower worm burden or alternatively from the immunity induced by the vaccination itself. The slightly greater lymphocyte SI during exposure *in vitro* to 10% ES products of groups A4 and A1, compared with cells of unprotected INF lambs (Fig. 4.3), supports different responsiveness of lymphocytes from prior *in vivo* exposure to parasite burdens and vaccination procedures. On the other hand, direct antagonists of proliferation are likely to be present in ES products, as ES products have been shown to inhibit mitogen-stimulated proliferation (Gill et al., 1991, Wang et al., 2014a). Their identity remains to be determined, although the male worm galectin Hco-gal-m (Wang et al., 2014a) is particularly interesting, as it is known that anti-mitogenic lectins bind to co-stimulatory molecules and can antagonise mitogens in co-culture (Singh and Walia, 2014).

These experiments support general lymphocyte hypo-responsiveness during chronic helminth infections and are consistent with *H. contortus* promoting its long term survival by excreting and/or secreting immunomodulators which act on both lymphocytes and dendritic cells.

Chapter 5

GENERAL DISCUSSION

ES products of parasitic nematodes are the main route of communication between gastrointestinal parasites and tissues of their host. These chemicals initiate both an immune response and the pathophysiology, but are also responsible for manipulation of the host to suppress inflammatory pathways leading to worm expulsion. Vaccination of susceptible hosts against these parasite chemicals may lead to alternative, or supplementary, therapies to chemical anthelmintic drenches to which parasites are becoming increasingly resistant. The most common antigens being included in trial vaccines are parasite metabolic enzymes, however, novel approaches could be vaccines incorporating parasite immunomodulators (Maizels et al., 2004) or antigens in ES products which are responsible for causing host pathology.

This project focused on two aspects of the host-parasite interaction: the initiation of host pathology associated with *H. contortus* infection and suppression of host immune responses. The experiments reported in Chapter 2 confirmed that adult *H. contortus* ES products increase the permeability of Caco-2 cell monolayers and that it could be blocked by phage displaying anti-*Hc*ES scFvs. Recombinant *H. contortus* enolase mimicked the action of ES products and may be one of its active components. The second part of the study showed direct immunomodulation by ES products *in vitro* of the phenotypic and functional properties of human mdDCs (Chapter 3) and hyporesponsive lymphocytes in abomasal lymph nodes collected from infected and vaccinated sheep (Chapter 4).

5.1 MUCOSAL PERMEABILITY

Genetic differences between and within breeds of sheep affect parasite burdens and the severity of their effects on the individual animal. Resistant animals are better able than susceptible sheep to limit establishment and survival of parasites (Albers et al., 1987; Woolaston and Barker, 1996; McEwan et al., 1997), however, resistance can reduce body growth (Riffkin and Dobson, 1979). In contrast, resilient (tolerant) sheep maintain productivity during infection, often with large numbers of worms (Riffkin and Dobson, 1979; Albers et al., 1987; Bisset and Morris, 1996; Bisset et al., 2001). There is no consensus on whether tolerance and resistance to parasitism are mutually exclusive (Råberg et al., 2009; Jackson et al., 2014; Athanasiadou et al., 2015). As individuals expressing both characteristics would be highly desirable in production animals, it may be possible to induce both resistance and resilience to nematodes by vaccination with appropriate antigens. It is in this context that experiments have been directed at identifying worm chemicals which increase mucosal permeability, which may be able to be blocked to induce a state of parasite resilience and perhaps also interfere with worm establishment.

The involvement of parasite ES products in mediating the effects of luminal parasites has been inferred from the onset of pathophysiological changes coinciding with the presence of luminal worms, either after a larval infection or very soon after adult worm transplantation (McKellar et al., 1986, 1987; Lawton et al., 1996; Simpson et al., 1997). To have an effect on the parietal and chief cells located deep in the glands, ES products probably cross the surface epithelium and act on the basolateral membranes of gastric gland cells. This entry of worm chemicals into the mucosa is likely to be facilitated by increased permeability, which may be actively promoted by components of ES products.

The experiments reported in Chapter 2 confirmed the preliminary findings of Büring (2009) that adult *H. contortus* ES products disrupt tight junctions of cultured epithelial cells, resulting in reduced TEER (Fig. 2.17). Other parasites also increase mucosal permeability. This was observed *in vivo* in tissues of rats infected with *H. diminuta* (Kosik-Bogacka et al., 2011) and mice infected with *H. polygyrus* (Su et al., 2011) and *in vitro* in *H. diminuta*-infected colonic tissues (Kosik-Bogacka et al., 2010). Faecal cysteine protease of the house dust mite increased the paracellular permeability of confluent lung epithelial cells *in vitro* (Wan et al., 1999), probably by cleavage of tight junction proteins. Tight junctional disruption also often accompanies cancers (Turksen, 2010; Somi et al., 2012), autoimmune conditions (Clayburgh et al., 2004), microbial infections (O'Hara and Buret, 2008; Zihni et al., 2014).

There are many specific mechanisms by which parasites and microbial pathogens alter epithelial permeability. There can be direct effects on tight junctional integral and peripheral proteins or indirect actions through the cytoskeleton, which attaches to tight junctional proteins. *H. contortus* ES products may act via the cytoskeleton, as they are known to cause cytoskeletal rearrangement in HeLa cells, another immortal epithelial line (Büring, 2009). Some pathogens may act via inflammatory responses. In addition, cytokines, hypoxia, bacterial toxins and cytotoxic compounds are known to affect tight junction function (reviewed by Bonazzi and Cossart, 2011). Inflammation has been implicated in the studies of parasites of rodents (Kosik-Bogacka et al., 2010; Su et al., 2011). Many cytokines increase the permeability of tight junctions (reviewed by Bonazzi and Cossart, 2011): overproduction of TNF-α, IFN-γ and IL-1β increase epithelial permeability (Clayburgh et al., 2004; Al-Sadi et al., 2009); IFN-γ causes endocytosis of occludin, JAM-1 and claudin-1; TNF-α down-regulates ZO-1 expression and redistributes ZO-1 away from the tight junction (Forster, 2008) and IL-1β down-regulates occludin expression (Al-Sadi and Ma, 2007).

Phage displaying anti-*Hc*ES scFvs prevented the decrease in TEER by adult *H. contortus* ES products (Fig. 2.9), showing that the immune host produces blocking antibodies against the components responsible for permeabilisation of epithelia. Recombinant *Hc*ENO, but not *Hc*AK, mimicked the effect of ES products in opening tight junctions (Fig. 2.13). This suggests that enolase could be one of the active components of ES products which increase the permeability of epithelial cells in culture and probably also abomasal tissues *in vivo*. As native enolases of abomasal nematodes are recognised by the immune host (Yatsuda et al., 2003; Kiel et al., 2007) and the recombinant enzymes are recognised by immune serum (pers. comm. Dr S. Umair), a direct effect of enolase on tight junctions is more likely than a response dependent on inflammation. This cannot be discounted, however, as inflammation and down-regulation of a protective host response also accompany the pathophysiology *in vivo* and the two may be inseparable.

Enolase may not be the principal active component in parasitised sheep *in vivo*, as there are several other candidates amongst the many metabolic enzymes with secondary or “moonlighting” activities which have been implicated in diseases and pathogen virulence (Huberts and van der Klei, 2010). These enzymes include most of the glycolytic and tricarboxylic acid (TCA) cycle enzymes, glutamate racemase and the glyoxylate cycle enzymes. In addition, many of the proteases released into ES products are capable of increasing epithelial permeability by degrading tight junction proteins, as has been shown for a house dust mite cysteine protease (Wan et al., 1999). Moonlighting enzymes which are most likely to be involved in permeabilising epithelial tissues are those which, like enolase, bind to extracellular matrix proteins, actin or plasminogen. These enzymes are possible candidates for inclusion in a vaccine to reduce host pathophysiology, which may have the added benefit of reducing worm burdens. Blocking access of ES products to the mucosal tissues may create an unsuitable environment for the parasites, in the case of the stomach, by maintaining a low pH and inhibiting the establishment or survival of acid-sensitive adult worms (Lawton et al., 2002; Haag et al., 2005)

5.2. IMMUNOSUPPRESSION IN *H. CONTORTUS* INFECTED SHEEP

Hypo-responsiveness of the immune system accompanies the T_H2 response induced by parasitism (van Riet et al., 2007; Hewitson et al., 2009; Everts et al., 2010; Maizels et al., 2012b; McSorley et al., 2013; McNeilly and Nisbet, 2014) and other chronic infections, such as leprosy (Kumar et al., 2013). Suppressed of the response to mitogens was apparent in lymphocytes collected from infected sheep (Chapter 4). ConA-stimulated T cell proliferation was reduced in younger lambs infected with *H. contortus* ($p < 0.05$) and vaccination with some combinations of *H. contortus* antigens which reduced parasite burdens also resulted in a trend for partially restored T cell blastogenic activity (Figs. 4.2, 4.4 and 4.5). In older animals, lymphocyte SI were about 10-fold higher than in the younger animals, but variation between individuals in each group was large (Fig. 4.6). Again, vaccinated groups with the least mean worm counts and FEC (B1 and B6) had the highest lymphocyte SI in response to ConA (Figs. 4.6, 4.8 - 4.9).

Exposure to *H. contortus* ES products during culture had little effect on the proliferation of lymph node cells in the older sheep (Fig. 4.7), the greatest SI being approximately 1.3. The response was greater in the younger animals (Fig. 4.3), but there were no significant group differences in SI. The slightly greater lymphocyte SI with both ConA and 10% ES products of groups A4 and A1 compared with unprotected INF lambs (Figs 4.2 - 4.3) is consistent with greater responsiveness due to prior *in vivo* exposure to lower parasite burdens and/or immunity induced by the vaccination. The T cells of the older animals appeared to have become almost unresponsive to ES products, despite a much greater capacity to respond to ConA, suggesting the specific effect of parasite antigens may decline with greater length of exposure to them *in vivo.*

Direct antagonists in ES products of T cell proliferation may be partly responsible for the very low responses to ES products, which are known to reduce the effects of mitogens in co-culture (Gill et al., 1991; Wang et al., 2014a). The male worm galectin Hco-gal-m (Wang et al., 2014a) is of particular interest, as anti-mitogenic lectins can bind to co-stimulatory molecules and antagonise mitogens in co-culture (Singh and Walia, 2014).

5.3. IMMUNOMODULATION BY *H. CONTORTUS* ES PRODUCTS

Parasites target many points in the inflammatory cascade for active suppression or evasion of host immunity (Fig. 1.12) to prolong their survival in the host (Harnett and Harnett, 2006; van Riet et al., 2009; McSorley et al., 2013; Grencis et al., 2014). These strategies include: changing surface antigens with each moult; attaching host molecules to their surface; expressing host mimics; degradation of host immune products; blocking complement; secreting molecules which bind to cytokines; suppressing DC, T cell and B cell function and inducing T_{regs}, suppressive AAMs and the secretion of the anti-inflammatory cytokines IL-10 and TGF-ß. The experiments reported in Chapter 3 have shown for the first time that adult *H. contortus* ES products modulate the phenotypic characteristics of human mdDCs, consistent with studies of other helminths chemicals.

Human mdDCs were not fully activated by exposure to *H. contortus* ES products, similar to the reports of "semi-mature" DCs exposed to chemicals of other helminths, including *N. brasiliensis* (Balic et al., 2004), *T. spiralis* (Gruden-Movsesijan et al., 2011), *T. crassiceps* (Terrazas et al., 2011), *A. lumbricoides* (Dowling et al., 2011), *F. hepatica* (Hamilton et al., 2009) and *E. granulosus* (Casaravilla et al., 2014). *H. contortus* ES products also down-regulated cytokine/chemokine release by LPS-stimulated mdDCs, but had no significant group effects on the expression of surface molecules (Figs 3.13 - 3.15). Low ES concentrations further increased MIP-1α, IL-8 and IL-6 secretion induced by LPS, but this effect was reversed as the ES concentration was raised to 10% (Fig. 3. 22). Release of other cytokines was significantly reduced (Fig. 3.21) or unchanged (Figs 3.23 - 3.24).

ES products alone moderately increased the surface expression of the MHCII antigen-presenting molecule HLA-DR by some donor mdDcs (Figs 3.2 - 3.3), although changes were small compared with the increases produced by LPS (Fig. 3.12). The weak up-regulation of the co-stimulatory molecules CD40 and CD80, but not CD86 and CD83, is consistent with only a semi-mature tolerance-inducing status (Lutz and Schuler, 2002; Frick et al., 2010). Increased expression of surface CD32, CD305 and galectin-1 (Figs 3.1, 3.3 and 3.5) is consistent with the development of more tolerogenic DCs in the presence of worm chemicals. The highly individual mdDCs responses, particularly for the combination of LPS and ES products, biased the group data and there were even opposite effects on mdDCs of individual donors. Whilst this diversity of responses can make it difficult to detect the effects of experimental treatments, studies with heterogeneous human cell populations highlight the genetic diversity of the immune system.

5.4. VACCINES INCORPORATING PARASITE SECRETIONS

Immunity to gastro-intestinal helminths is slow to develop, leaving lambs susceptible to repeated infection from weaning to about 6 months-of-age (Smith et al., 1985; Watson et al., 1994). Vaccination would be most useful in sheep if it conferred protection before this resistant state ends. Development of a vaccine requires selection of appropriate antigens and adjuvant and delivery system to generate a protective response. This has proved elusive for gastro-intestinal helminths, as there has been difficulty in producing recombinant proteins with the same immunogenicity as the native molecules (Newton and Munn, 1999; Meyvis et al., 2007; Roberts et al., 2013), however, more recently there have been encouraging results (Nisbet et al., 2013; Dicker et al., 2014).

The present experiments were directed at understanding the host-parasite interaction, leading to identifying protective antigens. Very young and unweaned lambs have a thick layer of highly sulphated and sialylated gastric mucins (Hoang et al., 2010a,b), which may contribute to resistance to parasitism until weaning (Zeng et al., 2001). Expression of the SMC MUC5AC gene is decreased during parasitism, less so in susceptible than in resistant sheep (Ingham et al., 2008; Rowe et al., 2009). Reduced mucus secretion may contribute to loss of the barrier to worm chemicals crossing the epithelium and reaching host tissues. Maintaining the mucosal barrier is likely to be protective against infection, therefore, vaccination with antigens responsible for permeabilising the gastric mucosa may have a dual effect of increasing resilience and limiting the parasite burden.

Enolase, one of the moonlighting enzymes implicated in invasion, pathogenesis and establishment of pathogens, in the present experiments was able to mimic the increase in permeability of epithelial cell monolayers induced by ES products (Fig. 2.13). Enolase is recognised in helminth ES products by host antibodies and has been identified as a major immunostimulatory protein in visceral leishmaniasis (Gupta et al., 2007). This suggests it may be a useful antigen for inclusion in a vaccine to induce resilience to abomasal parasites. Several vaccination trials have shown that glycolytic enzymes, including enolase, provide partial protection against parasites. Immunisation of mice with *A. suum* enolase reduced the number of larvae reaching the lungs and liver after a challenge infection (Chen et al., 2012b). Anti-enolase antibodies provided protection against *P. yoelii* in mice (Pal-Bhowmick et al., 2007) and enolase has been proposed as a vaccine candidate against *Candida albicans* (van Deventer et al., 1996; Montagnoli et al., 2004; Shibasaki et al., 2014), *Chlamydia pneumoniae* (Finco et al., 2005) and S*treptococcus sobrinus* (Dinis et al., 2009). Other enzymes in ES products may be equally useful protective antigens: vaccination with recombinant phosphoglucose isomerase, another glycolytic enzyme, induced resistance in mice to secondary infection with *Echinococcus multilocularis* (Stadelmann et al., 2010).

Another approach to protective vaccination is to attempt to block parasite immunomodulators in order to reproduce the immunity which naturally develops in most ruminants over about 6 months-of-age (Mulcahy et al., 2004). This may have added benefits in countering impaired responses in parasitic infection to other vaccines (Nookala et al., 2004; Kizito et al., 2013) or co-infections which require a T_H1 response, such as tuberculosis, malaria or viral infections (Nacher et al., 2000; Ezenwa et al., 2010; Ezenwa and Jolles, 2011; Metenou et al., 2012; Karadjian et al., 2014; Kinung'hi et al., 2014; Thumbi et al., 2014).

A feature of the experiments on mdDCs (Chapter 3) was the great variability in responses to the same treatment of cells from different donors and on different days. Sheep too have individual genetically based susceptibility to parasites and immune responses. It would be difficult, therefore, to predict the outcome in individual animals of manipulating the complex immune response to parasites, involving the innate system (neutrophils, AAMs, eosinophils, mast cells and basophils), secretion of cytokines (IL-4, IL-5 and IL-13) and the generation of an acquired antibody response (IgG, IgE and mucosal IgA) (Smith, 1988; Meeusen, 1999; Balic et al., 2000b; Gasbarre et al., 2001; MacDonald et al., 2002). Anti-parasite responses probably should include physiological responses, such as changes in mucins (Hoang et al., 2010a,b; Hasnain et al., 2011), gut motility (Maizels et al., 2012b) and perhaps in gut microbial populations (Simcock et al., 1999).

Despite the complexities of the immune system and the numerous immunomodulators so far identified (Chapter 1, Table 1.1), there is optimism for a positive outcome to their use as antigens. Some immunomodulators have already been considered as useful antigens because of their other biological actions, so their inclusion in a vaccine may have more than one benefit. An example is the glycolytic enzyme aldolase, a possible permeabilising agent, which is also an immunomodulator. Santos et al. (2013) observed that *T. trichiura* aldolase, macrophage migration inhibitory factor homologue and HSP 70 all induced IL-10 secretion *in vitro* from PBMC; this is used as a screening test, because serum IL-10 increases during *T. trichiura* infections used as a therapy for atopic and autoimmune diseases.

5.5. HUMAN THERAPIES USING PARASITE SECRETIONS OR PRODUCTS

Clinicians are increasingly evaluating specific parasite chemicals as immunosuppressants to treat autoimmune and allergic diseases, such as ulcerative colitis, multiple sclerosis and asthma (Harnett and Harnett, 2010; McSorley and Maizels, 2012; Finlay et al., 2014). Recombinant or purified helminth molecules showed promise are ES-62 from the filarial nematode *A. viteae* (McInnes et al., 2003; Harnett et al., 2010; Rzepecka et al., 2013), LNFPIII from *S. mansoni* (Atochina and Harn, 2006; Dutta et al., 2010) and AvCystatin from *A. viteae* (Schnoeller et al., 2008). The use of active chemicals may reduce the pathological changes associated with a live worm infection, however, immunomodulatory molecules may more easily be neutralised by the host immune system than the mixture of substances released by the parasites (Pritchard, 2012).

The variation in response to parasite chemicals of mdDCs from the four human donors studied most intensively in the present study (Chapter 3) suggests that clinical treatments based on helminth immunomodulators may need careful patient evaluation, depending on their genetic makeup and clinical history, as some may not respond as predicted to worm-based therapy and either adverse reactions or failures in the treatment could occur. No obvious relationships between the levels of activation surface marker expression and cytokine release in non-stimulated mdDCs and their responses to stimulants emerged from the four cell donors in the main part of the study (Chapter 3, 3.4.4). Donor 2 cells were more responsive to *H. contortus* ES products and less so to LPS than the other three cell populations, particularly on one of the two days. These cells had a high NS expression of activation surface molecules, cellular galectin-1 and secretion of cytokines, but small responses of surface molecules to LPS and LPS plus ES products, although there were large increases in many cytokines. In contrast, before stimulation, immature Donor 7 mdDCs released almost no cytokines and Donor 1 NS cells had low expression levels of some surface molecules, but average cytokine release. Donor 4 NS cells had average levels of expression of surface molecules, but almost no cytokines/chemokines were secreted by either NS or ES-stimulated cells. Donor 2 is usually resident in a South Asian country and has experienced infections with helminths and malaria, which may be related to his responses to ES products. No distinctive features of the other donors were apparent, apart from Donor 1 having a rare blood group (AB negative).

The ability of helminths to induce regulatory immune responses by suppressing overreacting T_H1/T_H17 and T_H2 responses that mediate autoimmunity and allergy respectively has provided a basis for both the "hygiene hypothesis" and immunosuppressive therapies for inflammatory diseases in humans. The hygiene hypothesis is based on observations that parasitic infections in early life provide protection against allergy (Strachan, 1989), as perinatal helminth infection results in production of helminth-specific antibodies (King et al.,1998). Previous exposure to parasites may be very important in a target population for immunomodulatory therapy. Not all studies have shown beneficial effects of helminths or their products, and in some cases the symptoms were exacerbated (van Riet et al., 2007) or ineffective, as in the case of *H. polygyrus* in *Citrobacter rodentium*-induced colitis (Weng et al., 2007). Maizels and Gause (2014) have recently highlighted the complexity of such immunotherapy, as helminth infection and a T_H2 environment can allow latent viral infections to be reactivated.

5.6. FUTURE WORK

These experiments have shown that components of ES products are capable of increasing epithelial tissue permeability and thus may facilitate the development of host pathophysiology. Enolase was the only enzyme studied and several other moonlighting enzymes (aldolase, GAPDH, phosphoglycerate isomerase, phosphoglycerate mutase, AK and proteases) have properties making them as good or better candidates for inclusion as vaccine antigens. Some of these may act in more than one way and their blocking could disrupt worm metabolism and also their ability to cross the mucosal barrier and act as immunomodulators.

In vitro screening tests to identify active compounds to be used for anti-parasite or other therapies may better be carried out on sheep, not human, mdDCs dendritic cells and epithelial cells other than the intestinal Caco-2 cells. This would require further development of protocols for culture of sheep immune cells, as well as for normal gastric mucosal cells (RGM1 cells) or immortal cell lines which form tight junctions.

It is expected that a better understanding of the ways that worms interact with their host and identifying the chemicals responsible will lead to novel methods of blocking their effects on livestock and humans. The aims can be twofold: preventing establishment and survival of the parasites or reducing the associated pathophysiology. As a result, treated animals could become both resistant and resilient to parasitism.

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Appendix 1: Parasitology

1.1. Faecal egg flotation

1. Sheep faecal samples (2g) were ground in 10ml saturated saline and transferred into a 50ml narrow necked bottle.
2. The bottle was filled to the top with saturated saline and covered with a cover slip and left for 4 - 5min.
3. The cover slip was placed on a microscope slide and checked under the light microscope for attached parasite eggs.

1.2. Faecal egg counts

The method was the modified McMaster method of Stafford et al. (1994).

1. 2g of faeces were passed through a small sieve into 30ml of saturated NaCl solution.
2. Aliquots of the resulting suspension were transferred with a Pasteur pipette into the chambers of a McMaster slide. The suspension was continuously stirred while aliquots were being removed to assure an even distribution of eggs.
3. After 2 min, eggs were counted under a microscope. Each egg represented 50 e.p.g.

1.3. Larval culture stock

Sheep were regularly infected with *H. contortus* for maintenance of larval culture stocks. Faeces were collected in faecal bags from Day 21 p. i. onward.

1. Nematode eggs were confirmed and quantified as described in Appendix 1.2. Faecal pellets were emptied from the bags and

evenly distributed into plastic trays. The pellets were kept moist with tap water using a squeeze bottle until the pellets were shiny. Pellets were aerated by gentle shaking and the inside of the tray was irrigated with water using a squeeze bottle.

2. Step 1 step was repeated daily for 7 - 10 days, until the L_3 formed a beige–pink ring just above the level of the faeces.
3. The Baermannising apparatus consisted of an inner mesh-bottomed tray inside a collection tray. Tap water (20 - 25°C) was added to a level about 3cm above the mash. The mash tray was lifted and covered with a single layer of wet paper towels. Faecal pellets were spread over the wet paper towels to a maximum depth of 3cm. More water was added, if necessary, to cover completely the faecal pellets on the mesh-bottomed tray. A second mesh-bottomed tray was placed on top of faecal pellets and covered with a lid or another tray. The apparatus was left overnight to allow larvae to pass through the tissue into the collection tray.
4. Both mesh-bottomed trays along with the lid were lifted up and propped up on the collection tray and left for 1h to drain the water off the faecal material. The drained water was transferred into a 5L glass beaker and the tray was rinsed thoroughly. The beaker was left undisturbed at 10°C overnight to settle the L_3 on the bottom. The upper half of the water was replaced with clean tap water and left undisturbed for 3h to settle. This washing step was repeated till the water became clear.
5. L_3 were washed though a 20µm sieve and transferred into a kitchen sieve, lined with a single layer of moist tissue, sitting on a funnel attached to a rubber tube with the end clamped. The water level in the funnel was adjusted to touch the bottom of the sieve. The Baermann apparatus was left overnight.
6. The following day, L_3 which had settled at the bottom of the rubber tube were collected on a 20µm sieve and L_3 were washed with

copious amounts of RO water. L_3 on the sieve surface were washed off into a clean 250ml beaker. If the L_3 were mixed with traces of faecal slurry, step 4 - 5 was repeated.

1.4. Recovery of adult worms

1. Sheep were killed 21 days p.i. using a captive bolt, followed by exsanguination.
2. The abdomen was opened and the abomasum was ligated and removed immediately after euthanasia.
3. The abomasum was opened along its greater curvature and the luminal surface of the mucosa was washed with pre-warmed 0.9% saline.
4. The abomasal contents and washings were mixed and collected into a graduated beaker and the volume was determined
5. A 3% agar solution was made on the same day by dissolving 30g of agar (Bacto Agar, DIFCO Laboratories, USA) in 0.9% saline and heating for 8 - 10min in a microwave oven until dissolved. The agar solution was allowed to cool to 37 - 45°C.
6. Abomasal contents and washings were mixed 2:1 (v/v) with warm agar in a graduated beaker and thoroughly mixed. This mixture was poured in a thin layer (~1cm) depth into plastic trays and allowed to set.
7. Pre-warmed saline (37°C) was poured on to the agar blocks to a height of 1 - 2cm. The trays were kept in the dark at 37°C for 10 - 30min.
8. The worms migrated out of the agar blocks into the saline and aggregated. Aggregated worms were carefully picked up and placed into saline for further experiments.

1.5. RP2 medium for collecting ES products

RPMI-1640 (31800-022) (Life Technologies, USA)	1 sachet
$NaHCO_3$	2g
HEPES	5.958g
Antibiotics 100x (see below)	10ml

RPMI-1640 was dissolved in 950ml of deionised water at room temperature, $NaHCO_3$ and then HEPES were added and mixed until dissolved. Antibiotics were added last. The pH was adjusted to 7.0 with 1N HCl or 1N NaOH if required and made up to 1L with deionised water. The medium was filtered through a 0.2µm disposable filter.

Antibiotics 100x stock solution

Penicillin G sodium	1g
Streptomycin sulphate	1g

Penicillin and streptomycin were dissolved in 100ml of deionised water; 20ml aliquots were placed in tubes and stored at –20°C until use.

Appendix 2: Molecular Biology

2.1 cDNA synthesis

cDNA synthesis was performed using the iSCRIPT select cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions.

Reaction Mixture

Ingredients	Quantity (µl)
RNase free water	To adjust final volume of 20µl
5x iScript select reaction mix	4
Oligo $(dT)_{20}$ primer mix	**1**
Random primer mix	**1**
Total RNA	up to 1µg in up to 13µl
iScript reverse transcriptase	**1**

The ingredients, including RNA (~1µg), were gently mixed and incubated at 42°C for 90min, followed by 85°C for 5min to heat-inactivate the reverse transcriptase.

2.2 Media

2.2.1 Luria broth (LB)

Tryptone	10g
Yeast Extract	5g
NaCl	5g

All ingredients were dissolved in 950ml of deionised water, the pH adjusted to 7.0 with 2N NaOH if required and made up to 1L with deionised water. The medium was autoclaved for 20min at 121°C.

2.2.2 LB Agar

Bacto agar (15g per L) was added to LB broth prior to autoclaving.

2.2.3. Yeast extract and tryptone (YT) (2X)

Tryptone...............16g
Yeast Extract...........5g
NaCl......................5g

All ingredients were dissolved in 950ml deionised water, the pH adjusted to 7.00 with 2N NaOH if required and made up to 1L with deionised water. The medium was autoclaved for 20min at 121°C.

2.3. Solutions

2.3.1. 5X Native purification buffer (250ml)

Sodium phosphate monobasic (8.75g) and sodium chloride (36.5g) were dissolved in 180ml of sterile deionised water; the pH was adjusted to 8.0 with NaOH before adjusting the volume to 250ml. The solution was stored at room temperature.

2.3.2. 1X Native purification buffer (100ml)

5X Native purification buffer (20ml) was diluted with 80ml of sterile deionised water; the pH was adjusted to 8.0 with NaOH or HCl. The solution was stored at 4°C.

2.3.3 3M Native imidazole (100ml)

Imidazole (20.6g) was added to 8.77ml of stock solution A and 1.23ml of stock solution B in approximately 80ml sterile deionized water. The solution was slowly heated to dissolve the imidazole and the pH adjusted to 6.0 with NaOH or HCl before adjusting the volume to 100ml. The solution was stored at room temperature.

- **Stock solution A (200mM NaH_2PO_4, 5M NaCl)**

NaH_2PO_4 (13.8g) and NaCl (146.5g) were dissolved in 400ml of deionised water, the volume was adjusted to 500ml and stored at room temperature.

- **Stock solution B (200mM Na_2HPO_4, 5M NaCl)**

Na_2HPO_4 (14.2g) and NaCl (146.5g) were dissolved in 400ml of deionised water, the volume was adjusted to 500ml and stored at room temperature.

2.3.4 Native binding buffer containing imidazole

- **10mM Imidazole (250ml)**

Imidazole (833µl of 3M stock solution) was added to 50ml of 5X native purification buffer, the volume made up to 250ml with sterile deionised water and the pH adjusted to 8.0 with 5M NaOH. The solution was stored at 4°C.

- **40mM Imidizole (100ml)**

Imidazole (1.33ml of 3M stock solution) was added to 20ml of 5X native purification buffer,the volume made up to 100ml with sterile deionised water and the pH was adjusted to 8.0 with 5M NaOH. The solution was stored at 4°C.

- **250mM Imidizole (100ml)**

Imidazole (8.33ml of 3M stock solution) was added to 20ml of 5X native purification buffer, the volume made up to 100ml with sterile deionised water and the pH was adjusted to 8.0 with 5M NaOH. The solution was stored at 4°C.

2.3.5. Dialysis buffer (10x PBS)

NaCl (80g), KCl (2g), Na_2HPO_4 (11.5g) and KH_2PO_4 (2g) were dissolved in 800ml of sterile deionised water, the pH was adjusted to 7.2 and the volume was made up to 1L. 400ml of 10xPBS was mixed in 3.6L of sterile deionised water to make 4L of PBS dialysis buffer.

2.3.6. 2X SDS-PAGE sample buffer

0.5M Tris-HCl, pH 6.8	2.5ml
Glycerol (100%)	2.0ml
β-Mercaptoethanol	0.4ml
Bromophenol Blue	0.02g
Sodium dodecyl sulphate (SDS)	0.4g

The volume was made up to 10ml with sterile water and 500µl aliquots were stored at –20 °C.

2.3.7. Reagents used in western blotting

- **10x TBS**

  Tris base (12.2g) and NaCl (153g) were dissolved in 1L of sterile deionised water and made up to 2L.

- **1x TBS**

  10x TBS was diluted 10 times in sterile deionised water

- **TBS/0.1% Tween-20**

  Tween-20 (10ml) was added to 1L of 1x TBS

- **0.05 Tris-HCl**

  Tris base (6.1g) was added in 300ml of sterile deionised water, the pH was adjusted to 7.6 with 1M HCl and the volume was made to 1L.

- **AEC Stock solution**

  1g of 3-amino-9-ethylcarbazole (AEC) was added to 100ml DMFA (N, N-dimethylformamide).

- **50mM acetate buffer**

  14.8ml of 0.2M acetic acid and 35.2ml of 0.2M sodium acetate were made up to 750ml with sterile deionised water.

- **0.2M Sodium acetate**

  Sodium acetate (13.6g) was dissolved in 500ml of sterile deionised water.

- **0.2M Acetic acid**

  Glacial acetic acid (11.5ml) in 1L of sterile deionised water.

Appendix 3: Cell culture

3.1 Cell culture media

3.1.1. Cell culture medium for RGM1 cells

DMEM/F12 (1:1) 1x (1132-033)	78ml
FBS	20ml
PSN100x	1ml
Non-essential amino acids	1ml

3.1.2. Cell culture medium for Caco-2 cells

DMEM 1x (11965-092)	88ml
FBS	10ml
PSN100x	1ml
Non-essential amino acids	1ml

All ingredients were obtained from Life Technologies, USA. The pH of media was adjusted to 7.2 if needed with 1M HCl or 0.1M NaOH.

- **PSN 100x (Penicillin-Streptomycin-Neomycin)**

1ml aliquots of PSN liquid were stored at –20°C

- **FBS (Foetal Bovine Serum)**

FBS was thawed at 37°C and 10ml aliquots were stored at –20°C.

- **Non-essential amino acids**

10ml aliquots of non-essential amino acids were stored at 4°C.

3.2. Cell passage

3.2.1. RGM1 cell passage

RGM1 cells were grown in cell culture medium (Appendix 3.1) in 75cm^2 cell culture flasks and split every 3 to 6 days when they reached ~80% confluency. The cell culture medium was removed and the cells were washed with 10ml warmed PBS. TrypleE express enzyme (1.5ml) (Life Technologies, USA) was added and the cells were incubated in 5% CO_2 and 95% humidified air at 37°C until the cells were detaching from the culture flask (~5min). After detachment, 10ml of RGM1 cell medium was added to neutralise the enzyme. Cells suspended in culture medium were spun for 2min at 500*g*, the supernatant was discarded and cells were re-suspended gently in 500µl of medium. Cells were split at 1:8 in 15ml of warmed complete medium into a new warmed flask and cultivated in an incubator at 37°C in 5% CO_2 and 95% humidified air.

3.2.2. Caco-2 cell passage

Caco-2 cell passage followed the same protocol as for RGM1 cells (Appendix 3.2.1) using the cell culture medium described in Appendix 3.1.2.

3.3. Cryopreservation of cells

Recovery cell culture freezing medium (12648-010) (Life Technologies, USA) was thawed and placed at 4°C until used. A Mr Frosty freezing container (Thermo scientific, USA) was placed at 4°C overnight before use. Cells were detached as for cells passage, counted, washed and re-suspended at 2x10^6/ml in recovery cell culture freezing medium. Aliquots (1ml) of re-suspended cells were placed in cryovials, loaded into a Mr Frosty freezing container and kept at –80°C overnight before storing in liquid nitrogen.

3.4.Thawing of cryopreserved cells

Cell culture medium (35ml) was transferred to a culture flask and warmed in an incubator at 37°C in 5% CO_2 and 95% humidified air. Cryovials were removed from liquid nitrogen and transported on dry ice to a water bath and thawed at 37°C. Thawed cells were transferred very gently to 50ml falcon tubes using low speed settings on a pipette. 1ml of warmed medium was added drop-wise into the cell suspension and the tube was left in a tissue culture hood for 5min. 2ml of warmed cell culture medium was added very gently to the cell suspension and the tube was left in the tissue culture hood for 5min. Pre-warmed cell culture medium (4ml) was added gently (fast drops) to the cell suspension and the tube was left in the tissue culture hood for 5min. 8ml of pre-warmed cell culture media was added slowly (slow stream) to cell suspension and tube was left in the tissue culture hood for 5min. The cell suspension was centrifuged for 5min at 100*g*, the supernatant was discarded and the cells were re-suspended in 5ml of pre-warmed medium and transferred to a culture flask. A further 10ml of pre-warmed cell culture medium was added to the flask and incubated at 37°C in 5% CO_2 and 95% humidified air until the cells were ~80% confluent and then passaged.

Appendix 4: Immunohistochemistry

4.1. Paraformaldehyde (PFA) 4%

The solution was prepared in a fume hood. PFA (4g) was added to 80ml sterilised deionised water and heated at 60°C while stirring (care was taken not to overheat the solution). The pH was raised slowly by adding 1M NaOH drop-wise until the solution became clear. After cooling to room temperature, the solution was filtered through a 0.2µm filter, the volume was made up to 100ml and the pH was adjusted to 7.2-7.4. Aliquots (5ml) were stored at –20°C until used.

4.2. Blocking buffer

1g BSA was diluted in 100ml PBS and 5ml aliquots were stored at –20°C. Final concentrations of 2% goat serum (Brand) and 0.05% Tween-20 were added prior to use.

4.3. Primary and secondary antibodies

- **Rabbit anti-ZO-1 Ab**

10µl aliquots of rabbit anti-ZO-1 (0.25mg/ml) were stored at –20°C and diluted with blocking buffer to a final concentration of 2.5µg/ml before use.

- **Alexa Fluor 488 goat anti-rabbit Ab**

Alexa Fluor 488 goat anti-rabbit IgG (2mg/ml) was diluted with blocking buffer to a final concentration of 1µg/ml prior to use.

- **Mouse anti-occludin Ab**

5µl aliquots of mouse anti-occludin (0.5mg/ml) were stored at –20°C and diluted with blocking buffer to a final concentration of 2.5µg/ml before use.

- **Biotin-XX goat anti-mouse IgG**

Biotinylated goat anti-mouse IgG (2mg/ml) was diluted in blocking buffer to a final concentration of 1µg/ml prior to use.

- **Alexa Fluor 546 conjugate**

Alexa Fluor 546 conjugate (2mg/ml) was diluted in blocking buffer to a final concentration of 1µg/ml prior to use.

- **Alexa Fluor 647 goat anti-mouse IgG**

Alexa Fluor 647 goat anti mouse IgG (2mg/ml) was diluted in blocking buffer to a final concentration of 1µg/ml prior to use.

Appendix 5: Immunology

5.1. Staining for surface antigens

All work was performed in a tissue culture hood and plates were covered with parafilm or plate seal during centrifugation.

1. Plates were spun at 800*g* for 2min and supernatants were removed by flicking the plate.
2. Cells were equally distributed in two 96 well plates for Panel A and B staining.
3. Antibodies for surface antigens were diluted in PBS (concentrations are given in Table 3.1.). Cocktails of antibodies for Panel A and B were prepared.
4. 50µl of staining cocktail was applied to each sample and incubated for 30min at room temperature on a rocker, while protected from light.
5. Cells were washed with 200µl PBS three times by centrifugation at 800*g* for 2min after each wash.
6. Cells were re-suspended after each wash by rolling forceps underneath the plate.
7. Washed cells were fixed in 100µl Fluorofix (BioLegend, USA) and incubated for 20min at room temperature on a rocker while protected from light.
8. Fixed cells were washed twice in 200µl PBS, followed by centrifugation at 800*g* for 2min and re-suspension of cells after each wash.
9. Finally, cells were re-suspended either in 150µl PBS for Flow cytometery or in 100µl permeabilisation buffer for intracellular staining.
10. Single stains for each antibody and unstained samples were also prepared with the same concentration of antibodies. For live/dead staining cells were used, while OneComp eBead (eBioscience, San Diego, USA) were used for surface antibodies.

11. Fluorescence minus ones (FMOs) for each antibody were also prepared at the same concentrations of antibody cocktails, while replacing the specific antibody with PBS.

5.2. Intracellular and surface staining for galectin-1

Galectin-1 staining was performed either after surface antibody staining or on separate samples.

1. After surface antibody staining, cells were fixed in 100µl of 1x Fluorofix (fixation buffer) (BioLegend, San Diego, USA) and incubated for 20min at room temperature on a rocker, while protected from light, followed by washing with 200µl PBS (centrifugation at 800*g* for 2min).
2. For permeabilisation, fixed cells were washed twice in 200µl permeabilising buffer by centrifugation at 800*g* for 2min and re-suspension of cells after each wash.
3. Primary and secondary antibodies were diluted in permeabilising buffer at 1:20 and 1:50 respectively. Rabbit monoclonal anti-galectin-1 (Abcam, Cambridge, UK) was used as primary antibody and FITC goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, USA) as secondary antibody.
4. 50µl of primary antibody was applied to each sample and incubated for 30min at room temperature on a rocker, while protected from light.
5. Cells were washed with 200µl permeabilising buffer three times by centrifugation at 800*g* for 2min and re-suspension of cells after each wash.
6. 50µl of secondary antibody was applied to each sample and incubated for 30min at room temperature on a rocker, while protected from light.
7. After staining, cells were washed three times, as described in step 5.

8. Cells were re-suspended in 150µl PBS for flow cytometry.

5.3 Cytokine assay

The assay was performed according to the recommended standard procedure from the manufacturer (eBioscience Bender MedSystems, Austria).

5.3.1. Preparation of reagents and samples

- **Assay buffer**
  Assay buffer concentrate (10x) (provided with the kit) was warmed gently at 30°C for 5min to dissolve any crystals. Assay buffer was then diluted 1:10 in RO water. Diluted assay buffer was stored at 4°C up to 30 days.

- **Preparation of standards**
  Lyophilised standard was reconstituted in freshly prepared 1x assay buffer and serial dilutions of reconstituted standard were prepared.

- **Preparation of bead mixture**
  The bead mixture vial was vortexed briefly for 5sec. The required volume of bead mixture (25µl per sample) was pipetted into a new vial and centrifuged at 3000*g* for 5min. The supernatant was removed carefully leaving 50µl of liquid in the vial. A volume of reagent dilution buffer (provided in the kit) equal to that removed was added to the vial and mixed by vortexing for 5sec.

- **Preparation of streptavidin-PE solution**
  Streptavidin-PE was diluted according to the manufacturer's recommendation. For 96 tests, 176µl of concentrated streptavidin-PE was diluted with 5324µl of freshly prepared 1x assay buffer.

5.3.2. Staining procedure

- 25µl of samples, standards, blanks (1x assay buffer), negative controls (cell culture medium) and dilutions of ES products were placed in 96 well plates in duplicate.
- 25µl of bead mixture was added to all wells.
- 50µl of Biotin-Conjugate mixture (provided with the kit) was added to all wells.
- Contents of all wells were mixed properly by pipetting and incubated at room temperature for 120min in the dark.
- After incubation, all samples were washed twice with 200µl of 1x assay buffer.
- 50µl of Streptavidin-PE was added to all samples.
- The contents of all wells were mixed properly by pipetting and incubated at room temperature for 60min in the dark.
- Samples were washed twice with 200µl of 1x assay buffer.
- The cells were re-suspended in 500µl of 1x assay buffer for flow cytometer.

Appendix 6: Cell proliferation assay

6.1. Cell harvesting

Cells were harvested and radioactive measurements were performed at the radioactive handling facility at the Hopkirk Research Institute.

- A Harvester96 Mach III (Tomtec, USA) was used for cell harvesting, with the instrument vacuum set at 2PSI.
- A system wash was performed using Filtermat A (thin filters) (PerkinElmer, USA).
- The thin filters were replaced with Filtermat B (thick filters) (PerkinElmer, USA), pre-wet by a pulse wash.
- Culture plates were loaded on the stage and pulse washes were performed five times to harvest cells on to filter mats.
- Filtermats with harvested cells were dried in a microwave oven on full power for 60sec.
- A system wash was performed between samples, using thin filter mats.
- Dried filtermats were scintillated with 9ml of betaplate scintillation liquid (PerkinElmer Life and Analytical Sciences, USA) and sealed in microbeta trilux sample bags (PerkinElmer).
- Sealed filtermats were put into 96 well format Microbeta loading cassettes and the cassettes loaded on a Microbeta Trilux 1450 (PerkinElmer, USA) for detection of radioactivity. Data were exported in excel files for analysis.

6.2. Reagents and solutions

6.2.1. ELISA buffer

- **Solution A:** Sodium dihydrogen phosphate dehydrate ($NaH_2PO_4.2H_2O$) (4.68g) was dissolved in 300ml of distilled water
- **Solution B:** Disodium phosphate (Na_2HPO_4) (11.2g) was dissolved in 800ml of distilled water
- Solution A was added to solution B gradually until the pH of the solution B was 7.2.
- The solution was mixed with approximately 7L of distilled water in a 10L carboy and 380g NaCl, 25g BSA and 55g Tween-10 were added.
- The solution was stirred until the BSA was fully dissolved and the final volume made to10L with distilled water.
- The solution was filter sterilised through a Sartolab P20 0.2μm pressure filter (Sartorius, Spain) and aliquots were stored in sterilised 500ml Schott bottles.

6.2.2. TMB buffer

- Citric acid (31.52g) was dissolved in 1.5L of distilled water.
- Sodium acetate (69.72) was dissolved in 8.5L of distilled water.
- The two solutions were mixed and the pH adjusted to 5.2 with citric acid.
- Aliquots of the solution were placed in 500ml Schott bottles and autoclaved for 20min at 121°C.

6.2.3. TMB substrate

Tetra methyl benzidine (TMB) (AppliChem, Germany) (10mg) was dissolved in 1ml of dimethyl sulfoxide and diluted 1:100 with TMB buffer. H_2O_2 (1μl/ml) was added to the solution prior to use.

6.2.4. PBS-EDTA

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.15g
KH_2PO_4	0.2g
EDTA	1.86g

All ingredients were dissolved in 950ml of deionised water, the pH adjusted to 7.2 with 10N NaOH if required and made up to 1L with deionised water. The solution was filtered with 0.2µm disposable filters into sterilised 500ml bottles.

Appendix 7: Statistical analyses

7.1 Experiment 1: effects of ES on mdDC surface markers

mdDC derived from 8 donors. ES 0.4%, 2% and 10% compared with ES 0 as 100% using LSD comparisons and calculated LSD for 5%, 10% and 15% significance.

7.1.1. CD305

Calculated LSD of means (df = 14): 28.36, 5% level; 23.29, 10% level; 20.14, 15% level

Table of means:

ES (%)	0.4	2.0	10.0
Mean (%)	94.5	143.8	134.1
P	>0.15	<0.05	<0.05

Both ES 2% and 10% means were greater than for ES 100%. ES 2% and ES 10% means not different from each other but greater than ES 0.4% (P<0.05).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Donor	7	19350.0	2764.3	3.95	
ES	2	10902.2	5451.1	7.79	0.005
Linear	1	2454.2	2454.2	3.51	0.082
Quadratic	1	8448.0	8448.0	12.08	0.004
Residual	14	9792.4	699.5		
Total	23	40044.6			

7.1.2. CD80

Calculated LSD of means (df = 14): 24.02, 5% level; 19.73, 10% level; 17.06, 15% level

Table of means:

ES (%)	0.4	2.0	10.0
Mean (%)	110.0	160.6	163.4
P	>0.15	<0.05	<0.05

Both ES 2% and 10% means greater than for ES 100%. ES 2% and ES 10% means not different from each other but greater than ES 0.4% (P<0.05).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Donor	7	8980.7	1283.0	2.56	
ES	2	14451.6	7225.8	14.40	<0.001
Linear	1	6362.9	6362.9	12.68	0.003

Quadratic	1	8088.7	8088.7	16.12	0.001
Residual	14	7025.1	501.8		
Total	23	30457.3			

7.1.3. CD86

Calculated LSD of means (df = 14): 50.79, 5% level; 41.71, 10% level; 36.07, 15% level

Table of means:

ES (%)	0.4	2.0	10.0
Mean (%)	86.0	114.9	96.1
P	>0.15	>0.15	>0.15

No significant effects of ES.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Donor	7	23323	3332.	1.49	
ES	2	3434	1717	0.77	0.484
Linear	1	1	1	0.00	0.986
Quadratic	1	3434	3434	1.53	0.236
Residual	14	31398	2243		
Total	23	58156			

7.1.4. HLA-DR (MHCII)

Calculated LSD of means (df = 14): 69.7, 5% level; 57.3, 10% level; 49.5, 15% level

Table of means:

ES (%)	0.4	2.0	10.0
Mean (%)	122	144	202
P	>0.15	>0.15	<0.05

ES 10% mean higher than ES 100% and ES 0.4% means (P<0.05) and ES 2% mean (P<0.1).

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Donor	7	56083	8012	1.90	
ES	2	26961	13481	3.19	0.072
Linear	1	26606	26606	6.29	0.025
Quadratic	1	356	356	0.08	0.776
Residual	14	59189	4228		
Total	23	142233			

7.1.5. CD32

Calculated LSD of means (df = 14): 38.14, 5% level; 31.32, 10% level; 27.09, 15% level

Table of means:

ES (%)	0.4	2.0	10.0
Mean (%)	110.8	238.0	233.6
P	>0.15	<0.05	<0.10

Both ES 2% (P<0.05) and ES 10% (P<0.1) means greater than for ES 100%.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Donor	7	20763	2966	2.34	
ES	2	83493	41747	33.00	<.001
Linear	1	30538	30538	24.14	<.001
Quadratic	1	52955	52955	41.86	<.001
Residual	14	17712	1265		
Total	23	121969			

7.1.6. CD40

Calculated LSD of means (df = 14): 37.76, 5% level; 31.00, 10% level; 26.81, 15% level

Table of means:

ES (%)	0.4	2.0	10.0
Mean (%)	115.5	123.6	154.4
P	>0.15	>0.15	<0.05

ES 10% mean higher than ES 100% and ES 0.4% means (P<0.05)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Donor	7	19835	2834	2.29	
ES	2	6728	3364	2.71	0.101
Linear	1	6715	6715	5.42	0.035
Quadratic	1	13	13	0.01	0.921
Residual	14	17353	1240		
Total	23	43915			

7.2 Experiment2: Effects of ES and LPS on mdDC surface markers

RELM analysis of effects of ES compared with ES 0 at different concentrations of LPS on mdDC derived from 4 donors, each studied twice. ES 0.5%, 1.25%, 2.5%, 5% and 10% and LPS 10 and 30 mg/ml were tested.

REML variance components analysis consisted generally of:

Fixed model:	Constant + ES + LPS + ES.LPS
Random model:	Donor
Number of units:	115

7.2.1. CD32

5 observations (RB ES 10% data) were removed as very large, leaving 110 units.

Approximate LSD of REML means: ES 3.5 (P=0.05), 2.9 (P=0.1), 2.6 (P=0.15); LPS 2.8, 2.4, 2.1; ES.LPS 6.25, 5.3, 4.6

There was generally little change from 100%, except LPS 0 and ES 2.5%, 5% and 10% (P<0.05).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	101.9	100.4	103.6	103.8	106.0

Predicted means for LPS:

LPS	0	10	30
	105.5	102.3	101.6

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	104.7	100.1	101.1
1.25	102.1	99.9	99.3
2.50	106.5	102.8	101.4
5.00	106.6	103.6	101.1
10.00	107.6	105.4	105.1

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	11.49	4	2.87	88.2	0.027
LPS	9.89	2	4.95	90.7	0.009
ES.LPS	1.30	8	0.16	88.2	0.995

7.2.2. CD80

Approximate LSD of REML means: ES 6.3 (P=0.05), 5.3 (P=0.1), 4.6 (P=0.15); LPS 5.0, 4.2, 3.7; ES.LPS 11.1, 9.3, 8.1.

Little evidence of any change from 100% for any treatment combination. One anomalous result for ES 2.5% and LPS 10.

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	101.39	101.68	94.85	98.10	94.29

Predicted means for LPS:

LPS	0	10	30
	102.77	93.61	97.82

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		102.82	97.34	104.00
1.25		104.35	98.18	102.51
2.50		103.30	84.97	96.29
5.00		103.06	96.06	95.19
10.00		100.30	91.48	91.09

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	9.39	4	2.35	3.1	0.060
LPS	13.33	2	6.67	94.1	0.002
ES.LPS	6.38	8	0.80	93.1	0.606

7.2.3. CD86

Total units = 80

Approximate LSD of REML means: ES 27.4 (P=0.05), 22.9 (P=0.1), 20 (P=0.15); LPS 21.6, 19.4, 15.8; ES.LPS 52, 38.2, 33.3.

Very little evidence of changes from 100% for any treatment.

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	93.3	101.5	102.9	102.3	106.6

Predicted means for LPS:

LPS	0	10	30
	112.0	97.6	94.4

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		97.5	89.0	93.5
1.25		107.8	98.0	98.7
2.50		120.1	97.3	91.4
5.00		117.0	96.6	93.2
10.00		117.4	107.0	95.3

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f	F statistic	d.d.f.	F pr
ES	1.60	4	0.40	58.7	0.807
LPS	3.15	2	1.57	62.5	0.215
ES.LPS	1.03	8	0.13	58.7	0.998

7.2.4. CD83

Total units = 95

Approximate LSD of REML means: ES 15 (P=0.05), 12.5 (P=0.1), 10.9 (P=0.15); LPS 12.4, 10.3, 9; ES.LPS 27.7, 26, 22.5.

Very little evidence of changes from 100% for any treatment.

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	97.29	98.09	99.84	93.56	96.73

Predicted means for LPS:

LPS	0	10	30
	90.11	98.57	102.62

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	91.10	95.94	04.84
1.25	85.00	102.87	106.39
2.50	88.25	109.20	102.08
5.00	87.72	91.59	101.36
10.00	98.47	93.27	98.44

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F stat	d.d.f.	F pr
ES	1.24	4	0.31	80.0	0.870
LPS	4.25	2	2.12	80.0	0.126
ES.LPS	3.32	8	0.41	80.0	0.909

7.2.5. CD305

Total units = 115

Approximate LSD of REML means: ES 7.2 (P=0.05), 6.0 (P=0.1), 5.3 (P=0.15); LPS 5.6, 4.7, 4.1; ES.LPS 12.5, 10.5, 9.4.

Very little evidence of changes from 100% for any treatment; some evidence (P<0.1) for ES 10% and LPS 0 and 30.

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	101.1	102.6	104.9	103.8	106.3

Predicted means for LPS:

LPS	0	10	30
	109.1	98.0	104.0

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		108.0	92.8	102.6
1.25		106.5	97.8	103.5
2.50		110.1	102.7	101.8
5.00		109.0	100.1	102.3
10.00		112.0	96.7	110.0

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	2.43	4	0.61	93.1	0.659
LPS	15.44	2	7.72	95.5	<0.001
ES.LPS	3.59	8	0.45	93.1	0.888

7.2.6. CD40

Total units = 80

Approximate LSD of REML means: ES 14.4 (P=0.05), 12.0 (P=0.1), 10.5 (P=0.15); LPS 11.9, 9.9, 8.9; ES.LPS 27.3, 22.8, 19.9

Some evidence (P<0.1) that for LPS 30 the level of CD40 decreases from 100% and for LPS 0 it is higher for ES 5% only.

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	87.18	88.95	90.55	96.10	83.57

Predicted means for LPS:

LPS	0	10	30
	108.15	84.23	75.44

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		97.44	90.46	73.66
1.25		107.65	89.46	69.76
2.50		109.65	83.81	78.21
5.00		126.27	79.16	82.86
10.00		99.71	78.28	72.71

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	6.60	4	1.65	57.7	0.174
LPS	32.83	2	16.41	61.0	<0.001
ES.LPS	6.99	8	0.87	57.7	0.544

7.2.7. HLA-DR (MHCII)

Total units = 80

Approximate LSD of REML means: ES 15.4 (P=0.05), 12.8 (P=0.1), 11.2 (P=0.15); LPS 13, 10.9, 9.3; ES.LPS 29.2, 24.3, 21.2

In each of the LPS 0 and 30 data sets, there were means significantly (P<0.05) different from 100% (increased by LPS 0, ES 5% and decreased by LPS 30, ES 1.25%) For P<0.1, there were significant decreases at LPS 10, ES5% and 10%; LPS 30, all ES concentrations.

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	86.76	88.88	92.77	95.77	86.66

Predicted means for LPS:

LPS	0	10	30
	114.73	3.62	72.15

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		98.66	91.15	70.45
1.25		111.44	88.00	67.20
2.50		117.98	83.60	76.73
5.00		135.14	77.78	74.40
10.00		110.41	77.58	71.98

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	5.83	4	1.46	57.7	0.227
LPS	48.66	2	24.33	60.9	<0.001
ES.LPS	9.54	8	1.19	57.7	0.319

7.3. Experiment 2: Effects of ES and LPS on mdDC cytokine/chemokines

RELM analysis of effects of ES compared with ES 0 at different concentrations of LPS on mdDC derived from 4 donors, each studied twice. ES 0.5%, 1.25%, 2.5%, 5% and 10% and LPS 10 and 30 mg/ml were tested.

REML variance components analysis consisted generally of:
Fixed model: Constant + ES + LPS + ES.LPS
Random model: Donor
Number of units: 115

There were a number of missing values, either because the maximum concentration was reached or the cytokine was not secreted.

7.3.1. IFN-γ

Observations 3 for LPS 0, 5 for LPS 10 and 7 for LPS 30.

Approximate LSD of REML means: ES 36.1 (P=0.05)

There was no evidence of change from ES 0

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		88.1	96.7	98.0
1.25		94.5	100.1	101.3
2.50		93.9	88.1	100.4
5.00		92.5	87.3	94.7
10.00		121.3	.82.1	.86.7

ANOVA:

Fixed term	n.d.f.	F statistic	d.d.f.	F pr
ES	4	0.16	59.0	0.96
LPS	2	0.39	59.0	0.68
ES.LPS	8	0.74	59.0	0.66

7.3.2. MIP-1ß

Only 1 level of LPS in data, so LPS ignored.

Approximate LSD of REML means: ES 19.4 (P=0.05), 16.2 (P=0.1), 14.2 (P=0.15)

There was little evidence of change from ES 0.

Predicted means:

ES (%)	
0.50	96.8
1.25	95.1
2.50	89.3
5.00	85.1
10.00	88.7

ANOVA:

Fixed term	n.d.f.	F statistic	d.d.f.	F pr
ES	4	0.50	69.0	0.74

7.3.3. IL-10

No observations for LPS 0, 5 for LPS 10 and 6 for LPS 30.

ES 5% less than ES 0 for both LPS 10 and 30 and ES 10% for LPS 30 (p<0.05).

Approximate LSD of REML means: ES.LPS 27.4 (P=05), 22.4 (P=0.1), 19.9 (P=0.15)

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	84.56	84.12

Predicted means for ES.LPS:

ES (%) / LPS	0	10	30
0.50	-	90.09	93.31
1.25	-	99.89	107.54
2.50	-	79.79	89.46
5.00	-	69.21	59.49
10.00	-	83.81	70.81

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	22.14	4	5.54	38.6	0.001
LPS	0	1	0	42.7	0.95
ES.LPS	2.53	8	0.63	38.6	0.64

7.3.4. MIP-1α

Observations: 4 for LPS 0, 6 for LPS 10 and 8 for LPS 30.

ES 0.5-5% greater than ES 0 for LPS 10; ES 1.25 and 2.5% for LPS 30 (p<0.05).

Approximate LSD of REML means: ES 30.3 (P=05), 25.4 (P=0.1), 22.1 (P=0.15); LPS 27.9 (P=05), 23.3 (P=0.1), 20.3 (P=0.15); ES.LPS 57.7 (P=05), 51.7 (P=0.1), 45.1 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	146.8	177.6	158.8	156.8	84.5

Predicted means for LPS:

LPS	0	10	30
	107.7	186.1	140 9

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	118.9	189.8	131.5
1.25	133.3	222.2	177.3
2.50	101.9	219.0	155.5
5.00	114.5	209.8	146.2
10.00	69.9	89.6	94.0

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	49.88	4	12.47	68.1	<0.001
LPS	34.07	2	17.04	69.7	<0.001
ES.LPS	9.30	8	0.63	68.1	0.34

7.3.5. IL-8

Observations: 5 for LPS 0, 7 for LPS 10 and 8 for LPS 30.

ES 1.25 and 2.5% greater than ES 0 for LPS 10 and LPS 30 (p<0.05) and for 5% for both LPS 10 and 30 (P<0.1).

Approximate LSD of REML means: ES 28 (P=05), 23.4 (P=0.1), 20.5 (P=0.15); LPS 24.5 (P=05), 20.6 (P=0.1), 18 (P=0.15); ES.LPS 54.9 (P=05), 45.9 (P=0.1), 40.1 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	126.7	150.8	144.4	125.3	101.3

Predicted means for LPS:

LPS	0	10	30
	86.1	156.0	147.0

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	106.0	141.5	132.4
1.25	87.8	197.3	167.3
2.50	92.5	174.8	166.0
5.00	80.1	148.8	147.0
10.00	63.9	117.4	122.5

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	17.47	4	4.37	78.0	0.003
LPS	36.18	2	18.09	79.3	<0.001
ES.LPS	5.76	8	0.72	78.0	0.67

7.3.6. ICAM-1

Observations: 6 for LPS 0, 7 for LPS 10 and 8 for LPS 30.

No differences from ES 0.

Approximate LSD of REML means: ES 15.7 (P=05), 13.1 (P=0.1), 11.4 (P=0.15); LPS13.3 (P=05), 11 (P=0.1), 9.7 (P=0.15); ES.LPS 29.1 (P=05), 24.3 (P=0.1), 21.3 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00
	99.7	98.3	100.5	96.5	89.7

Predicted means for LPS:

LPS	0	10	30
	93.6	90.9	106.4

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	98.3	89.7	111.2
1.25	94.6	98.2	102.0
2.50	94.1	88.9	118.4
5.00	99.3	86.9	103.4
10.00	81.6	90.6	96.9

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	2.52	4	0.63	83.0	0.64
LPS	7.85	2	3.93	83.9	0.023
ES.LPS	3.70	8	0.46	83.0	0.88

7.3.7. IL-6

No observations for LPS 0, 6 for LPS 10 and 8 for LPS 30.

ES 0.5% and 1.25% greater than ES 0 for LPS 10 and ES 1.25% and 2.5% for LPS 30 (p<0.05).

Approximate LSD of REML means: LPS 16.0 (P=05), 13.3 (P=0.1), 11.6 (P=0.15); ES.LPS 27.4 (P=05), 22.4 (P=0.1), 19.9 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	114.9	120.1

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	-	127.4	116.5
1.25	-	143.4	145.2
2.50	-	113.9	127.0
5.00	-	107.3	120.6
10.00	-	82.6	91.2

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	24.03	4	6.01	52.9	<0.001
LPS	0.42	1	0.42	54.7	0.518
ES.LPS	1.46	4	0.36	52.9	0.833

7.3.8. IL-1α

No analysis, as only 2 observations.

7.3.9. IFN-α

No observations for LPS 0, 5 for LPS 10 and 6 for LPS 30.

No differences from ES 0.

Approximate LSD of REML means: LPS 10.4 (P=05), 8.6 (P=0.1), 7.5 (P=0.15); ES.LPS 23.4 (P=05), 19.5 (P=0.1), 16.4 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	95.9	94.3

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		-	104.2	101.2
1.25		-	103.6	97.2
2.50		-	92.5	92.0
5.00		-	86.9	93.2
10.00		-	92.6	87.8

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	4.54	4	1.14	40.0	0.35
LPS	0.11	1	0.42	41.2	0.75
ES.LPS	0.81	4	0.36	40.0	0.94

7.3.10. IL-13

No observations for LPS 0, 2 for LPS 10 and 3 for LPS 30.

No differences from ES 0.

Approximate LSD of REML means: LPS 19.1 (P=05), 15.8 (P=0.1), 13.6 (P=0.15); ES.LPS 43.2 (P=05), 35.4 (P=0.1), 30.6 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	87.3	98.9

Predicted means for ES.LPS:

	LPS	0	10	30
ES (%)				
0.50		-	95.7	111.8
1.25		-	103.0	100.7

2.50	-	71.5	92.8
5.00	-	81.2	98.5
10.00	-	85.1	90.3

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	4.01	4	1.00	13.0	0.44
LPS	1.69	1	1.69	13.4	0.22
ES.LPS	1.14	4	0.29	13.0	0.88

7.3.11. IP-10

No observations for LPS 0, 5 for LPS 10 and 6 for LPS 30.

ES 5% and 10% less than ES 0 for LPS 10 and 30 (p<0.05).

Approximate LSD of REML means: LPS 13.8 (P=05), 11.5 (P=0.1), 10 (P=0.15); ES.LPS 31.1 (P=05), 25.9 (P=0.1), 22.5 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	82.4	76.0

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	-	107.1	93.0
1.25	-	95.7	89.4
2.50	-	77.1	81.0
5.00	-	70.0	60.6
10.00	-	62.4	55.7

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	22.4	4	5.60	40.1	0.001
LPS	0.91	1	0.91	41.2	0.35
ES.LPS	0.80	4	0.20	40.1	0.94

7.3.12. IL-17A

No observations for LPS 0, 1 for LPS 10 and 3 for LPS 30.

No differences from ES 0.

Approximate LSD of REML means: LPS 29.5 (P=05), 23.9 (P=0.1), 20.5 (P=0.15); ES.LPS 68.4 (P=05), 55.1 (P=0.1), 47.2 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	103.3	97.3

Predicted means for ES.LPS:

	LPS 0	10	30
ES (%)			
0.50	-	112.2	115.4
1.25	-	105.4	104.8
2.50	-	101.4	92.1
5.00	-	102.2	89.5
10.00	-	95.6	84.6

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	4.33	4	1.08	8.0	0.43
LPS	0.22	1	0.22	8.7	0.65
ES.LPS	0.33	4	0.08	8.0	0.99

7.3.13. TNF-α

No observations for LPS 0, 2 for LPS 10 and 5 for LPS 30.

ES 5% and 10% less than ES 0 for LPS 10 and 30 (p<0.05).

Approximate LSD of REML means: LPS 22.8 (P=05), 19.7 (P=0.1), 17.1 (P=0.15); ES.LPS 59.2 (P=05), 49.0 (P=0.1), 42.5 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	66.1	59.3

Predicted means for ES.LPS:

	LPS 0	10	30
ES (%)			
0.50	-	100.2	83.4
1.25	-	76.6	89.5
2.50	-	57.0	74.5
5.00	-	46.5	24.6
10.00	-	50.0	24.4

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	27.66	4	6.92	20.8	0.001
LPS	0.35	1	0.35	24.7	0.56
ES.LPS	2.94	4	0.74	20.8	0.58

7.3.14. E-Selectin

No observations for LPS 0, 4 for LPS 10 and 6 for LPS 30.

No differences from ES 0.

Approximate LSD of REML means: LPS 10.3 (P=05), 8.5 (P=0.1), 7.4 (P=0.15); ES.LPS 23.1 (P=05), 19.2 (P=0.1), 16.8 (P=0.15).

Predicted means for ES:

ES	0.50	1.25	2.50	5.00	10.00

Predicted means for LPS:

LPS	0	10	30
	-	91.7	95.5

Predicted means for ES.LPS:

LPS	0	10	30
ES (%)			
0.50	-	100.2	83.4
1.25	-	76.6	89.5
2.50	-	57.0	74.5
5.00	-	46.5	24.6
10.00	-	50.0	24.4

Tests for fixed effects:

Fixed term	Wald statistic	n.d.f.	F statistic	d.d.f.	F pr
ES	8.07	4	2.02	35.0	0.11
LPS	0.57	1	0.57	35.9	0.46
ES.LPS	2.26	4	0.57	35.0	0.69

7.4 Lymphocyte proliferation Trial 1

Data were analysed by ANOVA and LSD with Tukey's 95% confidence intervals. Data were logarithmically transformed because of skewed measurements.

7.4.1. ConA

ANOVA:

Source of variation	d.f.	s.s	m.s.	v.r.	F pr.
Treatment	6	6.0211	1.0035	2.45	0.037
Residual	51	20.9040	0.4099		
Total	57	26.9251			

Table of means (SI):

Group	Log Mean	Backtranformed means	n	Differences
NI	1.979	7.236	6	a
INF	1.121	3.068	7	b
ADJ	0.832	2.298	9	b
A1	1.321	3.749	9	b
A2	1.055	2.873	9	b
A3	1.139	3.124	9	b
A4	1.518	4.563	9	b

7.4.2. 10% ES products

No significant differences between groups

ANOVA:

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	49.890	8.315	1.19	0.325
Residual	51	355.335	6.967		
Total	57	405.225			

Table of means (SI):

Group	Log Mean	n
NI	2.445	6
INF	1.734	7
ADJ	1.218	9
A1	3.855	9
A2	1.406	9
A3	2.692	9
A4	3.184	9

7.4.3. 5% ES products

No significant differences between groups

ANOVA:

Treatment	6	1.0943	0.1824	1.09	0.380
Residual	51	8.5248	0.1672		
Total	57	9.6191			

Table of means (SI):

Group	Log Mean	n
NI	0.285	6
INF	0.094	7
ADJ	0.076	9
A1	0.429	9
A2	0.111	9
A3	0.429	9
A4	0.379	9

7.4.4. 2.5% ES products

No significant differences between groups

ANOVA:

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	0.8010	0.1335	0.73	0.628
Residual	51	9.3398	0.1831		
Total	57	10.1408			

Table of means (SI):

Group	Log Mean	n
NI	0.173	6
INF	0.031	7
ADJ	0.079	9
A1	-0.159	9
A2	-0.003	9
A3	0.114	9
A4	0.217	9

7.4.5. 1.2.5% ES products

Not log transformed. No significant differences between groups

ANOVA:

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	6	0.3516	0.0586	0.51	0.797
Residual	51	5.8517	0.1147		
Total	57	6.2033			

Table of means (SI):

Group	Mean	n
NI	1.069	6
INF	1.039	7
ADJ	1.139	9
A1	0.927	9
A2	1.057	9
A3	1.145	9
A4	1.157	9