THE MUCUS BARRIER

IMMUNE DEFENCE AGAINST GASTROINTESTINAL NEMATODES

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ABSTRACT

Trichuriasis, caused by the intestinal nematode *Trichuris*, is a disease that affects up to a billion people worldwide. To date, most of our knowledge of this disease comes from the mouse model, *Trichuris muris*, which has been successfully used to dissect the immune-mediated effector mechanisms that elicit the expulsion of the nematode.

Numerous studies have shown a temporal association between intestinal nematode expulsion and goblet cell hyperplasia; however their precise role in response to nematode infection remains elusive. Goblet cells found at mucosal surfaces secrete many constituent components of the mucus barrier, including the gel-forming mucins (Muc2 in the intestine); mucins are large multifunctional glycoproteins that provide the structural framework of the barrier. The studies presented in this thesis demonstrate that the mucosal barrier and in particular its mucin components, changes in response to acute and chronic *T.muris* infection.

In animals resistant to chronic *T. muris* infection, IL-13-mediated increase in Muc2 production and secretion was observed at the site of infection. Critically, expulsion of the nematode was significantly delayed in the absence of Muc2. Further investigation subsequently showed that Muc5ac, a mucin normally expressed in non-intestinal mucosa was, in fact, expressed in the intestine following nematode infection and was associated with nematode expulsion in the resistant mice. Moreover, mice deficient in Muc5ac were susceptible to chronic infection, despite a strong underlying T_H2-type immune response which is essential to eliminate the nematodes, suggesting that Muc5ac acts as a critical effector molecule. Several qualitative changes in the mucins were also noted during resistance: mucins were more highly charged and more sulphated during nematode expulsion. Overall, the changes within the mucus barrier during resistance result in altering the rheological properties of the mucus layer making it less porous and mucins were shown to directly ‘damage’ the nematodes during nematode expulsion as reflected by a significant reduction in ATP levels. Chronic infection was accompanied by decreased levels of low charged and highly sialylated Muc2. Additionally, we demonstrated that the excretory secretory products released by the nematode consist of serine proteases capable of depolymerising the Muc2 mucin network, which may be part of the nematodes regime to improve its niche and/or aid movement through the mucus layer. Overall, this resulted in a porous mucus layer and a favourable environment for the parasite.

Data is presented to show that the intestinal mucus barrier and its constituent mucins are an integral part of the co-ordinated expulsion mechanisms that occur in animals resistant to *T.muris* infection and we identify a mechanism whereby the nematode exerts its effects on the mucin environment to promote its survival within the host.

DECLARATION

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Dedicated to my mum…

ABBREVIATIONS

APC	Antigen Presenting Cells
Asn	Asparagine
CCL	CC Chemokine Ligand
CD	Cluster of Differentiation
DBA	*Dichloris Biflorus*
ER	Endoplasmic Reticulum
ESPs	Excretory Secretory Products
GABA	gamma amino-butyric acid
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
HD	High Dose
HID-AB	High Iron Diamine-Alcian Blue
IFN-γ	Interferon-γ
IBD	Inflammatory bowel disease
Ig	Immunoglobulin
IL	Interleukin
LD	Low Dose
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
NaS	Na^{+} sulphate transporter
NF-κB	Nuclear factor-Kappa Beta
PAS	Periodic acid-Schiff's
pi.	post infection
Relm	Resistin-like molecule
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
Sat1	Sulphate anion transporter
Ser	Serine
SCID	Severe combined immunodeficient
Spdef	SAM pointed domain containing ETS transcription factor
STP	Serine-threonine-proline
Tff3	Intestinal trefoil factor-3
TGF	Transformation growth factor
T_H	T helper cells
Thr	Threonine
TLR	Toll-like receptors
T. muris	*Trichuris muris*
TNF	Tumour necrosis factor
T. trichiura	*Trichuris trichiura*
vWF	von Willebrand Factor
WT	Wild-type

INTRODUCTION

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1.1 The intestinal mucus barrier

The large intestine is the final part of the digestive system and is responsible for the efficient absorption of water, sodium and chloride ions as well as the secretion of bicarbonate ions and mucus. The main components of the large intestine are the caecum, a pouch like structure, and the colon (Barrett, 2006; Hedrich, 2004), which consist of deep crypts that open onto the luminal surface as depicted in **Figure 1.1A**. The intestinal epithelial cell layer is continuously being regenerated by stem cells found at the bottom of the crypts (Brittan and Wright, 2004). The epithelial cell layer consists of several different cell types: enterocytes are the main cell type which are absorptive cells; these are interrupted by specialised secretory cells called goblet cells because of their shape as illustrated by **Figure 1.1B.**

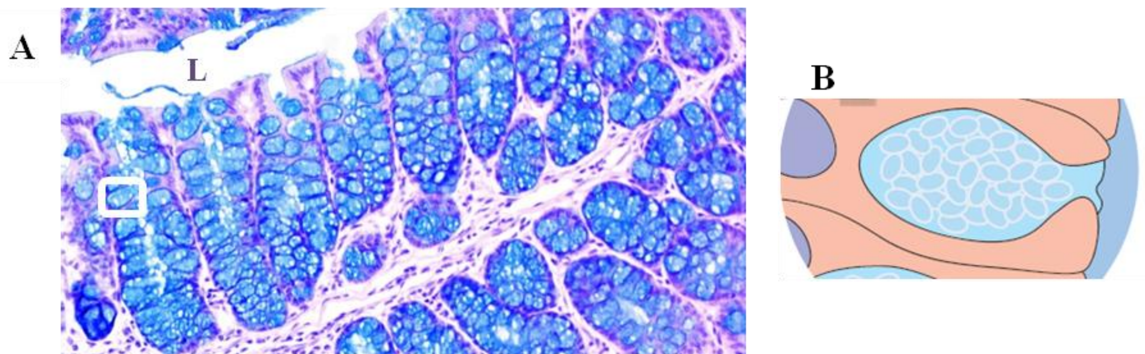

Figure 1.1: (A) The specialised mucin-secreting goblet cells (blue), within crypts that open onto the luminal surface (L), can be seen in the histological section of the large intestine. (B) Diagram shows that on a closer look these goblet cells have granules which store mucins, that can be secreted into the mucus barrier and directly influence the mucus barrier properties. (Diagram adapted from Hasnain, 2010).

The epithelial cell layer is protected by a layer of mucus, which is an essential part of the innate immune system, enabling the host to prevent access of pathogens. Mucus primarily consists of mucins, water, proteins and inorganic salts. The viscous and gel-like properties of the mucus barrier, which enable it to physically protect and lubricate the mucous membranes, are conferred mainly by mucins (Thornton and Sheehan, 2004). Goblet cells within the crypts of the epithelia synthesise and store mucins within their granules (**Figure 1.1B**), which can then be released via exocytosis in response to specific stimuli (Hasnain, 2010; Thornton *et al*, 2008). Many factors can stimulate secretion of mucins and mucus (e.g. cytokines, growth factors, bacterial products and viruses) and, of

particular relevance to this project, parasitic Excretory Secretory Products (ESPs) (Matsuo *et al*, 1997).

1.1.1 Mucins

Mucins are large heavily glycosylated proteins and typically have a molecular mass in excess of 10^6 Da. These large proteins are densely substituted with short glycan chains which have many important functional roles (Thornton *et al*, 2008). For example, it is well known that glycans have fundamental roles in many biological processes such as cell interaction, cell growth, cell signalling, cell adhesion and immune defence (Singh and Hollingsworth, 2006). Furthermore, glycan chains are also thought to modulate the structural and functional activity of proteins, which is discussed in detail below.

1.1.1.1 Glycosylation

There are two main types of glycosylation in mucins: N-glycosylation and O-glycosylation. Synthesis and transfer of N-glycans, which are important in intracellular processes (e.g. protein trafficking), occurs within the rough endoplasmic reticulum, where they are attached to asparagines (Asn) in the protein sequence Asn-X-Serine/Threonine (where X can be any amino acid except proline) through N-acetylglucosamine (GlcNAc) (Taylor and Drickamer, 2003). Mucins, however, are predominantly decorated with O-glycan sugars, which accounts for up to 80% of their molecular weight. The O-glycan sugars attach through N-acetlygalactosamine (GalNAc) to a serine or threonine residue in the serine-threonine-proline (STP) rich tandem repeats in the protein core (Taylor and Drickamer, 2003). Glycosylation of the mucin tandem repeat depends on several factors such as the presence of proline adjacent to serine/threonine, steric hindrance by neighbouring glycans and the availability of glycosyltransferases (Thornton *et al*, 2008). This results in diverse site-specific and mucin-specific glycosylation patterns which influence the properties of the mucin and therefore the mucus gel (Robbe *et al*, 2004). This extensive O-glycosylation occurs in the Golgi complex and is essential to the extracellular function of the protein. It is involved in the hydration, extension and expansion of the protein in solution which results in a reduction in the flexibility of the protein (Hollingsworth and Swanson, 2004). The dense O-glycan substitution of mucins shields the underlying polypeptide from degradation by proteases. Furthermore, these glycans can act as decoy extracellular receptors for viruses, bacteria and parasites, consequently

removing the pathogens and limiting the potential danger of infection (Hollingsworth and Swanson, 2004). The changes in glycosylation and its potential role of glycosylation in intestinal disease will be discussed later (*refer to section 1.3*).

At least 20 genes that encode for mucins have been identified and coined, MUC genes; nomenclature is presented as MUC/Muc for human/mice respectively. Mucins have been classified into two major groups: cell surface mucins and secreted mucins (Thornton and Sheehan, 2004).

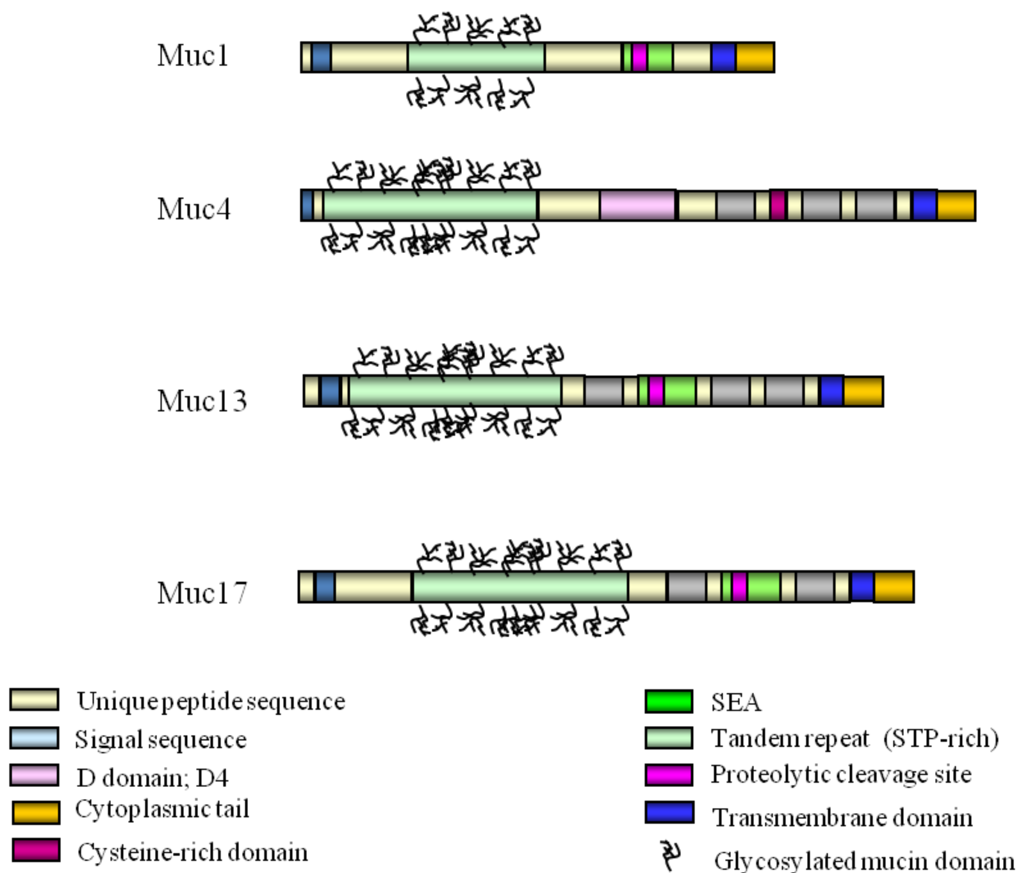

Figure 1.2: Schematic representation of the major intestinal cell surface mucins; Muc1, Muc4, Muc13, and Muc17.

1.1.1.2 Cell surface mucins

Cell surface mucins are intercalated in the plasma membrane through a transmembrane domain. The large extracellular mucin domain which contains the STP-rich region, is extensively O-glycosylated (**Figure 1.2**). The cytoplasmic domain is thought to play a role as a molecular sensor and is involved in signal transduction pathways. In mice, Muc1, Muc4, Muc12, Muc13, Muc15, Muc16, Muc17 (orthologue of human MUC3) and

Muc20 are all classed as cell surface mucins (Linden *et al*, 2008a; Singh and Hollingsworth, 2006). In the intestine, Muc1, Muc4, Muc13 and Muc17 have been shown to be expressed at high levels (**Figure 1.2**) (Linden *et al*, 2008a). Although membrane bound, the cell surface mucins can be released as they are susceptible to post-translational proteolytic cleavage at the conserved SEA domain (**Figure 1.2**). It is thought (but not proven) that the cell surface mucins also directly contribute to the physical properties of the mucus layer (Singh and Hollingsworth, 2006).

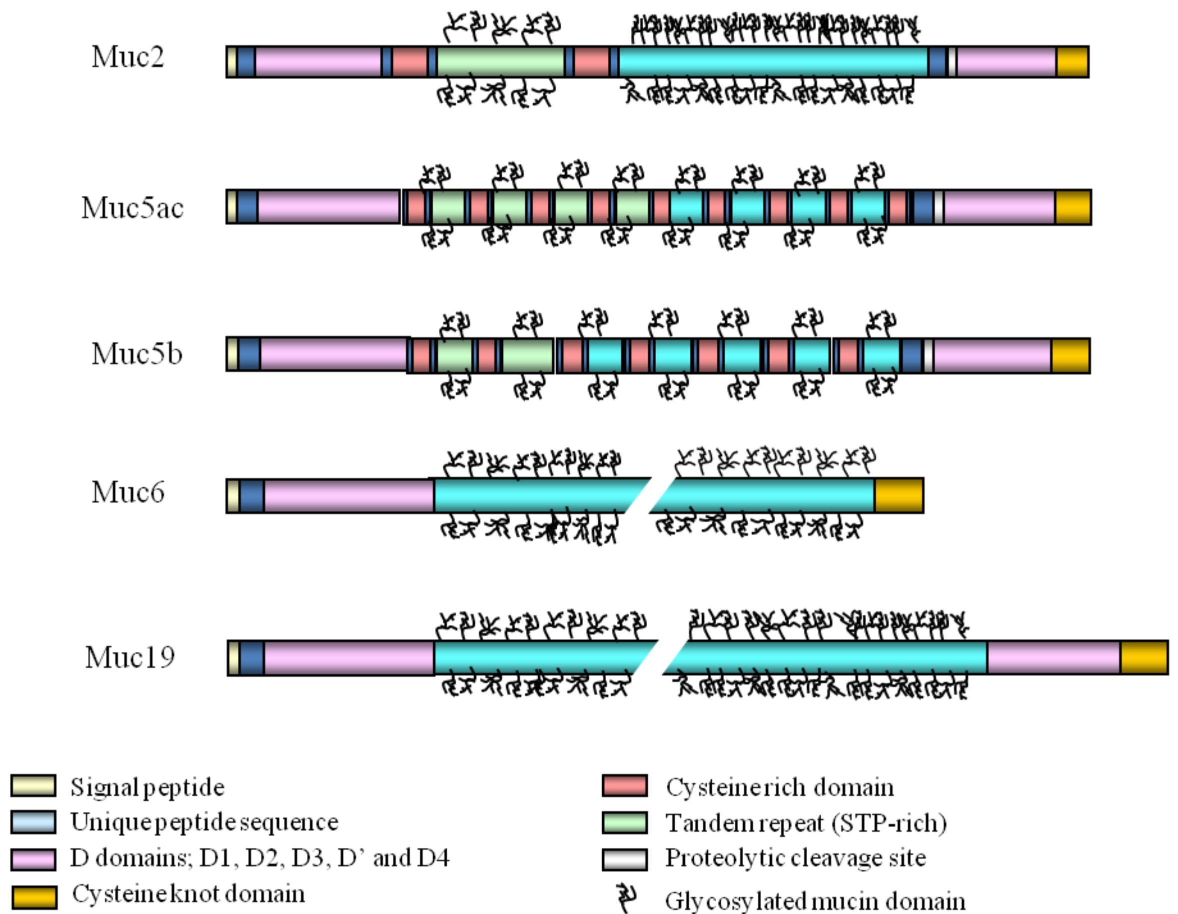

Figure 1.3: Schematic representation of the secreted polymeric (gel-forming) mucins; Muc2, Muc5ac, Muc5b, Muc6 and Muc19.

1.1.1.3 Secreted mucins

Epithelial surfaces are covered with a mucus gel layer which provides protection, and lubrication. The viscoelastic and barrier properties of mucus are mainly due to the polymeric secreted mucins. Of the secreted mucins, MUC2/Muc2, MUC5AC/Muc5ac, MUC5B/Muc5b, MUC6/Muc6 and MUC19/Muc19 are classed as polymeric (gel-forming)

mucins (Escande *et al*, 2004; Thornton *et al*, 2008; Thornton and Sheehan, 2004), whereas, MUC7 and MUC8 are non-polymeric mucins (**Figure 1.3**). Gel-forming mucins share a common evolutionary ancestor and, with the exception of MUC19/Muc19, are located on chromosome 11p15.5 in humans and on chromosome 7 in mice (Escande *et al*, 2004; Pigny *et al*, 1996). It is predominantly Muc2/MUC2 that is expressed in the normal adult intestine, however, Muc5ac/MUC5AC which is predominantly a gastric/respiratory mucin, has been shown to be produced in the intestine during foetal development (Buisine *et al*, 1998).

1.2 Assembly of mucins

The assembly of the secreted polymeric mucins is thought to be very similar to that of another O-glycosylated polymeric protein, von Willebrand factor (vWF) (Sheehan *et al*, 1996). Although the whole pathway has not yet been elucidated, vWF has been shown to form 20MDa polymers through disulphide bond linkage of 250kDa subunits (Sadler, 1998). Similar to vWF, N-glycosylation of mucin monomers occurs on the polypeptide chain in the endoplasmic reticulum (ER), this is followed by each linear mucin monomer (500nm) forming disulphide bonds with other monomers, which is mediated by the cysteine residues in the C-terminus (cysteine knot domain) (**Figure 1.4**) (Sheehan *et al*, 1995; Sheehan *et al*, 1996). The dimers are then translocated to the Golgi where the tandem repeat domains are O-glycosylated. Polymers, which can extend up to 10μm in length, are subsequently formed by disulphide bonds between the D-domains[1] present at the N-terminus. Once secreted, interactions between the mucin polymers form a network of large entangled structures (2-100MDa) (Hollingsworth and Swanson, 2004; Perez-Vilar and Hill, 1999; Sheehan *et al*, 1995).

Glycosylation, polymerisation and entanglement are all important factors in determining the gel-forming properties of the mucins, and hence the properties of the mucus gel. However, it is not yet clear whether these are the only essential factors. It is possible that carbohydrate-carbohydrate, mucin-carbohydrate, mucin-mucin or mucin-protein interactions contribute towards the gel-forming properties. Electron microscopy studies on airway mucus have shown unreduced mucins to form extensively entangled networks (Sheehan *et al*, 1996; Thornton and Sheehan, 2004). However, whether there are

[1] D-domains are very similar to and have been named after the D (dimerisation) domains from von Willebrand factor (vWF).

specific ‘cross-links’, or whether this occurs due to the sheer size and volume occupancy of the mucins is undetermined. It is important to acknowledge that, in the intestine, the mechanism of mucin assembly appears to differ from that observed in other mucus gels (Herrmann *et al*, 1999).

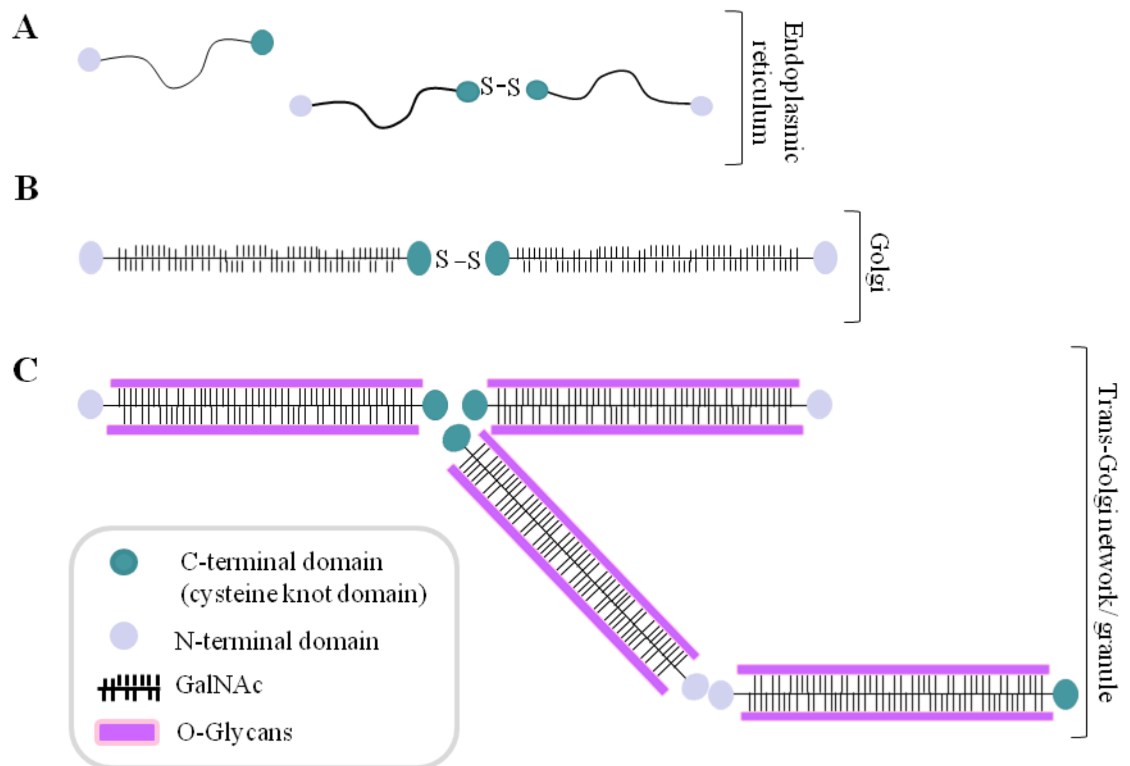

Figure 1.4: (A) In the endoplasmic reticulum mucin polypeptides dimerise through the disulphide linkages of their C-terminal cysteine knot domains. Dimers translocate to the Golgi (B) where O-glycosylation occurs followed by multimerisation of dimers (C).

Intestinal polymeric mucins are assembled via non-reducible linkages as well as disulphide bonds (Asker *et al*, 1995). Axelsson *et al.* hypothesise that the non-reducible ‘extra’ linkage is either between amino acids in the primary mucin sequence or possibly between smaller linking proteins (Axelsson *et al*, 1998); the details of the nature of this bond have not yet been elucidated. However, it was recently demonstrated that Fc-gamma binding protein is covalently bound to the N-terminus of MUC2/Muc2 (Johansson *et al*, 2009) and may act as a mucin cross-linker. In the intestine, the mucins are proposed to form a heterogeneous mucus barrier which is comprised of two distinct layers (Johansson *et al*, 2008): a ‘loose’ outer layer that the commensal flora can penetrate and an adherent inner layer that excludes the commensal flora from direct contact with the underlying epithelia (**Figure 1.5**). The adherent layer is densely packed with mucins and, upon

proteolytic cleavage, forms the loose outer layer hence the components within the layers are similar (Johansson *et al*, 2008). The different nature of the assembly of the mucins in tissues may reflect the different functional requirements at the sites: for instance, in the airways a transportable gel is required for clearance, whereas in the intestine, a non-transportable gel provides mechanical protection and lubrication.

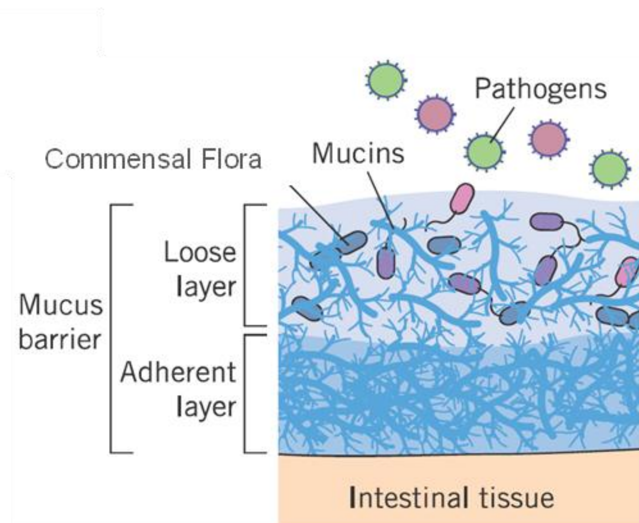

Figure 1.5: Illustration depicts the intestinal mucus barrier forming two layers, the outer 'loose' layer and the inner 'adherent' layer. The mucus layer protects against pathogens and the loose layer provides an environment for the commensal flora. (Diagram adapted from Hasnain, 2010).

1.3 Role of mucins in disease

Changes in the mucus layer underlie many common human disorders. For instance in respiratory diseases, such as asthma, cystic fibrosis (CF) and Chronic Obstructive Pulmonary Disease (COPD) an overproduction of thick viscous mucus impairs airway clearance (Thornton *et al*, 2008; Thornton and Sheehan, 2004). Since mucins are the main determinants of the properties of the mucus layer, changes in these complex glycoproteins are a feature of such disorders. The major gel-forming mucins in normal airway secretions are MUC5AC and MUC5B which exists in two different glycosylated forms (Thornton and Sheehan, 2004). In airway disease, an increase in mucin content and a change in mucin composition is observed: which imparts different charge to the molecule. Interestingly, the ratio of MUC5B charge form is altered and the low charge form predominates in disease conditions (Kirkham *et al*, 2002). Overall, these changes can lead to inflammation and increased coincidence of secondary bacterial infections (Thornton *et al*, 2008). Therefore, this suggested that the changes in mucin production could be associated with the

inflammation in infection and the alteration in mucins may lead to changes in mucus properties and, in turn, an inability to effectively clear pathogens.

As this project is concerned with the intestinal mucus barrier, a short review of what is known about changes in mucin expression, mucin production and mucin structure in intestinal diseases and parasitic infections is discussed below.

1.3.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a combined term used to describe a group of inflammatory conditions of the intestine, including ulcerative colitis and Crohn's disease. In contrast to mucin overproduction seen in airway disease, it is a decrease in mucin production, specifically of Muc2/MUC2, that is observed in intestinal diseases (McGuckin *et al*, 2009). Evidence from human and animal model studies (**Table 1.1**) has revealed that a diminished intestinal mucus barrier in the intestine can lead to direct bacterial contact with the epithelial cell layer, resulting in colitis-like inflammation (Heazlewood *et al*, 2008; Johansson *et al*, 2008). Aberrant expression of the respiratory/gastric mucin MUC5AC within the intestine has also been reported from studies on patients with ulcerative colitis (Forgue-Lafitte *et al*, 2007). Furthermore, qualitative changes in the glycosylation of mucins, in particular a loss in sulphation of mucins (Corfield *et al*, 1996), is associated with IBD and are thought to play a role in intestinal disease (An *et al*, 2007; Dawson *et al*, 2009; Tobisawa *et al*, 2010).

1.3.2 Colonic adenocarcinomas

A role for Muc2 has been suggested in the suppression of colorectal carcinoma: several murine models with alterations in intestinal secreted mucins result in developing invasive adenocarcinomas (**Table 1.1**). In particular, Muc2-deficient mice support this hypothesis, as these mice spontaneously develop adenomas which develop into invasive adenocarcinomas (Velcich *et al*, 2002). Furthermore, gastric and colonic tumours result in a loss of glycosylation on mucins, which could lead to altering the protective properties of the mucus barrier (Lidell *et al*, 2008). Similar to the changes in IBD, *de novo* expression of MUC5AC correlates with colon tumour progression and is used as a early marker for colonic carcinogenesis (Forgue-Lafitte *et al*, 2007).

Murine Model	Description	Phenotype	Reference
Muc2-deficient	In the absence of Muc2, goblet cells lose their goblet-like morphology, however the goblet cell lineage remains unaltered.	Mice spontaneously develop adenomas that progress to invasive adenocarcinomas. Mice also spontaneously develop colitis, are more susceptible to DSS-induced colitis and bacterial infections.	Van der Sluis *et al* 2006 Velcich *et al* 2002 Bergstorm *et al* 2010
Winnie/Eeyore	Missense mutations in the Muc2 gene results in aberrant Muc2 polymerisation, leading to ER stress.	Mice spontaneously develop colitis and intestinal permeability increases which results in high inflammation. Increased susceptibility to DSS-induced colitis.	Heazlewood *et al* 2008
Agr2-deficient	Mice deficient in Agr2, the disulphide isomerase secreted with Muc2, which results in abrogating Muc2 biosynthesis.	Spontaneous severe intestinal inflammation and goblet cell apoptosis.	Park *et al* 2009 Zhao *et al* 2010
C3GnT transferase-deficient	Loss of the core 3-derived O-glycans on Muc2, which results in decreased production of Muc2.	Mice have a diminished mucus barrier, increased permeability, are susceptible to DSS-induced colitis and developed colorectal tumours.	An *et al* 2007
GlcNAc6ST2-deficient	Mice deficient in N-acetyl glucosamine-6O-sulphotransferase, which catalyses the transfer of sulphates on mucins.	Reduced mucin sulphation, susceptibility to DSS-induced colitis and increased leukocyte inflammation.	Tobisawa *et al* 2010
NAS1-deficient	Mice deficient in Na^+ sulphate transporter 1 which is involved in sulphate homeostasis.	Reduced mucin sulphation, increased susceptibility to colitis. Mice are more susceptible to systemic infection with *Campylobacter jejuni*.	Dawson *et al* 2009

Table 1.1: Murine models of alterations in intestinal secreted mucins.

1.3.3 Protozoan infection

Entamoeba histolytica, a protozoan parasite that traverses the intestinal epithelium to reach the blood stream, expresses at least four cysteine proteases. These proteases have been shown to degrade MUC2 polymers *in vitro* (using CHO-K1 and LS174T cell lines), subsequently disrupting the gel-forming ability of the mucins and hence the protective nature of the mucus gel. The cleavage sites have also been identified in the C terminal domain which lies between two threonines in the sequence Arg/Lys-Thr-Thr (Lidell *et al*, 2006; Moncada *et al*, 2003; Moncada *et al*, 2000).

1.3.4 Bacterial and Fungal infections

Streptococcus pneumoniae (*S. pneumoniae)* is a gram-negative bacterium that resides in the middle ear, where *Muc2* is the major mucin expressed: infection induces acute otitis and hyperplasia of goblet cells (Lin *et al*, 2002). The level of the gel-forming mucin *Muc2* was more prominent in *S. pneumoniae* infected rats. Moreover, aberrant expression of *Muc5ac* was also observed post infection, which remained elevated until day 14 of the infection (Lin *et al*, 2002).

Lipopolysaccharide (LPS), a gram-negative bacterial product, can induce mucus hypersecretion. *In vitro* stimulation of murine-derived biliary epithelial cells with LPS induced a significant increase in *Muc2* and *Muc5ac* expression (Zen *et al*, 2002). Importantly, Muc2 has been shown to directly bind to *Citrobacter rodentium* and clear infection (Linden *et al*, 2008a). Furthermore, recently, it was demonstrated that the absence of Muc2 results in enhanced colonisation of *C. rodentium* (Bergstrom et al, 2010). *Campylobacter jejuni*, which is the main cause of gastroenteritis, can more readily cause systemic infections in mice with reduced mucin (Muc2) sulphation (Dawson *et al*, 2009) and in the absence of Muc2 (Bergstrom *et al*, 2010).

Helicobacter pylori is a gram-negative bacterium present in the stomach. Infection is usually asymptomatic, however it can cause peptic ulcers and stomach cancer. *H. pylori* is thought to degrade mucins through proteases, which leads to a reduction in the viscoelasticity of the mucus gel and hence allowing *H. pylori* to enter the mucus gel (Celli *et al*, 2009). Furthermore, sialyl and sulphate groups on gastric mucins play an important role in adhesion of the bacterium (Linden *et al*, 2004; Linden *et al*, 2002; Sutton, 2001). In addition, *H. pylori* has been found to reduce mucus secretion by directly impairing the function of goblet cells. Normally MUC5AC and MUC6 are the secreted mucins present in

the stomach and MUC2 expression is non-detectable. However, studies have shown that in MUC5AC and MUC6 expression decrease and MUC2 expression increases as the disease progresses (Byrd and Bresalier, 2000; Byrd *et al*, 1997; Byrd *et al*, 2000). MUC6 has been found to exhibit anti-bacterial activity, therefore the bacterium could possibly down-regulate its expression to make its own survival possible. Importantly, the properties of the mucus layer return to normal when the bacterium is eradicated.

Haemophilus influenzae was used in an *in vitro* model to study the signalling pathways involved in *MUC2* and *MUC5AC* expression. Transforming Growth Factor-β (TGF-β) receptor II and Toll-like receptor-2 (TLR-2) are utilised by the virus for the induction of *MUC2* and *MUC5AC* expression through different pathways. TGF-β signals through the Smad binding proteins, which translocate into the nucleus and act as transcription factors to mediate NF-κB dependent (initiated by TLR-2) induction of *MUC2* (Jono *et al*, 2002; Jono *et al*, 2003). In contrast to *MUC2, MUC5AC* is negatively regulated by TGF-β-Smad signalling through p38 MAPK. The role of TGF-β in influenza infection as an immune response mediator has not been investigated apart from its role in macrophage activation. Induction in the levels of TGF-β and *MUC2* has also been shown to correlate with resistance in the infection of *Candida albicans* (Jono *et al*, 2003), highlighting a protective function of MUC2.

1.3.5 Helminth infection

The most convincing data for the importance of mucus and mucins in nematode rejection comes from studies on the *Trichinella spiralis* (*T. spiralis*) and *Nippostrongylus brasiliensis* (*N. brasiliensis*). The nematodes *T. spiralis* and *N. brasiliensis* were shown to be enveloped in mucus just before their rejection, which lead to the 'mucus-trap' hypothesis (Bell *et al*, 1984; Carlisle *et al*, 1990; Miller *et al*, 1981).

Primary infection with the intestinal nematode *T. spiralis* results in goblet cell hyperplasia by day 8 of infection, enhancement of mucus secretion and glycocalyx[2] thickness. Another study showed that the mRNA expression of the mucins present in the small intestine, *Muc2* and *Muc17*, increases significantly in *T. spiralis* infected mice compared to the uninfected mice (Shekels *et al*, 2001). A reduction in glycosylation is observed in the *T. spiralis* infected mice, which may be due to the release of partially

[2] Glycocalyx is a "carbohydrate rich zone" found at the surface of the cell consisting of glycoproteins (including cell-surface mucins that project from the apical surface of the cells).

synthesised mucins or possibly due to the glycosidases activity exhibited by the parasite. Overall, this suggests that mucin expression and the mucin phenotype is different in different life cycle stages of the nematode and is regulated in a specific way, perhaps by the nematode or by the immune response (Khan, 2008).

Detailed studies by Yamauchi *et al.* have observed an increase in α-2-3-sialyltransferase IV and *Muc2* expression on day 2-3 after *T. spiralis* infection (Kawai *et al*, 2007; Yamauchi *et al*, 2006). Maturity of the *T. spiralis* helminth results in a marked increase in *Muc17* and 3-0 sulphotransferase-1 expression in the intestine, which remains high until the rejection of the parasite from the intestine on day 14, after which the levels start to decrease and return to normal by day 18. The up-regulation of sialomucins observed in *T. spiralis* infection has been found to be under the regulation of thymus derived T cells (Kawai *et al*, 2007). The alterations in mucin O-glycans (sulphate and sialyl groups) are thought to play an important role in the establishment of the nematodes, however, their exact role is unknown. One study illustrated that inducing sulphation *in vivo* leads to a reduction in the establishment of the intestinal nematode *Strongyloides venezuelensis* but not *N. brasiliensis* (Ishikawa *et al*, 1995). However, sulphotransferases up-regulated just before the rejection of the *N. brasiliensis* parasites are thought to play a protective role in worm expulsion.

1.4 Trichuriasis

The *Trichuris* nematode, which belongs to the phylum Trichuridae, is near ubiquitous as there have been about 90 to 95 species identified in different vertebrate host species worldwide (Lee, 2002); a few examples of these are shown in **Table 1.2**. These nematodes, coined whipworms because of their characteristic morphology, are unlike any other helminth as they do not undergo any tissue migration and are found only in the large intestine (Bethony *et al*, 2006; Lee, 2002). The distinctive bipolar eggs of *Trichuris spp.* can remain dormant but potentially infective for long periods. Once ingested through contaminated food or soil, the embryonated *Trichuris* eggs accumulate within the caecum (Bethony *et al*, 2006). After hatching the L1 *Trichuris* larvae embed into the epithelium of intestinal crypts and develop into cylindrical adult nematodes after moulting four times. The posterior end of the nematode protrudes in the lumen, where adults mate and a single female can lay up to 20,000 eggs per day that are shed in the faeces (Bethony *et al*, 2006). The anterior ends of the nematodes have a buccal cavity that is believed to secrete

digestive enzymes enabling the worm to burrow into the intestinal mucosa by forming a syncytial tunnel.

Species	Infects
Trichuris tenuis	Camel
Trichuris parvispicularis	Cane rat
Trichuris bovis	Cattle
Trichuris campanula	Cat
Trichuris vulpis	Dog
Trichuris giraffae	Giraffe
Trichuris globulosa	Goat
Trichuris muris	Mouse
Trichuris dediphis	Opossum
Trichuris suis	Pig
Trichuris trichiura	Primate
Trichuris leporis	Rabbit
Trichuris ovis	Sheep
Trichuris discolor	Zebra

Table 1.2: Species of *Trichuris* affecting their corresponding vertebrate hosts

1.4.1 Response to *Trichuris trichiura*

Trichuris trichiura affects up to a billion people worldwide; heavy infections can lead to chronic diarrhoea, intermittent stomach pain and weight loss. In heavily infected individuals, the trauma of the whipworm burrowing into the intestinal epithelium can cause a chronic haemorrhage that may result in anaemia (Bundy *et al*, 1987b). *T. trichiura* infection can last for up to 4 years and can also lead to secondary bacterial infections, which coupled with allergic responses, can result in colitis, proctitis, rectal prolapse, insomnia and malnutrition (Bundy and Cooper, 1989). The spectrum of differences observed in disease pathology in humans in terms of the intensity of infection are thought to be due to differences in age, genetic background, physiology and nutrition. Although infections are usually low level and asymptomatic, heavy infections correlate with impaired cognitive and physical development in children (Cooper and Bundy, 1988). These insidious *Trichuris* nematodes are extremely successful within the host, because

they not only have the ability to evade an immune attack but are thought to actively subvert the immune responses generated by the host (which will be discussed later on).

Trichuriasis is a disease still largely neglected by the medical and international community, despite its impact on economy, education and public health. This may be due to the geographical distribution of *T. trichiura* infection and possibly because infection does not cause sudden onset of serious debilitating symptoms. New strategies are at present being introduced to control transmission and develop vaccines as part of a multi-disease programme. Currently, treatment with multiple courses of benzimidole is common practice in the affected areas (Bethony *et al*, 2006). However, re-infections to the same level can easily occur as individuals remain in endemic areas and continuous intervention can lead to resistance to anti-helmintics (Turner *et al*, 2002). The chronic immune-activated state in nematode infections has been hypothesised to render the host susceptible to infections such as HIV and tuberculosis by impairing its ability to generate protective immunity (Borkow and Bentwich, 2000). Studies have demonstrated that nematode infections can modify responses to other vaccinations required in affected areas (Cooper *et al*, 1999; Duff *et al*, 1999). Other studies have shown that repetitive anti-helmintics treatment can lead to an increase in allergic reactions (van den Biggelaar *et al*, 2004). Therefore, infections with nematodes are not only debilitating themselves, but also have a profound effect on subsequent infections and treatment of other diseases. Nematode infections are generally associated with a T_H2-type immune response that down-regulates a T_H1-type immune response. Analysis of whole blood culture from a range of age groups (4-36 years) in Cameroon revealed that only 17% of that group produced IL-4, IL-9 and IL-13, in response to *Trichuris* infection. However, the majority of the group produced high levels of parasitic specific IL-10, TNF-α and IFN-γ (Jackson *et al*, 2004; Turner *et al*, 2002). A study by Turner *et. al.* illustrated that children infected with *T.trichiura* secrete more immunoregulatory cytokines, such as IL-10 and TGF-β (Turner *et al*, 2002). Other field studies have shown that chronic infection by *T. trichiura* in children is characterized by high concentrations of IgE, degranulating mast cells and the release of histamine. Furthermore, an age-dependent relationship was observed with infection intensity as antibody levels rise with the acquisition of infection (Bundy *et al*, 1987a; Bundy *et al*, 1988). The intensity of infection declines in adulthood which lead to the suggestion that continued exposure could potentially result in acquired immunity.

The data from field studies is limited and the interpretations of these correlations are subject to several variables, especially due to concurrent infections that occur in the

affected areas. Overall, the protective immune response to *T. trichiura* in humans remains largely unresolved. Most of our understanding about Trichuriasis is derived from the studies in animal model systems of this disease; mainly with the *Trichuris muris* (*T. muris*) model in mice and some with the *Trichuris suis* (*T. suis*) model in pigs.

1.4.2 Lessons from animal models

According to Wessler 'a living organism with an inherited, naturally acquired or induced pathological process that in one or more respects resembles the same phenomenon in man' will serve as a model system for studying a human disease (Wessler, 1976). *T.muris* infection occurs naturally in wild mice and using defined inbred mouse strains is utilised as a well-established laboratory model. *T. muris* is closely related to the human *T.trichiura* whipworm at a morphological and antigenic level (Cliffe and Grencis, 2004). Studies on the *T. muris* mouse model have shown that once ingested, the eggs accumulate in the caecum and predominantly at this site hatch. The L1 larvae establish infection within the epithelium at the base of the crypts of Lierberkühn. By day 35 the larvae develop into 1.5cm long cylindrical adult worms in the large intestine after moulting 4 times at days 9-11, day 21, day 24-28 and day 32-34 (Lee, 2002) (**Figure 1.6**).

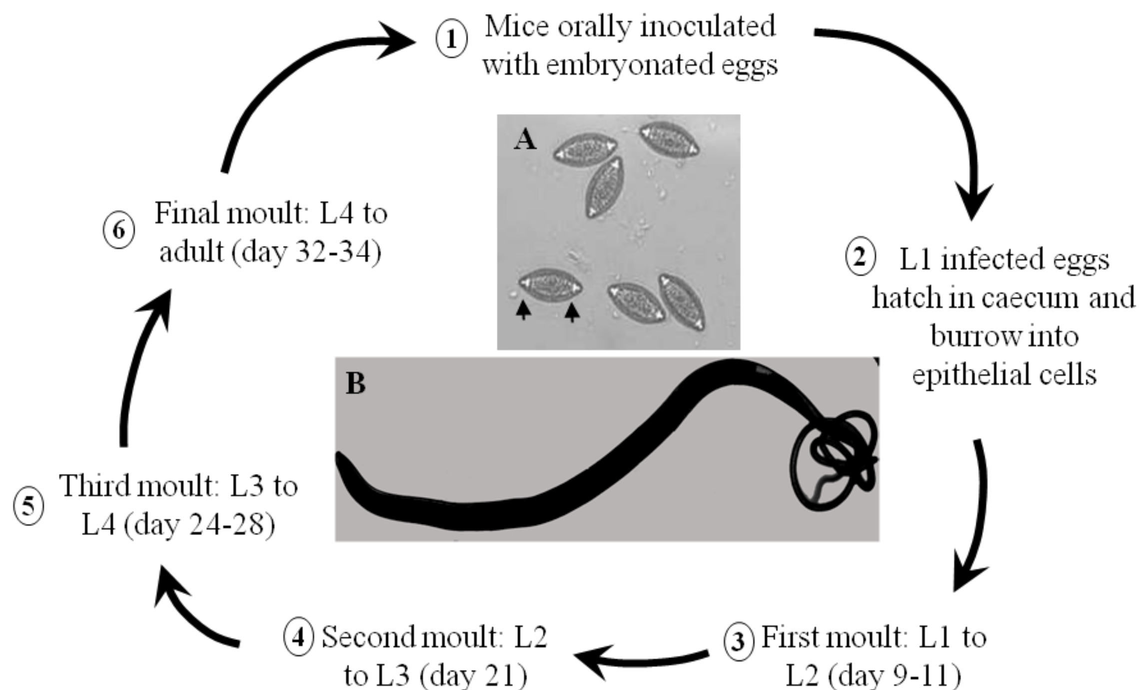

Figure 1.6: Life cycle of *T. muris* in mice. Mice are orally inoculated with embryonated eggs consisting of two distinctive polar plugs (A; highlighted by arrows). The larvae hatch in the caecum, burrow into the intestinal crypts. The adults (B) can develop up to 1.5cm in length by day 35 after four moults at days 9-11, day 21, days 24-28 and day 32-34.

The infection variability (susceptibility or resistance to chronic infection) seen in the human population can be replicated using different inbred strains of mice or by varying the dose of infection (Cliffe and Grencis, 2004; Else and deSchoolmeester, 2003). The *T.muris* model not only provides a manipulable system that can be used to investigate the host-parasite interactions, but it can be used to examine the immune mechanisms operating during acute and chronic infection.

1.4.3 Immunological basis of infection

Antigens from extracellular pathogens, such as *Trichuris* living in the intestinal crypts, are shed. These can be degraded and presented on major histocompatibility complex (MHC) Class II molecules by antigen presenting cells, such as dendritic cells and macrophages, to CD4 T cells. This subsequently stimulates their proliferation and differentiation into effector T helper (T_H) cells (Else and Grencis, 1991).

CD4 T cells have been shown to be extremely important in resistance to *T. muris* as neutralisation leads to the failure to expel the nematode (Koyama *et al*, 1995). On the basis of the differing cytokine secretion patterns, CD4 T cells have been characterised as T_H1 and T_H2 cells. More recently other subsets of CD4 T helper cells, T_H17 and regulatory T cells (T_{REG}), are also being investigated in this setting (D'Elia *et al*, 2009a). Several studies have focussed on the immune response that develops and, the cytokines and chemokines involved as a result of exposure to *Trichuris* (Artis and Grencis, 2008; Grencis, 2001). What initiates and promotes either a T_H1 or T_H2-type immune response is still largely undetermined. It is however, well-established that animal models resistant to chronic infection predominantly exhibit a T_H2 response, whilst the strains that are susceptible to chronic infection exhibit a T_H1 response (**Figure 1.7**).

1.4.3.1 T_H1 vs. T_H2 immune response

T_H2 responses, associated with resistance, are characterized by the production of IL-4, IL-5, IL-9 and IL-13 by CD4 T cells where both IL-4 and IL-13 operate through a STAT6 dependent pathway (Bancroft *et al*, 1998; Else *et al*, 1994; Faulkner *et al*, 1998). Therefore, it was concluded that IL-4 and IL-13 were important in mediating resistance as their production correlated with expulsion. Else *et al*. demonstrated this by the obstruction of the IL-4 receptor (IL-4R) in normally resistant mice which resulted in the animals mounting an appropriate T_H1 response and subsequent survival of the *T. muris* nematode.

Administration of the IL-4 cytokine complex into a susceptible model early on in infection resulted in worm expulsion (Else *et al*, 1994; Else *et al*, 1992). Furthermore, although the IL-13-deficient mice were shown to be able to mount a T_H2 response (characterised by the production of IL-4 and IL-9), they were unable to expel the parasites, demonstrating the importance of the IL-13 cytokine. Additionally, IL-13 can also mediate *Trichuris* expulsion in IL-4-deficient mice as IL-13 administration leads to expulsion (Bancroft *et al*, 2000; Bancroft *et al*, 1998). Additionally, a gender based difference with regards to susceptibility to chronic infection were observed in the IL-4/IL-4R-deficient mice (discussed later in detail) (Hepworth *et al*, 2009). IL-4 and IL-13 production can be enhanced by the release of chemotactic chemokines such as IL-5 (Bancroft *et al*, 1994), CCL2 (deSchoolmeester *et al*, 2003), CCL3 (Cruickshank *et al*, 2009) and CCL7 (deSchoolmeester *et al*, 2003); in fact, CCL2-deficient mice are susceptible to chronic *T. muris* infection. In contrast, the CCL11 chemokine involved in recruiting eosinophils acting in synergy with IL-5 has been shown not to be critical for *T. muris* worm expulsion (Dixon *et al*, 2006).

Neutralisation of IL-9, another T_H2-type cytokine, rendered resistant mice susceptible to *T. muris* infection, reduced colonic muscle contractility and goblet cell hyperplasia which accompany worm expulsion and will be discussed in detail later (Faulkner *et al*, 1998; Khan *et al*, 2003). It was demonstrated that IL-1 signalling is essential for $CD4^+$ T_H2-mediated protective immunity (Humphreys and Grencis, 2009). Furthermore, the IL-1-deficient mice were unable to expel the *T. muris* nematode. Another IL-1 superfamily member, IL-33 was shown to be up-regulated early during infection and skew the immune response towards T_H2-type immunity by inducing the release of IL-4, IL-13 and IL-9 (Humphreys *et al*, 2008). The recent addition to the IL-17 cytokine gene family, IL-25, is also critical in worm expulsion, as it promotes T_H2 cytokine-dependent immunity and limits heightened expression of T_H1-type cytokines, IFN-γ and IL-17 (Owyang *et al*, 2006). The IL-7-like cytokine, thymic stromal lymphopoietin (TSLP), known to promote T_H2-type immune responses by influencing DC responses in allergic models, has recently been shown to be essential to *T. muris* expulsion (Massacand *et al*, 2009; Taylor *et al*, 2009).

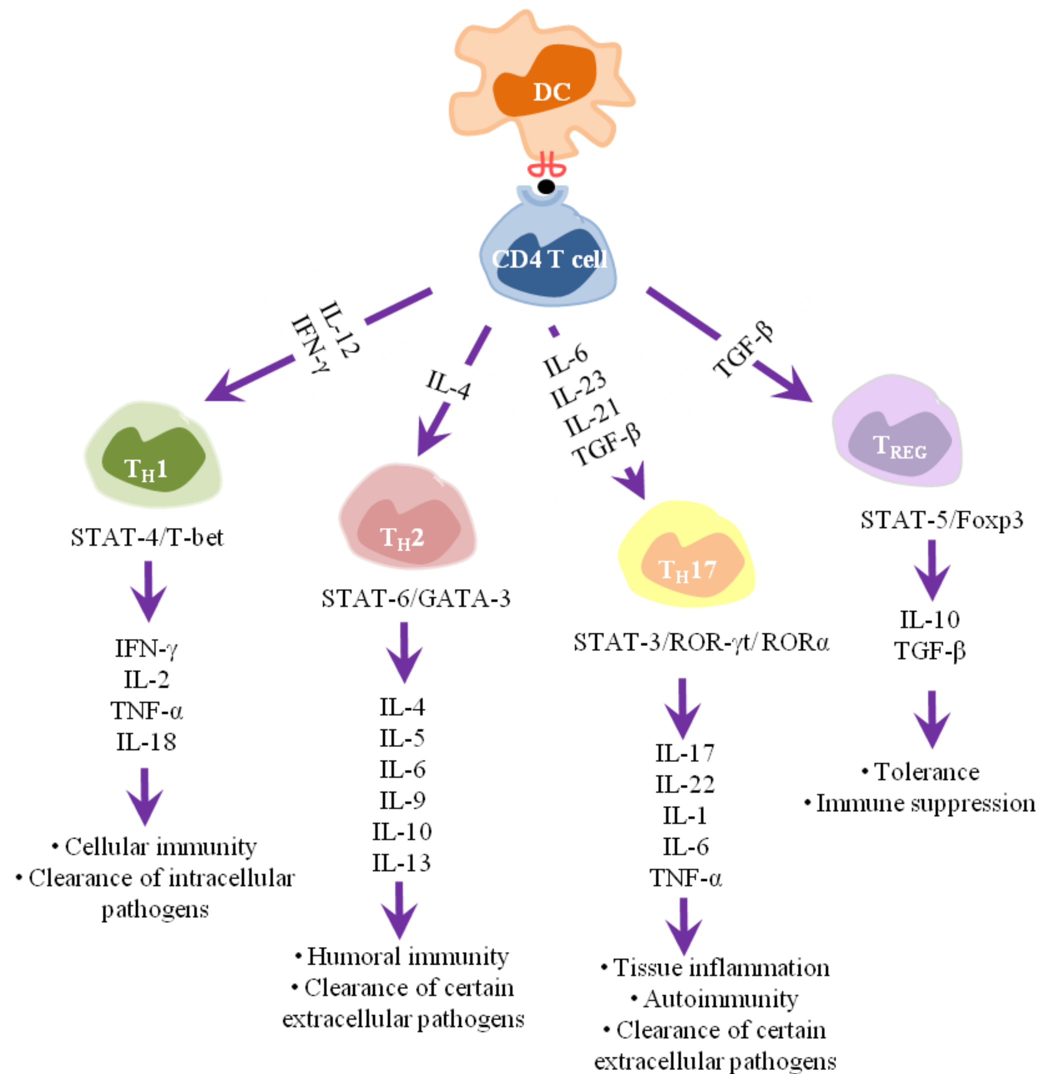

Figure 1.7: CD4 T cell activation by dendritic cells acting as antigen presenting cells and their differentiation into T helper cells, T_H1, T_H2, T_H17 or T_{REG}, dependent on the cytokines present and the signalling pathway activated. The T helper subsets produce cytokines which can then stimulate specific responses to pathogens/diseases.

The T_H1 immune response is mediated through the predominant production of IFN-γ, IL-18 and IL-12. IL-12 is mainly produced by macrophages and dendritic cells in response to bacterial products such as LPS via signal transducer and activator of transcription factors; STAT3 and STAT4. Indeed, the introduction of IL-12 into the resistant *T. muris* model was shown to result in susceptibility providing evidence that the T_H1 cytokine environment promotes *T. muris* chronicity (Bancroft *et al*, 1997). IL-18 gene deletion results in resistance in the susceptible mouse strains as it usually down regulates T_H2-type cytokine (IL-4 and IL-13) production (Helmby *et al*, 2001). The transcription factor, T-bet, which directs T_H1 lineage commitment is up-regulated by the cytokine IL-27. Moreover, IL-27R-deficiency in mice resulted in a strong T_H2-type immune response

which enables these mice to clear *T. muris* infection (Bancroft *et al*, 2004; Hunter *et al*, 2004).

Significant levels of IL-10 have been observed in chronic *T. muris* models; although IL-10-deficient mice suffer severe pathology and do not survive, suggesting IL-10 is critical in regulating both T_H1 and T_H2 responses. The role of IL-10, however, seems to be dependent on IL-12 as the IL-10/IL-12-deficient mice were resistant to *T. muris* infection (Schopf *et al*, 2002). Moreover, administration of broad spectrum antibiotics in the IL-10-deficient mice resulted in improving survival rates (Schopf *et al*, 2002). This suggests that secondary bacterial infections were contributing to the morbidity and mortality observed with *T. muris* infection.

1.4.3.2 T_H17 and T_{REG} immune reponse

Recently, a focus of attention has been the T helper subset T_H17 cells (which were discovered in autoimmune murine models) and T_{REG} cells. T_H17 cells have been shown to be essential for the clearance of extracellular bacteria such as *Citrobacter rodentium* (Mangan *et al*, 2006). IL-17, produced by T_H17 cells, is significantly up-regulated *in vivo* if challenged by *Borrelia burgdorferi* and blocking IL-17 results in abolishing the arthritis-like symptoms caused by this parasite (Weaver *et al*, 2006). Moreover, DC-derived TGF-β can direct a T_H1 cell to a T_H17 cell and IL-23 has been shown to induce differentiation of CD4 T cells into T_H17 in the absence of IFN- γ (IFN-γ-deficient mice) (Tato *et al*, 2006); however, their role in *T. muris* remains to be defined.

T_{REG} cells act as suppressors to control the immune response and interestingly, a correlation has been observed between *T. muris* infection and elevation in T_{REG} immune cells. They have been shown to protect the host from pathology induced by nematodes, however further studies are underway exploring this arm of the immune response (D'Elia *et al*, 2009a).

1.5 Host-mediated Immunomodulation

1.5.1 Immune effector cells

1.5.1.1 Antibodies: Infection with *Trichuris* has been shown to correlate with high levels of serum antibodies. There is some suggestion that FcγRI and FcγRII mediate the internalisation of IgG1, IgG2a, IgG2b and IgE. Therefore FcγR-deficient mice were used

to explore the role of antibodies in host protection. However, the FcγR-deficient mice were able to clear infection similarly to the wild-type mice (Betts and Else, 1999). In contrast, studies using B-cell-deficient μMT mice, showed that these mice were unable to expel the *T. muris* nematode. This study illustrated the importance of B cells in conditioning and maintaining a T_H2-type immune response (Blackwell and Else, 2001). Interestingly, a more recent study showed that antibodies in the *T. muris* setting may play a role in vaccination mediated immunity as opposed to natural immunity (Dixon *et al*, 2008).

1.5.1.2 Eosinophils: Moderate eosinophilia in the caecum accompanies *Trichuris* infection (Dixon *et al*, 2006). Coupled with this, eosinophils are generated by the T_H2 type cytokine IL-5 and hence were proposed to play an important role in resistance. As discussed previously, mice deficient in CCL11 (which acts synergistically with IL-5) to recruit eosinophils did not alter the worm expulsion kinetics (Dixon *et al*, 2006). Furthermore, the ablation of eosinophil production by blocking IL-5 in resistant mice still resulted in expulsion, confirming that eosinophils are not essential for protection (Betts and Else, 1999).

1.5.1.3 Mast cells: Local mastocytosis is also a feature of *Trichuris* infections. Mast cells too were considered to be significant as they are controlled by T_H2 type cytokines (IL-3, IL-4, IL-9 and IL-10) and in related systems such as *T. spiralis* infection mastocytosis is known to play a protective role (Grencis *et al*, 1993). *In vivo* administration of a monoclonal antibody against stem cell factor receptor (c-kit) was used to reduce the mast cell numbers in *T. muris* infected mice; however, this had no effect on the expulsion of the parasite (Betts and Else, 1999).

1.5.1.4 Dendritic cells and Basophils: Dendritic cell (DC) infiltration to the caecum has been shown to correlate with resistance in the *T. muris* model (Cruickshank *et al*, 2009). Cruickshank *et al* demonstrated that the elevation in DC markers occurs faster in resistant mice as compared to susceptible mice. This subsequently leads to differences in the chemokine pattern between resistance and susceptibility (Cruickshank *et al*, 2009). A recent study by Artis and colleagues have demonstrated that mice with restricted Cd11c expression on MHC class II presenting cells (classically defined as DC) were unable to induce a T_H2-type immune response, goblet cell hyperplasia and mice are subsequently unable to clear *T. muris* infection (Perrigoue *et al*, 2009). However, this study highlighted

that after nematode infection, basophils act as potential antigen presenting cells that express MHC class II and are able to drive and maintain a T_H2-type immune response.

1.5.1.5 <u>Macrophages:</u> Recent focus has been on the role of macrophages in a T_H2 setting, although macrophages do not usually initiate an immune response, they can amplify and possibly influence it by releasing several factors and acting as antigen presenting cells. It has been well documented that exposure to T_H1 (IFN-γ or LPS) or T_H2 (IL-4/IL-13) stimuli results in 'classically' (CAMØ) or 'alternatively' (AAMØ) activated macrophages respectively (Reyes and Terrazas, 2007). AAMØ in a T_H2 setting have been linked to roles in wound repair, anti-inflammation, and expulsion of parasite. Studies by Allen *et al.* have shown that AAMØ are crucial in killing the nematode, *Brugia malayi* (*B. malayi*) (Falcone *et al*, 2001). In infection with *Heligmosomoides bakeri/polygyrus* AAMØs produce arginase which is involved in clearing infection (reviewed in detail by Reyes and Terrazas, 2007).

Recent reports, however, suggest that AAMØs have an anti-inflammatory role and are induced to dampen down the immune response, as seen in *B. malayi* (Loke *et al*, 2000; MacDonald *et al*, 1998) and *Schistosoma mansoni* (Smith *et al*, 2004) infections. Furthermore, AAMØs produce IL-6, TGF-β (implicated to have fibrogenic properties) and CC chemokines, CCL6 and CCL9/10 associated with the polarisation towards a T_H2 immune response (Gordon, 2003). Immune effector molecules such as IL-1, IL-12, and nitric oxide, possibly secreted by macrophages, have also been associated with distorting the immune response to become predominantly T_H1; however, further studies are still needed to confirm these observations.

1.5.1.6 <u>Natural Killer cells:</u> Natural killer (NK) cells can produce high levels of IL-13 and therefore were proposed to play an important role in resistance to *Trichuris*. In the *T.spiralis* mouse model, IL-13 produced by NK cells had been shown to responsible for the disease pathology and changes in intestinal architecture (McDermott *et al*, 2005). However, no major differences in *Trichuris* worm expulsion kinetics were observed in the NK1.1-deficient mice (Cliffe and Grencis, 2004; Koyama, 2002). Recently, however using anti-asialo GM1 antibody which abolishes NK cell activity, a slight delay in *T. muris* worm expulsion was observed (Hepworth and Grencis, 2009). This suggested that NK cells may indeed play an active role in worm expulsion but are not essential for worm expulsion.

1.6 Intestinal effector mechanisms

1.6.1 Epithelial cells

Intestinal cell turnover plays an important role in modulating the fine balance that can cause resistance or susceptibility. Pulse chase experiments with bromodeoxyuridine (BrdU), which is incorporated into the DNA of proliferating cells, have shown that intestinal epithelial cell turnover is higher in resistant mice (BALB/c) compared to susceptible mice (AKR) (Cliffe *et al*, 2005). IFN-γ induces the production of the chemokine CXCL10, which reduces the rate of epithelial cell turnover resulting in the survival of the *T. muris* parasite in susceptible mouse models (Cliffe *et al*, 2005). Increasing the rate of epithelial cell turnover, by blocking CXCL10 in SCID (severe combined immunodeficient) mice, was sufficient for the expulsion of parasite. Furthermore, the CXCL10 receptor (CXCR3) was only expressed in susceptible mice (Cliffe *et al*, 2005).

As previously described, the intestinal epithelial cell-derived cytokine, TSLP, was also shown to be important in immunity to *Trichuris*. Recent studies illustrated that deletion/neutralisation of TSLP disrupts the T_H2 immune response (potentially by disrupting basophilia) and impairs resistance to *T. muris* (Massacand *et al*, 2009; Perrigoue *et al*, 2009; Taylor *et al*, 2009).

1.6.2 Enteroendocrine and Enterochromaffin cells

5-hydroxytryptamine (5-HT), mainly produced by enterochromaffin cells in the gut, is known to alter gut physiology, namely motility and secretory function, in gastrointestinal disorders (reviewed in detail by Akiho *et al*, 2007). Expression is induced by $CD4^+$ T cells and an increase in expression is observed in a resistant environment (T_H2-type immune response) (Wang *et al*, 2007), although its exact role in *Trichuris* infection is unknown.

As previously described, gender biased differences have been observed between acute and chronic *T. muris* infection. Recently, Hepworth *et al* demonstrated that IL-18 influences the expression of androgenic hormones (probably secreted by enteroendocrine cells), which are shown to be important in determining the balance between a T_H1 or a T_H2-type immune response (Hepworth *et al*, 2009). In this study, female-associated hormones induced and male-associated hormones suppressed T_H2 cytokine production, resulting in resistance and susceptibility to *T. muris*, respectively.

1.6.3 Smooth muscle

In other helminth models such as *T. spiralis*, a significant increase in intestinal motility and muscle contractility is associated with worm expulsion. At a cellular level, increases in actin and myosin are also observed in *T. spiralis* infection, which is thought to aid the expulsion of the parasite and are regulated through T_H2-type cytokines (for details refer to the review by Khan and Collins, 2006). In the *Nippostrongylus brasiliensis* infection model, using IL-4αR-smooth muscle specific conditional transgenic mice it was shown that administration of IL-12 using an adenovirus vector expressing the T_H1 cytokine resulted in delayed worm expulsion and significantly decreased hypercontractility (Khan and Collins, 2006; Zhao *et al*, 2003). Studies by the same group demonstrated that IL-9 directly influenced intestinal muscle function and interestingly, an increase in contractility was associated with accelerated *T. muris* clearance (Khan and Collins, 2006; Khan *et al*, 2003).

1.6.4 Mucus barrier

The *Trichuris* worm shares a very intimate relationship with the mucus barrier as it lives potentially within the layer of mucus that blankets the epithelial surface (**Figure 1.8**). In the *T. muris* model, goblet cell hyperplasia is associated with worm expulsion in several resistant settings (Artis and Grencis, 2008). Nuclear factor kappa B (NF-κB) transcription factor is thought to play a direct role in regulating goblet cell hyperplasia through the induction of IL-4 and IL-13 (Artis, 2006). Several studies have proposed bioactive goblet cell factors, secreted into the mucus barrier, act as potential candidates involved in resistance to *T.muris*; these are discussed below.

1.6.4.1 Gel-forming mucins: As introduced previously, the large polymeric mucin glycoproteins are the major product of goblet cells and determine gel-like properties of the mucus barrier. However, the role of mucins in *T. muris* remains largely unexplored.

1.6.4.2 Relm-β: Gene expression of Relm-β (resistin-like molecule-β), a goblet cell protein specifically expressed in the caecum and distal colon, was found to be highly induced in the T_H2 environment. It was suggested that Relm-β impairs survival of *T. muris* by affecting their chemosensory apparatus and hence impairing chemotaxis (Artis *et al*, 2004).

A recent study, however, revealed that Relm-β-deficient mice still have the ability to expel *T. muris* (Artis and Grencis, 2008).

1.6.4.3 <u>Tff3:</u> Intestinal goblet cells release another small protease-resistant cysteine-rich protein called intestinal trefoil factor (Tff3). Tff3 has been found to be important in goblet cell differentiation and proliferation and it has been shown to enhance mucus viscosity in mixtures with mucin glycoproteins (Thim *et al*, 2002). The expression of Tff3 usually correlates with the increase in goblet cell numbers and therefore was thought to be released as a selective effector molecule to protect the damaged mucosa by increasing mucus production and altering the mucus gel properties. It is possible that Tff3 is up-regulated in response to the damage caused by the nematode as it is known to be involved in mucosal repair (Podolsky *et al*, 2009).

1.6.4.4 <u>Intelectin:</u> Microarray analysis revealed that an anti-parasitic protein, intelectin, produced by goblet cells in the epithelium cell compartment, was up-regulated in the resistant (BALB/c) *T. muris* model (D'Elia *et al*, 2009b). Although supplementary studies are needed to define the precise role of intelectin in a T_H2 setting, there was no increase in intelectin expression in a susceptible environment (D'Elia *et al*, 2009b).

1.6.4.5 <u>Angiogenin-4:</u> Else *et al* proposed Angiogenin-4 (Ang-4) as a potential effector molecule as it was up-regulated in resistant mice (D'Elia *et al*, 2009b). Ang-4 has been known to be secreted into the intestinal lumen and exert its bactericidal activity against intestinal microbes (Ganz, 2003). Further immunohistochemical analysis identified goblet cells as a novel cellular source of Ang-4; however its role in resistance to *Trichuris* remains unknown.

1.6.5 Commensal Flora

An impressive number of commensal flora (10^{14}) resides in the niche of the *T.muris* nematode (Guarner and Malagelada, 2003). The adherent mucus layer in the intestine protects the epithelial cells from direct contact by bacteria (Johansson *et al*, 2008). A recent study has demonstrated an essential role for the commensal gut flora during *T. muris* infection (Hayes *et al,* 2010). This study shows that commensal flora, such as *Escherichia coli* bind to the opercula at the poles of the *Trichuris* egg to induce hatching. Moreover, it

was demonstrated that *in vivo* treatment of immunocompetent and immunodeficient mice with antibiotics significantly reduced the establishment of *T. muris* infection, therefore, highlighting the essential role of the microflora in the intestine during nematode infection.

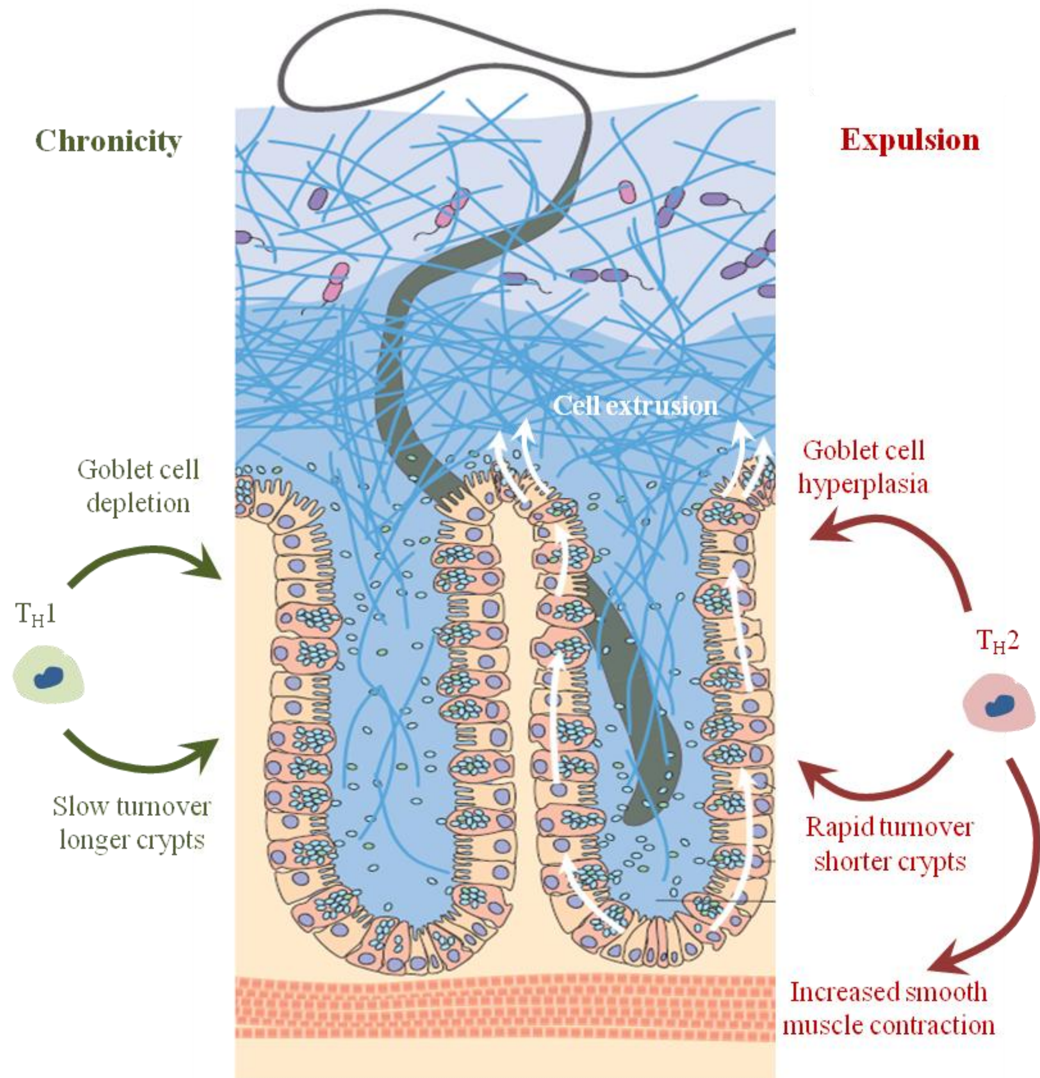

Figure 1.8: Simplified diagram depicts an adult *Trichuris* nematode embedded within intestinal crypts. The loose outer layer of the mucus barrier (light blue) consists of the bacteria and the nematode is in close contact with the mucus barrier. In resistance, the T_H2-type immune response activates the epithelial cell escalator by increasing cell turnover resulting in increased cell extrusion. Increased smooth muscle contractility and goblet cell hyperplasia is also observed during worm expulsion. In susceptibility, the T_H1-type immune response leads to slow turnover of epithelial cells, therefore longer crypts and chronic infection. Adapted from Hasnain 2010.

1.7 Nematode-mediated Immunomodulation

Even though a considerable amount is known about the immune response and the effector mechanisms that may mediate worm expulsion, how the nematode itself modulates the immune response to promote chronic infection is still elusive. Interestingly, more than

one isolate of *T. muris* exists: Edinburgh (E), Japan (J) and the Sobreda (S) isolates (Else, 2005). The S isolate influences infection to chronicity more readily than other strain and although the exact mechanism by which it evades the immune response is unknown, it provides evidence for nematode-mediated immunodulation (D'Elia *et al*, 2009a).

The excretory secretory products (ESPs) released from *Trichuris spp.* are thought to be very well-conserved and similar in terms of antigenicity (Roach *et al*, 1988). Interestingly, the major product from *T. muris* ESPs has homology to the T_H1-type cytokine IFN-γ, therefore it may be secreted to mimic and hence manipulate the host's immune response (Grencis and Entwistle, 1997). As discussed previously the *T. trichiura* nematode forms a syncytical tunnel and embeds itself into the caecal epithelium. Drake *et al* have shown that the major protein secreted by the *Trichuris* nematode has the ability to induce ion-conducting pores in a lipid bilayer, which in turn may aid nematode invasion (Drake *et al*, 1994). Other studies have attributed tunnel formation to the protease activity (zinc metalloprotease, thiol protease and phenol oxidase) detected in *Trichuris* ESPs (Hill and Sakanari, 1997). *T. suis* ESPs have been shown to contribute to epithelial cell damage within the niche and have anti-bacterial activity against *Campylobacter jejuni* (Abner *et al*, 2002). Although several studies have shown that the ESPs released through the course of infection are highly immunogenic (Drake *et al*, 1994), their function *in vivo* remains largely unknown.

1.7.1 Therapeutic uses of *Trichuris*

The fact that *Trichuris* induces a strong T_H2-type immune response while dampening the T_H1-type immune response has been exploited to treat disease. Recent data suggests that an immunoregulatory response (T_{REG}) is also generated in response to nematode infection. Therefore, diseases like inflammatory bowel disease, Crohn's disease and even allergies, where an imbalance in the immune response leads to pathology can be manipulated by utilising nematodes themselves or their ESPs. Most clinical studies have utilised the *T. suis* whipworm as it can transiently infect humans but cannot permanently colonise within the caecum.

Several clinical studies by Weinstock *et al* have shown that *T. suis*, can be successfully used in Crohn's disease to reduce mucosal inflammation (Summers *et al*, 2003; Summers *et al*, 2005a; Summers *et al*, 2005b; Summers *et al*, 2006). Furthermore, studies with *T. suis* ESPs in mice have been shown to prevent the onset of airway

hyperactivity observed in asthma (Hepworth *et al*, 2010). It was also reported that IgE levels in allergic rhinitis were dampened with treatment (Summers *et al*, 2010) however, contradicting data has also been reported (Bager *et al*, 2010). Although, the treatment with *T. suis* seems to be well tolerated in humans and safe at therapeutic doses, its long term effects are unknown.

1.8 Conclusion

The *Trichuris* nematode has co-evolved with its host and is finely tuned to its specific niche, where it minimises damage to prolong its survival. Animal models of trichuriasis provide a foundation of knowledge that defines the immunological basis of resistance and susceptibility to chronic infection. Overall, it is thought that, at least in mice, an amalgamation of mechanisms (under the T_H2 immunological control) result in worm expulsion (**Figure 1.8**). Although much is known about the immune regulation and potential effector mechanisms in mice, as discussed, important pieces of the puzzle remain unknown.

1.9 Aims of the project

Our knowledge concerning the role of the mucus barrier is limited when it comes to nematode infections. This project utilises both glycobiology and immunology approaches to explore the relationship of mucus barrier and the nematode, *T. muris*. Therefore, the major aims of the thesis are:

- To investigate the changes in the mucus barrier during the different life stages of the *T. muris* nematode by utilising models resistant or susceptible to chronic infection.
- To determine whether mucins, the major macromolecular component of the mucus barrier, play a role in the rejection of the *T. muris* nematode.
- To gain insight into the qualitative changes in mucins, specifically glycosylation, during infection.
- To investigate how the changes above are regulated by the host immune response and whether these changes have an impact on the physical properties of the mucus gel.

- To assess whether the nematodes can exert any effects on the mucus gel and whether the changes in the mucus barrier have an effect on the nematode itself.

1.10 Outline of this thesis

Changes in the mucosal barrier during acute and chronic *T. muris* infection (Chapter 2)

The glycoconjugate components of the intestinal epithelium provide physical protection and, by sequestering host defence factors provide a 'chemical' barrier (Thornton *et al*, 2008). The dynamic mucus barrier is the first line of defence, underneath this barrier is the 'glycocalyx' which consists of the cell-surface mucins intercalated into the apical surface of the epithelial cell layer (Linden *et al*, 2008b). The epithelial cell layer itself is an intrinsic barrier, consisting of goblet cells and enterocytes that secrete 'bioactive' factors. In this study we comprehensively characterise the changes within the mucosal barrier components in response to infection during acute and chronic *T. muris* infection (Hasnain *et al*, 2010a). Mucosal thickening, altered goblet cell differentiation resulting in depleted goblet cell numbers and glycoprotein secretion, were noted in the mice susceptible to chronic infection (Hasnain *et al*, 2010a). In contrast, a prominent feature during acute *T. muris* infection was a localised response of goblet cell hyperplasia (induced by the T_H2-type cytokine, IL-13). In addition to the synthesis of glycoproteins within the goblet cells of the caecum (the niche of the parasite), an increase in the secretion of glycoproteins into the mucus barrier, mediated by the gamma amino-butyric acid receptor-α3 (GABA-α3) was observed during nematode rejection.

Role of secreted gel-forming mucins during *T. muris* infection (Chapter 3 and 4)

Goblet cell hyperplasia and hypersecretion correlated with worm expulsion; however, the role of the major product of the intestinal goblet cells, Muc2 mucin, had not been addressed previously. Muc2 is a polymeric glycoprotein which forms a network within the mucus barrier and is responsible for its gel-like and viscous properties. Along with the increase in goblet cell number, the expression of Muc2 was also up-regulated during worm expulsion (Hasnain *et al*, 2010b). In resistance, the changes in the mucin network affect the physical nature of the mucus barrier and affect the vitality of the worm, thus aid worm expulsion. To address the protective capacity of Muc2 during *T. muris* worm expulsion, mice deficient in Muc2 were infected with a high dose of *T. muris* eggs.

A functional role for Muc2 was uncovered, as the absence of Muc2 significantly delayed worm expulsion despite a T_H2-type immune response against *T. muris*. Surprisingly, the *de novo* expression of Muc5ac was observed in the caecum of the Muc2-deficient mice and mice resistant to chronic infection, prior to worm expulsion (**Chapter 3**).

We subsequently addressed the importance of the IL-13 induced Muc5ac in the caecum during *T. muris* expulsion, by challenging Muc5ac-deficient mice with a high dose of *T. muris* eggs. Muc5ac-deficient mice still displayed an induction in T_H2-type cytokines leading to goblet cell hyperplasia, increased levels of Muc2 and an increase in epithelial cell turnover during *T. muris* infection. Importantly, however, the absence of Muc5ac rendered the mice susceptible to chronic *T. muris* infection. This study uncovers a novel role for Muc5ac, not just as a macromolecular component of the intestinal mucus barrier, but also as a crucial effector molecule acting independently of the underlying immune response to facilitate worm expulsion (**Chapter 4**).

Changes in mucin glycosylation during *T. muris* infection (Chapter 5)

The heavily glycosylated mucin proteins were shown to be critical to *T. muris* worm expulsion, as discussed above. The degree of sulphation on mucins in the niche of the parasite, i.e. the caecum, is higher than in any other site in the body. Therefore, in order to further dissect the mechanisms by which mucins may confer protection we characterised the changes in mucin glycosylation, in particular sulphation, during *T. muris* infection. Histological analysis revealed increased mucin sulphation during resistance; in contrast, mucins were highly sialylated during chronic *T. muris* infection. In light of these findings we hypothesised that mucin sulphation may affect the kinetics of worm establishment/expulsion. Therefore, to this end we infected mice deficient in the sulphate transporter NaS1, which have disturbed sulphate homeostasis and hence depleted intestinal mucin sulphation, with a high dose of *T.muris* eggs. Depleted levels of mucin sulphation did not affect worm establishment. However, as the demand of cellular sulphate increases after infection, mucin sulphation recovered in the NaS1-deficient mice by up-regulating the sulphate anion transporter1 (Sat1). This study indicated that increased sulphomucin levels are a physiological response against intestinal parasitic infection.

Effects of nematode Excretory Secretory Products (ESPs) on the mucus gel (Chapter 6)

In order for the nematode to successfully survive within the host, it releases ESPs to actively exert its immunomodulatory functions. As the *Trichuris* nematode lives under a

blanket of mucus during chronic infection, we speculated that the adult nematode may directly affect the mucus gel to promote its own survival within the host. Using biochemical techniques we demonstrate that the ESPs released by the nematode have the ability to alter the mucins within the mucus gel. Moreover, the changes in the mucus barrier in resistance prevented the ESPs from degrading the mucins; however, mucins extracted from susceptible mice post infection were more prone to degradation. Overall, our experiments illustrate that ESPs contain a serine protease that not only had the ability to depolymerise the mucin network but may also potentially affect the 'non-reducible' linkage specific to the Muc2 protein. Therefore, the *T. muris* nematode may secrete ESPs to degrade the mucus gel as part of its regime to aid its movement and promote its own survival within the host, during chronic infection.

REFERENCES

Abner SR, Hill DE, Turner JR, Black ED, Bartlett P, Urban JF, Mansfield LS (2002). Response of intestinal epithelial cells to *Trichuris suis* excretory-secretory products and the influence on *Campylobacter jejuni* invasion under in vitro conditions. *J Parasitol* **88**(4)**:** 738-745.

Akiho H, Khan WI, Al-Kaabi A, Blennerhassett P, Deng Y, Collins SM (2007). Cytokine modulation of muscarinic receptors in the murine intestine. *Am J Physiol Gastrointest Liver Physiol* **293**(1)**:** G250-255.

An G, Wei B, Xia B, McDaniel JM, Ju T, Cummings RD, Braun J, Xia L (2007). Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. *J Exp Med* **204**(6)**:** 1417-1429.

Artis D (2006). New weapons in the war on worms: identification of putative mechanisms of immune-mediated expulsion of gastrointestinal nematodes. *Int J Parasitol* **36**(6)**:** 723-733.

Artis D, Grencis RK (2008). The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunol* **1**(4)**:** 252-264.

Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, Schad GA, Scott P, Wu GD (2004). RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc Natl Acad Sci U S A* **101**(37)**:** 13596-13600.

Asker N, Baeckstrom D, Axelsson MA, Carlstedt I, Hansson GC (1995). The human MUC2 mucin apoprotein appears to dimerize before O-glycosylation and shares epitopes with the 'insoluble' mucin of rat small intestine. *Biochem J* **308 (Pt 3):** 873-880.

Axelsson MA, Asker N, Hansson GC (1998). O-glycosylated MUC2 monomer and dimer from LS 174T cells are water-soluble, whereas larger MUC2 species formed early during

biosynthesis are insoluble and contain nonreducible intermolecular bonds. *J Biol Chem* **273**(30)**:** 18864-18870.

Bager P, Arnved J, Ronborg S, Wohlfahrt J, Poulsen LK, Westergaard T, Petersen HW, Kristensen B, Thamsborg S, Roepstorff A, Kapel C, Melbye M (2010). Trichuris suis ova therapy for allergic rhinitis: a randomized, double-blind, placebo-controlled clinical trial. *J Allergy Clin Immunol* **125**(1)**:** 123-130 e121-123.

Bancroft AJ, Artis D, Donaldson DD, Sypek JP, Grencis RK (2000). Gastrointestinal nematode expulsion in IL-4 knockout mice is IL-13 dependent. *Eur J Immunol* **30**(7)**:** 2083-2091.

Bancroft AJ, Else KJ, Grencis RK (1994). Low-level infection with *Trichuris muris* significantly affects the polarization of the CD4 response. *Eur J Immunol* **24**(12)**:** 3113-3118.

Bancroft AJ, Else KJ, Sypek JP, Grencis RK (1997). Interleukin-12 promotes a chronic intestinal nematode infection. *Eur J Immunol* **27**(4)**:** 866-870.

Bancroft AJ, Humphreys NE, Worthington JJ, Yoshida H, Grencis RK (2004). WSX-1: a key role in induction of chronic intestinal nematode infection. *J Immunol* **172**(12)**:** 7635-7641.

Bancroft AJ, McKenzie AN, Grencis RK (1998). A critical role for IL-13 in resistance to intestinal nematode infection. *J Immunol* **160**(7)**:** 3453-3461.

Barrett K (2006). *Physiology of the gastrointestinal tract*, 4th edn. Elsevier Academic Press.

Bell RG, Adams LS, Ogden RW (1984). Intestinal mucus trapping in the rapid expulsion of Trichinella spiralis by rats: induction and expression analyzed by quantitative worm recovery. *Infect Immun* **45**(1)**:** 267-272.

Bergstrom KS, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, Ryz N, Huang T, Velcich A, Finlay BB, Chadee K, Vallance BA (2010). Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathog* **6**(5)**:** e1000902.

Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* **367**(9521)**:** 1521-1532.

Betts CJ, Else KJ (1999). Mast cells, eosinophils and antibody-mediated cellular cytotoxicity are not critical in resistance to *Trichuris muris*. *Parasite Immunol* **21**(1)**:** 45-52.

Blackwell NM, Else KJ (2001). B cells and antibodies are required for resistance to the parasitic gastrointestinal nematode *Trichuris muris*. *Infect Immun* **69**(6)**:** 3860-3868.

Borkow G, Bentwich Z (2000). Eradication of helminthic infections may be essential for successful vaccination against HIV and tuberculosis. *Bull World Health Organ* **78**(11)**:** 1368-1369.

Brittan M, Wright NA (2004). Stem cell in gastrointestinal structure and neoplastic development. *Gut* **53**(6)**:** 899-910.

Buisine MP, Devisme L, Savidge TC, Gespach C, Gosselin B, Porchet N, Aubert JP (1998). Mucin gene expression in human embryonic and fetal intestine. *Gut* **43**(4)**:** 519-524.

Bundy DA, Cooper ES (1989). *Trichuris* and trichuriasis in humans. *Adv Parasitol* **28:** 107-173.

Bundy DA, Cooper ES, Thompson DE, Anderson RM, Didier JM (1987a). Age-related prevalence and intensity of *Trichuris trichiura* infection in a St. Lucian community. *Trans R Soc Trop Med Hyg* **81**(1)**:** 85-94.

Bundy DA, Cooper ES, Thompson DE, Didier JM, Anderson RM, Simmons I (1987b). Predisposition to *Trichuris trichiura* infection in humans. *Epidemiol Infect* **98**(1)**:** 65-71.

Bundy DA, Cooper ES, Thompson DE, Didier JM, Simmons I (1988). Effect of age and initial infection intensity on the rate of reinfection with *Trichuris trichiura* after treatment. *Parasitology* **97 (Pt 3):** 469-476.

Byrd JC, Bresalier RS (2000). Alterations in gastric mucin synthesis by H*elicobacter pylori*. *World J Gastroenterol* **6**(4)**:** 475-482.

Byrd JC, Yan P, Sternberg L, Yunker CK, Scheiman JM, Bresalier RS (1997). Aberrant expression of gland-type gastric mucin in the surface epithelium of *Helicobacter pylori*-infected patients. *Gastroenterology* **113**(2)**:** 455-464.

Byrd JC, Yunker CK, Xu QS, Sternberg LR, Bresalier RS (2000). Inhibition of gastric mucin synthesis by *Helicobacter pylori*. *Gastroenterology* **118**(6)**:** 1072-1079.

Carlisle MS, McGregor DD, Appleton JA (1990). The role of mucus in antibody-mediated rapid expulsion of *Trichinella spiralis* in suckling rats. *Immunology* **70**(1)**:** 126-132.

Celli JP, Turner BS, Afdhal NH, Keates S, Ghiran I, Kelly CP, Ewoldt RH, McKinley GH, So P, Erramilli S, Bansil R (2009). *Helicobacter pylori* moves through mucus by reducing mucin viscoelasticity. *Proc Natl Acad Sci U S A* **106**(34)**:** 14321-14326.

Cliffe LJ, Grencis RK (2004). The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol* **57:** 255-307.

Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* **308**(5727)**:** 1463-1465.

Cooper ES, Bundy DA (1988). *Trichuris* is not trivial. *Parasitol Today* **4**(11)**:** 301-306.

Cooper PJ, Espinel I, Wieseman M, Paredes W, Espinel M, Guderian RH, Nutman TB (1999). Human onchocerciasis and tetanus vaccination: impact on the postvaccination antitetanus antibody response. *Infect Immun* **67**(11)**:** 5951-5957.

Corfield AP, Myerscough N, Bradfield N, Corfield Cdo A, Gough M, Clamp JR, Durdey P, Warren BF, Bartolo DC, King KR, Williams JM (1996). Colonic mucins in ulcerative colitis: evidence for loss of sulfation. *Glycoconj J* **13**(5)**:** 809-822.

Cruickshank SM, Deschoolmeester ML, Svensson M, Howell G, Bazakou A, Logunova L, Little MC, English N, Mack M, Grencis RK, Else KJ, Carding SR (2009). Rapid dendritic cell mobilization to the large intestinal epithelium is associated with resistance to *Trichuris muris* infection. *J Immunol* **182**(5)**:** 3055-3062.

D'Elia R, Behnke JM, Bradley JE, Else KJ (2009a). Regulatory T cells: a role in the control of helminth-driven intestinal pathology and worm survival. *J Immunol* **182**(4)**:** 2340-2348.

D'Elia R, DeSchoolmeester ML, Zeef LA, Wright SH, Pemberton AD, Else KJ (2009b). Expulsion of *Trichuris muris* is associated with increased expression of angiogenin 4 in the gut and increased acidity of mucins within the goblet cell. *BMC Genomics* **10:** 492.

Dawson PA, Huxley S, Gardiner B, Tran T, McAuley JL, Grimmond S, McGuckin MA, Markovich D (2009). Reduced mucin sulfonation and impaired intestinal barrier function in the hyposulfataemic NaS1 null mouse. *Gut* **58**(7)**:** 910-919.

deSchoolmeester ML, Little MC, Rollins BJ, Else KJ (2003). Absence of CC chemokine ligand 2 results in an altered Th1/Th2 cytokine balance and failure to expel *Trichuris muris* infection. *J Immunol* **170**(9)**:** 4693-4700.

Dixon H, Blanchard C, Deschoolmeester ML, Yuill NC, Christie JW, Rothenberg ME, Else KJ (2006). The role of Th2 cytokines, chemokines and parasite products in eosinophil recruitment to the gastrointestinal mucosa during helminth infection. *Eur J Immunol* **36**(7)**:** 1753-1763.

Dixon H, Johnston CE, Else KJ (2008). Antigen selection for future anti-Trichuris vaccines: a comparison of cytokine and antibody responses to larval and adult antigen in a primary infection. *Parasite Immunol* **30**(9)**:** 454-461.

Drake L, Korchev Y, Bashford L, Djamgoz M, Wakelin D, Ashall F, Bundy D (1994). The major secreted product of the whipworm, *Trichuris*, is a pore-forming protein. *Proc Biol Sci* **257**(1350)**:** 255-261.

Duff EM, Anderson NM, Cooper ES (1999). Plasma insulin-like growth factor-1, type 1 procollagen, and serum tumor necrosis factor alpha in children recovering from *Trichuris* dysentery syndrome. *Pediatrics* **103**(5)**:** e69.

Else KJ (2005). Have gastrointestinal nematodes outwitted the immune system? *Parasite Immunol* **27**(10-11)**:** 407-415.

Else KJ, deSchoolmeester ML (2003). Immunity to *Trichuris muris* in the laboratory mouse. *J Helminthol* **77**(2)**:** 95-98.

Else KJ, Finkelman FD, Maliszewski CR, Grencis RK (1994). Cytokine-mediated regulation of chronic intestinal helminth infection. *J Exp Med* **179**(1)**:** 347-351.

Else KJ, Grencis RK (1991). Helper T-cell subsets in mouse trichuriasis. *Parasitol Today* **7**(11)**:** 313-316.

Else KJ, Hultner L, Grencis RK (1992). Cellular immune responses to the murine nematode parasite *Trichuris muris*. II. Differential induction of TH-cell subsets in resistant versus susceptible mice. *Immunology* **75**(2)**:** 232-237.

Escande F, Porchet N, Bernigaud A, Petitprez D, Aubert JP, Buisine MP (2004). The mouse secreted gel-forming mucin gene cluster. *Biochim Biophys Acta* **1676**(3)**:** 240-250.

Falcone FH, Loke P, Zang X, MacDonald AS, Maizels RM, Allen JE (2001). A *Brugia malayi* homolog of macrophage migration inhibitory factor reveals an important link between macrophages and eosinophil recruitment during nematode infection. *J Immunol* **167**(9)**:** 5348-5354.

Faulkner H, Renauld JC, Van Snick J, Grencis RK (1998). Interleukin-9 enhances resistance to the intestinal nematode *Trichuris muris*. *Infect Immun* **66**(8)**:** 3832-3840.

Forgue-Lafitte ME, Fabiani B, Levy PP, Maurin N, Flejou JF, Bara J (2007). Abnormal expression of M1/MUC5AC mucin in distal colon of patients with diverticulitis, ulcerative colitis and cancer. *Int J Cancer* **121**(7)**:** 1543-1549.

Ganz T (2003). Angiogenin: an antimicrobial ribonuclease. *Nat Immunol* **4**(3)**:** 213-214.

Gordon S (2003). Alternative activation of macrophages. *Nat Rev Immunol* **3**(1)**:** 23-35.

Grencis RK (2001). Cytokine regulation of resistance and susceptibility to intestinal nematode infection - from host to parasite. *Vet Parasitol* **100**(1-2)**:** 45-50.

Grencis RK, Else KJ, Huntley JF, Nishikawa SI (1993). The *in vivo* role of stem cell factor (c-kit ligand) on mastocytosis and host protective immunity to the intestinal nematode Trichinella spiralis in mice. *Parasite Immunol* **15**(1)**:** 55-59.

Grencis RK, Entwistle GM (1997). Production of an interferon-gamma homologue by an intestinal nematode: functionally significant or interesting artefact? *Parasitology* **115 Suppl:** S101-106.

Guarner F, Malagelada JR (2003). Gut flora in health and disease. *Lancet* **361**(9356)**:** 512-519.

Hasnain SZ (2010). Mucus: a slippery slope for worms. In: Buttler DR (ed) *Biological Science Review*. Philip Allan. Vol. 23.

Hasnain SZ, Thornton DJ, Grencis RK (2010a). Changes in the mucosal barrier during acute and chronic *Trichuris muris* infection. *Parasite Immunology*. *In press.*

Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010b). Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* **138**(5)**:** 1763-1771.

Hayes KS, Bancroft AJ, Goldrick M, Portsmouth C, Roberts IS, Grencis RK (2010). Exploitation of the intestinal microflora by the parasitic nematode *Trichuris muris*. *Science* **328**(5984)**:** 1391-1394.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA (2008). Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* **5**(3)**:** e54.

Hedrich H (2004). *The Laboratory Mouse* Academic Press.

Helmby H, Takeda K, Akira S, Grencis RK (2001). Interleukin (IL)-18 promotes the development of chronic gastrointestinal helminth infection by downregulating IL-13. *J Exp Med* **194**(3)**:** 355-364.

Hepworth MR, Grencis RK (2009). Disruption of Th2 immunity results in a gender-specific expansion of IL-13 producing accessory NK cells during helminth infection. *J Immunol* **183**(6)**:** 3906-3914.

Hepworth MR, Hamelmann E, Lucius R, Hartmann S (2010). Looking into the future of Trichuris suis therapy. *J Allergy Clin Immunol*. *In press.*

Hepworth MR, Hardman MJ, Grencis RK (2009). The role of sex hormones in the development of Th2 immunity in a gender-biased model of *Trichuris muris* infection. *Eur J Immunol*.

Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, Carlstedt I (1999). Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. *J Biol Chem* **274**(22)**:** 15828-15836.

Hill DE, Sakanari JA (1997). *Trichuris suis*: thiol protease activity from adult worms. *Exp Parasitol* **85**(1)**:** 55-62.

Hollingsworth MA, Swanson BJ (2004). Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer* **4**(1)**:** 45-60.

Humphreys NE, Grencis RK (2009). IL-1-dependent, IL-1R1-independent resistance to gastrointestinal nematodes. *Eur J Immunol* **39**(4)**:** 1036-1045.

Humphreys NE, Xu D, Hepworth MR, Liew FY, Grencis RK (2008). IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol* **180**(4)**:** 2443-2449.

Hunter CA, Villarino A, Artis D, Scott P (2004). The role of IL-27 in the development of T-cell responses during parasitic infections. *Immunol Rev* **202:** 106-114.

Ishikawa N, Shi BB, Khan AI, Nawa Y (1995). Reserpine-induced sulphomucin production by goblet cells in the jejunum of rats and its significance in the establishment of intestinal helminths. *Parasite Immunol* **17**(11)**:** 581-586.

Jackson JA, Turner JD, Rentoul L, Faulkner H, Behnke JM, Hoyle M, Grencis RK, Else KJ, Kamgno J, Boussinesq M, Bradley JE (2004). T helper cell type 2 responsiveness predicts future susceptibility to gastrointestinal nematodes in humans. *J Infect Dis* **190**(10)**:** 1804-1811.

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* **105**(39)**:** 15064-15069.

Johansson ME, Thomsson KA, Hansson GC (2009). Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. *J Proteome Res* **8**(7)**:** 3549-3557.

Jono H, Shuto T, Xu H, Kai H, Lim DJ, Gum JR, Jr., Kim YS, Yamaoka S, Feng XH, Li JD (2002). Transforming growth factor-beta -Smad signaling pathway cooperates with NF-kappa B to mediate nontypeable Haemophilus influenzae-induced MUC2 mucin transcription. *J Biol Chem* **277**(47)**:** 45547-45557.

Jono H, Xu H, Kai H, Lim DJ, Kim YS, Feng XH, Li JD (2003). Transforming growth factor-beta-Smad signaling pathway negatively regulates nontypeable Haemophilus influenzae-induced MUC5AC mucin transcription via mitogen-activated protein kinase (MAPK) phosphatase-1-dependent inhibition of p38 MAPK. *J Biol Chem* **278**(30)**:** 27811-27819.

Kawai Y, Yamauchi J, Soga K, Yamada M, Uchikawa R, Tegoshi T, Arizono N (2007). T cell-dependent and -independent expression of intestinal epithelial cell-related molecules in rats infected with the nematode *Nippostrongylus brasiliensis*. *APMIS* **115**(3)**:** 210-217.

Khan WI (2008). Physiological changes in the gastrointestinal tract and host protective immunity: learning from the mouse-*Trichinella spiralis* model. *Parasitology* **135**(6)**:** 671-682.

Khan WI, Collins SM (2006). Gut motor function: immunological control in enteric infection and inflammation. *Clin Exp Immunol* **143**(3)**:** 389-397.

Khan WI, Richard M, Akiho H, Blennerhasset PA, Humphreys NE, Grencis RK, Van Snick J, Collins SM (2003). Modulation of intestinal muscle contraction by interleukin-9 (IL-9) or IL-9 neutralization: correlation with worm expulsion in murine nematode infections. *Infect Immun* **71**(5)**:** 2430-2438.

Kirkham S, Sheehan JK, Knight D, Richardson PS, Thornton DJ (2002). Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem J* **361**(Pt 3)**:** 537-546.

Koyama K (2002). NK1.1+ cell depletion *in vivo* fails to prevent protection against infection with the murine nematode parasite *Trichuris muris*. *Parasite Immunol* **24**(11-12)**:** 527-533.

Koyama K, Tamauchi H, Ito Y (1995). The role of CD4+ and CD8+ T cells in protective immunity to the murine nematode parasite *Trichuris muris*. *Parasite Immunol* **17**(3)**:** 161-165.

Lee DL (2002). *The Biology of Nematodes*. Taylor and Francis London.

Lidell ME, Bara J, Hansson GC (2008). Mapping of the 45M1 epitope to the C-terminal cysteine-rich part of the human MUC5AC mucin. *FEBS J* **275**(3)**:** 481-489.

Lidell ME, Moncada DM, Chadee K, Hansson GC (2006). *Entamoeba histolytica* cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. *Proc Natl Acad Sci U S A* **103**(24)**:** 9298-9303.

Lin J, Tsuboi Y, Pan W, Giebink GS, Adams GL, Kim Y (2002). Analysis by cDNA microarrays of altered gene expression in middle ears of rats following pneumococcal infection. *Int J Pediatr Otorhinolaryngol* **65**(3)**:** 203-211.

Linden S, Boren T, Dubois A, Carlstedt I (2004). Rhesus monkey gastric mucins: oligomeric structure, glycoforms and Helicobacter pylori binding. *Biochem J* **379**(Pt 3)**:** 765-775.

Linden S, Nordman H, Hedenbro J, Hurtig M, Boren T, Carlstedt I (2002). Strain- and blood group-dependent binding of Helicobacter pylori to human gastric MUC5AC glycoforms. *Gastroenterology* **123**(6)**:** 1923-1930.

Linden SK, Florin TH, McGuckin MA (2008a). Mucin dynamics in intestinal bacterial infection. *PLoS One* **3**(12)**:** e3952.

Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA (2008b). Mucins in the mucosal barrier to infection. *Mucosal Immunol* **1**(3)**:** 183-197.

Loke P, MacDonald AS, Allen JE (2000). Antigen-presenting cells recruited by *Brugia malayi* induce Th2 differentiation of naive CD4(+) T cells. *Eur J Immunol* **30**(4)**:** 1127-1135.

MacDonald AS, Maizels RM, Lawrence RA, Dransfield I, Allen JE (1998). Requirement for *in vivo* production of IL-4, but not IL-10, in the induction of proliferative suppression by filarial parasites. *J Immunol* **160**(8)**:** 4124-4132.

Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature* **441**(7090)**:** 231-234.

Massacand JC, Stettler RC, Meier R, Humphreys NE, Grencis RK, Marsland BJ, Harris NL (2009). Helminth products bypass the need for TSLP in Th2 immune responses by directly modulating dendritic cell function. *Proc Natl Acad Sci U S A* **106**(33)**:** 13968-13973.

Matsuo K, Ota H, Akamatsu T, Sugiyama A, Katsuyama T (1997). Histochemistry of the surface mucous gel layer of the human colon. *Gut* **40**(6)**:** 782-789.

McDermott JR, Humphreys NE, Forman SP, Donaldson DD, Grencis RK (2005). Intraepithelial NK cell-derived IL-13 induces intestinal pathology associated with nematode infection. *J Immunol* **175**(5)**:** 3207-3213.

McGuckin MA, Eri R, Simms LA, Florin TH, Radford-Smith G (2009). Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis* **15**(1)**:** 100-113.

Miller HR, Huntley JF, Wallace GR (1981). Immune exclusion and mucus trapping during the rapid expulsion of *Nippostrongylus brasiliensis* from primed rats. *Immunology* **44**(2)**:** 419-429.

Moncada D, Keller K, Chadee K (2003). *Entamoeba histolytica* cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. *Infect Immun* **71**(2)**:** 838-844.

Moncada D, Yu Y, Keller K, Chadee K (2000). *Entamoeba histolytica* cysteine proteinases degrade human colonic mucin and alter its function. *Arch Med Res* **31**(4 Suppl)**:** S224-225.

Owyang AM, Zaph C, Wilson EH, Guild KJ, McClanahan T, Miller HR, Cua DJ, Goldschmidt M, Hunter CA, Kastelein RA, Artis D (2006). Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. *J Exp Med* **203**(4)**:** 843-849.

Perez-Vilar J, Hill RL (1999). The structure and assembly of secreted mucins. *J Biol Chem* **274**(45)**:** 31751-31754.

Perrigoue JG, Saenz SA, Siracusa MC, Allenspach EJ, Taylor BC, Giacomin PR, Nair MG, Du Y, Zaph C, van Rooijen N, Comeau MR, Pearce EJ, Laufer TM, Artis D (2009). MHC class II-dependent basophil-CD4+ T cell interactions promote T(H)2 cytokine-dependent immunity. *Nat Immunol* **10**(7)**:** 697-705.

Pigny P, Guyonnet-Duperat V, Hill AS, Pratt WS, Galiegue-Zouitina S, d'Hooge MC, Laine A, Van-Seuningen I, Degand P, Gum JR, Kim YS, Swallow DM, Aubert JP, Porchet N (1996). Human mucin genes assigned to 11p15.5: identification and organization of a cluster of genes. *Genomics* **38**(3)**:** 340-352.

Podolsky DK, Gerken G, Eyking A, Cario E (2009). Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3 deficiency. *Gastroenterology* **137**(1)**:** 209-220.

Reyes JL, Terrazas LI (2007). The divergent roles of alternatively activated macrophages in helminthic infections. *Parasite Immunol* **29**(12)**:** 609-619.

Roach TI, Wakelin D, Else KJ, Bundy DA (1988). Antigenic cross-reactivity between the human whipworm, *Trichuris trichiura*, and the mouse trichuroids *Trichuris muris* and *Trichinella spiralis*. *Parasite Immunol* **10**(3)**:** 279-291.

Robbe C, Capon C, Coddeville B, Michalski JC (2004). Structural diversity and specific distribution of O-glycans in normal human mucins along the intestinal tract. *Biochem J* **384**(Pt 2)**:** 307-316.

Sadler JE (1998). Biochemistry and genetics of von Willebrand factor. *Annu Rev Biochem* **67:** 395-424.

Schopf LR, Hoffmann KF, Cheever AW, Urban JF, Jr., Wynn TA (2002). IL-10 is critical for host resistance and survival during gastrointestinal helminth infection. *J Immunol* **168**(5)**:** 2383-2392.

Sheehan JK, Hanski C, Corfield AP, Paraskeva C, Thornton DJ (1995). Mucin biosynthesis and macromolecular assembly. *Biochem Soc Trans* **23**(4)**:** 819-821.

Sheehan JK, Thornton DJ, Howard M, Carlstedt I, Corfield AP, Paraskeva C (1996). Biosynthesis of the MUC2 mucin: evidence for a slow assembly of fully glycosylated units. *Biochem J* **315 (Pt 3):** 1055-1060.

Shekels LL, Anway RE, Lin J, Kennedy MW, Garside P, Lawrence CE, Ho SB (2001). Coordinated Muc2 and Muc3 mucin gene expression in *Trichinella spiralis* infection in wild-type and cytokine-deficient mice. *Dig Dis Sci* **46**(8)**:** 1757-1764.

Singh PK, Hollingsworth MA (2006). Cell surface-associated mucins in signal transduction. *Trends Cell Biol* **16**(9)**:** 467-476.

Smith P, Walsh CM, Mangan NE, Fallon RE, Sayers JR, McKenzie AN, Fallon PG (2004). Schistosoma mansoni worms induce anergy of T cells via selective up-regulation of programmed death ligand 1 on macrophages. *J Immunol* **173**(2)**:** 1240-1248.

Summers RW, Elliott DE, Qadir K, Urban JF, Jr., Thompson R, Weinstock JV (2003). *Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory bowel disease. *Am J Gastroenterol* **98**(9)**:** 2034-2041.

Summers RW, Elliott DE, Urban JF, Jr., Thompson R, Weinstock JV (2005a). *Trichuris suis* therapy in Crohn's disease. *Gut* **54**(1)**:** 87-90.

Summers RW, Elliott DE, Urban JF, Jr., Thompson RA, Weinstock JV (2005b). *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* **128**(4)**:** 825-832.

Summers RW, Elliott DE, Weinstock JV (2006). Therapeutic colonization with *Trichuris suis*. *Arch Pathol Lab Med* **130**(12)**:** 1753; author reply 1753-1754.

Summers RW, Elliott DE, Weinstock JV (2010). *Trichuris suis* might be effective in treating allergic rhinitis. *J Allergy Clin Immunol*.

Sutton P (2001). Helicobacter pylori vaccines and mechanisms of effective immunity: is mucus the key? *Immunol Cell Biol* **79**(1)**:** 67-73.

Tato CM, Mason N, Artis D, Shapira S, Caamano JC, Bream JH, Liou HC, Hunter CA (2006). Opposing roles of NF-kappaB family members in the regulation of NK cell proliferation and production of IFN-gamma. *Int Immunol* **18**(4)**:** 505-513.

Taylor BC, Zaph C, Troy AE, Du Y, Guild KJ, Comeau MR, Artis D (2009). TSLP regulates intestinal immunity and inflammation in mouse models of helminth infection and colitis. *J Exp Med* **206**(3)**:** 655-667.

Taylor ME, Drickamer K (2003). *Introduction to Glycobiology*. Oxford University press Inc. .

Thim L, Madsen F, Poulsen SS (2002). Effect of trefoil factors on the viscoelastic properties of mucus gels. *Eur J Clin Invest* **32**(7)**:** 519-527.

Thornton DJ, Rousseau K, McGuckin MA (2008). Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* **70:** 459-486.

Thornton DJ, Sheehan JK (2004). From mucins to mucus: toward a more coherent understanding of this essential barrier. *Proc Am Thorac Soc* **1**(1)**:** 54-61.

Tobisawa Y, Imai Y, Fukuda M, Kawashima H (2010). Sulfation of colonic mucins by N-acetylglucosamine 6-O-sulfotransferase-2 and its protective function in experimental colitis in mice. *J Biol Chem* **285**(9)**:** 6750-6760.

Turner J, Faulkner H, Kamgno J, Else K, Boussinesq M, Bradley JE (2002). A comparison of cellular and humoral immune responses to trichuroid derived antigens in human trichuriasis. *Parasite Immunol* **24**(2)**:** 83-93.

van den Biggelaar AH, Rodrigues LC, van Ree R, van der Zee JS, Hoeksma-Kruize YC, Souverijn JH, Missinou MA, Borrmann S, Kremsner PG, Yazdanbakhsh M (2004). Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. *J Infect Dis* **189**(5)**:** 892-900.

Velcich A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S, Kucherlapati R, Lipkin M, Yang K, Augenlicht L (2002). Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science* **295**(5560)**:** 1726-1729.

Wang H, Steeds J, Motomura Y, Deng Y, Verma-Gandhu M, El-Sharkawy RT, McLaughlin JT, Grencis RK, Khan WI (2007). CD4+ T cell-mediated immunological control of enterochromaffin cell hyperplasia and 5-hydroxytryptamine production in enteric infection. *Gut* **56**(7)**:** 949-957.

Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM (2006). Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* **24**(6)**:** 677-688.

Wessler S (1976). Introduction: What is a model? *Institute of Laboratory animal resources*

Yamauchi J, Kawai Y, Yamada M, Uchikawa R, Tegoshi T, Arizono N (2006). Altered expression of goblet cell- and mucin glycosylation-related genes in the intestinal epithelium during infection with the nematode *Nippostrongylus brasiliensis* in rat. *APMIS* **114**(4)**:** 270-278.

Zen Y, Harada K, Sasaki M, Tsuneyama K, Katayanagi K, Yamamoto Y, Nakanuma Y (2002). Lipopolysaccharide induces overexpression of MUC2 and MUC5AC in cultured biliary epithelial cells: possible key phenomenon of hepatolithiasis. *Am J Pathol* **161**(4)**:** 1475-1484.

Zhao A, McDermott J, Urban JF, Jr., Gause W, Madden KB, Yeung KA, Morris SC, Finkelman FD, Shea-Donohue T (2003). Dependence of IL-4, IL-13, and nematode-induced alterations in murine small intestinal smooth muscle contractility on Stat6 and enteric nerves. *J Immunol* **171**(2)**:** 948-954.

CHANGES IN THE MUCOSAL BARRIER DURING ACUTE AND CHRONIC *Trichuris muris* INFECTION

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ABSTRACT

The intestinal mucosal barrier, part of the innate immune defence, is responsive to the external environment and changes in response to infection. There is disparate evidence for the epithelial and goblet cell products within the intrinsic barrier being part of a response to resolve infection. We comprehensively analysed the changes of mucosal glycoconjugates during acute and chronic infection by utilising the *Trichuris muris* (*T. muris*) model. Transcription factors, atonal homolog 1 (*Math-1*) and SAM pointed domain containing ETS transcription factor (*Spdef*) were activated during acute infection, which promoted stem cell fate towards a secretory cell phenotype. The thickness of the intermediate barrier, the carbohydrate-rich glycocalyx, composed of cell surface mucins increased with exposure to *T. muris*, with an increase in *Muc4*, *Muc13* and *Muc17*. Overall, hypersecretion of glycoproteins into the extrinsic barrier (mediated by IL-13) correlated with an induction in gamma amino-butyric acid-α3 receptor (GABA-α3) during acute infection. Furthermore, altered glycosylation was observed during acute and chronic infection; mucins were more highly charged during acute infection than during chronic infection. This study readdresses the changes within the mucosal barrier, in particular in the cell surface and secreted mucins during acute and chronic nematode infection.

ABBREVIATIONS

DBA, *Dolichos biflorus*; GABA-α3, gamma amino-butyric acid α-3; PAS, Periodic-Acid Schiff's; pi., post infection; Spdef, SAM pointed domain containing ETS transcription factor; T_H, T Helper; *T. muris*, *Trichuris muris*.

INTRODUCTION

The intestinal mucosa forms a dynamic defensive barrier that is responsive to the external environment (Thornton and Sheehan, 2004). The barrier has two major components: the intrinsic barrier which consists of the continuous epithelial cell lining and the extrinsic barrier which is the combination of the secretions from the goblet cells within the epithelium composing the mucus barrier (Linden *et al*, 2008b; Thornton and Sheehan, 2004). Moreover, cell surface mucins intercalated into the apical surface of the epithelium with their extracellular domain protruding out, are a dominant glycoconjugate component of the glycocalyx which forms an intermediate layer of defence between the extrinsic and intrinsic barriers (Linden *et al*, 2008b). Different components of the mucosal epithelium have been extensively studied in relation to parasitic infections, particularly intestinal dwelling nematodes (Hogan *et al*, 2006; Linden *et al*, 2008a). It is clear that innate and adaptive immune systems play a key role in resistance and susceptibility to chronic nematode infections (Artis and Grencis, 2008). Recent studies conducted on the nematode models have been focused on changes in non-mucin goblet cell products, such as resistin-like molecule-β and intelectin, in response to infection (Artis, 2006; Artis *et al*, 2004; Nair *et al*, 2008). Few studies, however, have fully characterised the prevalent glucoconjugate components of the goblet cells and the protective intestinal glycocalyx in response to nematode infection. In light of the recent data demonstrating a clear role for Muc2, the major goblet cell product, during resistance to the mouse whipworm *Trichuris muris* (*T.muris*) (Hasnain *et al*, 2010), the present work has comprehensively characterised this goblet cell and glucoconjugate axis in relation to resistance or susceptibility to chronic infection.

The *Trichuris muris* (*T. muris*) nematode survives in the host by eliciting a T_H1-type immune response; however, resistance is dependent on a T_H2-type immune response (Cliffe and Grencis, 2004). In BALB/c mice, a high dose infection (>150 eggs) results in the T_H2-mediated expulsion of the nematode; whereas, a low dose infection (<15 eggs) results in a T_H1-dominant immune response and consequently a chronic infection (Bancroft *et al*, 2001). Therefore, the nematode *T. muris* in mouse provides a manipulable system that can be used to investigate the changes in mucosal barrier operating during acute and chronic infection within a single system (Bethony *et al*, 2006; Cliffe and Grencis, 2004). It is thought that multiple effectors under immunological (T_H2) control orchestrate the responses involved in the expulsion of the nematode (Cliffe *et al*, 2005; Hasnain *et al*,

2010; Khan and Collins, 2004). The intestinal epithelium, the niche of the parasite, consists of four distinct cells types; enterocytes, paneth cells, enteroendocrine cells and goblet cells (Zaph *et al*, 2007). Stem cells at the bottom of the intestinal crypts can differentiate into absorptive cells (enterocytes) or secretory cells (paneth, enteroendocrine or goblet cells) (Noah *et al*, 2009). Absorptive cell differentiation is dependent on the transcription factor Hes-1 (hairy and enhancer of split 1), whereas differentiation towards secretory cell lineage is dependent on Math-1 (atonal homolog 1) (Shroyer *et al*, 2005). The recently discovered transcription factor, Spdef (SAM pointed domain containing ETS transcription factor), was shown to facilitate the terminal differentiation of secretory cells into goblet cells (Noah *et al*, 2009). During *T. muris* infection, within the niche of the parasite, hyperproliferation of cells in the intestinal crypts has been reported. Goblet cell hyperplasia has been described in models that are able to reject *T. muris* (Else, 2005; Hasnain *et al*, 2010), whereas hyperplasia of enterocytes is observed during chronic infection (Artis *et al*, 1999). Goblet cell hyperplasia is thought to be largely under the control of T_H2-type cytokines (Khan *et al*, 2001; McKenzie *et al*, 1998), although some studies have suggested that IL-4/IL-13 independent goblet cell hyperplasia can occur (Marillier *et al*, 2008).

In this study, we aimed to conduct a comprehensive analysis of the changes in the components of the mucosal barrier: the epithelial cell layer, cell surface mucins and the extrinsic mucus barrier during acute and chronic *T. muris* infection. We reconfirmed that goblet cell hyperplasia is a prominent feature of acute infection as an activation of transcription factors involved in goblet cell differentiation was observed. Expression of cell surface mucins, *Muc4*, *Muc13* and *Muc17* was elevated with the exposure to *T. muris* infection, resulting in an increase in the thickness of the apical glycocalyx. Interestingly, along with the increase in the storage and synthesis of glycoproteins within the goblet cells, which has been previously reported, an increase in secretion of these glycoproteins was observed during worm expulsion. The hypersecretion of glycoproteins was mediated by the gamma amino-butyric acid-α3 (GABA- α3) receptor under the control of the T_H2-type cytokine, IL-13. Furthermore, we demonstrate for the first time that along with the depleted mucin production, there is a general alteration of glycosylation on the mucins in chronic infection.

MATERIALS AND METHODS

Animals

Male BALB/c mice were purchased from Harlan Olac. Female wild-type BALB/c mice, IL-4-deficient (BALB/c-background) and IL-4R-deficient (BALB/c-background) mice, originally obtained from Dr. McKenzie (McKenzie *et al*, 1998), were maintained in the Biological Services Unit at The University of Manchester. The protocols employed were in accordance with guidelines by the Home Office Scientific Procedures Act (1986). All mice (6-12wk old) were kept in sterilized, filter-topped cages, and fed autoclaved food in the animal facilities.

Parasitological technique

The techniques used for *T. muris* maintenance and infection were described previously (Wakelin, 1967). Mice were orally infected with approximately 150 eggs for a high dose infection and <15 eggs for a low dose infection. Worm burdens were assessed by counting the number of worms present in the caecum.

Histology, Immunohistochemistry and Immunofluorescence microscopy

The whole caecum (rolled) was fixed in 95% ethanol and processed by using standard histological techniques. Sections were treated with 0.1M potassium hydroxide for 30 minutes prior to staining with periodic acid Schiff (PAS). Slides were counterstained with 1% fast-green. Standard immunohistochemical and immunofluorescent staining methods (Linden *et al*, 2008a; McGuckin and Thornton, 2000) were used to determine the levels of SAM pointed domain containing ETS transcription factor (Spdef) using the mspdef antibody (Abcam) and lectins (Heazlewood *et al*, 2008). Biotinylated lectins (*Sambucus nigra*, *Mackia amuresis*, Peanut agglutinin, *Maclura pomifera*, *Dolichos biflorus*, *Triticum vulgaris* and *Ulex europeaus*) were used for the detection of glycans.

Mucus extraction and agarose gel electrophoresis

The caecum was gently flushed with PBS and subsequently flushed 5 times with 3M Urea to obtain the secreted mucus; the tissue was fixed for histology before and after treatment to ensure only the secreted mucus within the intestinal lumen was isolated without caecal crypt disruption (data not shown). The mucus was solubilised in 8M guanidium chloride, reduced using 50mM dithiothreitol (DTT) and carboxylmethylated using 0.125M iodoacetamide prior to electrophoresis on a 1% (w/v) agarose gel. Mucins were detected

after western blotting using PAS-staining (Thornton *et al*, 1989). Subsequently, the staining intensity of the positive-bands was determined using the GS-800 BioRad Densitometer (Thornton *et al*, 1994; Thornton *et al*, 1989).

Anion-Exchange Chromatography

Crude mucus samples were reduced, carboxymethylated and treated with DNAse before being subjected to anion exchange chromatography as described previously (Linden *et al*, 2002; Thornton *et al*, 1996), using a Resource™ Q column. Samples were eluted with the starting buffer (10mM piperazine at pH 5 in 6M Urea containing 0.02% 3-[(chloamidopropyl) dimethylammonio]-1-propanosulphonate) for 15 minutes (0.5ml/min), followed by a linear gradient (60 mins) up to 0.4M Lithium perchlorate-10mM piperazine at pH 5 in 6M Urea containing 0.02% 3-[(chloamidopropyl) dimethylammonio]-1-propanosulphonate (Linden *et al*, 2002; Thornton *et al*, 1996). Fractions were blotted onto nitrocellulose membrane using a 72-well slot blot manifold (Schleicher & Schuell, Dassel, Germany) attached to a vacuum pump. Membranes were then assayed for glycoprotein using PAS reagent, as described previously (Thornton *et al*, 1989). Staining intensity was measured using a Bio-Rad GS-800 Calibrated densitometer.

Real Time PCR (RT-PCR)

Total RNA from epithelial cells was isolated using the previously described method (Cliffe *et al*, 2005). cDNA was generated using an IMPROM-RT kit (Promega) and Absolute QPCR SYBR Green (ABgene) was used for quantitative PCR. Primer efficiencies was determined using cDNA dilutions and genes of interest were normalised against the housekeeping gene, β-actin and expressed as a fold difference to uninfected naïve mRNA levels. mRNA expression was investigated using the primer pairs shown in **Supplementary Table 2.1**. Melting curves were analysed and products were directly sequenced to verify the identity of the amplified genes. In brief, products were digested with Exonuclease I and Calf Intestinal Phosphatase and subsequently sequenced using the ABIPRISM Big-Dye Terminator cycle sequencing reaction at the Sequencing Facility in The University of Manchester. The data was analysed using Chromas Pro v1.34 and the sequences obtained were compared against the GenBank database; http://www.ncbi.nlm.nih.gov/BLAST.

Quantification of histological staining

The number of goblet cells were counted in 100 longitudinally sectioned crypt units (expressed per crypt). The number of epithelial cells were counted in 20 longitudinal crypts and expressed as per crypt. 5 randomly selected fields of view were analysed to determine the levels of positive lectin staining; indicated as 90-100% (++++), 60-80% (+++), 10-50% (++) and 0-10% (+).

Statistical analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed using SPSS version 16.0. Statistical significance of different groups was assessed by using parametric tests; ONE-way Analysis of variance with post-test following statistical standards or paired student t test. $P<0.05$ was considered statistically significant.

RESULTS

Up-regulation of secretory cell lineage transcription factors during acute **T. muris** *infection*

It is well established that a T_H1-type immune response (characterised by IFN-γ) leads to a chronic *T. muris* infection, whereas a T_H2-type immune response (characterised by IL-4 and IL-13) leads to the expulsion of the nematodes (Cliffe and Grencis, 2004). Chronic infection leads to changing the gross morphology of the niche of the parasite (caecum). Furthermore, no changes were observed in the number of goblet cell and, an increase in epithelial cell numbers is associated with chronicity (**Supplementary Figure 2.1**). Stem cells located at the bottom of the intestinal crypts mature and differentiate into absorptive cells or secretory cells as migrate to the luminal surface (Noah *et al*, 2009). Therefore, to investigate whether there was a change in differentiation of stem cells that in turn promotes differentiation towards a certain cell lineage during acute or chronic infection, the expression of transcription factors involved in the differentiation of the intestinal progenitor stem cell into absorptive cells or secretory cells was determined using RT-PCR (**Figure 2.1**).

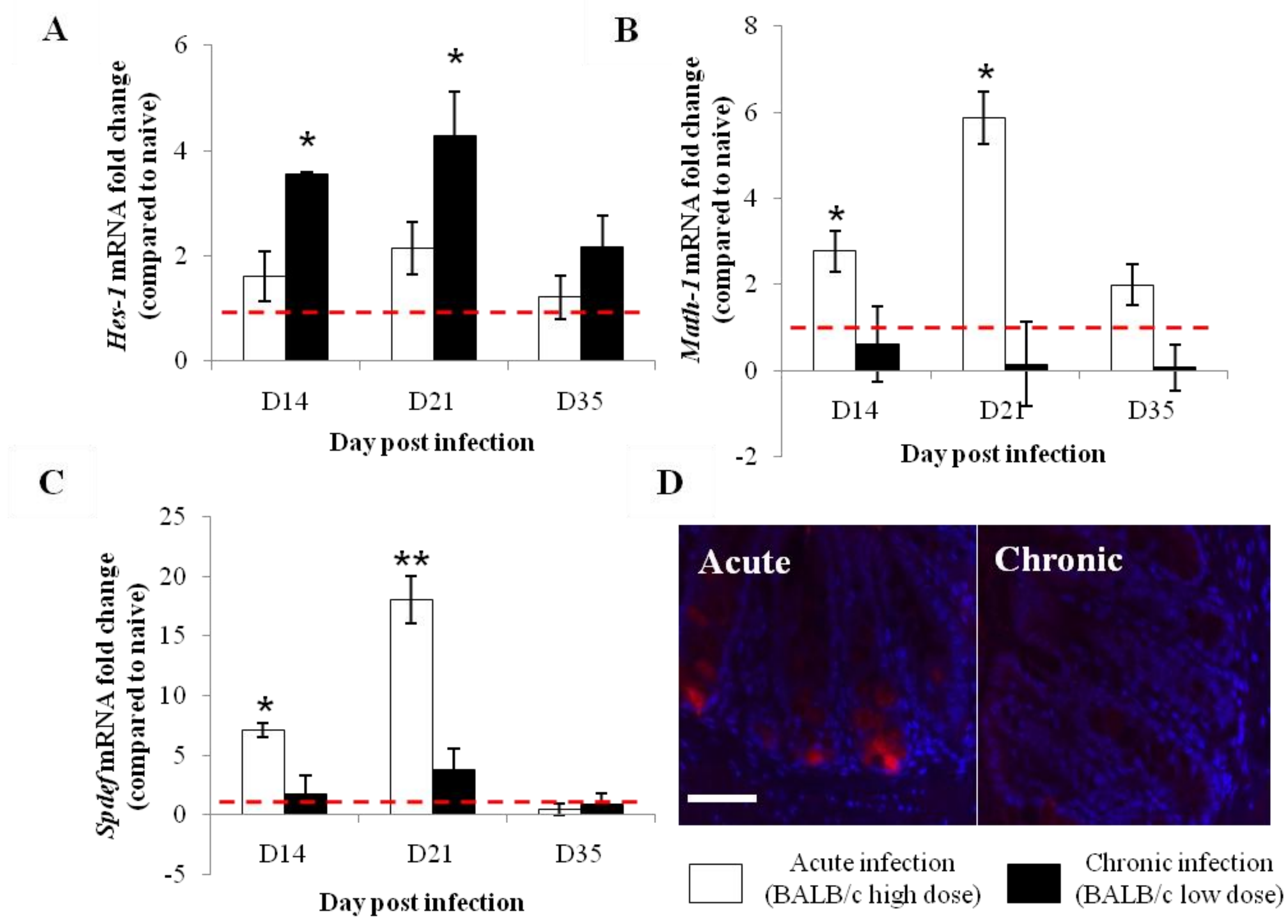


Figure 2.1: RT-PCR was used to determine the levels of *Hes-1* (A), *Math-1* (B) and *Spdef* (C) during acute (open bars) and chronic (closed bars) infection. Red dashed line = naïve levels. Immunofluorescence microscopy (D) was used to assess levels of Spdef on day 35 pi. Scale bar; 10μm. * = P<0.05, ** = P<0.001. Results represent the mean value of 5-10 mice per group ± SEM.

The enterocyte differentiation transcription factor Hes-1 (hairy and enhancer of split 1), the transcription factor for differentiation towards absorptive cell lineage Math-1 (atonal homolog 1) (Shroyer *et al*, 2005) and Spdef (SAM pointed domain containing ETS transcription factor), the transcription factor involved in the terminal differentiation of goblet cells (Noah *et al*, 2009) were chosen as markers of cell lineage. Levels of *Hes-1* were significantly increased on day 21 pi., explaining the increase in epithelial cell numbers (**Supplementary Figure 2.1G**), in chronic infection compared to acute infection and uninfected controls (**Figure 2.1A**). In contrast, *Math-1* and *Spdef* were only significantly up-regulated on day 14 and 21 pi. of acute infection (**Figure 2.1B** & **C**) when an increase in goblet cell numbers was also observed (**Supplementary Figure 2.1H**). Furthermore, immunofluorescence microscopy with the mSpdef antibody showed that

Spdef was up-regulated at the bottom of the intestinal crypts of mice with acute infection (**Figure 2.1D**; on day 21pi.).

The increase in glycocalyx thickness with exposure to* T. muris *infection

Glycocalyx is the "carbohydrate rich zone" found at the surface of cells, consisting of glycoproteins including cell surface mucins that project from the apical surface of the cells (Linden *et al*, 2008b). Using immunofluorescent microscopy with the *Dolichol Biflorus* (DBA) lectin, which recognises the D-GalNAc glycan, revealed that with exposure to *T. muris* infection there was an increase in the thickness of the glycocalyx layer, as compared to the uninfected naïve mice (**Figure 2.2A** & **B**). However, the glycocalyx was significantly thicker in mice during acute infection (day 21 pi.) as compared to during chronic infection.

To comprehensively characterise the changes in the composition of the glycocalyx during acute and chronic *T. muris* infection, the levels of cell surface mucins were determined using RT-PCR (**Figure 2.2**). In the intestine, *Muc1*, *Muc4*, *Muc13*, *Muc15*, *Muc17* and *Muc20* have previously been shown to be expressed (Singh and Hollingsworth, 2006). No major changes were observed in *Muc1*, *Muc15* and *Muc20* levels during chronic or acute infection when compared to uninfected control mice (day 21 pi. shown here; **Figure 2.2**). However, a marked increase in *Muc4*, *Muc13* and *Muc17* levels were observed in response to *T. muris* infection (**Figure 2.2**). As expected with the increased thickness of glycocalyx layer, the levels of *Muc4* and *Muc13* were significantly higher in the mice during acute infection. However, the increase in *Muc17* was more prominent in the mice during chronic infection. Similar trends in the levels of these cell surface mucins were observed on day 14 and 35 pi. (**Supplementary Table 2.2**).

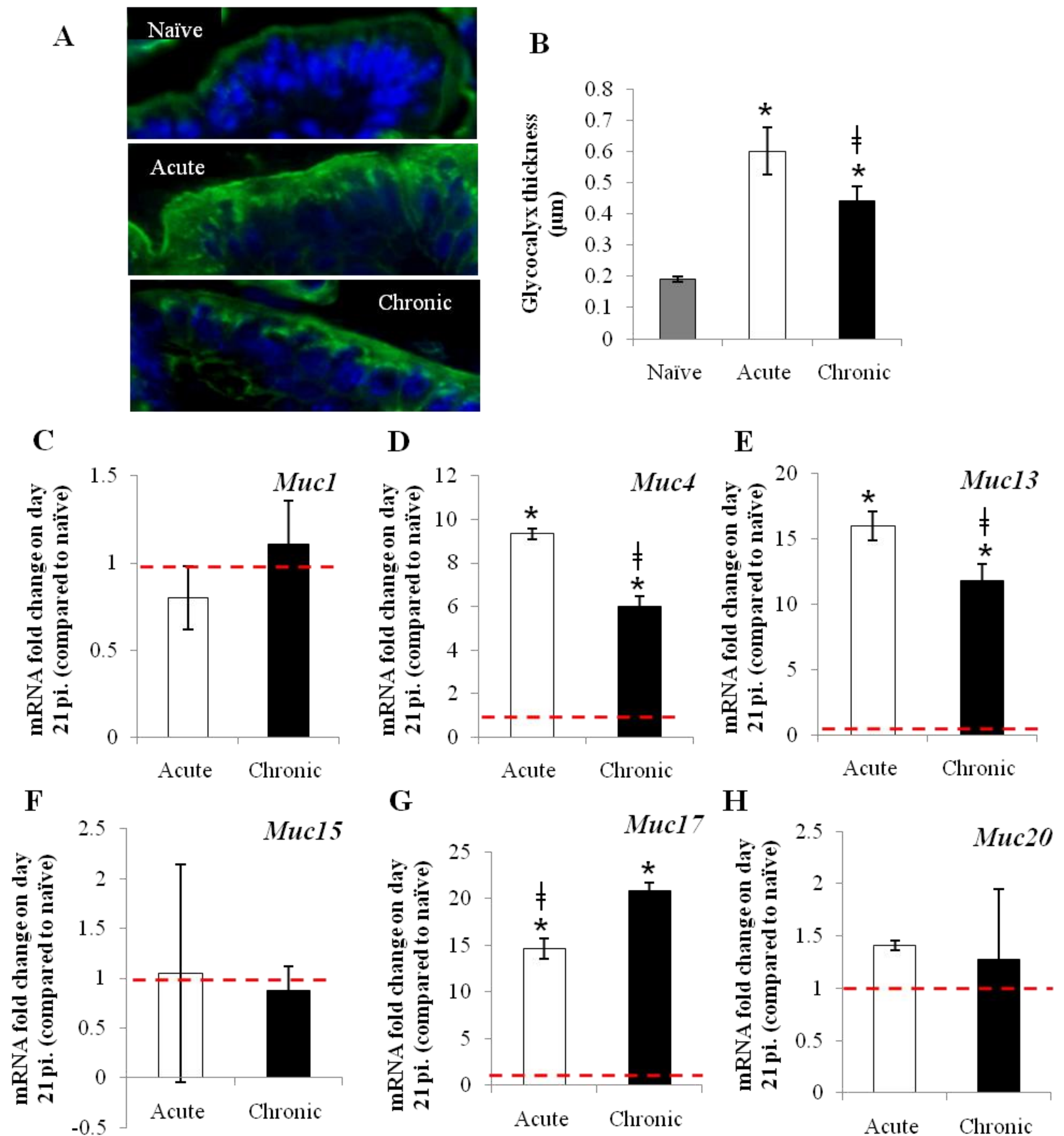


Figure 2.2: Changes in the glycocalyx were determined using immunofluorescence microscopy with the DBA lectin (A), the changes in the thickness were quantified (B). Levels of the cell surface mucins *Muc1* (C), *Muc4* (D), *Muc13* (E), *Muc15* (F), *Muc17* (G) and *Muc20* (H) were determined using RT-PCR during acute (open bars) and chronic (closed bars) infection. Red dashed line = naïve levels. * = P<0.05 compared to naïve. ǂ = P<0.05 compared to acute or chronic infection. Results represent the mean value of 5-10 mice per group ± SEM.

Hypersecretion of glycoproteins mediated by GABA-α3 receptor during worm rejection

An elevation in transcription factors that determine the secretory cell lineage was observed during acute infection, furthermore there was an increase in the number of goblet

cells within the caecal crypts (**Figure 2.1**). Therefore, to determine whether there was also an increase in the secretion of glycoproteins during acute infection, the mucus secreted into the intestinal lumen was isolated and subjected to agarose gel electrophoresis (**Figure 2.3A** & **B**). On day 14 pi., an increase in the secretion of glycoproteins was observed in both acute and chronic models. Interestingly, during acute infection, as well as an increase in the amount of glycoproteins stored within goblet cells (refer to **Supplementary Figure 2.1H**), higher amounts of glycoproteins were secreted into the mucus barrier (**Figure 2.3A** & **B**).

Studies have shown that GABA-α receptors mediate the hypersecretion of glycoproteins into the mucus barrier, under the control of IL-13 in asthma models (Xiang *et al*, 2007). Several studies have described GABA receptors, α-subunit 1-3 and β-subunit 1-3, to be expressed in the intestine (Nakajima *et al*, 1996; Zeiter *et al*, 1996). By utilising RT-PCR we investigated the basal expression of these GABA receptors in the uninfected BALB/c mice; all GABA subunits, with the exception of GABA-α2 and GABA-β3, were expressed at high levels in the caecum (**Figure 2.3C**). Interestingly, in response to infection only the expression of GABA-α3 was altered (**Figure 2.3D**); an increase in levels of GABA-α3 was observed during acute infection (day 21 pi.), and this correlated with the increase in glycoprotein secretion. The expression of all the other α and β isoforms remained unaltered when challenged with *T. muris* infection (data not shown). To dissect the role of the immune response in the up-regulation GABA-α3 receptor and mucus hypersecretion, we utilised the IL-4-deficient and IL-4R-deficient mice. The IL-4-deficient mice do not contain any active IL-4 but are able to expel the nematodes due to IL-13 mediated effector mechanisms (**Supplementary Figure 2.2;** (Bancroft *et al*, 1998)). In contrast, the IL-4Rα1 chain is missing in the IL-4R-deficient mice and as both chains in the heterodimeric receptor are required to initiate intracellular signalling (Ramalingam *et al*, 2008), IL-4 and IL-13 cannot act in the IL-4R-deficient mice, which are therefore susceptible to chronic infection ((Bancroft *et al*, 1998) **Supplementary Figure 2.2**).

Interestingly, the levels of GABA-α3 remained unaltered in the IL-4R-deficient mice (**Figure 2.3E**), where goblet cell depletion was also observed (**Supplementary Figure 2.2**). In the IL-4-deficient mice, however, along with the hyperplastic goblet cell response against *T. muris*, a 10-fold increase in levels of GABA-α3 was also observed post infection (**Figure 2.3E**).

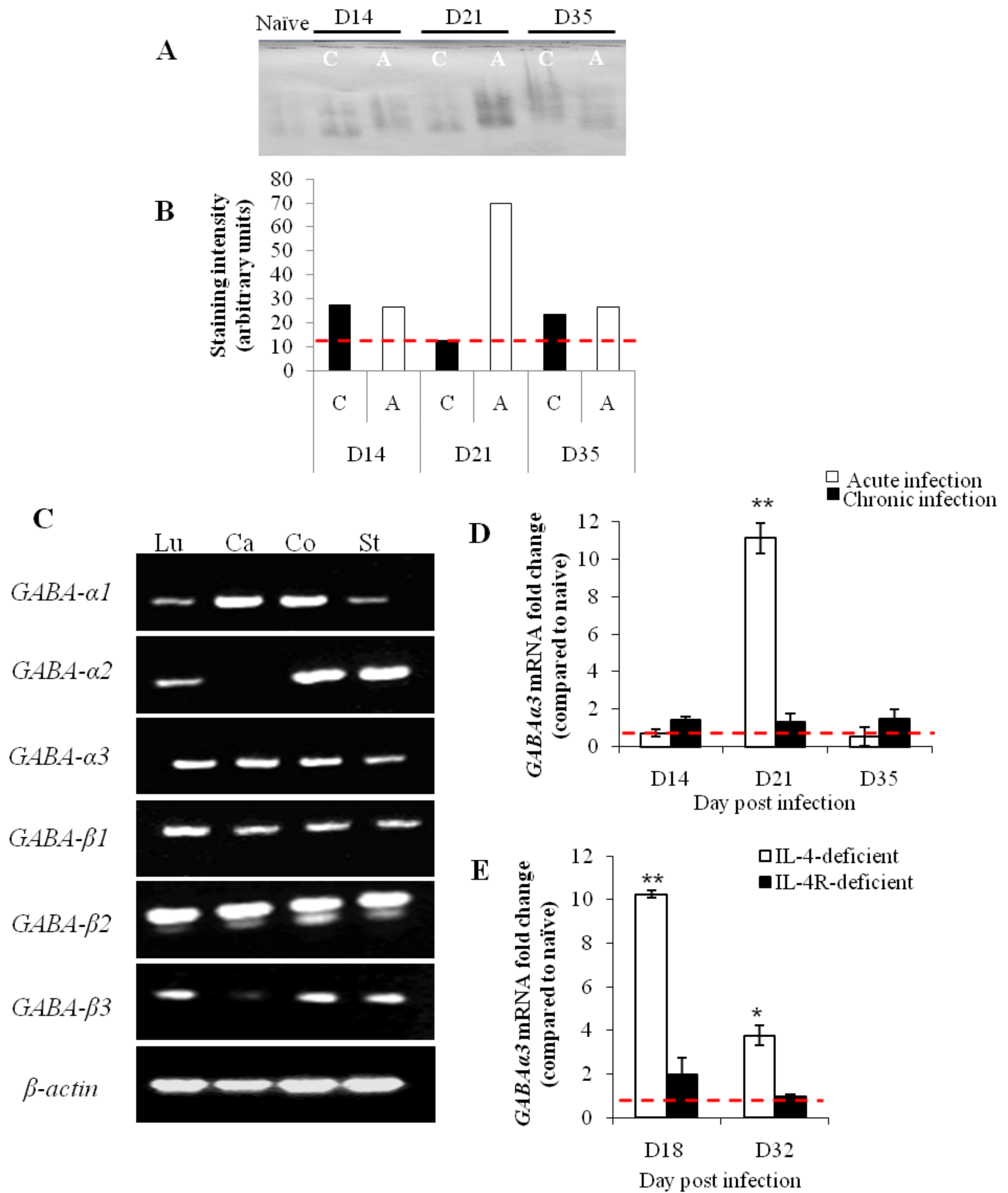
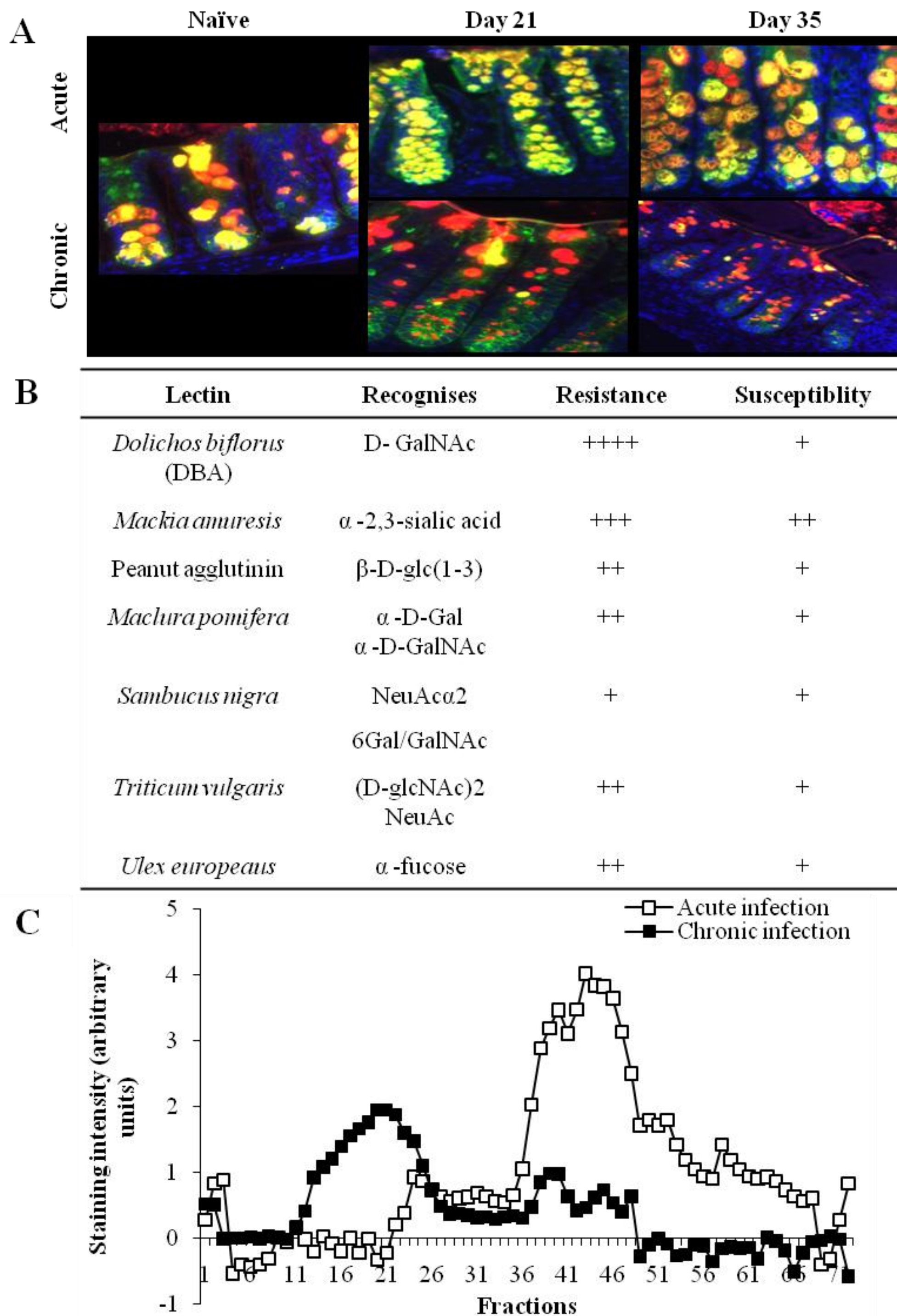


Figure 2.3: Mucus extracted into the intestinal lumen was isolated from mice with chronic (closed bars) and acute (open bars) infection on day 14, 21 and 35 pi. (pooled from 5 mice). Mucus was reduced and carboxymethylated, subjected to agarose gel electrophoresis, western blotted and stained with PAS (A) and staining intensity was measured (B) Representative 3 individual experiments. PCR was used to determine the basal expression of the GABA receptor in a range of tissues (C); Lung (Lu), Caecum (Ca), Colon (Co) and Stomach (St). The levels of GABAα3 in the caecal tissue were determined post infection in BALB/c (D) and, IL-4 and IL-4R-deficient mice (D) (Results represent the mean value of 3-8 mice per group ± SEM). Red dashed line = naïve levels. * = $P<0.05$. ** = $P<0.001$.

Altered glycosylation on goblet cell mucins during chronic* T. muris *infection

Interestingly, a slight difference in the electrophoretic migration of mucins, when subjected to agarose gel electrophoretic analysis (**Figure 2.3A**), was noted during acute infection which suggested a change in glycosylation. Therefore, to evaluate the variation in mucin glycoconjugates in response to acute and chronic *T. muris* infection, dual immunofluorescence labeling for Muc2 (Muc2 antibody, red) and glycans (lectin, green) was performed (**Figure 2.4A**) and the relative lectin-staining was quantified (**Figure 2.4B**). The most striking differences were observed in the staining with the DBA lectin, which recognises the D-GalNAc glycan (**Figure 2.4**). Most of the goblet cells within the caecal epithelium stained positive with the DBA lectin on day 21 pi. of acute infection, when the nematodes are rejected. In contrast, in the chronic infection only a few goblet cells stained with the DBA lectin. In fact, during chronic infection (day 21 pi.) less staining was observed with all lectins employed (**Figure 2.4A** & **Supplementary Figure 2.3**). Low levels of staining were observed using the *Sambucus nigra* lectin which detects the α-2,6-sialic acid compared to the *Mackia amuresis* lectin which detects the α -2,3-sialic acid. This suggested that mucins within the caecum contained mucins majorly containing the α-2,6-sialic acid.

Overall, lectin histochemistry analysis suggested that the mucins produced during acute infection are altered in their glycosylation compared to those produced by chronically infected mice. To assess whether the charge density of the mucins was also altered, the secreted mucus was isolated from the caecum of the two groups of mice on day 21 pi., reduced and carboxymethylated and analysed by anion exchange chromatography (**Figure 2.4C**). PAS-staining of fractions collected from the anion exchange chromatography showed that mucins isolated from the chronically infected mice (on day 21 pi.) eluted before the mucins from mice with acute infection (**Figure 2.4C**). This suggested that the mucins in the caecum of the chronically infected mice were less charged as compared to the mucins from the mice with acute infection.



Lectin	Recognises	Resistance	Susceptiblity
Dolichos biflorus (DBA)	D- GalNAc	++++	+
Mackia amuresis	α -2,3-sialic acid	+++	++
Peanut agglutinin	β-D-glc(1-3)	++	+
Maclura pomifera	α -D-Gal α -D-GalNAc	++	+
Sambucus nigra	NeuAcα2 6Gal/GalNAc	+	+
Triticum vulgaris	(D-glcNAc)2 NeuAc	++	+
Ulex europeaus	α -fucose	++	+

Figure 2.4: Dual immunofluorescent (A) staining with DBA lectin (green) and mMuc2 antibody (red) in caecal tissue from mice with acute and chronic infection on days 21 and 35pi. Merge (yellow) shows the co-localisation of Muc2 and lectins. (B) Quantitation scores of immunofluorescent staining of caecal tissue (Supplementary Figure 2.3) with lectins (DBA, *Mackia amuresis*, Peanut agglutinin, *Maclura pomifera*, *Sambucus nigra*, *Triticum vulgaris*, *Ulex europeaus*) on day 21 pi. of acute and chronic infection. Secreted mucus was isolated from acute (open bars) and chronically (closed bars) infected mice on day 21 pi., reduced and carboxymethylated and subjected to anion exchange chromatography. Fractions were collected, transferred to nitrocellulose membrane and stained with PAS (C). Results representative of 3 individual experiments.

DISCUSSION

The *T. muris* nematode survives within its host by eliciting a T_H1-type immune response in mice susceptible to chronic infection, whereas in an acute infection a T_H2-type immune response results in nematode expulsion (Cliffe and Grencis, 2004). The intestinal mucosa, the niche of the nematode, forms a dynamic barrier that is responsive to the external environment and changes in response to infection due to the inflammatory response. It has already been demonstrated that T_H2-type cytokine mediated regulation of epithelial cell turnover (Cliffe *et al*, 2005), smooth muscle contractility (Khan *et al*, 2003) and the mucus barrier (Hasnain *et al*, 2010) can contribute to efficient worm expulsion (Artis and Grencis, 2008). Recently, we have demonstrated a significant role for the intestinal mucin Muc2 (Hasnain *et al*, 2010), which is the major intestinal goblet cells product, during the expulsion of the *T. muris* nematode. In the present study we aimed to characterise the changes within the mucosal barrier, in particular in its glyconconjugate components that may play a role in parasite rejection, by using the acute and chronic mouse models of *T. muris* infection.

Surprisingly, the glycocalyx which forms a layer of defence underneath the mucus barrier on apical surface of epithelial cells has not been investigated in this setting previously. Cell surface mucins, which are anchored to the apical cell surface, are a major component of this glycocalyx layer and extend further than most other cell surface structures (Linden *et al*, 2008a; McAuley *et al*, 2007; Packer *et al*, 2004; Renes *et al*, 2002; Singh and Hollingsworth, 2006; Tu *et al*, 2008). Cell surface mucins provide both a physical barrier and are also involved in intracellular signalling events; their expression has been shown to be both infection promoting and infection limiting with regard to pathogenic bacteria (Linden *et al*, 2008a; Linden *et al*, 2008b). We observed a striking difference in the thickness of the glycocalyx in response to *T. muris* infection, with the increase being more prominent during acute infection. Analysis of the cell surface mucin components, of the glycocalyx during acute and chronic infection, revealed an increase in the levels of *Muc4*, *Muc13* and *Muc17*, which most likely explains the increase in the thickness of the glycocalyx. Although the up-regulation of cell surface mucins was not exclusive to acute infection, they may contribute to worm expulsion. Whether this is because cell surface mucins directly influence the physicochemical properties of the glycocalyx or whether they are shed into the mucus affecting its barrier properties or have

a role that is associated with the increased epithelial cell extrusion shown to be critical to worm expulsion (Cliffe *et al*, 2005), requires further attention.

The rejection of the whipworm was accompanied by increased differentiation of stem cells within the basal caecal crypts into goblet cells. *Math-1* is involved in the differentiation of a progenitor stem cell into a secretory cell type and Spdef subsequently regulates its terminal differentiation into a goblet cell (Noah *et al*, 2009; Shroyer *et al*, 2005). Both these transcription factors were up-regulated during acute *T. muris* infection, around the time of worm expulsion. In contrast, *Hes-1*, which is involved in the differentiation towards an enterocyte fate, was only up-regulated in the mice susceptible to chronic infection. This agrees with previously published data, where an increase in epithelial cell proliferation is observed during chronic infection (Artis *et al*, 1999; Cliffe *et al*, 2005). Overall, this clearly demonstrated that there is a bias towards goblet cell differentiation during acute infection which is not observed during chronic infection. The extrinsic mucosal barrier is composed of a mixture of products secreted by the goblet cells within the epithelial cell layer (Thornton *et al*, 2008). Therefore, during acute infection, along with the increased differentiation towards the goblet cell lineage, and hence the increased levels of glycoproteins within the goblet cells, we also observed an increase in the amount of glycoproteins in the mucus barrier. The intestinal mucus barrier forms a bilayer: a ‘loose’ outer layer containing the commensal flora and the inner adherent layer which is devoid of commensal flora (Johansson *et al*, 2008). It has been proposed that a diminished mucus barrier can result in inflammation in several mouse models (Heazlewood *et al*, 2008; Johansson *et al*, 2009; Park *et al*, 2009), due to the direct contact of the commensal flora with the epithelial cell surface. We show here that there is a diminished glycoprotein content within the mucus barrier during chronic infection. Therefore, during chronicity to *T. muris* the diminished mucus barrier could result in the commensal flora coming into contact with the epithelial cell layer which may result in exacerbation of epithelial inflammation.

Previously published data has described goblet cell hyperplasia to be under the control of a T_H2-type immune response. Although one study has shown goblet cell hyperplasia to be independent of IL-4/IL-13 (Marillier *et al*, 2008), our data supported the hypothesis of T_H2 cytokines controlling goblet cell hyperplasia post *T. muris* infection (Hayes *et al*, 2007; McKenzie *et al*, 1998; Woodruff *et al*, 2009). Studies have shown that GABA receptors expressed in the mucosal epithelium can mediate this hypersecretion of

mucus in asthma (Xiang *et al*, 2007). Our data demonstrated that within the caecum, GABA-α3 receptor expression is elevated in response to the increased IL-13 levels, which may mediate the secretion of glycoproteins into the mucus barrier. It is important to highlight that differences were observed in the expression of the GABA receptors between the caecum and the colon. This corroborates our previous findings, where we observed goblet cell hyperplasia in response to *T. muris* only in the caecum (the niche of the parasite) (Hasnain *et al*, 2010). If in fact, the GABA-α3 receptor is up-regulated to mediate glycoprotein hypersecretion during worm expulsion, it could provide a novel therapeutic target which could be manipulated to induce secretion of glycoproteins during chronic nematode infections to increase the thickness of the mucus barrier and potentially lessen inflammation.

Interestingly, lectin histochemistry has identified an alteration in glycosylation of mucins within the caecal niche of the parasite. Moreover, the mucins within the mucus barrier were less charged during chronic infection as compared to mice with acute infection. The presence of more highly charged mucins with an altered glycosylation pattern in acute infection may affect the physicochemical properties of the mucus barrier and contribute to worm rejection. For instance, mucin glycosylation may play an important role in the hydration of the mucus gel (Singh and Hollingsworth, 2006) and hence barrier properties of the mucus gel. Furthermore, mucin glycans are thought to provide a framework within the mucus gel which allows the sequestration and presentation of essential host defence molecules (Thornton *et al*, 2008).

In conclusion, in this study we have characterised the changes within the dynamic mucosal barrier during acute and chronic nematode infection. The glycocalyx that coats the apical surface of the intestinal lumen was thickened in response to infection, with an increase in the cell surface mucins, *Muc4*, *Muc13* and *Muc17*. Moreover, we have clearly demonstrated that along with goblet cell hyperplasia observed during worm expulsion, there was an increase in the secretion of more highly charged mucins into the mucus barrier. Furthermore, during chronicity the decrease in goblet cell numbers results in a depleted mucus barrier, containing predominantly lowly charged mucins. Taken together, this study highlights dynamic changes within the glycoconjugate components of the extrinsic and intrinsic mucosal barrier and, emphasises that the quality and the quantity of these components may be important during worm expulsion.

REFERENCES

Artis D (2006). New weapons in the war on worms: identification of putative mechanisms of immune-mediated expulsion of gastrointestinal nematodes. *Int J Parasitol* **36**(6)**:** 723-733.

Artis D, Grencis RK (2008). The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunol* **1**(4)**:** 252-264.

Artis D, Potten CS, Else KJ, Finkelman FD, Grencis RK (1999). *Trichuris muris*: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. *Exp Parasitol* **92**(2)**:** 144-153.

Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, Schad GA, Scott P, Wu GD (2004). RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc Natl Acad Sci U S A* **101**(37)**:** 13596-13600.

Bancroft AJ, Else KJ, Humphreys NE, Grencis RK (2001). The effect of challenge and trickle *Trichuris muris* infections on the polarisation of the immune response. *Int J Parasitol* **31**(14)**:** 1627-1637.

Bancroft AJ, McKenzie AN, Grencis RK (1998). A critical role for IL-13 in resistance to intestinal nematode infection. *J Immunol* **160**(7)**:** 3453-3461.

Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* **367**(9521)**:** 1521-1532.

Cliffe LJ, Grencis RK (2004). The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol* **57:** 255-307.

Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* **308**(5727)**:** 1463-1465.

Else KJ (2005). Have gastrointestinal nematodes outwitted the immune system? *Parasite Immunol* **27**(10-11)**:** 407-415.

Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010). Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* **138**(5)**:** 1763-1771.

Hayes KS, Bancroft AJ, Grencis RK (2007). The role of TNF-alpha in *Trichuris muris* infection II: global enhancement of ongoing Th1 or Th2 responses. *Parasite Immunol* **29**(11)**:** 583-594.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA (2008). Aberrant mucin assembly in mice causes endoplasmic

reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* **5**(3)**:** e54.

Hogan SP, Seidu L, Blanchard C, Groschwitz K, Mishra A, Karow ML, Ahrens R, Artis D, Murphy AJ, Valenzuela DM, Yancopoulos GD, Rothenberg ME (2006). Resistin-like molecule beta regulates innate colonic function: barrier integrity and inflammation susceptibility. *J Allergy Clin Immunol* **118**(1)**:** 257-268.

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* **105**(39)**:** 15064-15069.

Johansson ME, Thomsson KA, Hansson GC (2009). Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. *J Proteome Res* **8**(7)**:** 3549-3557.

Khan WI, Blennerhasset P, Ma C, Matthaei KI, Collins SM (2001). Stat6 dependent goblet cell hyperplasia during intestinal nematode infection. *Parasite Immunol* **23**(1)**:** 39-42.

Khan WI, Collins SM (2004). Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. *Parasite Immunol* **26**(8-9)**:** 319-326.

Khan WI, Richard M, Akiho H, Blennerhasset PA, Humphreys NE, Grencis RK, Van Snick J, Collins SM (2003). Modulation of intestinal muscle contraction by interleukin-9 (IL-9) or IL-9 neutralization: correlation with worm expulsion in murine nematode infections. *Infect Immun* **71**(5)**:** 2430-2438.

Linden S, Nordman H, Hedenbro J, Hurtig M, Boren T, Carlstedt I (2002). Strain- and blood group-dependent binding of Helicobacter pylori to human gastric MUC5AC glycoforms. *Gastroenterology* **123**(6)**:** 1923-1930.

Linden SK, Florin TH, McGuckin MA (2008a). Mucin dynamics in intestinal bacterial infection. *PLoS One* **3**(12)**:** e3952.

Linden SK, Sutton P, Karlsson NG, Korolik V, McGuckin MA (2008b). Mucins in the mucosal barrier to infection. *Mucosal Immunol* **1**(3)**:** 183-197.

Marillier RG, Michels C, Smith EM, Fick LC, Leeto M, Dewals B, Horsnell WG, Brombacher F (2008). IL-4/IL-13 independent goblet cell hyperplasia in experimental helminth infections. *BMC Immunol* **9:** 11.

McAuley JL, Linden SK, Png CW, King RM, Pennington HL, Gendler SJ, Florin TH, Hill GR, Korolik V, McGuckin MA (2007). MUC1 cell surface mucin is a critical element of the mucosal barrier to infection. *J Clin Invest* **117**(8)**:** 2313-2324.

McGuckin MA, Thornton DJ (2000). Detection and quantitation of mucins using chemical, lectin, and antibody methods. *Methods Mol Biol* **125:** 45-55.

McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN (1998). A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr Biol* **8**(6)**:** 339-342.

Nair MG, Guild KJ, Du Y, Zaph C, Yancopoulos GD, Valenzuela DM, Murphy A, Stevens S, Karow M, Artis D (2008). Goblet cell-derived resistin-like molecule beta augments CD4+ T cell production of IFN-gamma and infection-induced intestinal inflammation. *J Immunol* **181**(7)**:** 4709-4715.

Nakajima K, Tooyama I, Kuriyama K, Kimura H (1996). Immunohistochemical demonstration of GABAB receptors in the rat gastrointestinal tract. *Neurochem Res* **21**(2)**:** 211-215.

Noah TK, Kazanjian A, Whitsett J, Shroyer NF (2009). SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells. *Exp Cell Res* **316**(3)**:** 452-465.

Packer LM, Williams SJ, Callaghan S, Gotley DC, McGuckin MA (2004). Expression of the cell surface mucin gene family in adenocarcinomas. *Int J Oncol* **25**(4)**:** 1119-1126.

Park SW, Zhen G, Verhaeghe C, Nakagami Y, Nguyenvu LT, Barczak AJ, Killeen N, Erle DJ (2009). The protein disulfide isomerase AGR2 is essential for production of intestinal mucus. *Proc Natl Acad Sci U S A* **106**(17)**:** 6950-6955.

Ramalingam TR, Pesce JT, Sheikh F, Cheever AW, Mentink-Kane MM, Wilson MS, Stevens S, Valenzuela DM, Murphy AJ, Yancopoulos GD, Urban JF, Jr., Donnelly RP, Wynn TA (2008). Unique functions of the type II interleukin 4 receptor identified in mice lacking the interleukin 13 receptor alpha1 chain. *Nat Immunol* **9**(1)**:** 25-33.

Renes IB, Verburg M, Van Nispen DJ, Taminiau JA, Buller HA, Dekker J, Einerhand AW (2002). Epithelial proliferation, cell death, and gene expression in experimental colitis: alterations in carbonic anhydrase I, mucin MUC2, and trefoil factor 3 expression. *Int J Colorectal Dis* **17**(5)**:** 317-326.

Shroyer NF, Wallis D, Venken KJ, Bellen HJ, Zoghbi HY (2005). Gfi1 functions downstream of Math1 to control intestinal secretory cell subtype allocation and differentiation. *Genes Dev* **19**(20)**:** 2412-2417.

Singh PK, Hollingsworth MA (2006). Cell surface-associated mucins in signal transduction. *Trends Cell Biol* **16**(9)**:** 467-476.

Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK (1996). Respiratory mucins: identification of core proteins and glycoforms. *Biochem J* **316 (Pt 3):** 967-975.

Thornton DJ, Carlstedt I, Sheehan JK (1994). Identification of glycoproteins on nitrocellulose membranes and gels. *Methods Mol Biol* **32:** 119-128.

Thornton DJ, Holmes DF, Sheehan JK, Carlstedt I (1989). Quantitation of mucus glycoproteins blotted onto nitrocellulose membranes. *Anal Biochem* **182**(1)**:** 160-164.

Thornton DJ, Rousseau K, McGuckin MA (2008). Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* **70:** 459-486.

Thornton DJ, Sheehan JK (2004). From mucins to mucus: toward a more coherent understanding of this essential barrier. *Proc Am Thorac Soc* **1**(1)**:** 54-61.

Tu QV, McGuckin MA, Mendz GL (2008). *Campylobacter jejuni* response to human mucin MUC2: modulation of colonization and pathogenicity determinants. *J Med Microbiol* **57**(Pt 7)**:** 795-802.

Wakelin D (1967). Acquired immunity to *Trichuris muris* in the albino laboratory mouse. *Parasitology* **57**(3)**:** 515-524.

Woodruff PG, Modrek B, Choy DF, Jia G, Abbas AR, Ellwanger A, Koth LL, Arron JR, Fahy JV (2009). T-helper type 2-driven inflammation defines major subphenotypes of asthma. *Am J Respir Crit Care Med* **180**(5)**:** 388-395.

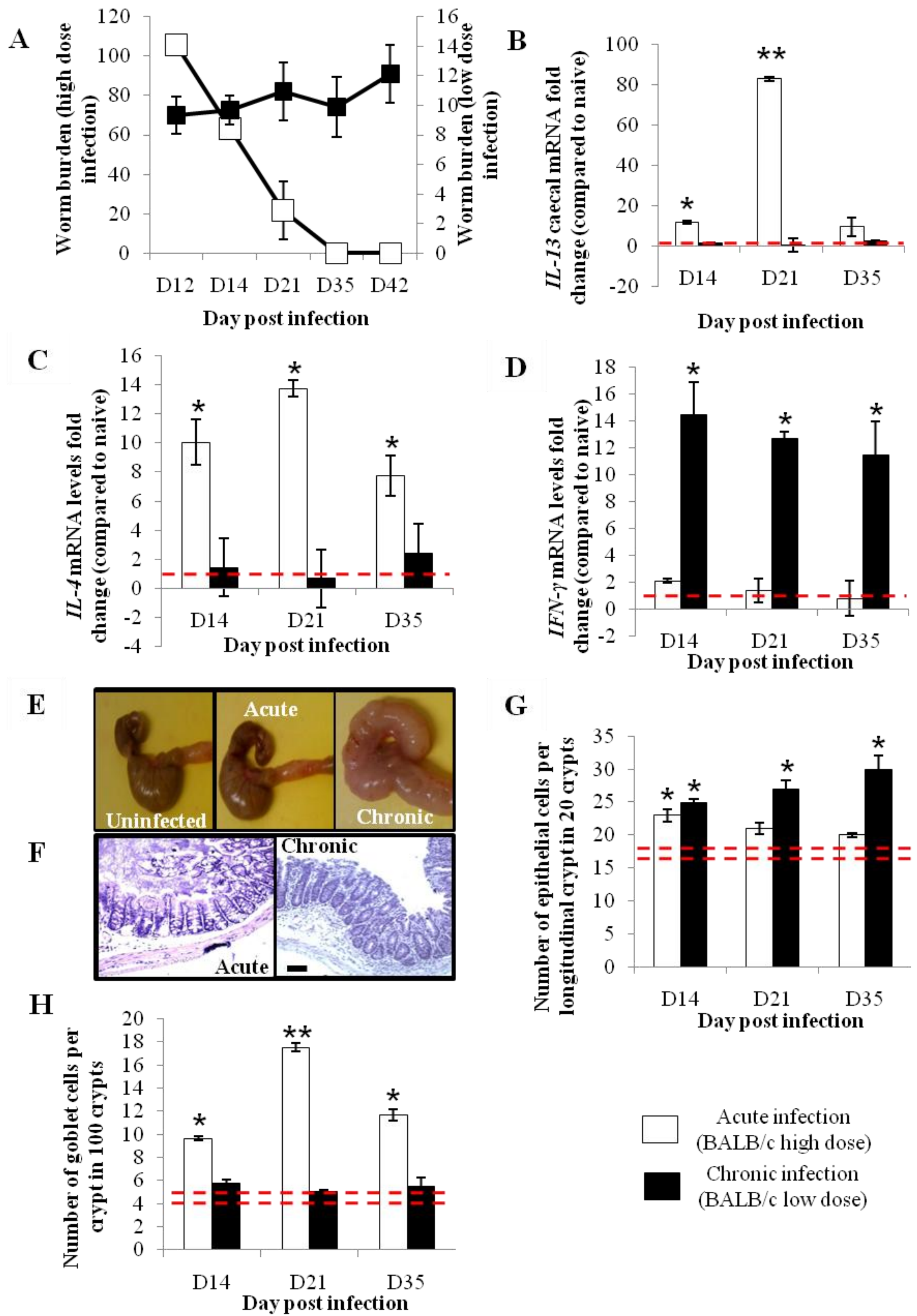
Xiang YY, Wang S, Liu M, Hirota JA, Li J, Ju W, Fan Y, Kelly MM, Ye B, Orser B, O'Byrne PM, Inman MD, Yang X, Lu WY (2007). A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. *Nat Med* **13**(7)**:** 862-867.

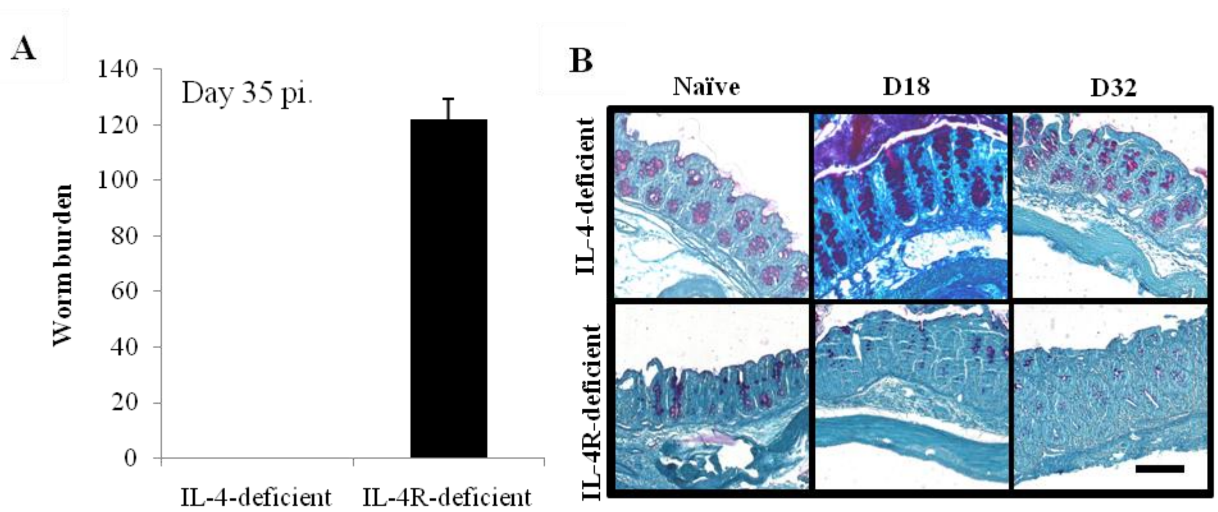
Zaph C, Troy AE, Taylor BC, Berman-Booty LD, Guild KJ, Du Y, Yost EA, Gruber AD, May MJ, Greten FR, Eckmann L, Karin M, Artis D (2007). Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* **446**(7135)**:** 552-556.

Zeiter DK, Li X, Broussard DL (1996). Identification of the GABAA receptor alpha-subunit mRNA in rat intestine. *Brain Res Mol Brain Res* **39**(1-2)**:** 241-244.

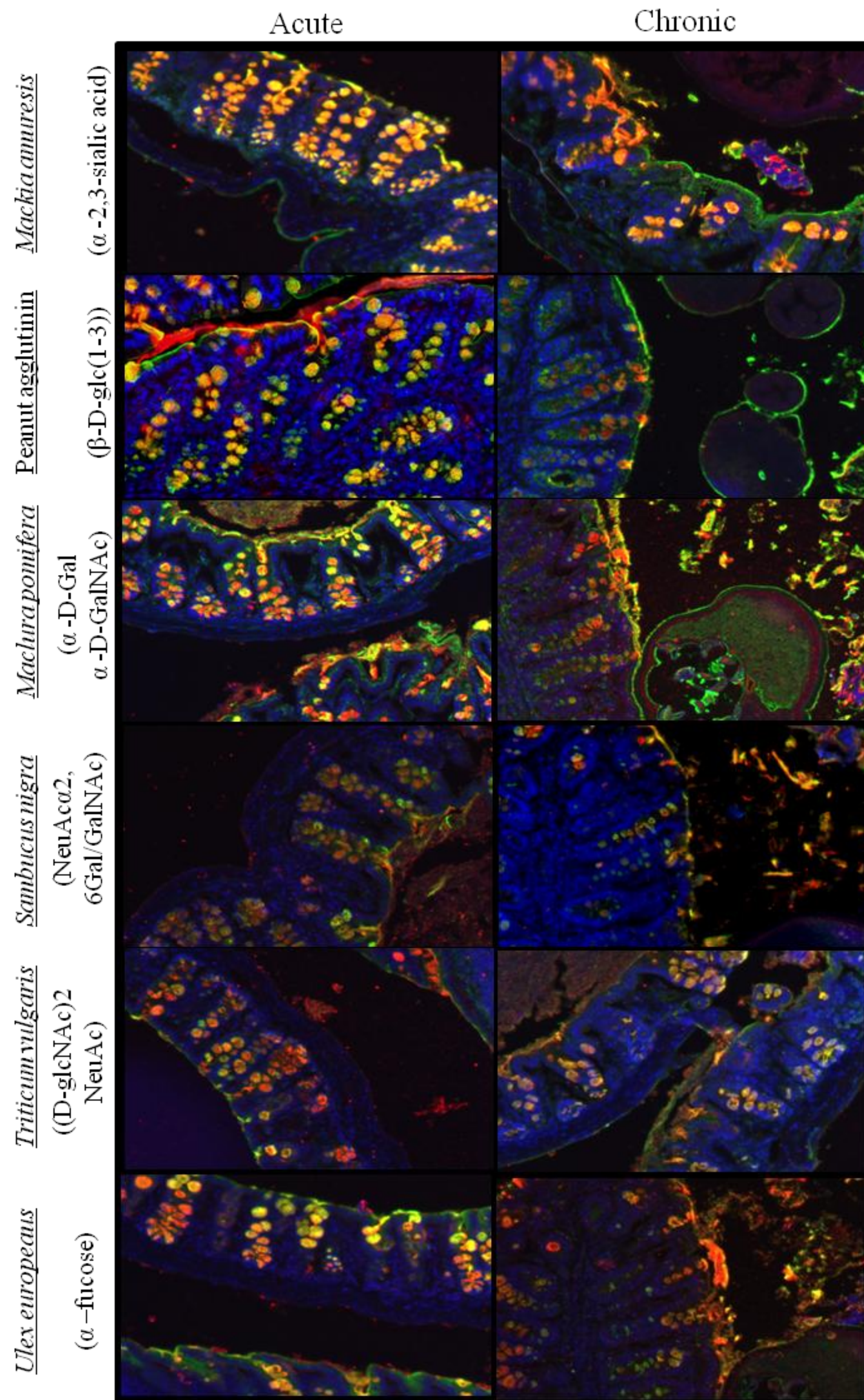
SUPPLEMENTARY FIGURES

Supplementary Figure 2.1: (A) BALB/c mice were infected orally with a high (acute infection) or a low dose (chronic infection) of *T. muris* eggs and worm burdens were assessed on day 12, 14, 21, 35 and 42 pi. Levels of *IL-13* (B), *IL-4* (C) and *IFN-γ* (D) in the caecal tissue was determined using RT-PCR (data presented as fold change compared to naïve (red dashed line)). Gross morphology (E) of the caecum of uninfected mice compared to during acute and chronic infection (day 35 pi.). H&E staining (F) of caecal tissue from mice with acute and chronic infection (day 35 pi.). The number of epithelial cells per crypt in 20 crypts were quantified (G). Quantification of the number of goblet cells (H) during the course of infection. Scale bar = 10µm. * = $P<0.05$, ** = $P<0.01$. Results represent the mean value of 8-10 mice per group ± SEM.


A
Worm burden (high dose infection)
Worm burden (low dose infection)
Day post infection
D12
D14
D21
D35
D42
B
IL-13 caecal mRNA fold change (compared to naive)
C
IL-4 mRNA levels fold change (compared to naive)
D
IFN-γ mRNA levels fold change (compared to naive)
E
Uninfected
Acute
Chronic
F
G
Number of epithelial cells per longitudinal crypt in 20 crypts
H
Number of goblet cells per crypt in 100 crypts
Acute infection (BALB/c high dose)
Chronic infection (BALB/c low dose)



Supplementary Figure 2.2: IL-4-deficient and IL-4R-deficientmice were infected with a high dose of *T. muris* eggs and worm burdens (A) were assessed on day 35 pi. PAS staining (B) of the caecum from IL-4-deficient and IL-4R-deficient naïve mice and on day 18 and 32 pi. Results represent the mean value of 4 mice per group ± SEM.



Supplementary Figure 2.3: Dual label immunofluorescence staining with lectins (green) was used to determine the changes in the glycans on the Muc2 (red) during acute and chronic infection (day 21 pi.). Positive staining of glycans on Muc2 = yellow (merge). Representative of 3 individual experiments.

SUPPLEMENTARY TABLES

Gene of interest	5'-Forward primer-3'	5'-Reverse primer-3'
β-actin	GTGGGCCGCTCTAGGCACCAA	CTCTTTGATGTCACGCACGATTTC
GABA-α1	GAGCCGGCAGCTACAGAGAT	GGTACCAGCCACTGGATCTG
GABA-α2	CTCTGAGTGACGGGACCTAC	GCCTCTGGGTTGCAGAAG
GABA-α3	CACACCCAGCTCCTCCTAGA	GGTTGCTCTCCTTGCCTCAG
GABA-β1	ATCCTGTCCTGGGTGTCG	GTCTCCCTGAGGTGAGTGCT
GABA-β2	GGTGCCTGACACCTACTTCC	GAGGCCATACAGGACAGTGC
GABA-β3	CACAGGTGCCTATCCTCGAC	CAGAAGGACACCCACGAGAG
Hes-1	GGTCCTAACGCAGTGTCACC	GAGAGGTGGGCTAGGGACTT
IL-4	GAGCTCGTCTGTAGGGCTTC	GCCCGAAAGAGTCTCTGC
IL-13	CTCCCTCTGACCCTTAAGGAG	GAAGGGGCCGTGGCGAAACAG
Math-1	GCTTCCTCTGGGGGTTACTC	GAAGGCGACAGGTCCTTCTG
Muc1	GGTTGCTTTGGCTATCGTCTATTT	AAAGATGTCCAGCTGCCCATA
Muc4	CCACCTCCTCGACCCTTACT	CTCCGACTTCAGACCCGTAG
Muc13	ACTGGCTCTGTCCTCTGTGG	CCTTGGCCTCTCTCTACCTC
Muc15	GAGTTCCAGACCCTCCTCCA	GGATTTGCGTCTGCTGTGG
Muc17	GTGGGACGGGCTCAAATG	TACGCTCTCCACCAGTTCCT
Muc20	CAGCAGCAGTCTCTCAGTGG	GTGGCTCTCTGTGAGGAAGG

Supplementary Table 2.1: Forward and reverse primer sequences for the genes of interest.

Cell surface mucins	Fold change (Day 14 pi.)		Fold change (Day 35 pi.)	
	Acute	Chronic	Acute	Chronic
Muc1	1.10 ± 0.28	1.06 ± 0.22	0.99 ± 0.038	0.86 ± 0.03
Muc4	7.34 ± 0.50	3.91 ± 0.15	7.44 ± 1.50	6.45 ± 0.87
Muc13	10.05 ± 1.21	8.93 ± 0.20	6.6 ± 2.2	3.36 ± 0.69
Muc15	1.15 ± 0.90	1.06 ± 0.53	1.12 ± 0.35	1.03 ± 0.36
Muc17	12.6 ± 1.51	13.87 ± 1.41	10.75 ± 1.09	12.42 ± 0.80
Muc20	1.11 ± 0.18	1.09 ± 0.27	1.61 ± 1.08	1.75 ± 1.02

Supplementary Table 2.2: Levels of cell surface mucins in response to *T. muris* acute and chronic infection on day 14 and 35 after infection. Results represent the mean value of 5 mice per group ± SEM.

MUCIN GENE DEFICIENCY IMPAIRS HOST RESISTANCE TO *Trichuris muris* INFECTION

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(All the work was carried out by S.Z.H, with the exception of Figure 4.2 and Figure 4.3A-D)

ABSTRACT

Background & Aims: Hyperplasia of mucin-secreting intestinal goblet cells accompanies a number of enteric infections including infections by nematode parasites. Nevertheless, the precise role of mucins in host defence in nematode infection is not known. We investigated the role of the mucin (Muc2) in worm expulsion and host immunity in a model of nematode infection.

Methods: Resistant (BALB/c, C57BL/6), susceptible (AKR), and Muc2-deficient mouse strains were infected with the nematode, *Trichuris muris* and worm expulsion, energy status of the whipworms, changes in mucus/mucins, and inflammatory and immune responses were investigated following infection.

Results: The increase in Muc2 production, observed exclusively in resistant mice, correlated with worm expulsion. Moreover, expulsion of the worms from the intestine was significantly delayed in the Muc2-deficient mice. Although a marked impairment in the development of PAS-stained intestinal goblet cells was observed in Muc2-deficient mice, as infection progressed a significant increase in the number of PAS-positive goblet cells was observed in these mice. Surprisingly, an increase in Muc5ac, a mucin normally expressed in the airways and stomach, was observed following infection of only the resistant animals. Overall, the mucus barrier in the resistant mice was less permeable than that of susceptible mice. Furthermore, the worms isolated from the resistant mice had a lower energy-status.

Conclusions: Mucins are an important component of innate defence in enteric infection; this is the first demonstration of the important functional contribution of mucins to host protection from nematode infection.

ABBREVIATIONS

ND, Not detectable; PAS, Periodic-Acid Schiff's; pi., Post infection; Relm, Resistin-like molecule; Tff, Trefoil factor; T_H, T Helper; *T. muris*, *Trichuris muris*; WT: Wild-type

INTRODUCTION

The mucus barrier is an essential part of the innate immune system which hydrates and protects the underlying epithelia. The gel-like properties of the barrier are mainly due to the polymeric mucins which are the major secretory products of epithelial goblet cells (Artis *et al*, 2004; Sheehan and Thornton, 2000; Thornton and Sheehan, 2004). The colonic epithelium expresses mainly MUC2/Muc2 in large amounts which is stored in bulky apical granules of the goblet cells and is the most important factor determining the goblet cell morphology (Chang *et al*, 1994; Tytgat *et al*, 1994; Van der Sluis *et al*, 2006). Muc2 forms a heterogeneous mucus barrier that is proposed to comprise of two distinct layers; a 'loose' outer layer that bacteria can penetrate and an adherent inner layer that excludes bacteria from direct contact with the underlying epithelia (Johansson *et al*, 2008).

Alterations or absence of MUC2 production can lead to many common human disorders such as colon carcinoma (Boland *et al*, 1982), ulcerative colitis (Buisine *et al*, 1999), and celiac disease (Crabtree *et al*, 1989). A role for Muc2 in the suppression of colorectal carcinoma has also been suggested as Muc2-deficient mice spontaneously develop colitis and adenomas that progress to invasive adenocarcinoma (Velcich *et al*, 2002), suggesting an important function for this mucin in colonic protection (Van der Sluis *et al*, 2006). Furthermore, missense mutations in the *Muc2* gene results in aberrant Muc2 oligomerisation leading to endoplasmic reticulum stress and subsequently increased susceptibility to colitis (Heazlewood *et al*, 2008).

Hyperplasia of mucin producing goblet cells has been described in a number of parasitic infections including *Nippostrongylus brasiliensis, Hymenolepsis diminuta*, *Trichinella spiralis* and *Trichuris muris* (Else and Finkelman, 1998; Khan et al, 2001; Miller and Nawa, 1979; Shekels et al, 2001; Webb et al, 2007). Putative mechanisms underlying the protective role of mucins against infectious agents include the demonstration of trapping of *Hymenolepsis diminuta* (Webb et al, 2007) and *Trichinella spiralis* (Carlisle et al, 1991) in the mucus and inhibition of parasite motility and feeding capacity (Carlisle *et al*, 1991; Ishiwata and Watanabe, 2007; Miller, 1987). Goblet cell response, in all four of these nematode models, is thought to be under the control of a T helper (T_H) 2-type immune response and is considered as a potential effector mechanism (Cliffe and Grencis, 2004; Deschoolmeester and Else, 2002; Else, 2005). Recently a number of goblet cell bioactive factors such as resistin-like molecule-β (Relm-β), intelectin and calcium-activated chloride channel-3 ($ClCa_3$) have been suggested to play an important role in nematode infection (Artis and Grencis, 2008; Hogan *et al*, 2006).

However, a definitive and precise role of mucins, the major secreted product of goblet cells, in host defence in intestinal nematode infection remains to be elucidated.

The nematode *T. muris* inhabits the caecum of mice and is closely related at the morphological, physiological and antigenic levels to *Trichuris trichiura,* the causative agent of chronic trichuriasis in humans (Else and Grencis, 1991). In this parasitic infection, strains resistant to chronic infection (BALB/c, C57BL/6) expel the parasites through the generation of a T_H2-type immune response whereas susceptible strains (AKR), that do not expel the worms, develop a T_H1-type immune response (Cliffe and Grencis, 2004; Cliffe *et al*, 2007). In this study, we demonstrated that the increase in Muc2, the major determinant of mucus barrier properties, correlates with worm expulsion. In the absence of Muc2 there is a delay in worm expulsion, but interestingly Muc5ac is up-regulated in the Muc2-deficient mice prior to expulsion. Moreover, Muc5ac is up-regulated in the wild-type mice that are resistant to infection, but not in those unable to expel. The physical properties of the mucus barrier are also altered during infection, resulting in a less porous network, with overall changes having a direct effect on the viability of the whipworm. Collectively, these data demonstrate for the first time a protective role for mucins in nematode infection.

MATERIALS AND METHODS

Animals

Breeding pairs of Muc2-deficient mice originally produced by gene mutation (Velcich *et al,* 2002) and their wild-type (C57BL/6) littermates (Albert Einstein Medical College, USA) were kept at the animal facilities of McMaster University, Hamilton, Ontario, Canada. AKR, BALB/c (Harlan Olac) and SCID mice were maintained in the Biological Services Unit at Manchester University. The protocols employed were in accordance with guidelines by the McMaster University Animal Care Committee, Canadian Council on the Use of Laboratory Animals and the Home Office Scientific Procedures Act (1986). All mice were kept in sterilized, filter-topped cages, and fed autoclaved food in the animal facilities. Only 6-10wk old male mice were used.

Parasitological techniques

The techniques used for *T. muris* maintenance and infection were described previously (Wakelin, 1967). Mice were orally infected with approximately 100-300 eggs for a high dose infection and <15 eggs for a low dose infection. Worm burdens were assessed by

counting the number of worms present in the caecum as described previously (Wakelin, 1967).

Histology, immunohistochemistry and immunofluorescence

A 1cm segment or the whole caecum (rolled) was fixed in 10% neutral buffered formalin or 95% ethanol and processed by using standard histological techniques. Sections were treated with 0.1M KOH for 30 minutes prior to staining with Periodic Acid Schiff's (PAS) reaction (Linden *et al*, 2008). Slides were counterstained with either haematoxylin and eosin or 1% fast-green. Standard immunohistochemical and immunofluorescent staining methods (Kirkham *et al*, 2008; Linden *et al*, 2008) were used to determine the levels of Muc2, Muc5ac, Relm-β and Tff3.

Antibodies

Immunodetection was carried out using a polyclonal antibody raised against a murine Muc2 (mMuc2) (Heazlewood *et al*, 2008). Commercially available 45M1 antibody was used for the detection of mouse Muc5ac (Lidell *et al*, 2008). The mouse Muc5b-specific antibody (Zhu *et al*, 2008) was a kind gift from Dr. Camille Ehre (University of North Carolina, Chapel Hill). Commercially available mRelm-β (Abcam, Cambridge, United Kingdom) and mITF (Santa Cruz Biotechnology Inc., CA, USA) antibodies were used to detect Relm-β and Tff3, respectively. Detection of BrdU incorporated into nuclei was carried out using a monoclonal anti-BrdU antibody (AbD Serotec,Oxford, United Kingdom) (Potten *et al*, 2002).

Mucus extraction and agarose gel electrophoresis

The caecum was gently flushed with PBS, scraped and mucus solubilised in 8M guanidium chloride. Subsequently, extracted mucus samples were reduced using 50mM dithiothreitol (DTT) and carboxylmethylated using 0.125M iodoacetamide prior to electrophoresis on a 1% (w/v) agarose gel. Mucins were detected after western blotting with mucin specific antisera (Thornton *et al*, 1996).

Analysis of mucus network properties

Caecal tissue isolated from BALB/c and AKR mice was cut longitudinally, washed with PBS and kept hydrated in a 6 well plate. 0.1μm blue fluorescently labelled polymer microspheres (Dukes Scientific, UK) were placed on top of the luminal surface of the

caecum (set as a reference) and their position analysed using the Nikon C1 Upright confocal microscope. 3D optical stacks were taken every 5µm and combined to obtain a Z-stack at the time points stated.

Worm isolation for ATP analysis

The caecum was longitudinally cut and segmented before incubation with 0.1M NaCl for 2 hours at 37°C with frequent shaking. Worms were counted after separation from debris and epithelial cells using a 0.7µm filter and kept in RPMI-1640 supplemented with 10% FCS. Live worms were subsequently homogenised using the FastPrep® homogeniser (MP Biomedicals, Inc.).

Energy status of worms

The CellTiter-Glo® luminescent cell viability assay was carried out according to manufacturer's instructions (Promega Corp., USA). Relative light units (RLUs) were calculated per worm: RLU = (sample light units − blank light units)/number of worms. Substrate only was used as a blank control whereas, worms were boiled before homogenisation for negative controls. To determine recovery of energy status, worms recovered on D19 pi. were washed extensively in DMEM, added to 6 well plates with LS174T cells (maintained as previously described by Hayes *et al.*(Hayes et al, 2007)) for 24 hours prior to measuring ATP levels.

RT-PCR

Total RNA from epithelial cells was isolated using the previously described method (Cliffe *et al*, 2005). cDNA was generated using an IMPROM-RT kit (Promega) and Absolute QPCR SYBR Green (ABgene) was used for quantitative PCR. Primer efficiencies was determined using cDNA dilutions and genes of interest were normalised against housekeeping gene, β-actin and expressed as a fold difference to uninfected naïve message levels. mRNA expression was investigated using primers 5'-GTGGGCCGCTCTAGGCACCAA-3' and 5'-CTCTTTGATGTCACGCACGATTTC-3' for β-actin; 5'-GTCCAGGGTCTGGATCACA- 3' and 5'-CAGATGGCAGTGAGCTGAGC-3' for Muc2; 5'-GTGATGCACCCAT GATCTATTTTG-3' and 5'-ACTCGGAGCTATAACAGGTCATGTC-3' for Muc5ac; GGTTGCTTTGGCTATCGTCTATTT and AAAGATGTCCAGCTGCCCATA for Muc1;

CCACCTCCTCGACCCTTACT and CTCCGACTTCAGACCCGTAG for Muc4; 5'-GTGGGACGGGCTCAAATG-3' and 5'-CTC TACGCTCTCCACCAGTTCCT-3' for Muc17; 5'-TTGCTGGGTCCTCTGGGATA-3' and 5'-GCCGGCACCATA CATTGG-3 for Tff3 and 5'-GCTCTTCCCTTTCCTTCTCCAA-3' and 5'-ACCACAGTGTAGGCTTCATGCTGTA-3' for Relm-β. RT-PCR products were directly sequenced to verify the identity of the amplified genes. In brief, products were digested with Exonuclease I and Calf Intestinal Phosphatase and subsequently sequenced using the ABIPRISM Big-Dye Terminator cycle sequencing reaction at the Sequencing Facility in the University of Manchester. The data was analysed using Chromas Pro v1.34 and the sequences obtained were compared against the GenBank database; http://www.ncbi.nlm.nih.gov/BLAST.

Rate of epithelial cell turnover

The rate of intestinal epithelial cell turnover was assessed by visualising BrdU incorporated into nuclei after mice were injected with 10mg of BrdU 16hrs before sacrifice, as described previously (Cliffe *et al*, 2005).

Evaluation of* in-vitro *cytokines production from splenocytes

Single-cell suspensions of spleen were prepared in RPMI 1640 containing 10% FCS, 5mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin, 25mM Hepes, 0.05mM 2-ME (all Gibco-BRL). Cells (10^7) were incubated in the presence of 5μg/ml Concavalin A (Con A). IL-4 and IL-13 levels in the supernatant were measured by enzyme immunoassay using a commercially available kit (R & D Systems; Minneapolis, MN).

Investigation of intestinal tissue cytokines levels

Frozen intestinal tissues were homogenized in lysis buffer containing protease inhibitor cocktail. The homogenates were freeze-thawed three times and centrifuged, and then supernatant was collected and stored at -20°C until analyzed. IFN-γ, IL-4 and IL-13 level in the supernatant was measured by enzyme immunoassay technique using a commercially available kit purchased from R&D Systems. Concentration of protein in the intestinal tissue was determined by a commercially available DC Protein Assay kit (Bio-Rad), and the amount of cytokines in the tissues was expressed per milligram of tissue protein.

Quantification of histological staining

The numbers of goblet cells expressed per crypt were counted in 50 longitudinally sectioned crypt units. The area stained (pixel/mm^2) per 100 crypts was determined by using the ImageJ software version 1.39a.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed using SPSS version 16.0. Statistical significance of different groups was assessed by using parametric tests (ONE-way Analysis of variance with post-test following statistical standards or paired Student t test). $P<0.05$ was considered statistically significant.

RESULTS

Increased Muc2 production correlates with worm expulsion

It has been well documented that susceptible (AKR) mice harbour the *T. muris* worms until patency (D35 post infection; **Figure 3.1A**). Whereas, the resistant (BALB/c) mice start expelling worms by D14 post infection (pi.) and expulsion is achieved by D21 pi. (Bancroft and Grencis, 1998; Cliffe and Grencis, 2004) (**Figure 3.1A**). Changes in the production of Muc2, the major gel-forming constituent of intestinal mucus, were explored within the caecum of AKR or BALB/c mice exposed to a high dose *T. muris* infection. Immunohistochemical staining and RT-PCR analysis for Muc2 (**Figure 3.1B** & **C**) showed that significantly higher amounts of Muc2 were expressed within the caecal crypts of the resistant mice on D21 pi. compared to naïve and susceptible mice; a similar staining pattern was observed with the PAS reagent (**Supplementary Figure 3.1**).This increase in goblet cell number and Muc2 levels was restricted to the niche of the parasite, was not observed in the colon (**Supplementary Figure 3.1**) and correlated with worm expulsion. Therefore, to further understand the role of Muc2 in *T. muris* infection we performed a high dose infection in Muc2-deficient mice on the resistant C57BL/6-background.

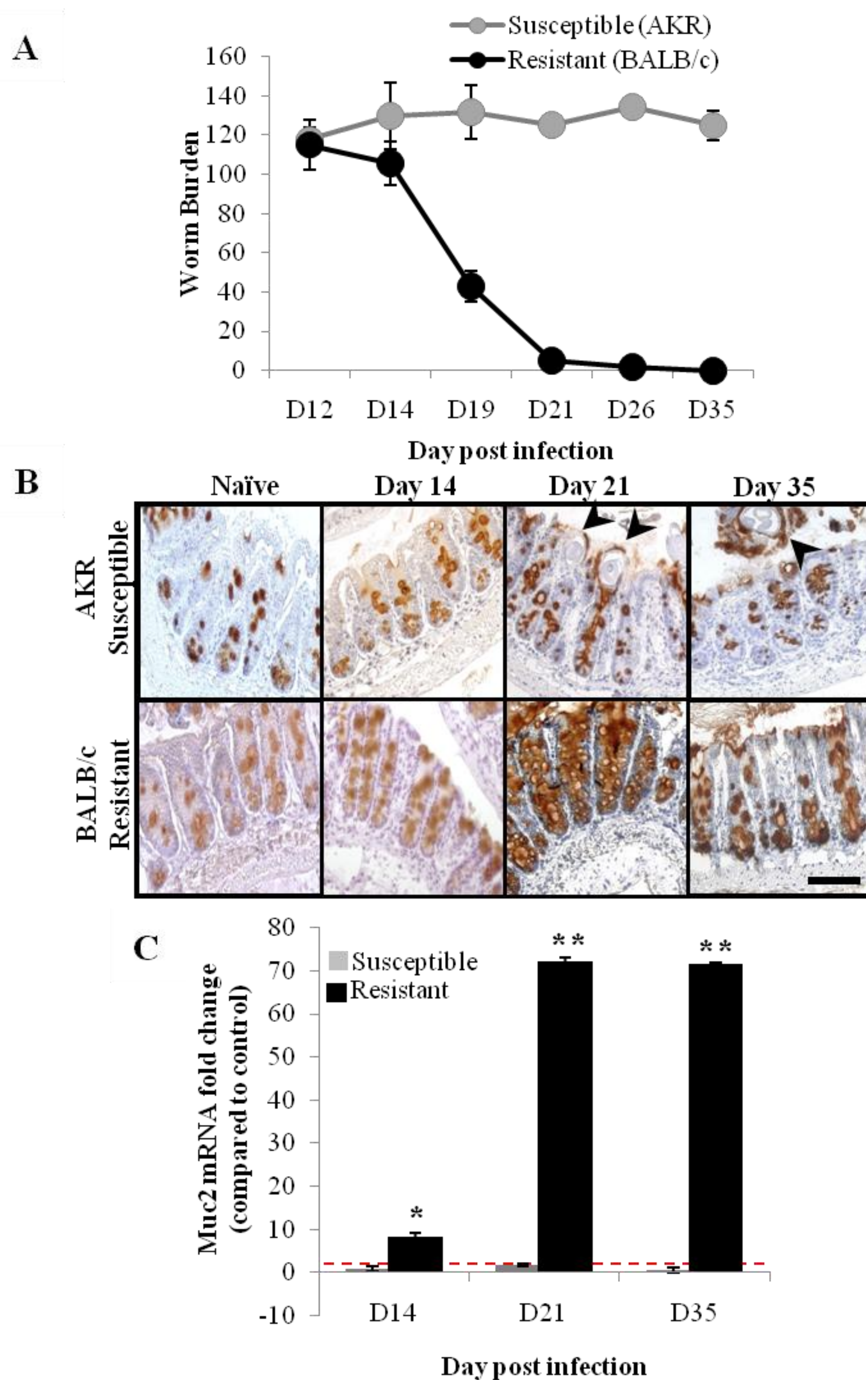


Figure 3.1: Worm burdens were assessed in both resistant (BALB/c) and susceptible (AKR) mice (A). Immunohistochemistry with mMuc2 antibody (B) and RT-PCR (C) were used to determine changes in Muc2 levels during infection. Nematodes are depicted by arrows (B). Red dashed line = naïve levels (C). Representative of 3 mice. Scale Bar = 50µm. * = $P<0.05$, ** = $P<0.01$.

Muc2 deficiency delayed* T. muris *worm expulsion from infected mice

A high dose *T. muris* infection established in both wild-type and Muc2-deficient mice; showed no marked difference in the number of worms at D13 pi. (**Figure 3.2**). However, as infection progressed, there was a significant decrease in worm burden in the

wild-type mice; evident by D15 pi. (46% reduction) and with a 84% decrease over establishment levels by D20 pi. In contrast, in the Muc2-deficient mice there was no decrease in worm burdens until after D20 pi. although mice did eventually expel their parasites.

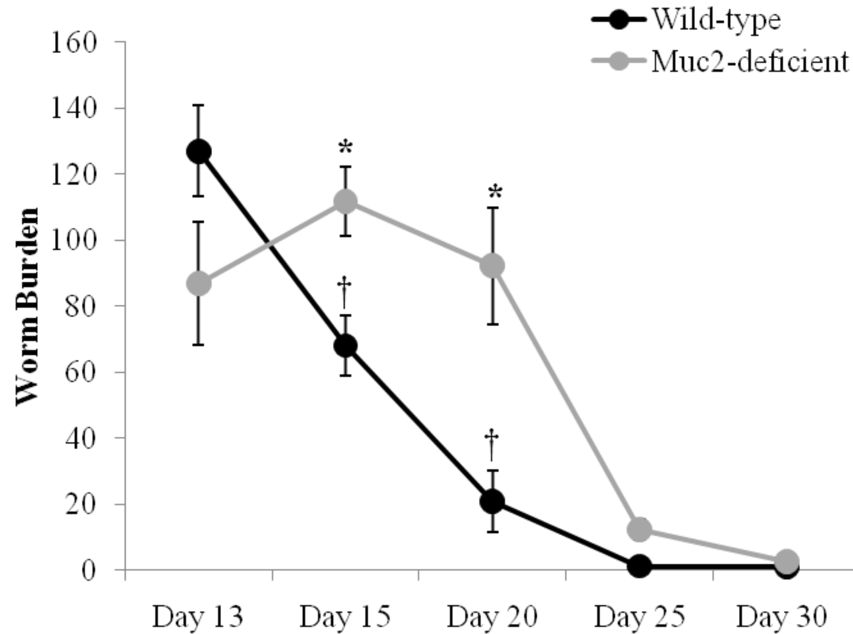


Figure 3.2: Muc2-deficient mice and their resistant wild-type (C57BL/6-background) littermates were infected orally with 300 eggs of *T. muris* and worm burdens were investigated on days 13, 15, 20, 25 and 30 pi. Representative of five mice. † = *P<0.05* compared to D13 pi. * = P<0.05 compared to wild-type mice.

Muc2 deficiency had no significant effect on T_H2-type immune response elicited by* T. muris *infection

We next sought to determine whether the delay in worm expulsion in the Muc2-deficient mice was due to an alteration to the adaptive immune response to *T. muris* infection. IL-4 and IFN-γ levels in intestinal tissue were not detectable in the naïve wild-type and Muc2-deficient mice. Furthermore, there was no significant difference in IL-4 or IFN-γ levels in intestinal tissues between both strains on D20 pi. (**Figure 3.3A** and **B**). Consistent with the local immune response, there was no significant difference in IL-4 and IL-13 production from *in vitro* ConA stimulated spleen cells (**Figure 3.3C** and **D**). Thus, in spite of the delay in worm expulsion Muc2 deficiency had no significant effect on generation of the T_H2-type immune response in *T. muris* infection. The crypt architecture, an indicator of inflammation, changed during infection; there was an increase in crypt

length on D15 pi. in wild-type and Muc2-deficient mice which was more pronounced in the Muc2-deficient mice (**Figure 3.3E**).

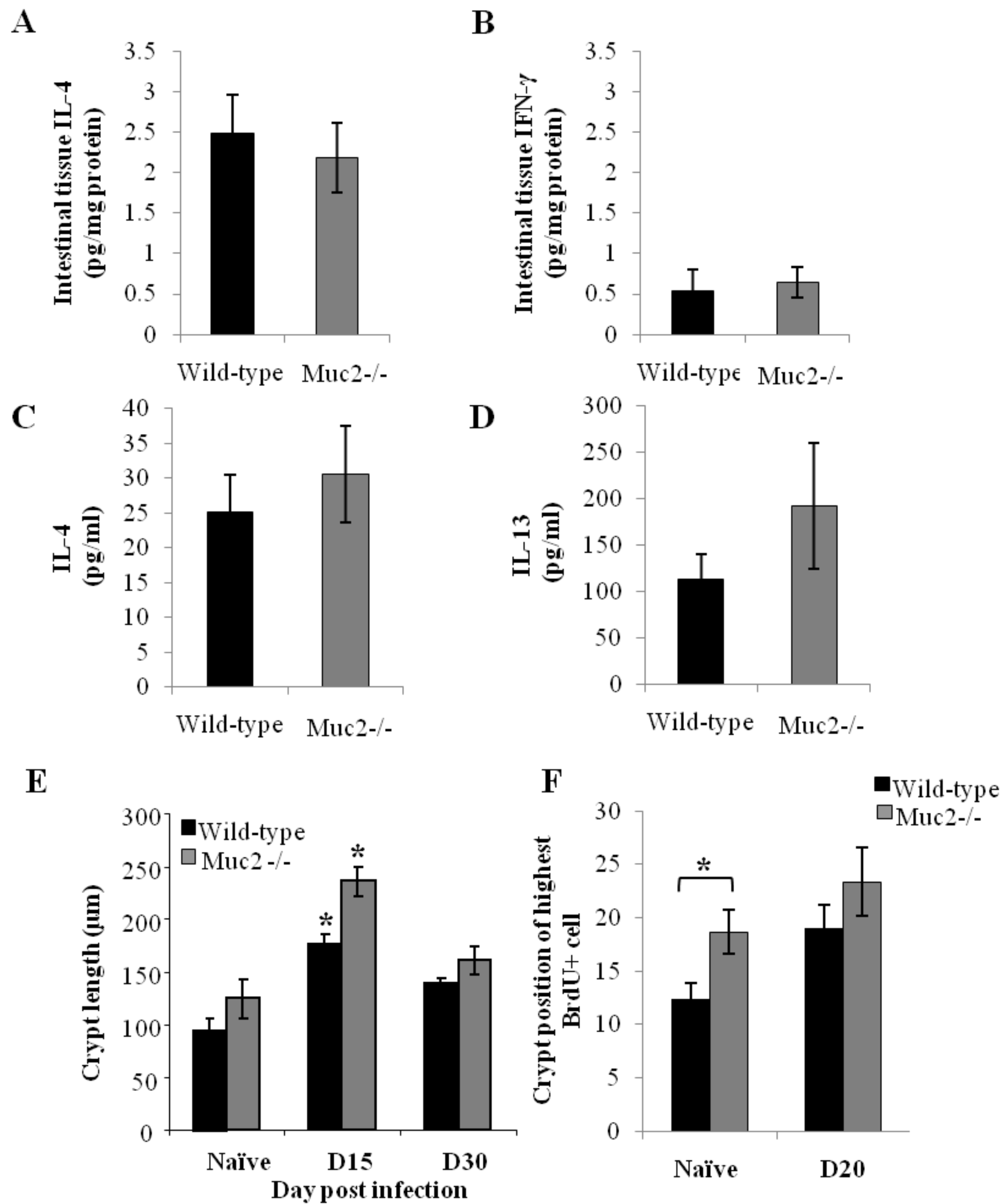


Figure 3.3: Cytokine levels were determined in intestinal tissues (pg/mg) or by ConA stimulation of spleen cells (pg/ml) from *T. muris* infected mice on D20 pi.; IL-4 (A, C), IFN-γ (B) and IL-13 (D). Caecal crypt length measured (E) and crypt position of the highest BrdU$^+$ cell (F) in Muc2-deficient (Muc2-/-) and wild-type mice was determined. Representative of five mice. * = $P<0.05$.

Using the highest position of BrdU-positive cells in the crypts as a measure of rate of epithelial cell turnover (Potten *et al*, 2002), it was clear that cell turnover was higher in the naïve Muc2-deficient mice compared to wild-type mice. However, there was no

significant difference in epithelial cell turnover between the Muc2-deficient and wild-type mice on D20 pi. (**Figure 3.3F**), indicating that the delay in worm expulsion in Muc2-deficient mice was not associated with an alteration of the "epithelial escalator" (Cliffe *et al*, 2005).

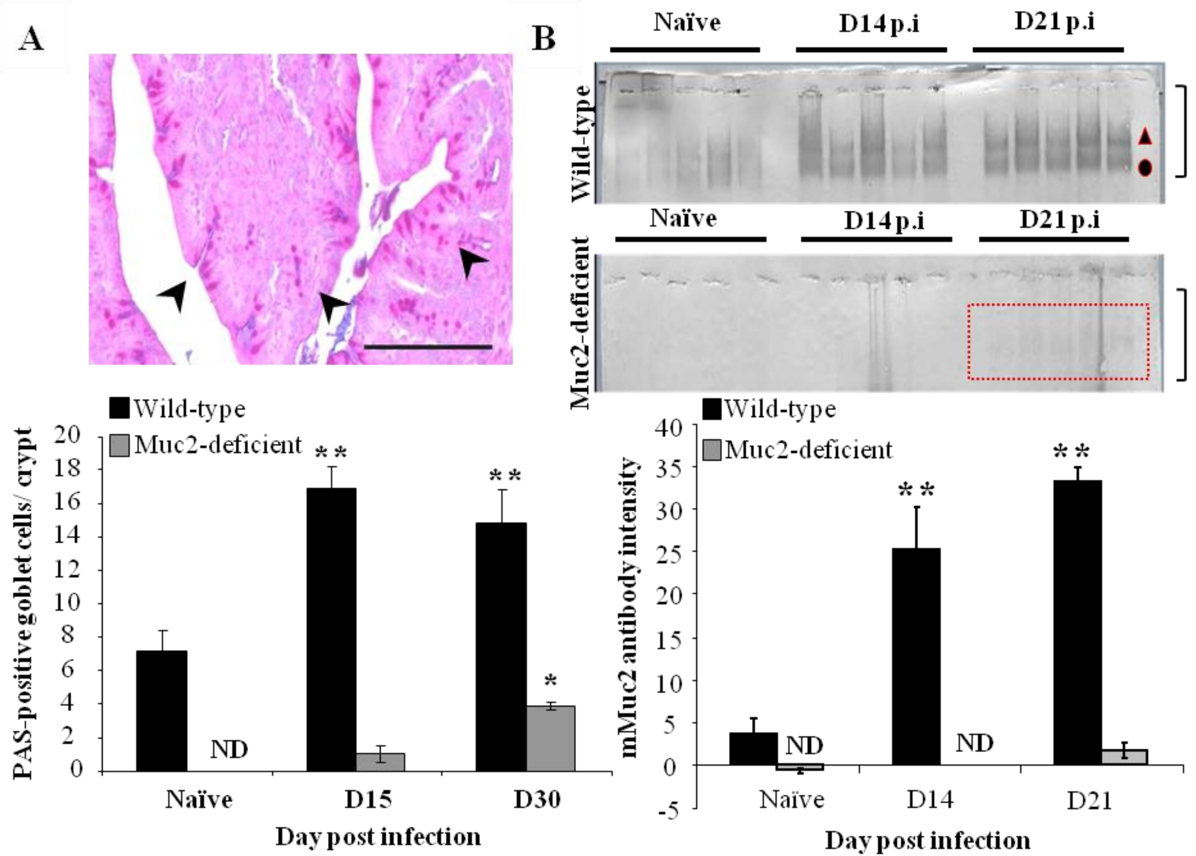


Figure 3.4: Quantification of goblet cell numbers in the caecum of wild-type and Muc2-deficient mice during infection (A); goblet cells marked by arrows in deficient mice can be visualised on D30 pi. (PAS-staining without fast-green counterstain). Total mucus scraped from wild-type and Muc2-deficient mice were reduced/alkylated, separated by agarose gel electrophoresis, western blotted and probed with the mMuc2 antibody (B). The relative staining intensity of the mMuc2 antibody in the portion of the blot indicated by brackets was measured. A faint band (red box highlighted) was observed on D21pi. in the Muc2-deficient mice. The two Muc2 bands in the wild-type animals most likely represent the monomeric (●) and dimeric (▲) forms of Muc2 (B). Representative of five mice. * = $P<0.05$, ** = $P<0.01$. ND = not detectable.

T. muris *infection induced expression of PAS-positive goblet cells in Muc2-deficient mice*

In spite of the similar number of goblet cells (as defined by Relm-β and Tff3; **Supplementary Figure 3.2**) in the infected and non-infected Muc2-deficient and wild-type mice, there was a significant difference between the number of PAS-positive goblet

cells (**Figure 3.4**). As with the resistant BALB/c mice, there was a significant increase in the numbers of PAS-positive goblet cells in wild-type mice post infection. Although there was significant impairment in the development of hyperplastic goblet cells in the Muc2-deficient mice, unexpectedly by D15 pi. there was an increase in PAS-positive goblet cells, with significant elevation by D30 pi. (**Figure 3.4** & **Supplementary Figure 3.3**).

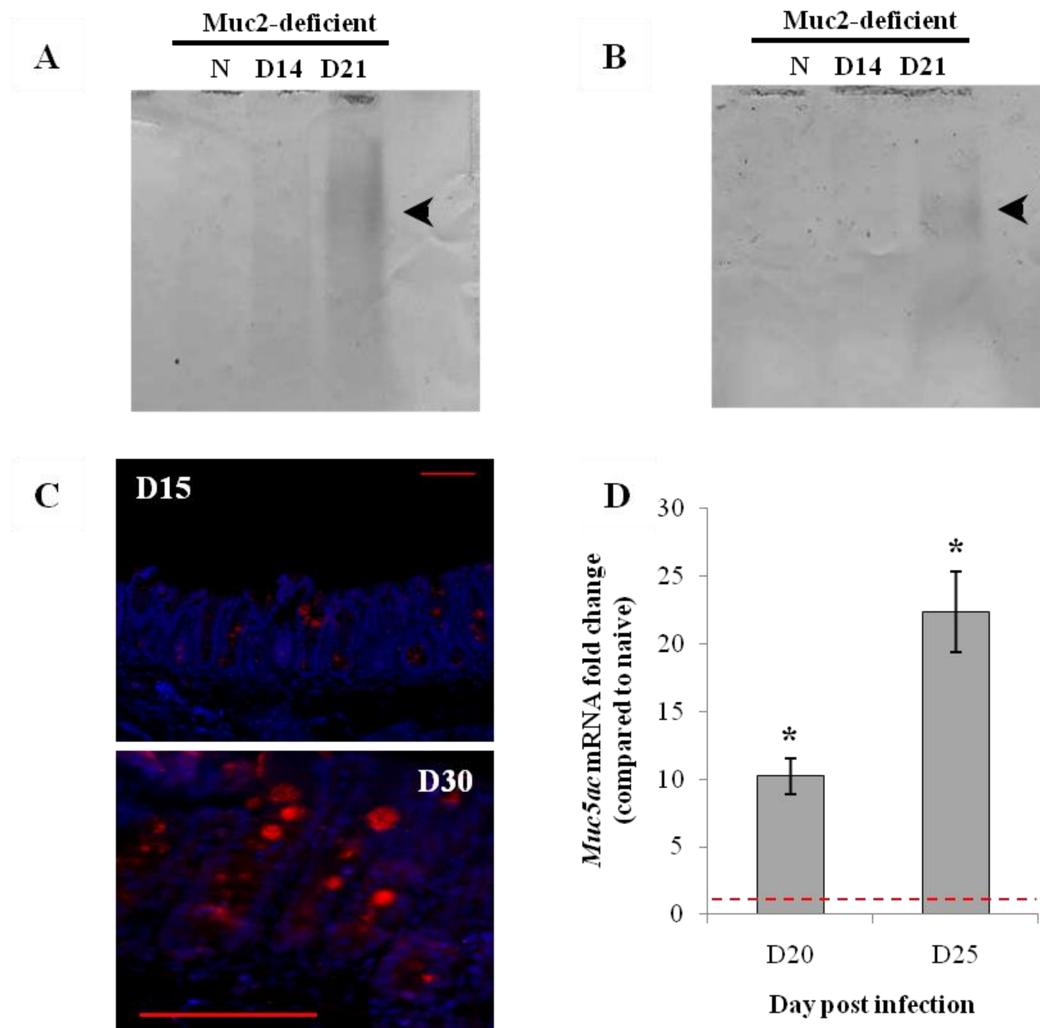


Figure 3.5: Muc5ac (A) and total glycoprotein (B) levels present in caecal mucus, determined by western blotting using 45M1 antibody and PAS staining respectively, in the Muc2-deficient mice. Immunofluorescence microscopy (C) and RT-PCR (D) illustrated Muc5ac was present in the Muc2-deficient mice pi. D; Red dashed line = naïve levels. Representative of 5 mice. Scale bar; 10µm. * = $P<0.05$.

T. muris *infection triggers Muc5ac mucin production*

Following exposure to *T. muris* the levels of Muc2 were significantly elevated in the wild-type mice (**Supplementary Figure 3.3**). As expected, no Muc2 positive goblet cells were seen in the Muc2-deficient mice. Similarly, higher amounts of Muc2 (assessed

by western blotting after agarose gel electrophoresis) were present in the content of mucus collected in the wild-type mice post infection (**Figure 3.4**).

Although, there was little evidence of mature, glycosylated Muc2 in the Muc2-deficient mice, interestingly, on D21 pi. there was a faint band consistent with the electrophorectic migration of Muc2 in these mice (red box; **Figure 3.4B**). This along with the PAS-positive goblet cells suggested the presence of another polymeric mucin post infection. To identify this mucin, the mucus (pooled from 5 Muc2-deficient mice) was analysed by western blotting after agarose gel electrophoresis. The mouse Muc5b-antiserum did not reveal any bands (data not shown). In contrast, a Muc5ac monoclonal antibody (Bara *et al*, 1991; Lidell *et al*, 2008), identified bands in the mucus samples from infected mice (**Figure 3.5A**). Immunofluorescence microscopy (**Figure 3.5C**), RT-PCR (**Figure 3.5D**) and tandem mass spectrometry (data not shown) confirmed the *de novo* expression of Muc5ac post infection in the Muc2-deficient mice. Furthermore, the PAS-stained material after agarose gel electrophoresis showed coincidence with the Muc5ac reactive band (**Figure 3.5B**; arrow), suggesting Muc5ac is a significant component of the mucus in the Muc2-deficient animals. No marked changes were observed in the expression of the cell surface mucins, *Muc1*, *Muc4* and *Muc17*, which are thought to contribute to mucosal protection (**Supplementary Figure 3.2**).

Muc5ac is up-regulated as part of the 'normal' response to worm expulsion

Unexpectedly, Muc5ac expression was also significantly up-regulated in the wild-type mice on D14 and 21 pi (**Figure 3.6**). In contrast to the Muc2-deficient mice, western blotting revealed that Muc5ac mucin was not the major component in the mucus, since the major PAS-bands migrated further than the broad, Muc5ac-reactive band (**Figure 3.6A** and **B**; arrow) and was coincident with Muc2 staining bands (data not shown). However, the *de novo* expression of *Muc5ac* was only observed in the resistant mouse models (high dose in C57BL/6 and BALB/c mice) and not in the susceptible models (low dose in BALB/c, high dose in AKR and SCID mice) (**Figure 3.6D**). Immunofluorescence microscopy and immunohistochemistry confirmed the expression of Muc5ac post infection (D15 and 21pi.) in the caecal crypts of the resistant models (**Figure 3.6C**). No reactivity was observed in the susceptible models (data not shown).

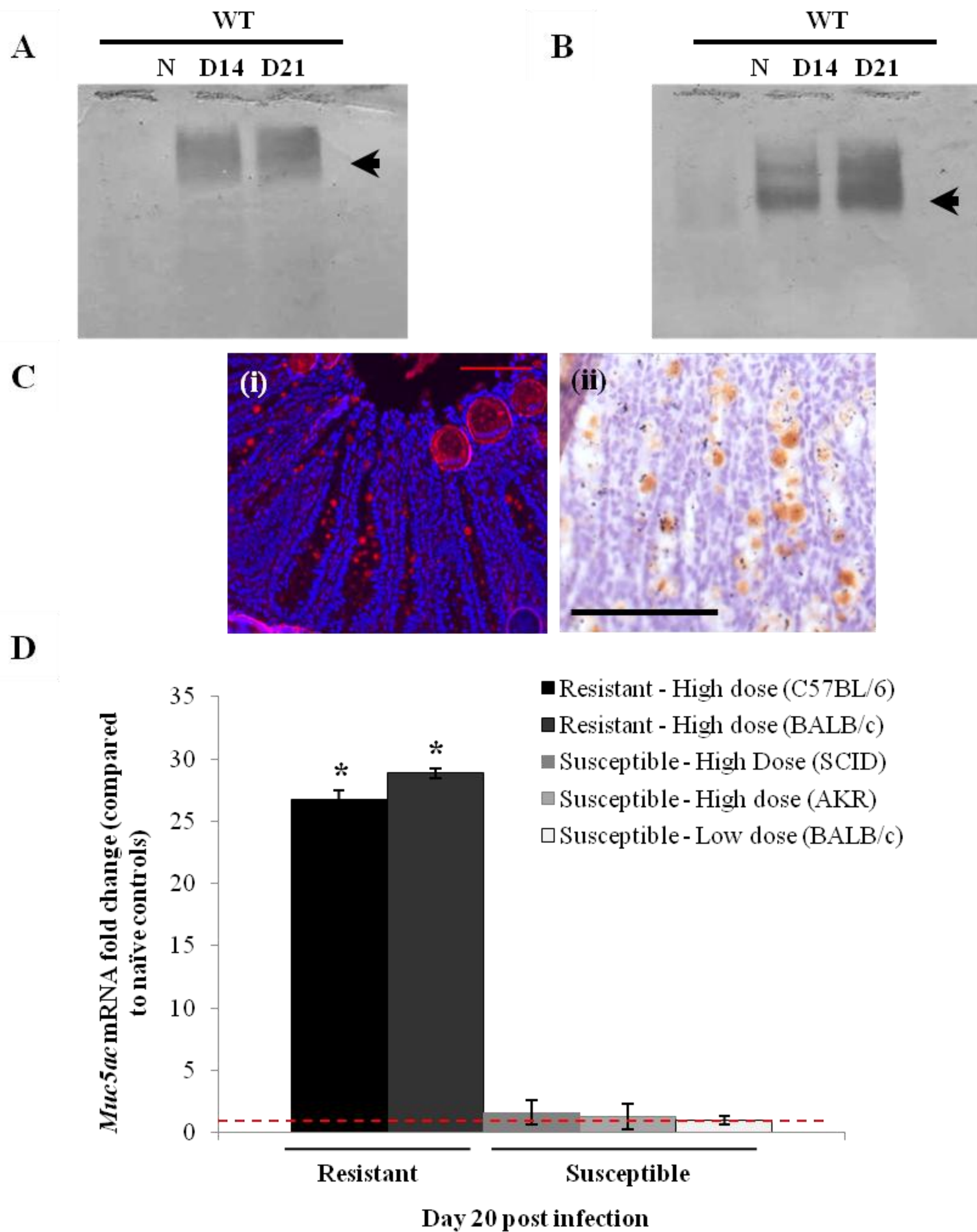


Figure 3.6: Muc5ac (A) and total glycoprotein (B) levels present in caecal mucus, determined by western blotting using 45M1 antibody and PAS staining respectively, in the wild-type resistant (C57BL/6) mice. Immunofluorescence microscopy and immunohistochemistry illustrated that Muc5ac was present in some of the goblet cells of resistant C57BL/6 (i) and BALB/c (ii) mice after infection; (C). RT-PCR illustrated that *Muc5ac* levels increase significantly only in the resistant models (high dose infection in BALB/c and C57BL/6 mice) and not in the susceptible models (low dose infection in BALB/c and high dose infection in AKR and SCID mice) (D; Red dashed line = naïve levels). Representative of 5 mice. Scale bar; 10μm. * = $P<0.05$.

Susceptibility is associated with altered mucus porosity

Fluorescently labelled beads were used to investigate mucus permeability post infection (D19) in BALB/c and AKR mice (**Figure 3.7A**). The beads travelled to a depth

of ~100μm over a 60 second period in both strains. Thereafter, there was a reduction in diffusion rate of the beads in the resistant (BALB/c) mice. However, the beads travelled significantly further in the mucus of susceptible (AKR) mice over a 20 minute period.

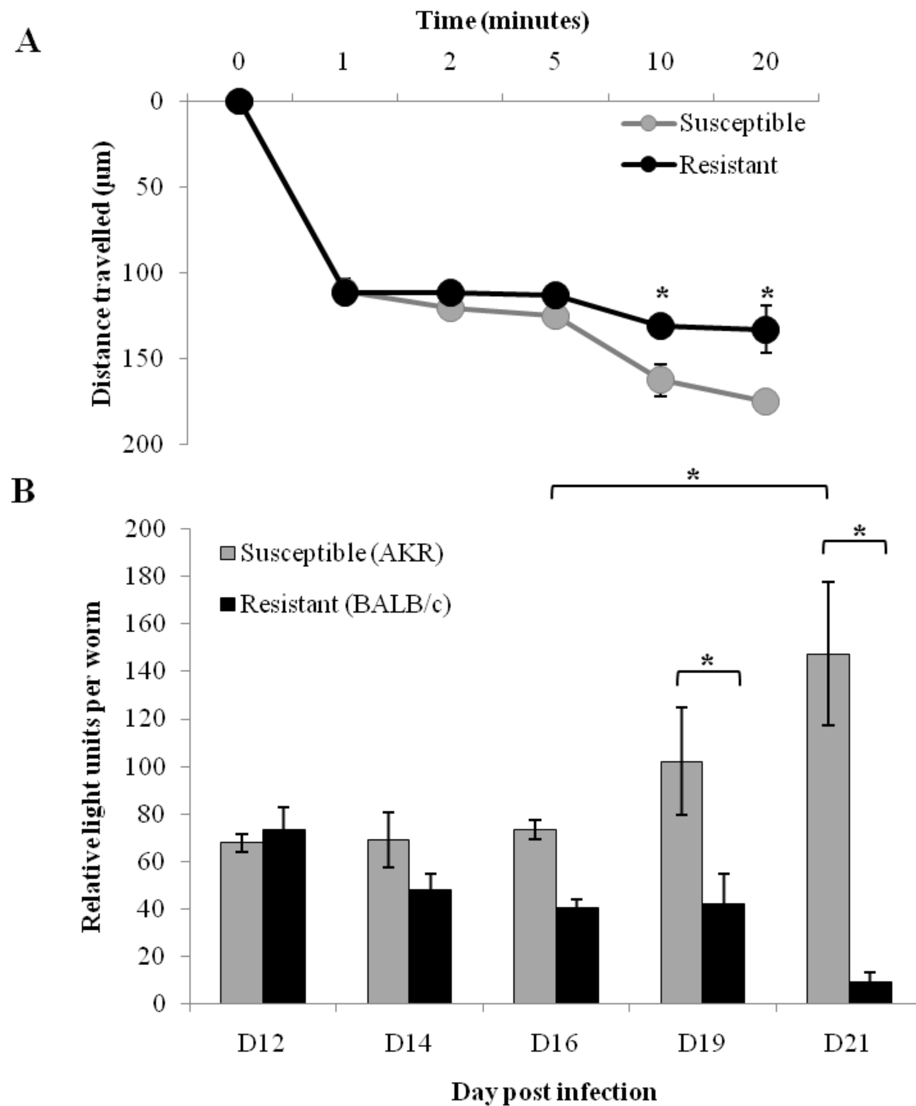


Figure 3.7: Fluorescent beads were used to determine the permeability of the mucus barrier of the susceptible (AKR) and resistant (BALB/c) mice on D19 pi.; represented as the distance travelled from the top of the mucus barrier in the time stated (A). Energy levels (data presented as relative light units per worm) were determined in worms extracted from BALB/c and AKR mice during infection (B). * = $P<0.05$.

Worms in a resistant environment have a reduced energy status

ATP measurements were carried out to determine the energy status of worms in resistant and susceptible mice as a measure of worm vitality. As infection progressed (D21 pi.) in the AKR mice, there was a significant increase in the ATP production of the worms (**Figure 3.7B**). In contrast, there was a marked reduction in ATP production in the worms

isolated from the BALB/c mice. However, these worms were not irreversibly damaged as they recovered their ATP production when transferred to *in vitro* culture with the colonic LS174T cell line for 24 hours (**Supplementary Figure 3.4A**). Importantly, worms taken from Muc2-deficient mice showed a comparable drop in energy status during worm expulsion (**Supplementary Figure 3.4B**).

DISCUSSION

It is well-established that *T. muris* survives by eliciting a T_H1 response in mice susceptible to chronic infection in the absence of a T_H2 response. In common with all other studies of intestinal helminth immunity, multiple effectors under immunological (T_H2) control are likely to be operating during worm expulsion. Whilst we already know that IL-13 mediated regulation of epithelial cell turnover and smooth muscle contractility can contribute to worm expulsion, little detail is known regarding the protective role of the secreted barrier i.e. mucus (Artis and Grencis, 2008; Cliffe and Grencis, 2004; Else and deSchoolmeester, 2003). Previously we have shown that the T_H2-type immune response in resistance plays an important role in the development of goblet cell hyperplasia (Khan *et al*, 2001; Khan *et al*, 2003). Some reports have also suggested mucus produced from goblet cells have an important role in trapping and removing nematodes from the intestine (Carlisle *et al*, 1991; Miller, 1987; Webb *et al*, 2007). The polymeric mucins are responsible for the physical properties of the mucus barrier (Sheehan *et al*, 1995; Thornton *et al*, 2008) and changes in mucins are associated with pathophysiology of a number of gastrointestinal disorders (Heazlewood *et al*, 2008; Van der Sluis *et al*, 2006). Recently it has also been shown that deficiency in the major component of the intestinal mucus barrier, Muc2, leads to an abnormal morphology of the colon and contributes to the onset and perpetuation of DSS-induced experimental colitis (Van der Sluis *et al*, 2006; Velcich *et al*, 2002).

In this study we demonstrated, using the *T. muris* model that Muc2 increased in resistance (restricted to the caecum; the niche of the parasite) which correlated with worm expulsion. However, this was not the case for the mice susceptible (AKR) to *T. muris* infection supporting the hypothesis that Muc2 contributed to host protection in nematode infection. A distinct functional role for Muc2 in host protective immunity in *T. muris* infection was demonstrated in the Muc2-deficient mice. These animals exhibited a significant delay in worm expulsion even though the adaptive immune response was unaltered; similar T_H2-type immune responses were shown in Muc2-deficient and wild-

type control mice following infection. Unexpectedly, *de novo* expression of Muc5ac was observed just before worm expulsion in the Muc2-deficient mice and resistant mouse models, but not in the susceptible models.

Overall, the network properties of the intestinal mucus barrier are different between resistance and susceptibility and the changes in the parasitic niche can have damaging effects on the vitality of the parasite. To our knowledge this is the first direct demonstration for a functionally protective role of gel-forming mucins in nematode infection. Analysis of caecal mucus from Muc2-deficient mice revealed that Muc5ac was the only polymeric mucin present in the mucus post infection. Moreover in wild-type mice, though not the major mucin (which is Muc2), for the first time in a nematode infection we show the up-regulation of Muc5ac post intestinal infection.

Several studies have elucidated that T_H2-type cytokines such as IL-13 have the ability to up-regulate MUC5AC/Muc5ac expression levels (Fujisawa *et al*, 2008; Kondo *et al*, 2006). Therefore, the up-regulation in Muc5ac expression observed post infection, in both wild-type and Muc2-deficient mice may be as a result of IL-13 production. Interestingly, this *de novo* expression of Muc5ac was observed in all the resistant models (T_H2-type response) but in none of the susceptible models (T_H1-type response) of *T. muris* infection. While this mucin is predominantly found in airway and stomach mucus (Ho *et al*, 1993; Thornton *et al*, 2008), studies on patients with ulcerative colitis and adenocarcinomas have shown MUC5AC expression in the intestine along with MUC2 (Forgue-Lafitte *et al*, 2007; Lidell *et al*, 2008). However, this is the first time that Muc5ac expression has been implicated in response to an enteric parasitic infection.

We observed no discernable PAS-positive goblet cells throughout the caecum of Muc2-deficient mice without infection. However, this was not due to the absence of goblet cell lineage as the expression of Tff3 and Relm-β was observed in the caecum of both infected and non-infected wild-type and Muc2-deficient mice. This observation corroborates with the findings of Van der Sluis *et al* where the expression of Tff3 was observed, despite the lack of PAS-positive goblet cells (Van der Sluis *et al*, 2006). Muc2 seems to be the major phenotypic determinant of goblet cells and in the absence of Muc2, goblet cells lose their characteristic goblet-like shape and specific staining but the goblet cell lineage is still present (Van der Sluis *et al*, 2006; Velcich *et al*, 2002). Interestingly, post infection there was an increase in PAS-positive goblet cells in the Muc2-deficient mice. Although, the size of the goblet cells in Muc2-deficient mice was smaller compared to those in wild-type mice, their emergence correlated with worm expulsion.

We have shown a functional role for the mucus barrier in host protective immunity to *T. muris* infection since in the absence of Muc2 worm expulsion is significantly delayed. Moreover, the physical properties of the mucus barrier are changed after infection; although the details of how these changes contribute to protection remains to be fully elucidated. However, one possibility is that in the susceptible mice, the lower levels of a Muc2 result in a network that may compromise defence due to inappropriate presentation or concentration of other host defence proteins in the environment of the worms. Whereas, in the resistant mice, these other proteins (such as Relm-β, Tff3 and angiogenins) may be retained and effectively concentrated at the sites of worm infection by specific interactions with Muc2 or with the infected induced Muc5ac, or by the physical constraints imposed by the mucin network; thus rendering the host interface unsuitable for worm reproduction and/or survival which results in expulsion (Ishiwata and Watanabe, 2007). Indeed, changes in the niche of the parasite do have a detrimental effect on the parasite, as worms extracted from mice during worm expulsion clearly have a reduced energy status as compared to those extracted from the susceptible mice. This reduction in the worm vitality is reversible if worms are transferred to a 'favourable' environment, supporting the notion that expulsion reflects damaged, but not killed parasites.

Another explanation supported by our finding, which is by no means mutually exclusive, is that the physical nature of the mucus barrier is changed in such a way as to facilitate worm expulsion. We have shown that around the time of worm expulsion the mucus barrier is less porous in the resistant mice as compared to the susceptible mice and this alteration in physical properties of the barrier post infection may directly impact on the niche of the worms. The intestinal mucus barrier is proposed to comprise of a 'loose' outer layer and a less porous, adherent inner layer (Johansson *et al*, 2008). The results from the bead penetration assay revealed that after 60 seconds the beads travelled to a ~100μm depth in the mucus from the susceptible and resistant mice, suggesting the properties of the 'loose' layer are similar in both. However, the beads travelled at different rates thereafter, suggesting that the differences in network properties observed between the resistant and susceptible mice is mainly in the inner adherent layer of the barrier. This alteration may physically constrain the worms thus affecting the niche.

What might be the role of the infection-induced mucin Muc5ac in protection against the worms? Muc5ac is assembled in a different manner to Muc2 and does not possess the disulphide-resistant cross-links present in Muc2 (Carlstedt *et al*, 1995; Herrmann *et al*, 1999; Sheehan *et al*, 2000; Sheehan *et al*, 1991), thus it may result in a

mucus gel with different rheological properties. Indeed, Muc5ac is a major component of airways mucus and unlike the intestinal barrier which is normally an adherent Muc2-rich gel, a specific functional requirement in the airways makes a transportable mucus gel. Thus Muc5ac may change the rheological nature of the mucus gel, and in conjunction with the intestinal muscle hypercontractility (controlled via T_H2 response (Khan *et al*, 2001; Khan *et al*, 2003)), could physically aid worm expulsion. This is consistent with the observations of mucus trapping in *N. brasiliensis* and *T. spiralis* infection, in which globules of mucus trap worms, which are then transported out of the intestine (Carlisle *et al*, 1991; Miller, 1987). Another interesting possibility raised by the data is that expulsion occurs in two phases: an early phase influenced by Muc2 and a final, clearance phase that occurs independently of Muc2, possibly involving Muc5ac.

In conclusion, this study clearly demonstrates that the mucus barrier is a significant component of a well co-ordinated response in the gut to worm expulsion. Even though *T.muris* has an intracellular niche within the gut epithelium, in resistance as the 'epithelial escalator' displaces worms, it may be that the overall changes in barrier have a subsequent significant detrimental effect on the worm itself and the additional changes in the physical properties of mucus contribute to the efficient elimination of the worms from the intestinal lumen. Moreover, it further highlights the functionally dynamic and highly regulated nature of the mucus barrier during immunologically mediated intestinal disease.

REFERENCES

Artis D, Grencis RK (2008). The intestinal epithelium: sensors to effectors in nematode infection. Mucosal Immunol **1**(4)**:** 252-264.

Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, Schad GA, Scott P, Wu GD (2004). RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. Proc Natl Acad Sci U S A **101**(37)**:** 13596-13600.

Bancroft AJ, Grencis RK (1998). Th1 and Th2 cells and immunity to intestinal helminths. Chem Immunol **71:** 192-208.

Bara J, Gautier R, Mouradian P, Decaens C, Daher N (1991). Oncofetal mucin M1 epitope family: characterization and expression during colonic carcinogenesis. Int J Cancer **47**(2)**:** 304-310.

Boland CR, Montgomery CK, Kim YS (1982). Alterations in human colonic mucin occurring with cellular differentiation and malignant transformation. Proc Natl Acad Sci U S A **79**(6)**:** 2051-2055.

Buisine MP, Desreumaux P, Debailleul V, Gambiez L, Geboes K, Ectors N, Delescaut MP, Degand P, Aubert JP, Colombel JF, Porchet N (1999). Abnormalities in mucin gene expression in Crohn's disease. Inflamm Bowel Dis **5**(1)**:** 24-32.

Carlisle MS, McGregor DD, Appleton JA (1991). Intestinal mucus entrapment of *Trichinella spiralis* larvae induced by specific antibodies. Immunology **74**(3)**:** 546-551.

Carlstedt I, Herrmann A, Hovenberg H, Lindell G, Nordman H, Wickstrom C, Davies JR (1995). 'Soluble' and 'insoluble' mucins--identification of distinct populations. Biochem Soc Trans **23**(4)**:** 845-851.

Chang SK, Dohrman AF, Basbaum CB, Ho SB, Tsuda T, Toribara NW, Gum JR, Kim YS (1994). Localization of mucin (MUC2 and MUC3) messenger RNA and peptide expression in human normal intestine and colon cancer. Gastroenterology **107**(1)**:** 28-36.

Cliffe LJ, Grencis RK (2004). The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode infection. Adv Parasitol **57:** 255-307.

Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. Science **308**(5727)**:** 1463-1465.

Cliffe LJ, Potten CS, Booth CE, Grencis RK (2007). An increase in epithelial cell apoptosis is associated with chronic intestinal nematode infection. Infect Immun **75**(4)**:** 1556-1564.

Crabtree JE, Heatley RV, Losowsky MS (1989). Glycoprotein synthesis and secretion by cultured small intestinal mucosa in coeliac disease. Gut **30**(10)**:** 1339-1343.

Deschoolmeester ML, Else KJ (2002). Cytokine and chemokine responses underlying acute and chronic *Trichuris muris* infection. Int Rev Immunol **21**(4-5)**:** 439-467.

Else KJ (2005). Have gastrointestinal nematodes outwitted the immune system? Parasite Immunol **27**(10-11)**:** 407-415.

Else KJ, deSchoolmeester ML (2003). Immunity to *Trichuris muris* in the laboratory mouse. J Helminthol **77**(2)**:** 95-98.

Else KJ, Finkelman FD (1998). Intestinal nematode parasites, cytokines and effector mechanisms. Int J Parasitol **28**(8)**:** 1145-1158.

Else KJ, Grencis RK (1991). Helper T-cell subsets in mouse trichuriasis. Parasitol Today **7**(11)**:** 313-316.

Forgue-Lafitte ME, Fabiani B, Levy PP, Maurin N, Flejou JF, Bara J (2007). Abnormal expression of M1/MUC5AC mucin in distal colon of patients with diverticulitis, ulcerative colitis and cancer. Int J Cancer **121**(7)**:** 1543-1549.

Fujisawa T, Ide K, Holtzman MJ, Suda T, Suzuki K, Kuroishi S, Chida K, Nakamura H (2008). Involvement of the p38 MAPK pathway in IL-13-induced mucous cell metaplasia in mouse tracheal epithelial cells. Respirology **13**(2)**:** 191-202.

Hayes KS, Bancroft AJ, Grencis RK (2007). The role of TNF-alpha in *Trichuris muris* infection II: global enhancement of ongoing Th1 or Th2 responses. Parasite Immunol **29**(11)**:** 583-594.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA (2008). Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. PLoS Med **5**(3)**:** e54.

Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, Carlstedt I (1999). Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. J Biol Chem **274**(22)**:** 15828-15836.

Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, Dahiya R, Kim YS (1993). Heterogeneity of mucin gene expression in normal and neoplastic tissues. Cancer Res **53**(3)**:** 641-651.

Hogan SP, Seidu L, Blanchard C, Groschwitz K, Mishra A, Karow ML, Ahrens R, Artis D, Murphy AJ, Valenzuela DM, Yancopoulos GD, Rothenberg ME (2006). Resistin-like molecule beta regulates innate colonic function: barrier integrity and inflammation susceptibility. J Allergy Clin Immunol **118**(1)**:** 257-268.

Ishiwata K, Watanabe N (2007). *Nippostrongylus brasiliensis*: reversibility of reduced-energy status associated with the course of expulsion from the small intestine in rats. Exp Parasitol **117**(1)**:** 80-86.

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. Proc Natl Acad Sci U S A **105**(39)**:** 15064-15069.

Khan WI, Blennerhasset P, Ma C, Matthaei KI, Collins SM (2001). Stat6 dependent goblet cell hyperplasia during intestinal nematode infection. Parasite Immunol **23**(1)**:** 39-42.

Khan WI, Richard M, Akiho H, Blennerhasset PA, Humphreys NE, Grencis RK, Van Snick J, Collins SM (2003). Modulation of intestinal muscle contraction by interleukin-9 (IL-9) or IL-9 neutralization: correlation with worm expulsion in murine nematode infections. Infect Immun **71**(5)**:** 2430-2438.

Kirkham S, Kolsum U, Rousseau K, Singh D, Vestbo J, Thornton DJ (2008). MUC5B is the major mucin in the gel phase of sputum in chronic obstructive pulmonary disease. Am J Respir Crit Care Med **178**(10)**:** 1033-1039.

Kondo M, Tamaoki J, Takeyama K, Isono K, Kawatani K, Izumo T, Nagai A (2006). Elimination of IL-13 reverses established goblet cell metaplasia into ciliated epithelia in airway epithelial cell culture. Allergol Int **55**(3)**:** 329-336.

Lidell ME, Bara J, Hansson GC (2008). Mapping of the 45M1 epitope to the C-terminal cysteine-rich part of the human MUC5AC mucin. FEBS J **275**(3)**:** 481-489.

Linden SK, Florin TH, McGuckin MA (2008). Mucin dynamics in intestinal bacterial infection. PLoS One **3**(12)**:** e3952.

Miller HR (1987). Gastrointestinal mucus, a medium for survival and for elimination of parasitic nematodes and protozoa. Parasitology **94 Suppl:** S77-100.

Miller HR, Nawa Y (1979). *Nippostrongylus brasiliensis*: intestinal goblet-cell response in adoptively immunized rats. Exp Parasitol **47**(1)**:** 81-90.

Potten CS, Booth D, Cragg NJ, Tudor GL, O'Shea JA, Booth C, Meineke FA, Barthel D, Loeffler M (2002). Cell kinetic studies in the murine ventral tongue epithelium: mucositis induced by radiation and its protection by pretreatment with keratinocyte growth factor (KGF). Cell Prolif **35 Suppl 1:** 32-47.

Sheehan JK, Brazeau C, Kutay S, Pigeon H, Kirkham S, Howard M, Thornton DJ (2000). Physical characterization of the MUC5AC mucin: a highly oligomeric glycoprotein whether isolated from cell culture or in vivo from respiratory mucous secretions. Biochem J **347 Pt 1:** 37-44.

Sheehan JK, Hanski C, Corfield AP, Paraskeva C, Thornton DJ (1995). Mucin biosynthesis and macromolecular assembly. Biochem Soc Trans **23**(4)**:** 819-821.

Sheehan JK, Thornton DJ (2000). Heterogeneity and size distribution of gel-forming mucins. **125:** 87-96.

Sheehan JK, Thornton DJ, Somerville M, Carlstedt I (1991). Mucin structure. The structure and heterogeneity of respiratory mucus glycoproteins. Am Rev Respir Dis **144**(3 Pt 2)**:** S4-9.

Shekels LL, Anway RE, Lin J, Kennedy MW, Garside P, Lawrence CE, Ho SB (2001). Coordinated Muc2 and Muc3 mucin gene expression in *Trichinella spiralis* infection in wild-type and cytokine-deficient mice. Dig Dis Sci **46**(8)**:** 1757-1764.

Thornton DJ, Carlstedt I, Sheehan JK (1996). Identification of glycoproteins on nitrocellulose membranes and gels. Mol Biotechnol **5**(2)**:** 171-176.

Thornton DJ, Rousseau K, McGuckin MA (2008). Structure and function of the polymeric mucins in airways mucus. Annu Rev Physiol **70:** 459-486.

Thornton DJ, Sheehan JK (2004). From mucins to mucus: toward a more coherent understanding of this essential barrier. Proc Am Thorac Soc **1**(1)**:** 54-61.

Tytgat KM, Buller HA, Opdam FJ, Kim YS, Einerhand AW, Dekker J (1994). Biosynthesis of human colonic mucin: Muc2 is the prominent secretory mucin. Gastroenterology **107**(5)**:** 1352-1363.

Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Buller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. Gastroenterology **131**(1)**:** 117-129.

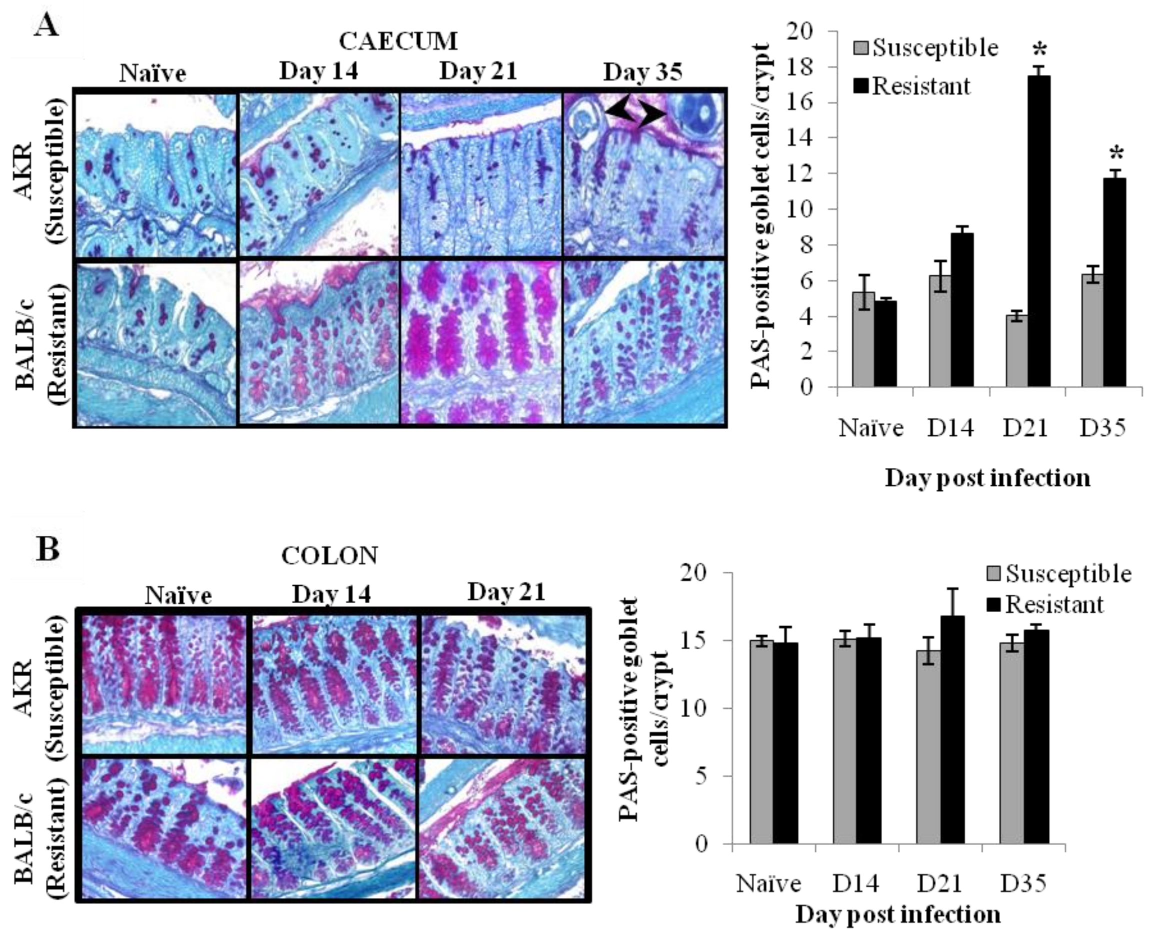
Velcich A, Yang W, Heyer J, Fragale A, Nicholas C, Viani S, Kucherlapati R, Lipkin M, Yang K, Augenlicht L (2002). Colorectal cancer in mice genetically deficient in the mucin Muc2. Science **295**(5560)**:** 1726-1729.

Wakelin D (1967). Acquired immunity to *Trichuris muris* in the albino laboratory mouse. Parasitology **57**(3)**:** 515-524.

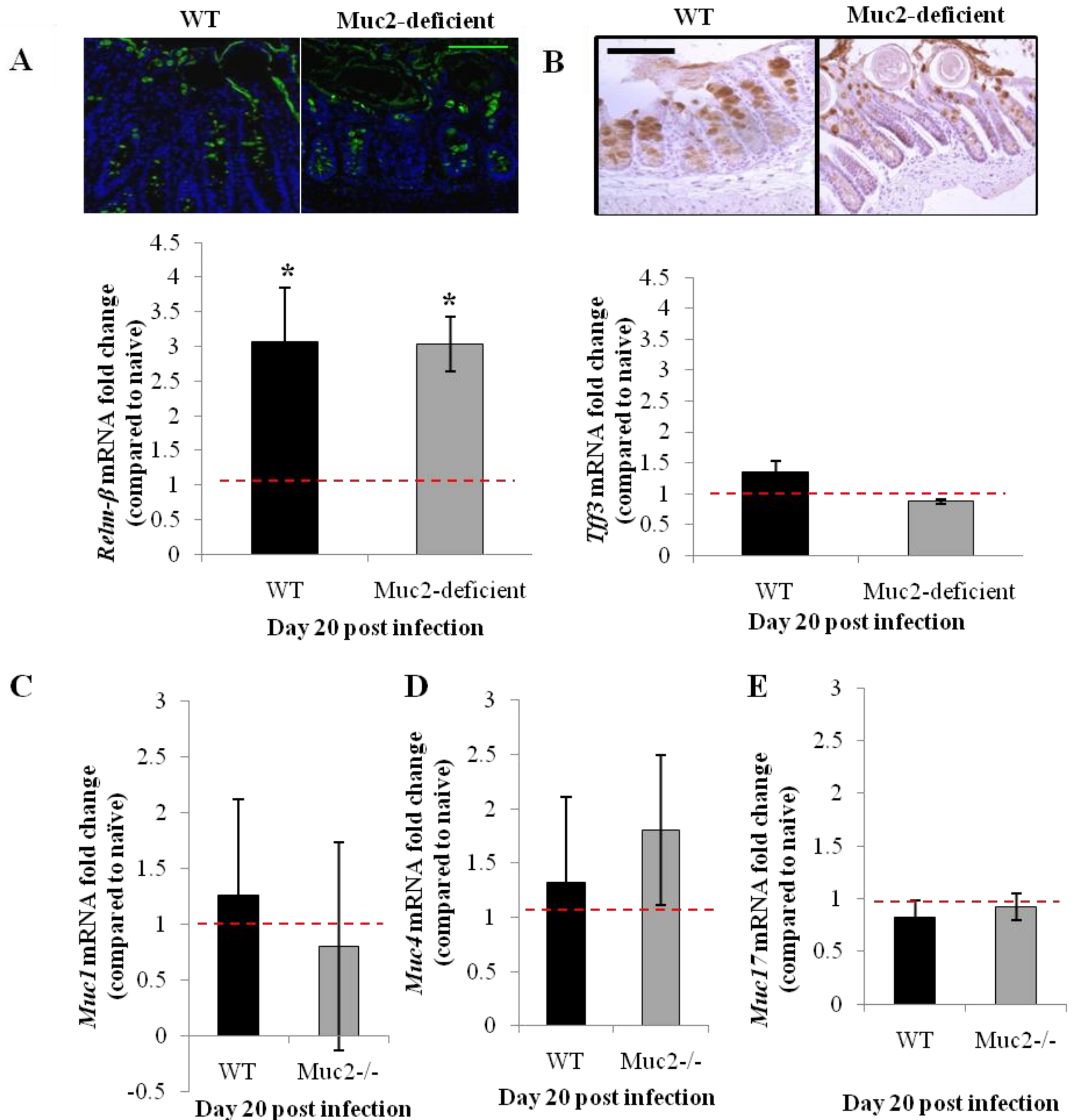
Webb RA, Hoque T, Dimas S (2007). Expulsion of the gastrointestinal cestode, Hymenolepis diminuta by tolerant rats: evidence for mediation by a Th2 type immune enhanced goblet cell hyperplasia, increased mucin production and secretion. Parasite Immunol **29**(1)**:** 11-21.

Zhu Y, Ehre C, Abdullah LH, Sheehan JK, Roy M, Evans CM, Dickey BF, Davis CW (2008). Munc13-2-/- baseline secretion defect reveals source of oligomeric mucins in mouse airways. J Physiol **586**(7)**:** 1977-1992.

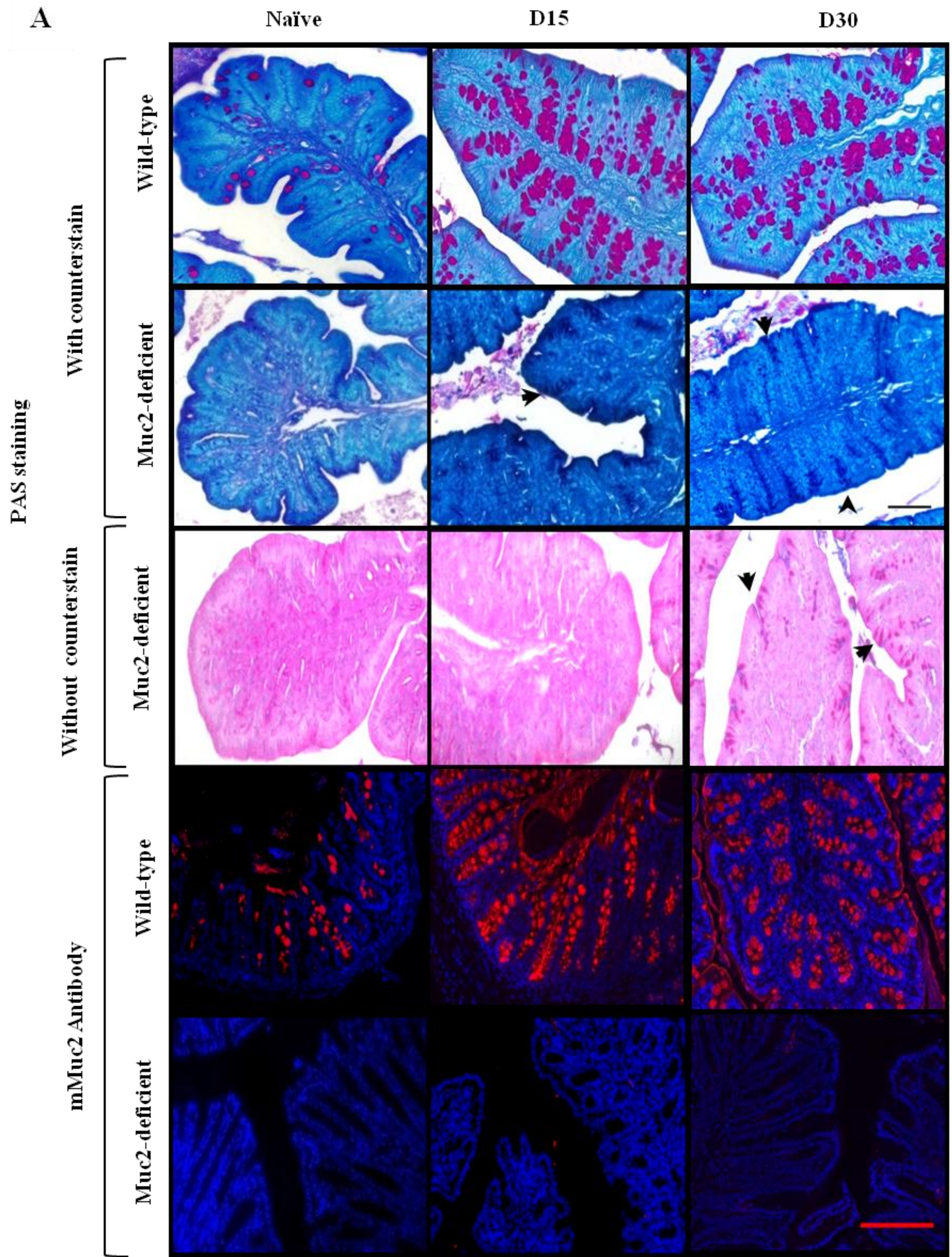
SUPPLEMENTARY FIGURES



Supplementary Figure 3.1: PAS staining in the caecum showed a significant increase in goblet cell numbers only in the resistant (BALB/c) mice with infection (A). Worms are highlighted by arrows visible in the sections from susceptible mice. No major changes in goblet cell numbers in the colon of resistant (BALB/c) and susceptible (AKR) mice on day 14 and day 21 post infection compared to naïve (B). Representative of 3 mice. * = P<0.05.



Supplementary Figure 3.2: Expression of Tff3 (A) and Relm-β (B), determined using immunofluorescence/immunohistochemistry and RT-PCR in caecal tissue of Muc2-deficient mice and their resistant wild-type littermates (C57BL/6) on day 15 and day 20 pi respectively. RT-PCR showed no major changes in the mRNA expression of cell surface mucins, *Muc1* (C), *Muc4* (D) or *Muc17* (E), in the wild-type and Muc2-deficient (Muc2-/-) mice on day 20 pi. Red dashed line = naïve levels. Scale bar; 10μm. Representative of 5 mice. * = $P<0.05$.


A
Naïve
D15
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PAS staining
With counterstain
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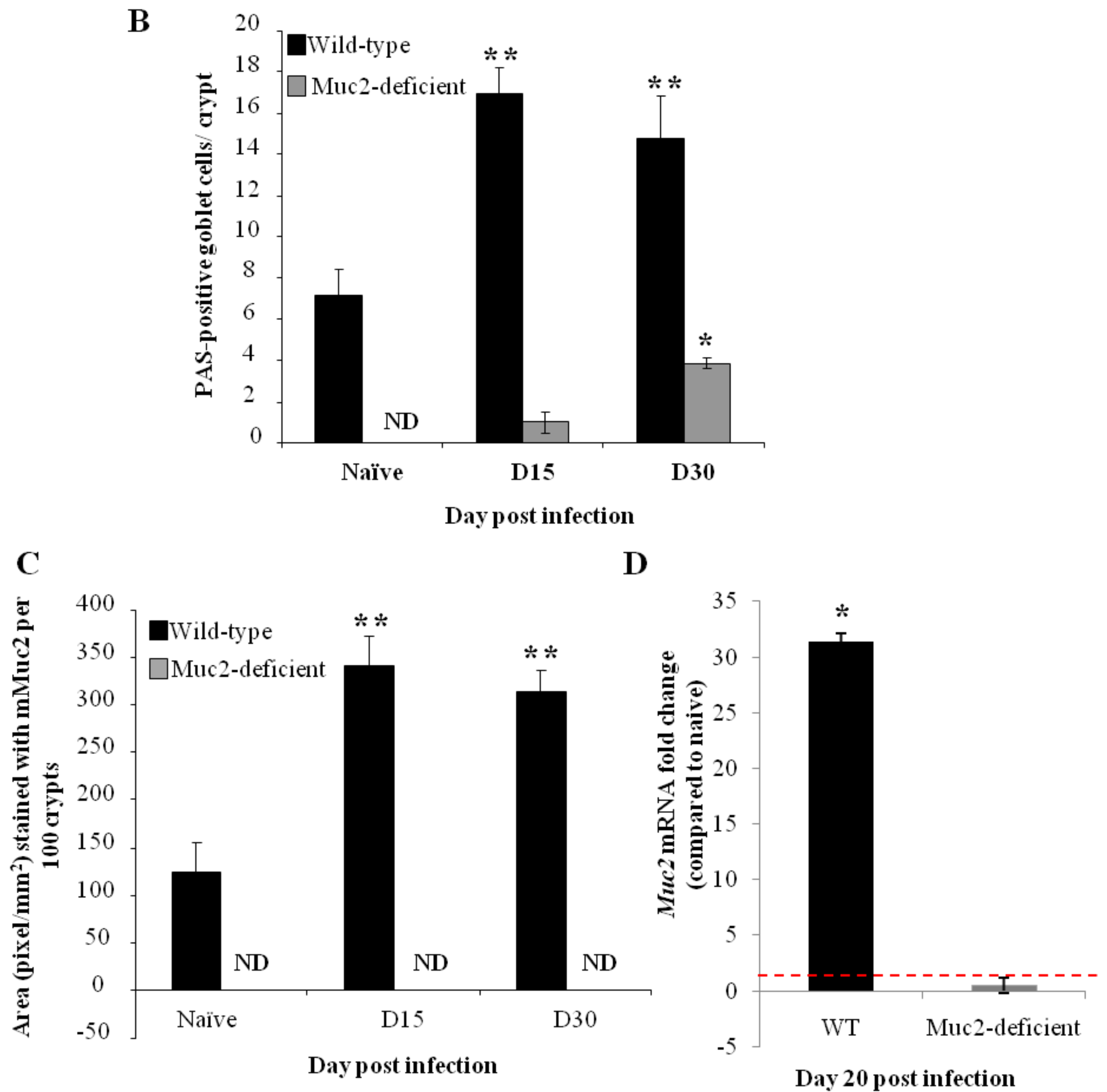
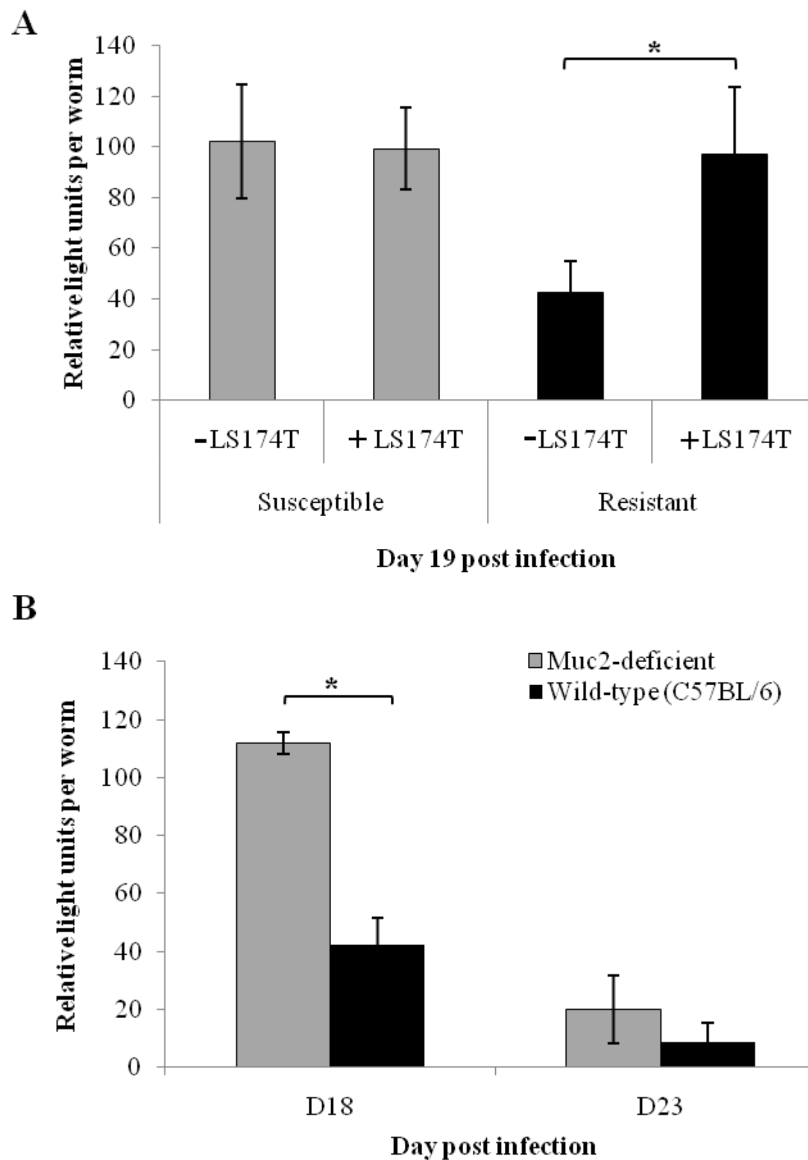


Supplementary Figure 3.3: PAS staining with and without fast-green counterstaining, and immunofluorescent staining with mMuc2 antibody of caecal tissue of Muc2-deficient mice and their resistant wild-type littermates (A). Arrows highlight the emergence of smaller PAS-positive goblet cells in the Muc2-deficient mice. Quantification of mMuc2 antibody staining represented as area stained in pixels per mm^2 (B). RT-PCR confirms the increase in *Muc2* levels post infection in resistant wild-type mice (C; Red dashed line = naïve levels). Representative of 5 mice. * = $P<0.05$.



Supplementary Figure 3.4: ATP production (data presented as relative light units per worm) was determined in the worms isolated from the resistant (BALB/c) or susceptible (AKR) mice; compared to isolated worms transferred onto LS174T cell culture on day 19 pi. (B) ATP production by worms isolated from Muc2-deficient mice and their wild-type littermates was determined on day 18 and 23 pi. Representative of 3 mice. * = $P<0.05$.

Muc5ac: A CRITICAL COMPONENT MEDIATING THE REJECTION OF THE ENTERIC NEMATODE, *Trichuris muris*

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(*Submitted manuscript*)

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(All the work was carried out by S.Z.H with the exception of Supplementary Figure 4.1)

ABSTRACT

De novo expression of Muc5ac, which is a mucin not normally expressed in the intestinal tract, is observed in the caecum of mice resistant to *Trichuris muris* infection. Therefore, in this study we investigated the role of Muc5ac, which is detected just before worm expulsion and associated with the development of interleukin-13 (IL-13) resistance to this nematode. We show that Muc5ac-deficient mice were incapable of expelling *T. muris* from the intestine and developed long term chronic infections, despite developing strong T_H2 responses. Muc5ac-deficient mice had elevated levels of IL-13; however, an increase in the T_H1-cytokine IFN-γ was also observed, which is thought to favour chronic nematode infection. Therefore, to determine whether IFN-γ was promoting susceptibility, IFN-γ was neutralized *in vivo*. In the absence of IFN-γ, Muc5ac-deficient mice developed even stronger T_H2-type immune responses, characterised by increased levels of IL-4, IL-9 and IL-13 and significant production of goblet cell bioactive molecules (Resistin-like molecule-β) Relm-β and (intestinal trefoil factor) Tff3. Nevertheless, despite developing even stronger T_H2 effector responses, the Muc5ac-deficient mice remained highly susceptible to chronic *T. muris* infection. Importantly, human MUC5AC had a direct detrimental effect on the nematode's vitality. Thus, for the first time we identify a single mucin, Muc5ac, as a direct and critical mediator of resistance during chronic intestinal nematode infection.

ABBREVIATIONS

PAS, Periodic-Acid Schiff's; pi., Post infection; T_H, T Helper; *T. muris*, *Trichuris muris*; WT, Wild-type.

INTRODUCTION

The intestinal epithelium is covered by a thick layer of mucus that enables the host to inhibit the access of pathogens to the underlying mucosa (Deplancke and Gaskins, 2001). Specialised secretory cells termed 'goblet cells' secrete gel-forming mucins, the major macromolecular components of the mucus barrier that are responsible for its viscoelastic properties (Thornton *et al*, 2008). In the adult intestine, MUC2/Muc2 is the major gel-forming mucin stored within the granules of goblet cells (Allen *et al*, 1998). Aberrant expression of MUC2 underlies many human gastrointestinal diseases, such as ulcerative colitis (Longman *et al*, 2006), colorectal carcinomas (Andrianifahanana *et al*, 2001) and parasitic infections (Lidell *et al*, 2006). MUC5AC, a mucin normally expressed in non-intestinal mucosa, has been reported to be expressed in the intestine along with MUC2, during inflammation in diseases such as ulcerative colitis and adenocarcinoma (Forgue-Lafitte *et al*, 2007). A host protective role for this mucin in the intestine following infection has not been demonstrated.

Trichuriasis, caused by the nematode *Trichuris trichiura*, is one of the most prevalent nematode infections causing significant morbidity and mortality in humans (Artis and Grencis, 2008). The nematode *Trichuris muris* (*T. muris*) is closely related to *T. trichiura* at a morphological and antigenic level and is thus used as a well-established mouse model of human Trichuriasis (Cliffe and Grencis, 2004). It is well-established that the expulsion of the *T. muris* nematode is critically dependent on T_H2-associated cytokines, whereas, a T_H1-type immune response is beneficial for the nematode, resulting in a chronic infection. Our recent studies have highlighted that the mucus barrier is a significant component of the well-coordinated response initiated against the nematode, influenced by the T_H2-type cytokines (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b) and, we have recently demonstrated that in the absence of the major intestinal mucin, Muc2, a significant delay in worm expulsion occurs (Hasnain *et al*, 2010b). Interestingly, MUC5AC/Muc5ac a mucin predominantly expressed in the adult airway, stomach and occular epithelia (Bara *et al*, 2003; Buisine *et al*, 1998; Inatomi *et al*, 1996) was expressed in the caecum of resistant mice and associated with worm expulsion. We hypothesise that Muc5ac may function as a pharmacological regulator to damage the nematodes directly or indirectly via associated "protective" molecules. In addition, the incorporation of Muc5ac within the mucus layer may influence the biochemical properties of the mucus gel and thus facilitate worm expulsion (Hasnain *et al*, 2010b). To investigate the role of Muc5ac in enteric parasitic

infection, we examined the etiology of *T. muris* infection in the targeted Muc5ac-deficient mice and the interaction of MUC5AC with *T. muris in vitro*.

In this study we demonstrate, for the first time, that Muc5ac induced by IL-13 is critical for worm expulsion. Loss of Muc5ac resulted in susceptibility to chronic *T. muris* infection, compared to wild-type mice which expelled their worm burden. These findings are particularly important, as previously described effector mechanisms were activated, including a T_H2-type immune response (Grencis, 1993), goblet cell hyperplasia , up-regulation of Muc2 (Hasnain *et al*, 2010b) and the activation of the epithelial cell escalator (Cliffe *et al*, 2005) without parasite expulsion. Together, these results identify a unique functional role for the Muc5ac mucin during intestinal infection.

RESULTS and DISCUSSION

Our recent studies have shown that Muc5ac was up-regulated during *T. muris* expulsion in a strong T_H2-type environment and therefore may be a critical component of the immune regulated response (Hasnain *et al*, 2010b). Muc5ac levels were measured in IL-4 and IL-4R deficient mice during *T. muris* infection to determine whether the induction of Muc5ac was regulated by IL-4 and IL-4/IL-13 pathways, respectively. The IL-4 deficient mice had a delay in worm expulsion and were able to mount a goblet cell response, whereas the IL-4R deficient mice (which have defective IL-4 and IL-13 signalling) were susceptible to chronic infection and did not exhibit goblet cell hyperplasia (Hasnain *et al*, 2010a). Expression of Muc5ac was assessed using immunofluorescence microscopy. *De novo* Muc5ac expression was observed in IL-4 deficient mice, on days 18 and 35 post infection (pi.), correlating with worm expulsion. However, Muc5ac was undetectable in caecal crypts during infection in IL-4R deficient mice, which manifest defects in both IL-4 and IL-13 signalling (**Figure 4.1**), indicating that Muc5ac in the caecum was regulated by IL-13 (Rose *et al*, 2000) and to a lesser extent IL-4.

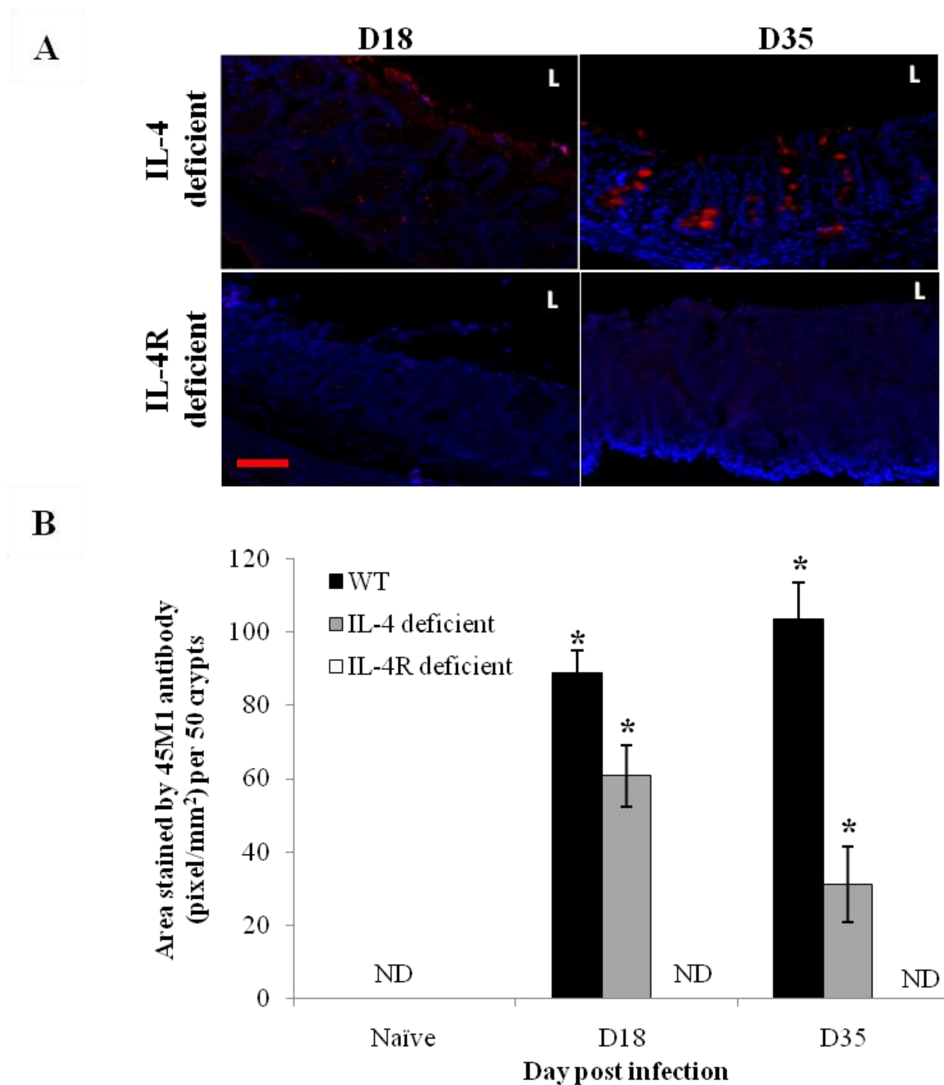


Figure 4.1: IL-13 induces Muc5ac as part of the 'normal' response to worm expulsion. Levels of Muc5ac were determined using immunofluorescence with the 45M1 antibody (A) in the IL-4 and IL-4R deficient mice (BALB/c-background) infected with *T. muris*. L marks the luminal side of the caecum. Area stained with 45M1 (B) was quantified. Results represent the mean value of 5 mice per group ± SEM. Scale bar; 10μm. * = $P<0.05$.

As Muc5ac expression correlated with worm expulsion in resistant mice even in the absence of Muc2 (Hasnain *et al*, 2010b), we investigated the role of Muc5ac during nematode expulsion. Muc5ac-deficient mice on the resistant C57BL/6-background were infected with a high dose of *T. muris* eggs. Infection became established similarly in the wild-type and Muc5ac-deficient mice as seen on day 14 pi. (**Figure 4.2**). As expected, wild-type (WT) mice cleared 70% of their worms by day 23 pi. and were essentially parasite free by day 45 pi. (**Figure 4.2**). However, there was no significant reduction in worm burdens in the Muc5ac-deficient mice at any time point (day 14, 23 and 45 pi.) demonstrating that these mice were susceptible to chronic infection (**Figure 4.2**).

Therefore, the inability of mice to express Muc5ac rendered them susceptible to chronic infection.

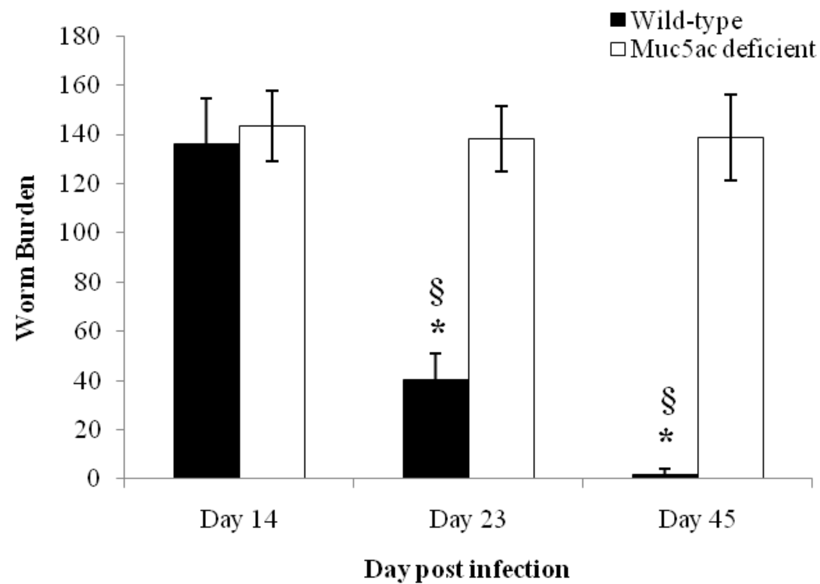


Figure 4.2 Muc5ac deficiency renders resistant mice susceptible to chronic *T.muris* infection. Muc5ac-deficient mice and the wild-type C57BL/6 mice were infected with a high dose of *T. muris* eggs and worm burdens were assessed post infection on day 14, 23 and 45 pi. Results represent the mean value of 3-5 mice per group ± SEM. * $P < 0.01$ compared to Day 14; § $P < 0.01$ compared to Muc5ac-deficient mice.

The 'epithelial cell escalator' (Cliffe *et al*, 2005) and goblet cell hyperplasia (Hasnain *et al*, 2010b) are immune-mediated effector mechanisms which contribute to worm expulsion. The rate of epithelial cell (EC) turnover was determined by measuring BrdU incorporation into ECs and measuring the length of longitudinal caecal crypts during infection (Cliffe *et al*, 2005). On day 23 pi., a marked increase in EC turnover was observed in Muc5ac-deficient mice compared to uninfected controls (**Figure 4.3A**). Furthermore, this was accompanied by an increase in caecal crypt length indicative of increased proliferation of ECs at the base of the crypt unit (**Figure 4.3B**). Despite the increase in epithelial cell turnover, the worms were not expelled from the intestine.

PAS staining and immunofluorescence microscopy with mMuc2 antibody were used to determine changes in the number of goblet cells in the caecal tissue of the Muc5ac-deficient mice during *T. muris* infection. The absence of Muc5ac in naïve and infected Muc5ac-deficient mice was confirmed with immunofluorescence microscopy with 45M1 (MUC5AC/Muc5ac) antibody (**Supplementary Figure 4.2**). During infection, an increase

in goblet cell numbers was apparent in Muc5ac-deficient mice infected with *T. muris* (day 23 and 45 pi.) compared to naïve mice (**Figure 4.4A-C**). However, the total number of PAS-positive goblet cells was ~20% lower in the Muc5ac-deficient mice compared to infected wild-type mice (**Figure 4.4A, B**); this was attributed primarily to the absence of Muc5ac, as similar amounts of Muc2 were present in both groups of mice (**Figure 4.4A, C**).

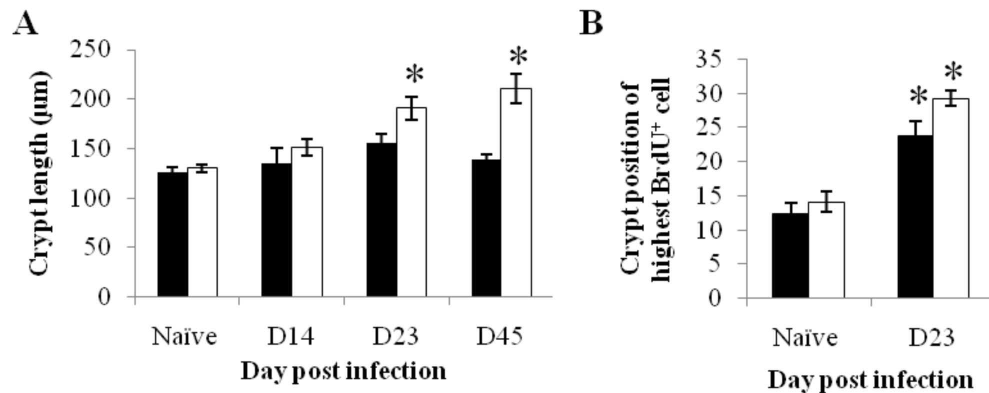


Figure 4.3 The epithelial cell escalator is active in the Muc5ac-deficient mice. Caecal crypt length measure (A) and crypt position of the highest $BrdU^+$ cell (B) in the wild-type (closed bars) and Muc5ac-deficient (open bars) mice was determined. Results represent the mean value of 3-5 mice per group ± SEM. * = $P<0.05$.

We have previously shown that during the development of resistance, the mucin network is less porous and the overall changes in the environmental niche can be detrimental to the worm's vitality (Hasnain *et al*, 2010b). To address whether changes in worm viability are affected by the absence of Muc5ac, we measured worm ATP levels. Interestingly, the worms extracted from Muc5ac-deficient mice on day 23 pi. had significantly higher levels of ATP compared those from the wild-type mice (**Figure 4.4D**), similar to the levels observed in other mice that develop chronic infections (Hasnain *et al*, 2010b). Furthermore, we demonstrated that fluorescent beads placed at the top of the mucus travelled a greater distance through the mucus barrier in Muc5ac-deficient mice as compared to wild-type mice (**Figure 4.4E**), suggesting that the absence of Muc5ac resulted in a more porous network. Whether this is due to the lower mucin content of the mucus or different organisation of the barrier due to the lack of Muc5ac remains to be elucidated. Nevertheless, these data clearly show that the absence of Muc5ac increases the viability of the worm and increases the porosity of the mucus network. We have also previously shown that Muc2 plays a significant role in protection against *T. muris* infection (Hasnain *et al*, 2010b). However, Muc2 deficiency delayed worm elimination by 1 week (Hasnain *et al*,

2010b). In contrast, Muc5ac deficiency prevented parasite expulsion, with little to no worm expulsion detected as late as 45 days pi., demonstrating a greater requirement for Muc5ac. Therefore, these data suggest that Muc2 and Muc5ac act in concert to promote worm expulsion, with Muc5ac playing the dominant role in IL-13-driven immunity.

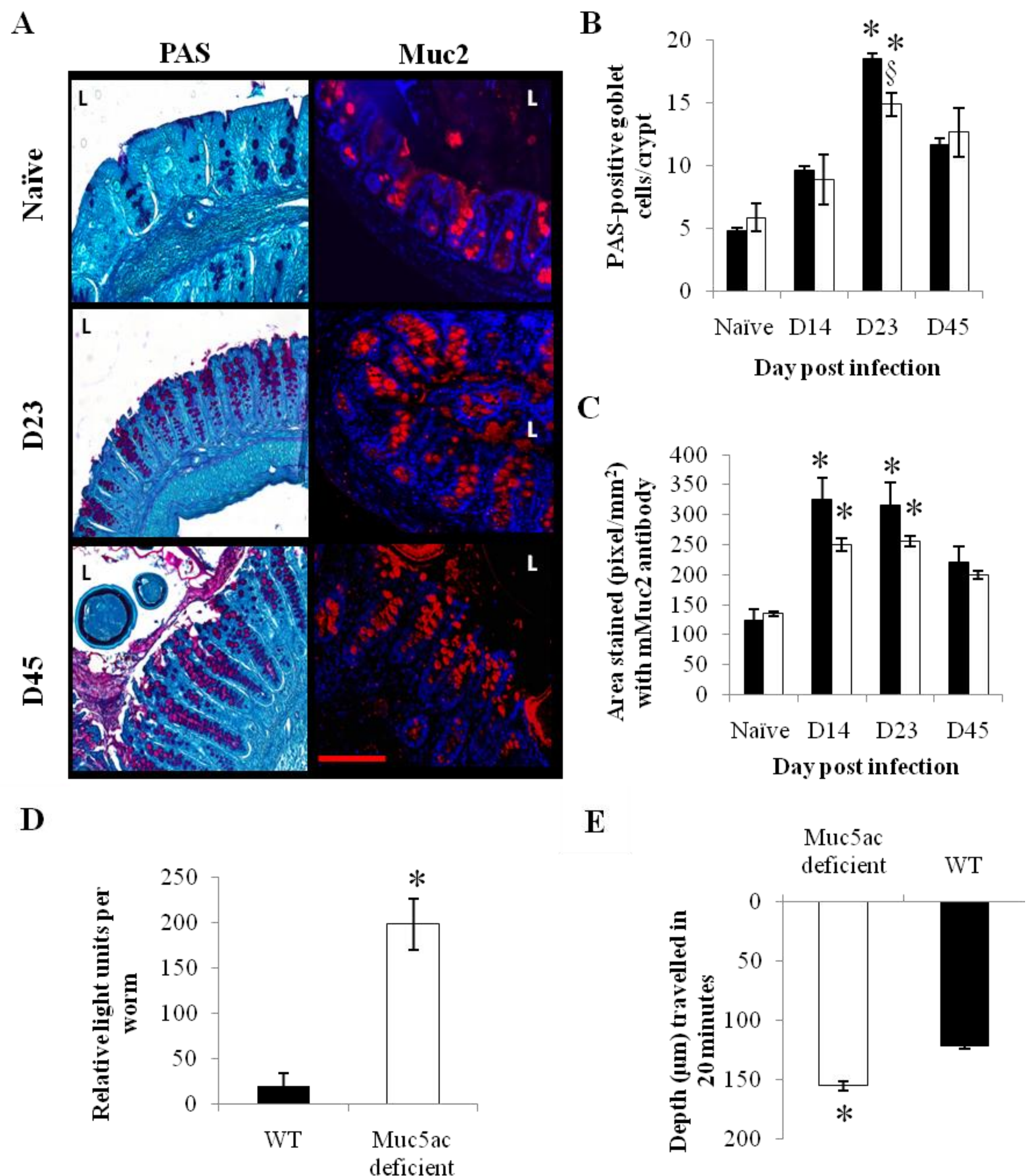


Figure 4.4 Goblet cell hyperplasia in Muc5ac-deficient mice. PAS staining and immunohistochemistry with mMuc2 of caecal tissue from naïve, day 23 and day 45 *T.muris* infected Muc5ac-deficient mice (A). L marks the luminal side of the caecum. Quantification of goblet cell numbers (B) and mMuc2 antibody (C) staining (represented as area stained in pixels per mm^2) in the caecum of wild-type (closed bars) and Muc5ac-deficient (open bars) mice during infection. Energy levels of worms (D; presented as relative light units per worm) and mucus permeability (E; using fluorescent beads) was measured in the wild-type and Muc5ac-deficient mice on day 23 pi. Results represent the mean value of 3-5 mice per group ± SEM. Scale bar; 10μm. * = P<0.01 compared to naïve mice. § = P<0.05 compared to wild-type mice.

It is well established that a strong T_H2-type immune response mediates worm expulsion, in particular, IL-13 is critical for resistance to *T. muris* infection (Artis and Grencis, 2008; Cliffe and Grencis, 2004). RT-PCR was used to determine the mRNA levels of the T_H2-type cytokines, *IL-4* and *IL-13* and the T_H1-type cytokine, *IFN-γ* in mesenteric lymph nodes (mLNs). In contrast to WT mice, a mixed response T_H1/T_H2-type response was observed in the Muc5ac-deficient mice following infection with a 4-fold increase in *IFN-γ* levels on day 14 pi., that further doubled by day 23 pi. (**Figure 4.5A**) accompanied by marked increase in *IL-4* and *IL-13* expression levels (**Figure 4.5B,C**). Similar cytokine gene expression was observed in the WT mice (**Supplementary Figure 4.3**). The increase in *IL-4* and *IL-13* levels likely explains the increase in goblet cell numbers, Muc2 expression, and the increase in epithelial cell turnover observed in the Muc5ac-deficient mice.

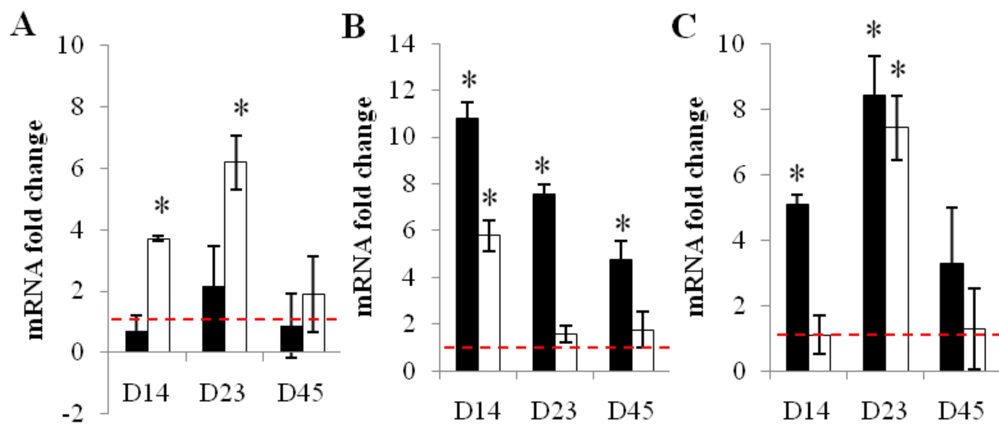


Figure 4.6: RT-PCR was used to determine the changes in the levels of cytokines, IFN-γ (A), IL-4 (B) and IL-13 (C) during infection in the mesenteric lymph nodes (mLN) of wild-type (black bars) and Muc5ac-deficient (white bars) mice. Red dashed line = naïve levels. Results represent the mean value of 3-5 mice per group ± SEM. * = $P<0.05$.

The unimpaired T_H2 response seen after infection was surprising given the susceptible phenotype of the Muc5ac-deficient mice; however, IFN-γ was also significantly elevated in these mice following infection. There was also a slight increase in IFN-γ in uninfected Muc5ac-deficient (**Supplementary Figure 4.3**). Increased levels of IFN-γ may skew the immune response towards T_H1-dominant environment that is known to promote chronic infection. Therefore, we sought to determine whether skewing the immune response of the Muc5ac-deficient mice to a more polarised T_H2-type immune response would return them to a more susceptible phenotype. To this end, mice were

treated with anti-IFN-γ antibody or an IgG control antibody, both before and during infection (every 2 days starting on 2 days before infection until day 26 pi.). Expression of IFN-γ, IL-4, IL-9 and IL-13 was measured after stimulation of mLNs with *T. muris* excretory/secretory (E/S) antigen (**Supplementary Figure 4.3**). Treatment with anti-IFN-γ antibody abolished IFN-γ expression and induced a much stronger T_H2-type immune response, characterised by increased production of IL-13 and lower IgG2a expression (**Supplementary Figure 4.3**). The Muc5ac-deficient mice treated with anti-IFN-γ remained highly susceptible to chronic *T. muris* infection, despite developing strong T_H2-type immune responses; indeed similar number of worms were observed in both control IgG and anti-IFN-γ-treated mice (**Figure 4.6**). In addition, we investigated whether deletion of Muc5ac altered the goblet cell lineage and affected the expression of goblet cell bioactive factors that may contribute to the expulsion of *T. muris*. Immunohistochemical and immunofluorescence microscopy were used to determine the expression of intestinal trefoil factor (Tff3) and resistin-like molecule-β (Relm-β), respectively. Staining for Tff3 and Relm- β were similar within the caecum of the WT and Muc5ac-deficient mice on day 35 after the neutralisation of IFN-γ (**Supplementary Figure 4.4**). These data clearly establish an important and indispensable role for Muc5ac in worm expulsion, even during chronic infection. Furthermore, it implies that Muc5ac functions as the key immune effector molecule during the development of T_H2-dependent immunity.

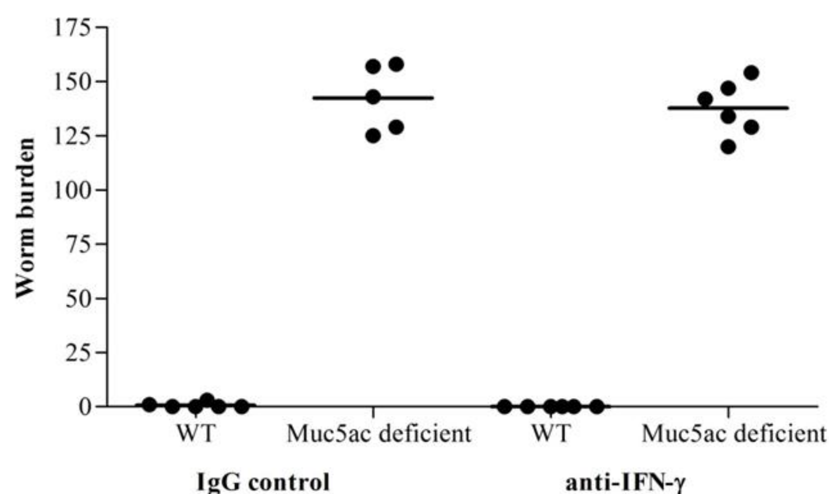


Figure 4.6 Neutralisation of IFN-γ induces a T_H2 immune response without altering the outcome of infection. Muc5ac-deficient and wild-type mice were infected with a high dose of *T. muris* eggs. Infected mice received either anti-IFN-γ or a control IgG antibody at 0.5mg/injection every 2/3 days (till day 26 of infection, starting on 2 days before infection). Worm burdens were assessed on day 35pi. Results represent the mean value of 5-6 mice per group.

The question that arises in light of this evidence is how does the absence of Muc5ac result in chronic *T. muris* infection despite an on-going T_H2-type immune response? To explore whether the Muc5ac present within goblet cells in the caecal crypts during worm expulsion has a direct effect on the nematode, we utilised human colonic cell lines producing either MUC5AC (HT-29) or MUC2 (LS174T). Worms were extracted from highly susceptible athymic nude mice and therefore, the worms extracted from this environment are in a low-stress state. Worms were then placed on either MUC2-producing LS174T cells or a sub-clone of MUC5AC-producing HT-29 cells (Kirkham *et al*, 2002) for 24 hours (**Figure 4.7**). Interestingly, the data showed that the nematodes placed on MUC2-producing cells produced significantly higher levels of ATP compared to those placed on the MUC5AC-producing cells, suggesting that MUC5AC may have a direct effect on the vitality of the nematode. The levels of ATP of worms extracted from a resistant environment were significantly lower than those observed *in vitro* (HT-29 cells) (**Figure 4.7**). This may be due to the differences in concentration or chemical composition between human and mouse MUC5AC/Muc5ac. In particular, the glycosylation pattern *in vivo* may differ from that *in vitro* and the protein core structure and assembly differs between the two mucins, where MUC2 consists of two cysteine-rich domains MUC5AC consists of nine (Hollingsworth and Swanson, 2004; Thornton *et al*, 2008). Nevertheless, MUC5AC had a deleterious effect on the vitality of the nematode. The exact mechanism by which this occurs is not yet known. Another interesting possibility by which Muc5ac/MUC5AC may exert its effects on the nematodes is via factors that are either produced from the same goblet cells and/or are specifically associated with this mucin in mucus. However, since Relm-β and Tff3, that are known to be active against nematodes (Herbert *et al*, 2009), were still expressed within the goblet cells of the Muc5ac-deficient mice (**Supplementary Figure 4.4**), our findings suggest that differences in the physical properties of the mucins themselves play the major role here.

In order to address whether MUC5AC was directly having a detrimental effect on the vitality of the nematode, we altered the concentration and structure of polymeric MUC5AC produced by HT-29 cells in culture (**Figure 4.7B**). A dose dependent decrease in the nematodes ATP levels was observed when they were treated with increasing concentration of MUC5AC. To test whether the intact polymer was required for the direct effect on the nematode, the MUC5AC preparation was depolymerised by treatment with DTT. Incubation of the nematodes with the monomeric, unfolded mucins did not have a

significant effect on the protein function in terms of reducing nematodes vitality (**Figure 4.7C**). Subsequently, the monomeric MUC5AC mucins were treated with trypsin which degrades the protein-rich N- and C-terminal domains and the internal cys-domains but leaves intact the highly-glycosylated tandem repeat domains. Importantly, the digested MUC5AC mucin did not reduce the worm vitality, highlighting the specific role of MUC5AC as an effector that acts to damage the nematode as part of the co-ordinated worm expulsion process.

Recently another study has also reported the up-regulation of Muc5ac in intestinal epithelial cells *in vitro* with exposure to the intestinal nematode *Nippostrongylus brasiliensis* (Takeda *et al*, 2010). Furthermore, *Muc5ac* mRNA was also shown to be up-regulated in the intestines during *Trichuris suis* infection in pigs (Kringel *et al*, 2006). Additionally, we have preliminary data that suggest that Muc5ac is up-regulated during *Trichinella spiralis* infection (data not shown). Therefore, we propose that *de novo* expression of Muc5ac observed as part of the IL-13 mediated immune response serves as a "universal protective mechanism" during worm expulsion. This study is the first to address the critical role of Muc5ac during an enteric parasitic infection functionally. Moreover, it highlights a novel role for Muc5ac not just as a structural component of the mucus barrier but as a crucial effector molecule during chronic infections of the intestine.

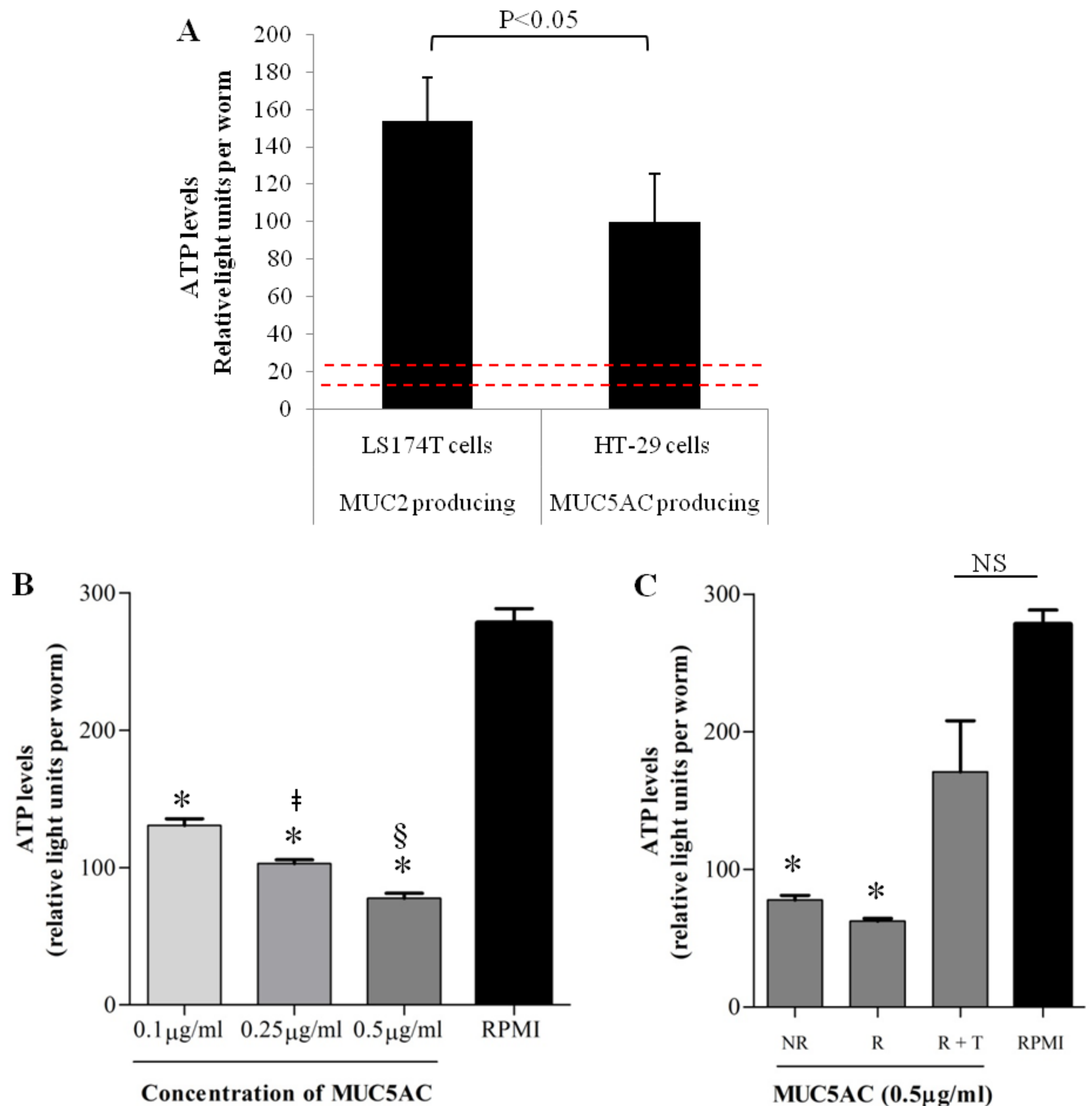


Figure 4.7 MUC5AC can directly affect nematodes vitality. Live worms extracted from a nude mouse on day 26 pi. were placed on HT-29 (MUC5AC–producing) cells or LS174T (MUC2-producing) cells for 24 hours prior to measuring ATP levels (A). Worms were treated with 0.1µg/ml, 0.25µg/ml or 0.5µg/ml of MUC5AC, or RPMI media only for 24 hours prior to measuring ATP levels (B). (C) Worms isolated were treated with non-reduced (NR), reduced and carboxymethylated (R) or reduced, carboxymethylated and trypsin-digested (R + T) 0.5µg/ml of MUC5AC, or RPMI media only for 24 hours prior to measuring ATP levels. ATP levels are presented as relative light units per worm. Red dashed line = worms extracted from resistant mice. Results represent the mean value of 100 worms per group ± SEM. * = $P<0.05$ compared to RPMI control. ǂ = $P<0.05$ compared to 0.1µg/ml MUC5AC. § = $P<0.001$ compared to 0.1µg/ml MUC5AC. NS = not significant.

MATERIALS AND METHODS

Animals

The wild-type (C57BL/6) mice were commercially bought from Harlan Olac, IL-4 deficient and their wild-type (BALB/c) littermates were originally obtained from Dr. Andrew McKenzie (McKenzie *et al*, 1998)). IL-4R deficient mice were obtained from Prof. F. Brombacher (Mohrs *et al*, 1999). Muc5ac deficient mice were originally produced by gene mutation at the University of Texas (by Dr. C. Evans). The Muc5ac allele was targeted by inserting LoxP sites into the 5' flanking region and intron 1. Neomycin was used for selection, and the selection cassette was removed by crossing animals with Rosa-FLPe knock-in mice (data not shown). Southern blot analysis using a probe located outside of the 5' targeting arm (**Supplementary Figure 4.1**) confirmed homologous insertion of the 5' LoxP site. Long range PCR, restriction endonuclease, and sequence analysis confirmed homologous insertion of the 3' LoxP site. EcoRI and BamHI (sites present in the recombinant but not in the wild type allele) were used to digest PCR products. DNA extracted from mouse tail biopsies were screened by PCR to identify +/+, +/-, and -/- animals. Quantitative RT-PCR was used to confirm loss of Muc5ac expression in the stomachs of knockout animals using methods described previously (Young *et al*, 2007). All the animals were maintained in the Biological Services Unit at the University of Manchester. The protocols employed were in accordance with guidelines by the Home Office Scientific Procedures Act (1986). All mice were kept in sterilized, filter-topped cages, and fed autoclaved food in the animal facilities. Only 6-12wk old male mice were used.

Parasitological techniques

The techniques used for *T. muris* maintenance and infection were described previously (Wakelin, 1967). Mice were orally infected with approximately 100-300 *T. muris* eggs for a high dose infection. Worm burdens were assessed by counting the number of worms present in the caecum as described previously (Wakelin, 1967).

Histology, immunohistochemistry and immunofluorescence microscopy

A 1cm segment or the whole caecum or small intestine (rolled) was fixed in 95% ethanol and processed by using standard histological techniques. Sections were treated with 0.1M KOH for 30 minutes prior to staining with periodic acid Schiff (PAS) reaction . Slides were counterstained with either haematoxylin and eosin or 1% fast-green. Standard

immunohistochemical and immunofluorescent staining methods were used to determine the levels of Muc2 and Muc5ac.

Antibodies

Immunodetection was carried out using a polyclonal antibody raised against a murine Muc2 (mMuc2) (Heazlewood *et al*, 2008). Commercially available 45M1 antibody was used for the detection of mouse Muc5ac (Lidell *et al*, 2008). Detection of BrdU incorporated into nuclei was carried out using a monoclonal anti-BrdU antibody (AbD Serotec,Oxford, United Kingdom). Commercially available mRelm-β (Abcam, Cambridge, UK) and mITF (Santacruz Biotech., USA) antibodies were used to detect Relm-β and Tff3, respectively. The rat immunoglobulin G1 monoclonal antibody XMG1.6 and GL113 antibody (for isotype control) were purified from supernantant by cell culture passage and protein G-Sepharose column and concentrated using a Centricon Centriprep tube. Antibody was administered at 0.5mg per 200µl of phosphate buffered saline (PBS) by intraperitoneal injection. Injections were given every 4 days starting from day -2 to day 26 post infection.

Analysis of mucus network properties

Caecal tissue isolated from mice was cut longitudinally, washed with PBS and kept hydrated in a 6 well plate. 0.1µm blue fluorescently labelled polymer microspheres (Dukes Scientific, UK) were placed on top of the luminal surface of the caecum (set as a reference) and their position analysed using the Nikon C1 Upright confocal microscope. 3D optical stacks were taken every 5µm and combined to obtain a Z-stack at the time points stated (Hasnain *et al*, 2010b).

Worm isolation for ATP analysis

The caecum was longitudinally cut and segmented before incubation with 0.1M NaCl for 2 hours at 37◦C with frequent shaking. Worms were counted after separation from debris and epithelial cells using a 0.7µm filter and kept in RPMI-1640 supplemented with 10% FCS. Alive worms were subsequently homogenised using the FastPrep® homogeniser (MP Biomedicals, Inc.).

Energy status of worms

The CellTiter-Glo® luminescent cell viability assay was carried out according to manufacturer's instructions (Promega Corp., USA). Relative light units (RLUs) were calculated per worm: RLU = (sample light units − blank light units)/number of worms. Substrate only was used as a blank control; whereas worms were boiled before homogenisation for negative controls. To determine recovery of energy status, *w*orms recovered on D19 pi. were washed extensively in DMEM, added to 6 well plates with LS174T cells (maintained as previously described by Hayes et al.) for 24 hours prior to measuring ATP levels (Hasnain *et al*, 2010b). MUC5AC was purified as previously described (Sheehan *et al*, 2000).

Rate of epithelial cell turnover

The rate of intestinal epithelial cell turnover was assessed by visualising BrdU incorporated into nuclei after mice were injected with 10mg of BrdU 16hrs before sacrifice, as described previously (Cliffe *et al*, 2005).

In-vitro ***cytokine analysis***

Mesenteric lymph nodes (mLNs) were removed, cells were isolated and resuspended at 5 x 10^6 cells/ml in RPMI 1640 with 10% FBS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cultures were stimulated with 50µg/ml of ESPs (Excretory secretory products) for 24 hours at 37°C and 5% CO_2. Cell free supernatants were stored at -80°C; cytokine analysis was carried out by cytometric bead assay (BD Biosciences) (Hayes *et al*, 2007). For the analysis of *T. muris* specific antibodies (IgG2a) was carried out by sandwich ELISA using homogenised intestinal tissue.

Quantification of histological staining

The numbers of goblet cells expressed per crypt were counted in 50 longitudinally sectioned crypt units. The area stained (pixel/mm^2) per 100 crypts was determined by using the ImageJ software version 1.39a.

Statistical analysis

All results are expressed as the mean ±SEM. Statistical analysis was performed using GraphPad PRISM version 3.2 (GraphPad software, Inc.). Statistical significance of

different groups was assessed by using parametric tests (ONE-way Analysis of variance with post-test following statistical standards or paired Student t test). $P<0.05$ was considered statistically significant.

REFERENCES

Allen A, Hutton DA, Pearson JP (1998). The MUC2 gene product: a human intestinal mucin. *Int J Biochem Cell Biol* **30**(7)**:** 797-801.

Andrianifahanana M, Moniaux N, Schmied BM, Ringel J, Friess H, Hollingsworth MA, Buchler MW, Aubert JP, Batra SK (2001). Mucin (MUC) gene expression in human pancreatic adenocarcinoma and chronic pancreatitis: a potential role of MUC4 as a tumor marker of diagnostic significance. *Clin Cancer Res* **7**(12)**:** 4033-4040.

Artis D, Grencis RK (2008). The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunol* **1**(4)**:** 252-264.

Bara J, Forgue-Lafitte ME, Maurin N, Flejou JF, Zimber A (2003). Abnormal expression of gastric mucin in human and rat aberrant crypt foci during colon carcinogenesis. *Tumour Biol* **24**(3)**:** 109-115.

Buisine MP, Devisme L, Savidge TC, Gespach C, Gosselin B, Porchet N, Aubert JP (1998). Mucin gene expression in human embryonic and fetal intestine. *Gut* **43**(4)**:** 519-524.

Cliffe LJ, Grencis RK (2004). The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol* **57:** 255-307.

Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* **308**(5727)**:** 1463-1465.

Deplancke B, Gaskins HR (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am J Clin Nutr* **73**(6)**:** 1131S-1141S.

Forgue-Lafitte ME, Fabiani B, Levy PP, Maurin N, Flejou JF, Bara J (2007). Abnormal expression of M1/MUC5AC mucin in distal colon of patients with diverticulitis, ulcerative colitis and cancer. *Int J Cancer* **121**(7)**:** 1543-1549.

Grencis RK (1993). Cytokine-mediated regulation of intestinal helminth infections: the *Trichuris muris* model. *Ann Trop Med Parasitol* **87**(6)**:** 643-647.

Hasnain SZ, Thornton DJ, Grencis RK (2010a). Changes in the mucosal barrier during acute and chronic *Trichuris muris* infection. *Parasite Immunology*. *In press.*

Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010b). Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* **138**(5)**:** 1763-1771.

Hayes KS, Bancroft AJ, Grencis RK (2007). The role of TNF-alpha in *Trichuris muris* infection I: influence of TNF-alpha receptor usage, gender and IL-13. *Parasite Immunol* **29**(11)**:** 575-582.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA (2008). Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* **5**(3)**:** e54.

Herbert DR, Yang JQ, Hogan SP, Groschwitz K, Khodoun M, Munitz A, Orekov T, Perkins C, Wang Q, Brombacher F, Urban JF, Jr., Rothenberg ME, Finkelman FD (2009). Intestinal epithelial cell secretion of RELM-beta protects against gastrointestinal worm infection. *J Exp Med* **206**(13)**:** 2947-2957.

Hollingsworth MA, Swanson BJ (2004). Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer* **4**(1)**:** 45-60.

Inatomi T, Spurr-Michaud S, Tisdale AS, Zhan Q, Feldman ST, Gipson IK (1996). Expression of secretory mucin genes by human conjunctival epithelia. *Invest Ophthalmol Vis Sci* **37**(8)**:** 1684-1692.

Kirkham S, Sheehan JK, Knight D, Richardson PS, Thornton DJ (2002). Heterogeneity of airways mucus: variations in the amounts and glycoforms of the major oligomeric mucins MUC5AC and MUC5B. *Biochem J* **361**(Pt 3)**:** 537-546.

Kringel H, Iburg T, Dawson H, Aasted B, Roepstorff A (2006). A time course study of immunological responses in Trichuris suis infected pigs demonstrates induction of a local type 2 response associated with worm burden. *Int J Parasitol* **36**(8)**:** 915-924.

Lidell ME, Bara J, Hansson GC (2008). Mapping of the 45M1 epitope to the C-terminal cysteine-rich part of the human MUC5AC mucin. *FEBS J* **275**(3)**:** 481-489.

Lidell ME, Moncada DM, Chadee K, Hansson GC (2006). Entamoeba histolytica cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. *Proc Natl Acad Sci U S A* **103**(24)**:** 9298-9303.

Longman RJ, Poulsom R, Corfield AP, Warren BF, Wright NA, Thomas MG (2006). Alterations in the composition of the supramucosal defense barrier in relation to disease severity of ulcerative colitis. *J Histochem Cytochem* **54**(12)**:** 1335-1348.

McKenzie GJ, Bancroft A, Grencis RK, McKenzie AN (1998). A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr Biol* **8**(6)**:** 339-342.

Mohrs M, Ledermann B, Kohler G, Dorfmuller A, Gessner A, Brombacher F (1999). Differences between IL-4- and IL-4 receptor alpha-deficient mice in chronic leishmaniasis reveal a protective role for IL-13 receptor signaling. *J Immunol* **162**(12)**:** 7302-7308.

Rose MC, Piazza FM, Chen YA, Alimam MZ, Bautista MV, Letwin N, Rajput B (2000). Model systems for investigating mucin gene expression in airway diseases. *J Aerosol Med* **13**(3)**:** 245-261.

Sheehan JK, Brazeau C, Kutay S, Pigeon H, Kirkham S, Howard M, Thornton DJ (2000). Physical characterization of the MUC5AC mucin: a highly oligomeric glycoprotein whether isolated from cell culture or in vivo from respiratory mucous secretions. *Biochem J* **347 Pt 1:** 37-44.

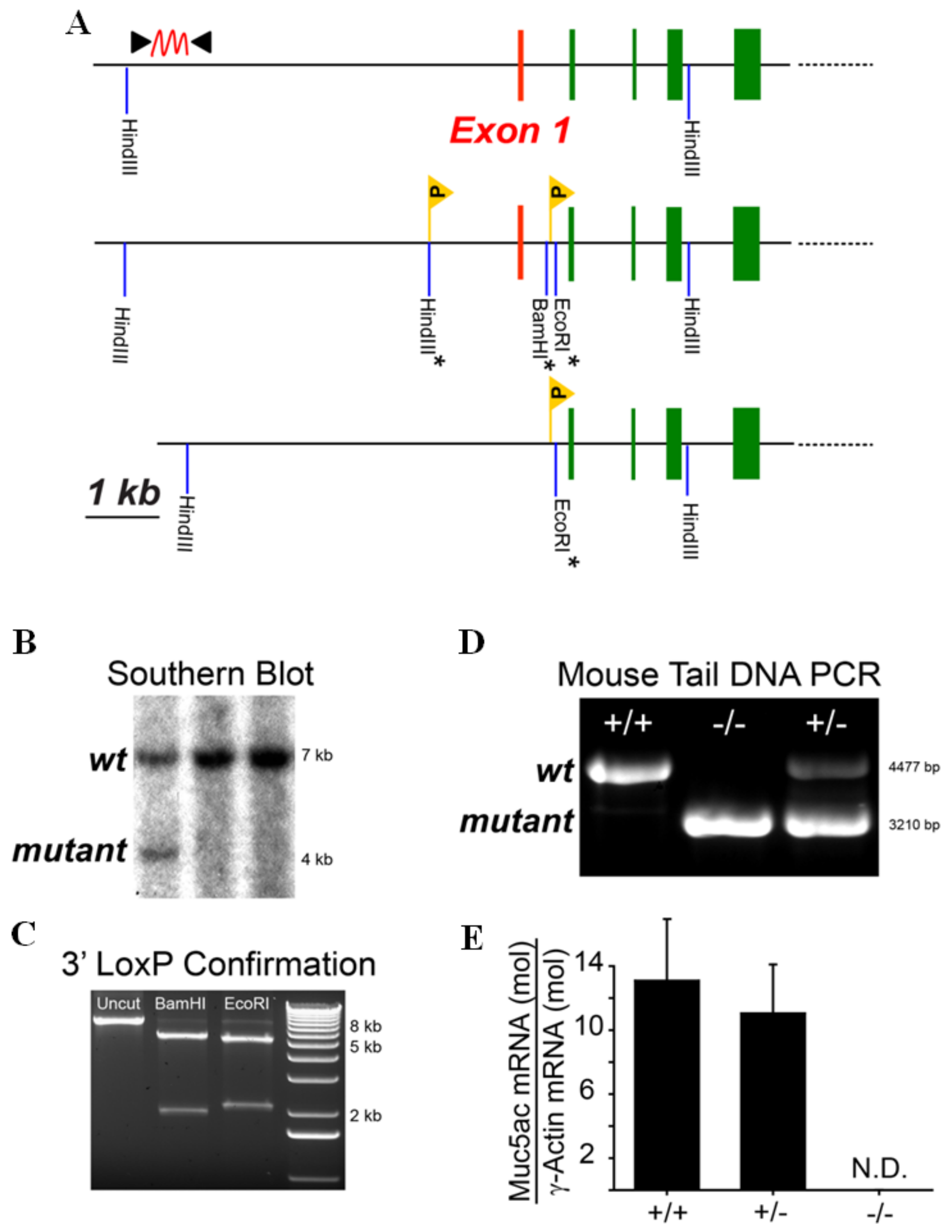
Takeda K, Hashimoto K, Uchikawa R, Tegoshi T, Yamada M, Arizono N (2010). Direct effects of IL-4/IL-13 and the nematode Nippostrongylus brasiliensis on intestinal epithelial cells in vitro. *Parasite Immunol* **32**(6)**:** 420-429.

Thornton DJ, Rousseau K, McGuckin MA (2008). Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* **70:** 459-486.

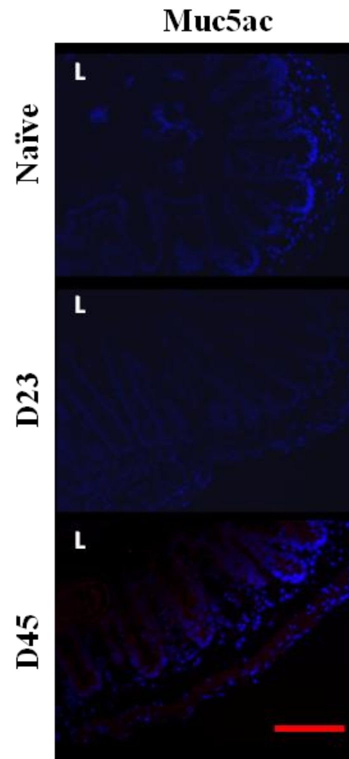
Wakelin D (1967). Acquired immunity to *Trichuris muris* in the albino laboratory mouse. *Parasitology* **57**(3)**:** 515-524.

Young HW, Williams OW, Chandra D, Bellinghausen LK, Perez G, Suarez A, Tuvim MJ, Roy MG, Alexander SN, Moghaddam SJ, Adachi R, Blackburn MR, Dickey BF, Evans CM (2007). Central role of Muc5ac expression in mucous metaplasia and its regulation by conserved 5' elements. *Am J Respir Cell Mol Biol* **37**(3)**:** 273-290.

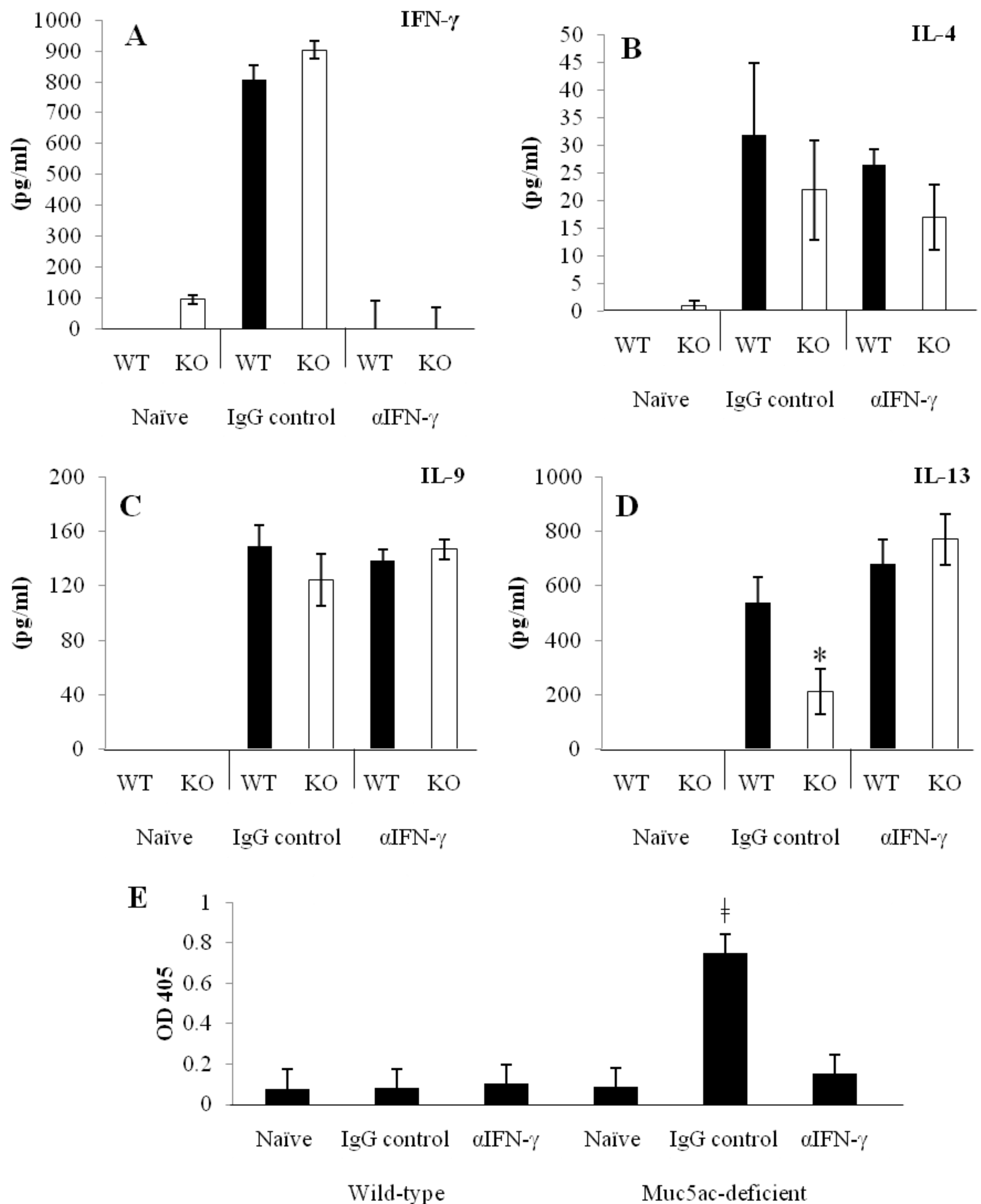
SUPPLEMENTARY FIGURES



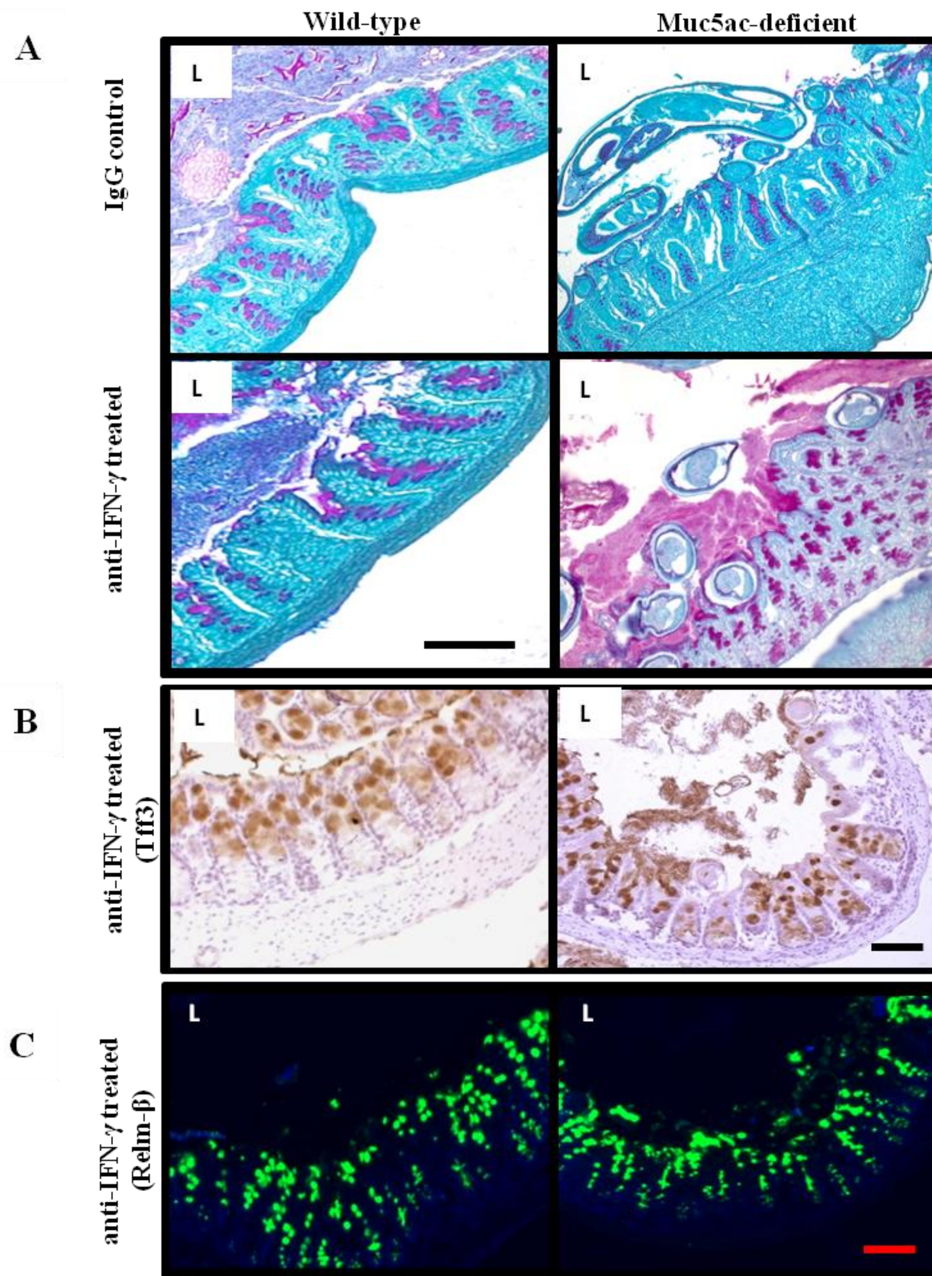
Supplementary Figure 4.1: Generation of Muc5ac-deficient mice. Southern blot analysis (B) using a probe located outside of the 5′ targeting arm (within the region surrounded by the black triangles in A). Long range PCR, restriction endonuclease, and sequence analysis (C). EcoRI and BamHI (sites present in the recombinant but not in the wild type allele) were used to digest PCR products (C). DNA extracted from mouse tail biopsies were screened by PCR (D) to identify +/+, +/-, and -/- animals. Quantitative RT-PCR (E) was used to confirm loss of *Muc5ac* expression in the stomachs of Muc5ac-deficient animals.



Supplementary Figure 4.2: Immunohistochemistry with 45M1 of caecal tissue from naïve, day 23 and day 45 *T. muris* infected Muc5ac-deficient mice. L marks the luminal side of the caecum. Results are representative of 3-5 mice per group. Scale bar; 10µm.



Supplementary Figure 4.3: Chronic *T. muris* infection despite a T_H2-type immune response. Muc5ac-deficient and wild-type mice were infected with a high dose of *T. muris* eggs. Infected mice received either anti-IFN-γ or a control IgG antibody at 0.5mg/injection. On day 35 pi., the levels of IFN-γ (A), IL-4 (B), IL-9 (C) and IL-13 (D) (pg/ml) were determined by *T. muris* E/S antigen stimulation of mesenteric lymph nodes or, antibody IgG2a levels were determined using sandwich ELISA on intestinal homogenates (E). Results represent the mean value of 4-5 mice per group ± SEM. * = P<0.05 compared to wild-type mice. ǂ = P<0.02 compared to all groups.



Supplementary Figure 4.4: Chronicity despite the presence of goblet cell bioactive factors. Histological section from Muc5ac-deficient and wild-type mice on day 35 pi. which received either anti-IFN-γ or a control IgG antibody were stained with PAS (A). Immunohistochemistry was used to determine the expression of Tff3 (B) and Relm-β (C) in the anti-IFN-γ treated wild-type and Muc5ac-deficient mice on day 35 pi. Representative of 4-5 mice. Scale bar; 10μm.

CHRONIC *Trichuris muris* INFECTION CAUSES LOSS OF MUCIN SULPHATION

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(All the work was carried out by S.Z.H with the exception of the oral inoculation of NaS1-deficient mice by M.A.M and help in the processing of samples by I.D, T.V.T, R.D.E, R.J.A and M.A.M)

ABSTRACT

Background & Aims: Mucins are heavily glycosylated proteins that have been shown to play an important role in rejection of the nematode, *Trichuris muris (T. muris)*. The glycans decorating the mucin protein core can alter the protective properties of the mucus barrier. Therefore, we investigated the changes in glycosylation during *T. muris* infection.

Methods: Using different infection doses and strains of mice, resistance (high dose infection in BALB/c or C57BL/6 mice) or susceptibility (high dose infection in AKR and low dose infection in BALB/c mice) to *T. muris* infection was modelled. The changes in mucin glycosylation, whether these alterations were controlled by the immune system and overall the affect of mucin glycosylation on the properties of the mucus barrier were investigated.

Results: During chronicity, within the close vicinity of the *T. muris* nematode the goblet cell thecae contained mainly sialylated mucins. In contrast, mucins were mainly sulphated in the goblet cells within the epithelial cell crypts in the resistant models. The mucin sulphation was controlled by a T_H2-type immune response in particular by IL-13 via the up-regulation of sulphotransferases. Moreover, the high sulphomucin content was less prone to degradation by *T. muris* ESPs. However, worm establishment or expulsion was unaffected in mice deficient in the Na^+ sulphate transporter (NaS1) which have depleted sulphomucin content. Unexpectedly, *T. muris* infection resulted in the recovery of sulphomucin content of the goblet cells at the time of expulsion via the sulphate anion transporter (Sat1).

Conclusions: This study highlights the complex process by which T_H2-mediated alterations in glycosylation may contribute in resistance to *T. muris*.

ABBREVIATIONS

NAS, Na^+ sulphate transporter; PAS, Periodic-Acid Schiff's; pi., Post infection; T_H, T Helper; *T. muris*, *Trichuris muris*; LD, Low dose infection; HD, High dose infection; WT, Wild-type; HID-AB, High-Iron Diamine-Alcian blue; ESP, Excretory secretory products.

INTRODUCTION

The intestinal epithelium is lined by a continuous mucus barrier which provides physical protection and, by sequestering important host defence factor within its complex matrix it chemically protects the epithelial cell layer (Thornton *et al*, 2008). Gel-forming mucins (Muc2 in the intestine) produced by goblet cells, give mucus its gel-like properties and play a significant role in protection against nematode infections (Hasnain *et al*, 2010b). Mucins are large heavily glycosylated proteins, predominantly consisting of O-glycans which account for at least 70% of their molecular weight (Thornton and Sheehan, 2004). The O-linked sugars are assembled progressively by glycosyltransferases on to a serine or threonine residue, found in the serine-threonine-proline rich tandem repeats in the mucin protein core (Thornton *et al*, 2008). It is well established that these glycan chains have fundamental roles in many biological processes including inflammatory response and parasitic infections (Theodoropoulos *et al*, 2001).

Changes in glycosylation have been well described in parasitic infections (Hasnain *et al*, 2010a; Holmen *et al*, 2002; Karlsson *et al*, 2000; Soga *et al*, 2008; Theodoropoulos *et al*, 2005; Tsubokawa *et al*, 2009; Yamauchi *et al*, 2006), whether these changes are important in the protective function of the mucus barrier however, has not yet been established. Several studies have investigated the direct role of glycans as protective elements that attach and clear pathogens, in particular bacteria, from the gut (Deplancke and Gaskins, 2001; Linden *et al*, 2004; Linden *et al*, 2008a; Szymanski and Wren, 2005). Most of the evidence for the protective role of the glycans present on mucin comes from studies on animal models. Alteration in glycans can lead to inflammation because of the alteration in the protective properties of the mucus barrier (Holmen *et al*, 2002; Hurd *et al*, 2005; Theodoropoulos *et al*, 2005; Yamauchi *et al*, 2006). For instance, the terminal sugar fucose has been shown to be crucial in host-pathogen interaction in the *Helicobacter pylori* infection (Ikehara *et al*, 2001). Moreover, inducing colitis in mice deficient in the N-acetylglucosamine-6-O-sulphotransferases-2 (Tobisawa *et al*, 2010) which is expressed highly in the colon, results in leukocyte infiltration and is thought to exacerbate inflammation. Furthermore, mice lacking the core 3-derived O-glycan chain have also been shown to produce less mucins (An *et al*, 2007), possibly due to defects in the processing of Muc2, which renders the mice more susceptible to colitis.

In the intestine, a rich commensal flora is maintained within the mucus layer and it is thought that the microflora can influence the level of sulphation on the mucins (Hill *et*

al, 1990). Paradoxically, mucin sulphation can also protect the mucin core from bacterial glycosidases (Theodoropoulos *et al*, 2001). Previous studies have shown that inducing sulphation *in vivo* leads to the reduction in the establishment of the intestinal nematode *Strongyloides venezuelensis* (Ishikawa et al, 1995). Sulphotransferases were also up-regulated shortly before the rejection of the nematode, *Nippostrongylus brasiliensis* and are thought to play a protective role during worm expulsion (Soga *et al*, 2008; Tsubokawa *et al*, 2009). In addition, the up-regulation of sialomucins observed in other nematode models such as *Trichinella spiralis*, have been found to be regulated by thymus derived T cells and are thought to be protective (Theodoropoulos *et al*, 2005; Yamauchi *et al*, 2006). Moreover, gastrointestinal disorders such as ulcerative colitis and Crohn's disease are associated with a loss of mucin sulphation (Corfield *et al*, 1996; Longman *et al*, 2006). Whether the changes observed in glycosylation occur as a consequence of disease or are an active alteration important in resolving the disease has yet to be fully elucidated. Recently it was shown that a reduction in mucin sulphation can lead to an increase in susceptibility to colitis and subsequently secondary bacterial infections (Dawson *et al*, 2009). Overall, it is thought that the changes in glycosylation can lead to reducing the effectiveness of the mucus barrier which in turn may exacerbate inflammation.

Trichuris muris (*T. muris*) infection in mice is used as a model to investigate the changes in glycosylation response to Trichuriasis, which affects up to a billion people worldwide (Bethony *et al*, 2006; Cliffe and Grencis, 2004). The *T. muris* model is useful as both resistant (high dose infection in BALB/c and C57BL/6 mice) and susceptible (high dose infection in AKR and low dose infection in BALB/c mice) mouse models exist, reflecting the situation in humans. It is well-established that the resistant mice predominantly exhibit a T_H2-type immune response, which is essential for the clearance of *T. muris*; the strains susceptible to chronic infection exhibit a T_H1-type immune response (Cliffe and Grencis, 2004). Previously we have shown that mucins play a significant role in worm expulsion (Hasnain *et al*, 2010b) and in the caecum, the niche of the parasite, the degree of sulphation on mucins is higher than in any other site in the body (Corfield *et al*, 1996). Therefore, mucin sulphation could be an important aspect of the protective nature of the mucus barrier and affect *T. muris* establishment and/or expulsion.

In this study we investigated the changes in mucin glycosylation (in particular sulphation and sialylation) during infection with *T. muris*. We demonstrated a clear switch during chronic infection: from sulphomucins to sialomucins. For the first time to our

knowledge, we demonstrate that sulphation on mucins is influenced by IL-13 and mucus with high sulphomucin content is more resistant to degradation by the *T. muris* excretory secretory products (ESPs). A reduced state of intestinal mucin sulphation (using NaS1-deficient mice) did not have an effect on worm establishment. However, we observed that *T. muris* infection was a strong inducer of sulphation of mucins and the sulphomucin content within the goblet cells recovered in a Na^+-independent manner, by the up-regulation of the sulphate anion transporter (Sat1).

MATERIALS AND METHODS

Animals

AKR, BALB/c (Harlan Olac), IL-4-deficient and IL-4R-deficient mice (originally obtained from McKenzie *et al*, 1998) were maintained in the Biological Services Unit at the University of Manchester. The protocols employed were in accordance with guidelines by the Home Office Scientific Procedures Act (1986). NaS1-deficient mice (4-12 weeks old male C57BL/6) and their wild-type littermates originally produced by gene mutation (Dawson *et al*, 2003) were housed at the Mater Medical Research Institute and experiments were approved by the University of Queensland Animal Experimentation Ethics Committee. All mice were kept in sterilized, filter-topped cages, and fed autoclaved food.

Parasitological technique

The techniques used for *T. muris* maintenance and infection were described previously (Wakelin, 1967). Mice were orally infected with approximately 100-300 eggs for a high dose infection and <15 eggs for a low dose infection. Worm burdens were assessed by counting the number of worms present in the caecum as described previously (Wakelin, 1967).

Histology, Immunohistochemistry and Immunofluorescence microscopy

A 1cm segment or the whole tissue (rolled) was fixed in 10% neutral buffered formalin or 95% ethanol and processed using standard histological techniques. Sections were treated with 0.1M potassium hydroxide for 30 minutes prior to staining with Periodic Acid Schiff's reagent (PAS). Slides were counterstained with either haematoxylin and eosin or 1% fast-green. To assess mucin sulphation, sections were stained with High Iron Diamine-Alcian Blue (HID-AB) as previously described (Spicer, 1965). Standard

immunohistochemical staining methods (Linden *et al*, 2008b) were used to detect Sat1 (Sulphate anion transporter 1), using the monoclonal mouse Sat1 antibody (Dawson *et al*, 2010).

RT-PCR

RNA from epithelial cells was isolated using the previously described method (Humphreys *et al*, 2008). cDNA was generated using an IMPROM-RT kit (Promega) or Superscript III (Invitrogen). Absolute QPCR SYBR Green (ABgene) was used for quantitative PCR. Primer efficiencies was determined using cDNA dilutions and genes of interest (**Supplementary Table 5.1**) were normalised against the housekeeping gene, β-actin, and expressed as a fold difference to uninfected naïve message levels. RT-PCR products were directly sequenced to verify the identity of the amplified genes. In brief, products were digested with Exonuclease I and Calf Intestinal Phosphatase and subsequently sequenced using the ABIPRISM Big-Dye Terminator cycle sequencing reaction at the Sequencing Facility in the University of Manchester. The data was analysed using Chromas Pro v1.34 and the sequences obtained were compared against the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Mucus extraction and analysis by agarose gel electrophoresis

Caeca from NAS1-deficient mice and their wild-type littermates were gently flushed with PBS to remove the faecal matter. The mucus was lightly scraped and lyophilized and subsequently solubilised in 6M urea, reduced using 50mM dithiothreitol (DTT) and carboxylmethylated using 0.125M iodoacetamide prior to electrophoresis on a 1% (w/v) agarose gel. Mucins were western blotted onto nitrocellulose membrane and detected using PAS or HID staining (Thornton *et al*, 1994).

Treatment with* T. muris *excretory secretory products

The excretory secretory products (ESPs) were collected using methods previously described (Else and Wakelin, 1989). Aliquots of crude mucus (in equal volumes of PBS) were incubated at 37°C with the ESPs at 50μg/ml for various time points (later specified). Control samples were not treated with the ESPs, but were incubated at 37°C for the maximum time point.

Rate zonal centrifugation

6–8M guanidinium chloride gradients were formed in centrifuge tubes using an MSE gradient maker connected to a Gilson Minipuls 2 peristaltic pump. Mucin samples (in 4M guanidinium chloride) were loaded onto the gradient and centrifuged in a Beckman Optima™ L-90K Ultracentrifuge using a Beckman SW40 rotor at 40,000RPM for 2.45 hours at 15°C. Fractions were taken from the top of the tubes, analysed by slot blotting and PAS-staining (Thornton *et al*, 1990). The refractive index of each fraction was measured using a refractometer to determine the guanidinium chloride concentration; the gradients were comparable (data not shown).

Quantification of histological staining

Sulphomucin containing crypts within the caecum (identified as black goblet cells) were quantified and compared to the total number of crypts. The numbers of goblet cells expressed per crypt were counted in 20-50 longitudinally sectioned crypt units. The area stained (pixel/mm^2) per 20-50 crypts and the goblet cell staining intensity (arbitrary units) were determined by using the ImageJ software version 1.39a.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed using SPSS version 16.0. Statistical significance of different groups was assessed by using parametric tests (ONE-way Analysis of variance with post-test following statistical standards or paired Student t test). P<0.05 was considered statistically significant.

RESULTS

Reduced sulphation of Muc2 during chronic **T. muris** *infection*

The degree of sulphation and sialylation were explored during *T. muris* infection using HID-AB staining in resistant (high dose (HD) infection in BALB/c mice) and susceptible (HD and low dose (LD) infection in AKR and BALB/c mice, respectively). Using HID-AB staining a distinction can be made between sulphomucins (in black) and sialomucins (in blue), present in the goblet cell thecae (Spicer, 1965). Normally in the caecum, the niche of the parasite, the mucins are majorly sulphated (**Figure 5.1A** – naïve levels). In the resistant mice, as infection progressed, there was an increase in goblet cell

numbers in the caecal crypts (Hasnain *et al*, 2010b), and these goblet cell predominantly contained sulphated mucins.

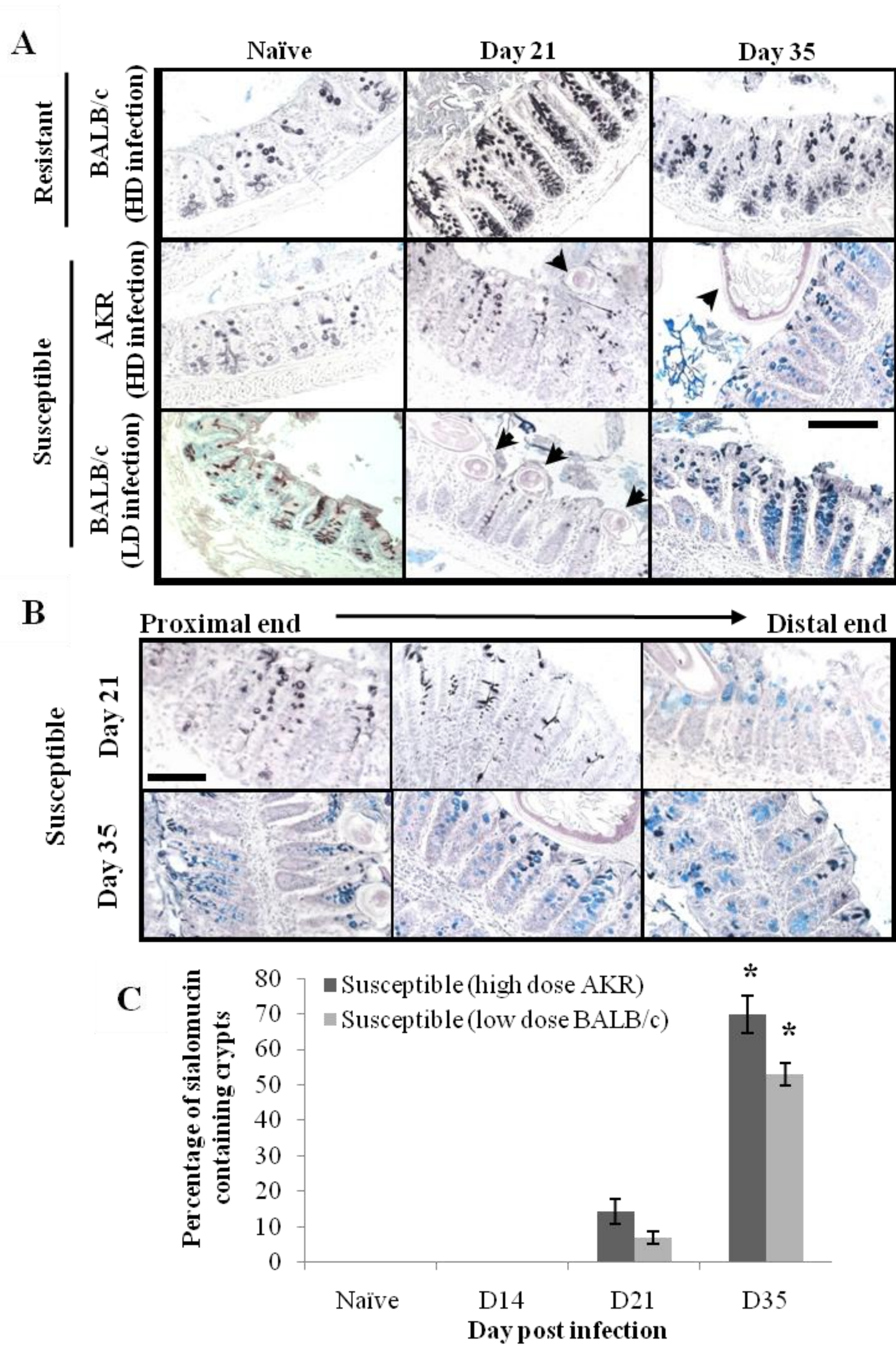


Figure 5.1: HID-AB (A) staining of the caecum of resistant mice (BALB/c high dose (HD) infection) and susceptible mice (AKR HD infection and BALB/c low dose (LD) infection) was used to differentiate between sulphomucins (black) and sialomucins (blue) during *T. muris* infection. Nematodes are highlighted by arrows. HID-AB staining of the caecum (B) of susceptible AKR mice from the proximal to the distal end on day 21 and 35 pi. Quantification (C) of crypts containing sialomucins per total number of crypts during the course of *T. muris* infection. Results represent the mean value of 3-5 mice per group ± SEM. Scale bar = 10µm. * = P<0.05.

However, in the susceptible models, there was a decrease in goblet cell numbers, accompanied by a loss of sulphated goblet cell thecae and an increase in sialomucins within the goblet cells. This switch in glycosylation was localised to the caecum; no major changes were observed in the colon (**Supplementary Figure 5.1**). Interestingly, the change from goblet cells predominantly containing sulphomucin to containing sialomucin was graded in the susceptible mice from the distal end to the proximal end of the caecum (**Figure 5.1B**). On day 21 pi., 20% of the total caecal crypts contained sialomucins which were all situated at the distal end of the caecum. However, by day 35pi., 75% of the crypts, throughout the caecum, contained sialomucins (**Figure 5.1C**).

Up-regulation of sulphotransferases in resistance

Mucin glycosylation is dependent on glycosyltransferases expressed within the goblet cells. Therefore, considering the differences identified, the changes in the expression of major sulpho- and sialo- transferases expressed within the caecum were determined using RT-PCR (**Figure 5.2**). Interestingly, mirroring the increase in sulphated mucins present in goblet cells, the galactose-O-sulphotransferases *Gal3St1*, *Gal3St2* and *Gal3St3* (**Figure 5.2A**) and glucosamine-O-sulphotransferases *GlcNAc6ST1*, *GlcNAc6ST2* and *GlcNAc6ST4* (**Figure 5.2B**) were up-regulated on day 21 pi. in the resistant mice but not in the susceptible mice. Furthermore, an up-regulation of sialyltransferases *ST3Gal1*, *ST3Gal2* and *ST8GalNAc* (**Figure 5.2C**) was observed, on day 21 and 35 pi., only in the susceptible mice.

Inducing resistance in susceptible mice reversed glycosylation from sialylated to sulphated

We wanted to determine whether the changes in glycosylation were as a direct result of infection itself and whether these changes were permanent. Therefore, to address these questions, BALB/c mice were infected with a low dose of *T. muris* eggs (Bancroft *et al*, 2001); this resulted in a chronic infection, as approximately 10-12 adult worms were present in the caeca of these mice on day 35 pi. (**Figure 5.3A** & **B**). As expected, no changes in the goblet cell numbers were observed in these mice (**Figure 5.3C**) and, by day 35 pi. goblet cells contained predominantly sialomucins (**Figure 5.3D**). Subsequently, at chronicity (day 35 pi.), these mice were re-challenged with a high dose (~150) of *T. muris* eggs (**Figure 5.3A**).

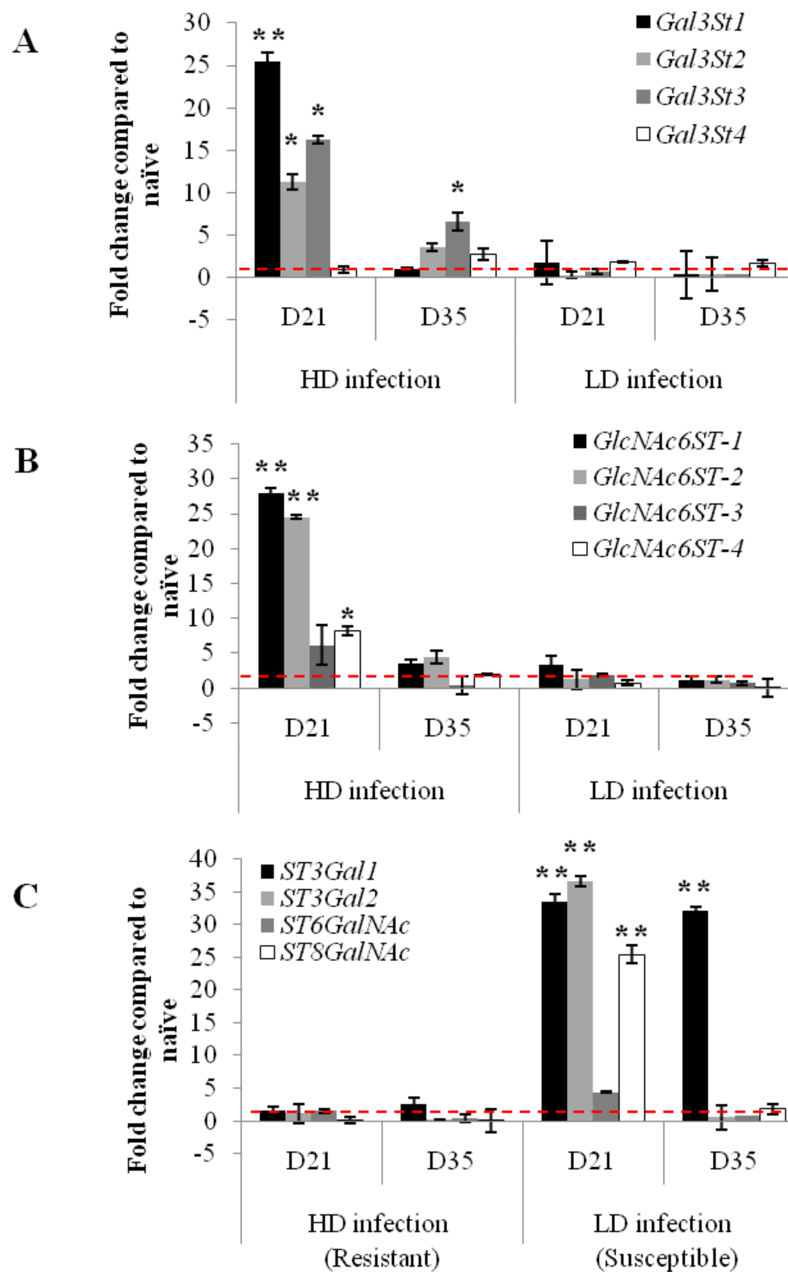


Figure 5.2: RT-PCR was used to determine the levels of sulphotransferases, *Gal3St1*, *Gal3St2* and *Gal3St3*(A) and *GlcNAc6ST1*, *GlcNAc6ST2*, *GlcNAc6ST3* and *GlcNAc6ST4* (B) and sialyltransferases, *ST3Gal1*, *ST3Gal2* and *ST8GalNAc* (C) in the resistance (BALB HD infection) and susceptible (BALB/c LD infection) mice on day 21 and 35 pi. (Red dashed line = naïve levels). Results represent the mean value of 3-5 mice per group ± SEM. * = P<0.05.** = P<0.01.

The infection established in these mice, as approximately 100 worms were present on after 12 days (day 47) of re-infection (**Figure 5.3B**). However, by day 21 (day 56) pi., infection was cleared (**Figure 5.3B**) and no worms were present in the caecum by day 35 (day 70) pi. Re-challenge with a high dose infection in the chronically infected mice also resulted in inducing goblet cell hyperplasia and elevated expression of IL-13 and IL-4 were observed (**Supplementary Figure 5.2**). Histological analysis with HID-AB staining

revealed that the change from goblet cells containing predominantly sulphomucins to sialomucins observed in chronic infection, could be reverted in the HD infection (**Figure 5.3D**) and the mucins were again predominantly sulphated. Furthermore, the change in sulphated mucins correlated with *T. muris* worm expulsion (**Figure 5.3B**).

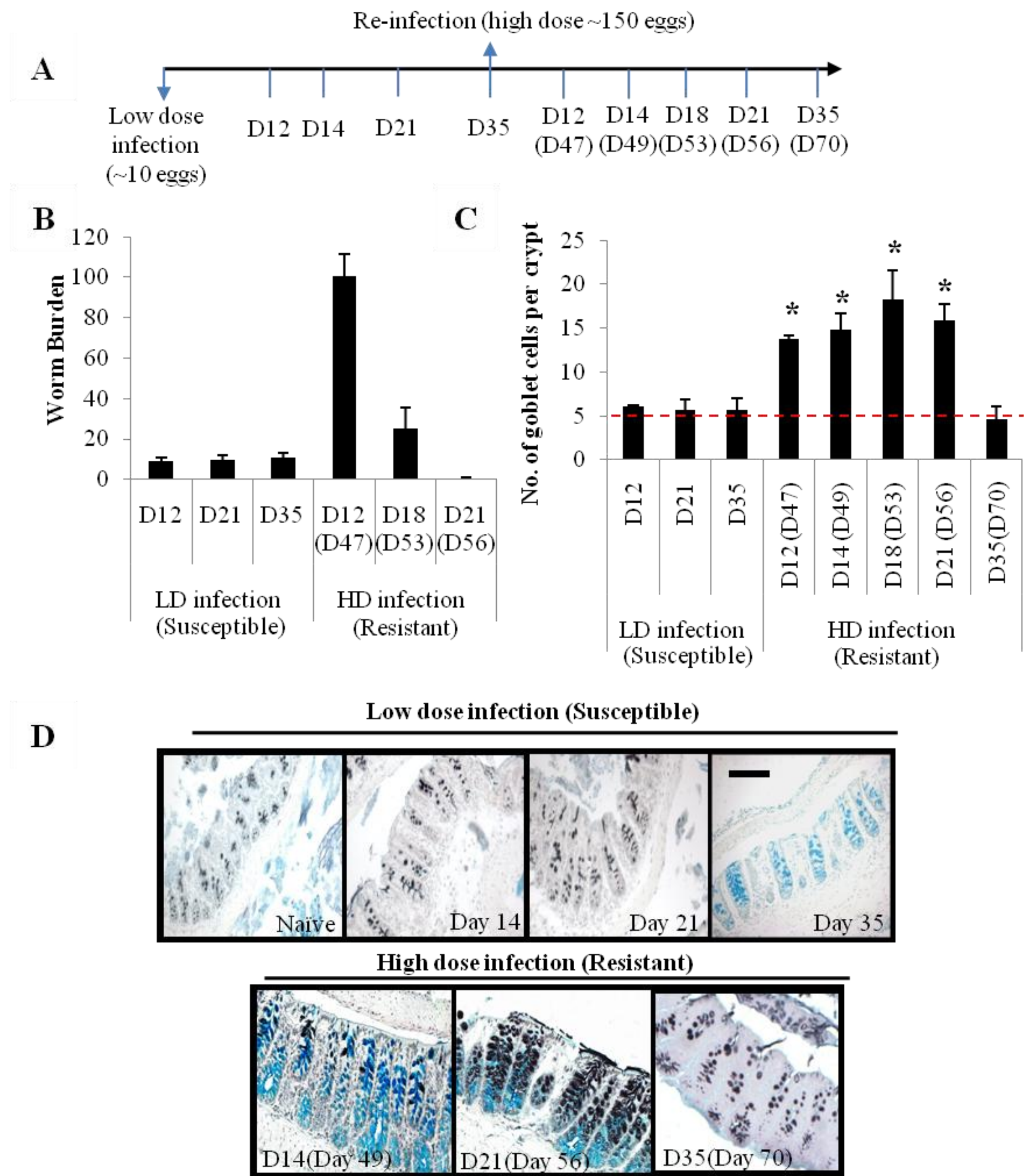


Figure 5.3: Schematic (A) depicting the time course of infection in BALB/c mice. Worm burdens (B) were assessed on Day 21, 21, 35, 47, 53 and 56 pi. Quantitation of goblet cell numbers (C) and HID-AB staining (D) was used to determine the change in glycosylation during the course of the infection. Red dashed line = naïve levels. Results represent the mean value of 3-5 mice per group ± SEM. Scale bar =10μm. * = P<0.05.

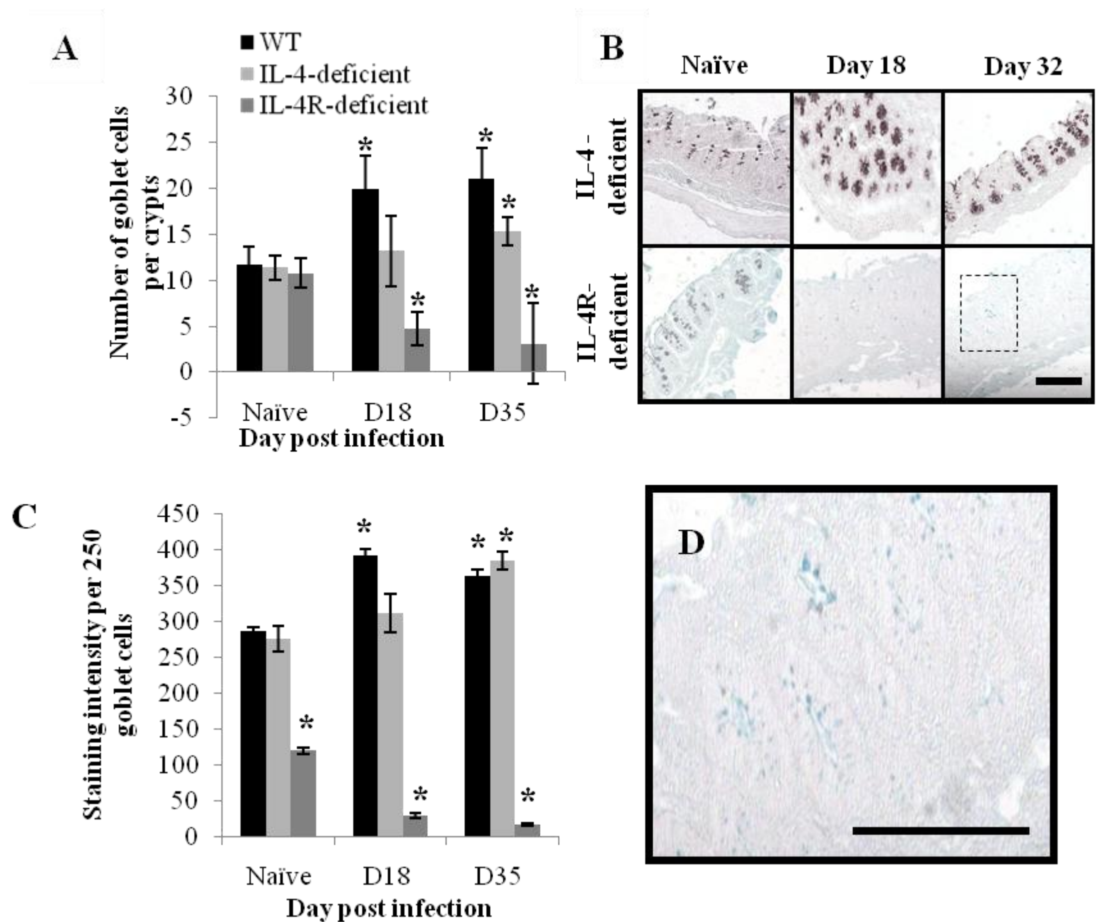


Figure 5.4: Goblet cell numbers (A) were quantified in wild-type (BALB/c), IL-4-deficient and IL-4R-deficient mice post infection. Caecal tissue from IL-4-deficient and IL-4R-deficient mice was stained with HID-AB (B). HID-AB staining intensity measured per 250 goblet cell (D). x20 magnification of dashed box in (B). Results represent the mean value of 3-5 mice per group ± SEM. Scale bar =10µm. * = $P<0.05$.

Sulphation of mucins occurs under the control of IL-13

It is well-established that the mice resistant to *T. muris* mount a strong T_H2-type immune response and those susceptible to chronic infections exhibit a T_H1-type immune (**Supplementary Figure 5.2**) (Cliffe and Grencis, 2004); high levels of sulphomucins were observed in the T_H2-type environment, during resistance. Therefore, to assess the role of the T_H2-type immune response and in particular, IL-4 and IL-13, in mucin sulphation, IL-4-deficient and IL-4R-deficient mice on the BALB/c background (resistant to high dose infection) were infected with a high dose of *T. muris* eggs (**Figure 5.4**). The IL-4-deficient mice are able to expel worms, however worm expulsion is slightly delayed in these as reported previously (Bancroft *et al*, 2000). On day 18 pi., goblet cell hyperplasia was less pronounced in the IL-4-deficient mice when compared to the wild-type mice, although this

was not significantly different. However, by day 35 pi. a significant increase in the number of goblet cells was observed in the IL-4-deficient mice. Furthermore, HID-AB staining showed that goblet cells, in the IL-4-deficient mice, predominantly contained sulphated mucins.

As IL-4 and IL-13 act through the IL-4R, the IL-4R-deficient mice are unable to mount a T_H2-type immune response and are susceptible to chronic infection (Bancroft *et al*, 2000). Strikingly, in contrast to the naïve IL-4-deficient mice, the naïve IL-4R-deficient mice had significantly lower levels of mucin sulphation (**Figure 5.4B** & **C**). As infection progressed, a decrease in the number of goblet cells was also observed in the IL-4R-deficient mice compared to naïve and IL-4-deficient mice. Moreover, the goblet cells lost their 'goblet like morphology', exhibiting very small thecae (**Figure 5.4D**) which stained blue with HID-AB staining, indicative of predominantly containing sialylated mucins (**Figure 5.4B**).

Sulphomucins are less prone to degradation by* T. muris *products

The excretory secretory products (ESPs) released by *T. muris*, thought to be a mixture of enzymes (Drake *et al*, 1994a; Drake *et al*, 1994b), have the potential to alter the intestinal mucus barrier. To test this mucus from wild-type mice and NaS1-deficient mice was treated with 50µg/ml of *T. muris* ESPs for 2 or 6 hours at 37°C. The intestinal mucins from these mice have depleted sulphation, due to the lack of a sulphate transporter (**Figure 5.5A;** Dawson *et al*, 2009). Mucus extracted from the uninfected wild-type and NaS1-deficient mice was subjected to agarose gel electrophoresis and western blotted before staining with PAS to confirm that comparable amounts of mucins were isolated (**Figure 5.5B**).

Staining with HID clearly showed that lower levels of sulphomucins were present in the NaS1-deficient mice compared to wild-type mice (**Figure 5.5A**, **B** & **C**). Treated or untreated mucus was subjected to rate zonal centrifugation to assess the change in size distribution of the mucins. The sedimentation profiles of mucins from untreated wild-type (high sulphomucin content) and NaS1-deficient (lower sulphomucin content) mucus were comparable showing a broad polydispersed distribution of mucins (fractions 9-20) (**Figure 5.5C** & **D**). After treatment with ESPs from *T. muris*, the sedimentation profile of mucins from NaS1-deficient mice was altered. The PAS-positive material from the NaS1-deficient mice shifted towards the top of the gradient, fractions 4-17 (**Figure 5.5D**), compared to PAS-positive material from the wild-type mice which did not change in sedimentation

even after 6 hours of treatment (**Figure 5.5C**). This suggested that mucins with lower sulphation are more susceptible to the degradative effects exerted by the *T. muris* ESPs.

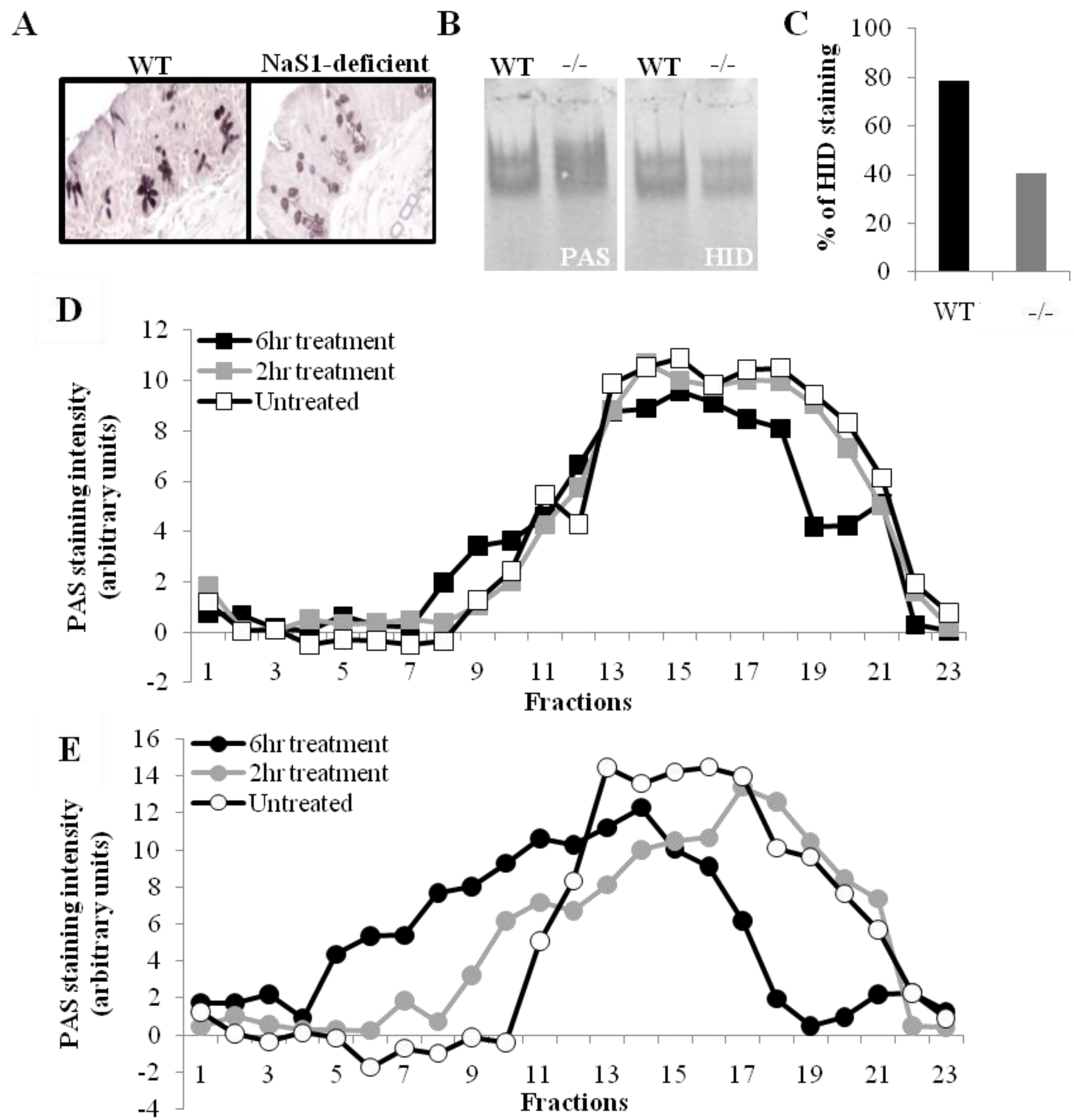


Figure 5.5: HID staining (A) was used to determine the levels of sulphation in the caecum in naïve wild-type (WT) (C57BL/6) and NaS1-deficient (-/-) mice. Caecal mucus extracted was analysed by agarose gel electrophoresis, western blotted and stained using PAS and HID (B). Percentage of HID staining (C) compared to the PAS staining was measured (presented as arbitrary units). Mucus from naïve wild-type (D) and NaS1-deficient (E) mice was subjected to rate-zonal centrifugation before or 2 or 6hrs after treatment with 50μg/ml of *T. muris* ESPs. Fractions were transferred to nitrocellulose membrane, stained with PAS and staining intensity was measured. Results represent the mean value of 3-5 mice per group ± SEM.

Lower mucin sulphation does not affect worm establishment or expulsion

High sulphomucin content that accompanies nematode rejection was partly controlled by IL-13 and therefore the sulphation of mucins in the caecum could affect *T.muris* infection in particular worm establishment and rejection. To address this possibility, NaS1-deficient and their wild-type littermates (C57BL/6-background) were infected with a high dose of *T. muris* eggs. As stated previously, the mucin sulphation in the caecum of the naïve NaS1-deficient mice was lower as compared to the wild-type mice (Dawson *et al*, 2009). This, however, did not have an effect on the establishment of infection (**Figure 5.6**); similar number of worms were present, on day 12 pi., in the NaS1-deficient and wild-type mice. Moreover, as infection progressed, worm expulsion kinetics were similar in the both wild-type and the NaS1-deficient mice (**Figure 5.6**).

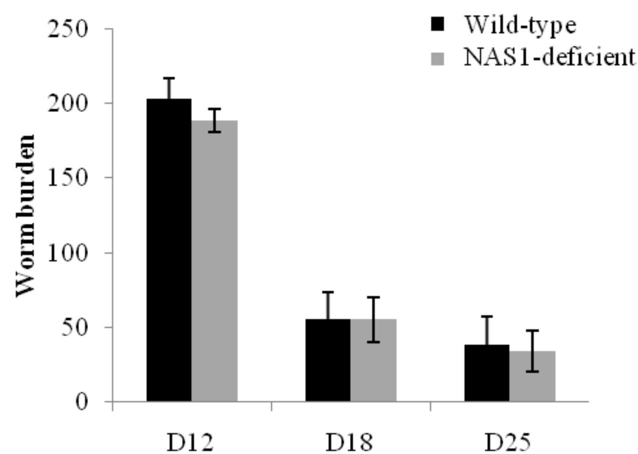


Figure 5.6: Wild-type (C57BL/6) and NaS1-deficient mice were infected orally with ~300 eggs of *T. muris* and worm burdens were investigated on days 12, 18 and 25 post infection. Results represent the mean value of 3-5 mice per group ± SEM.

Recovery of sulphomucins in NaS1-deficient mice after* T. muris *infection

As demonstrated, the NaS1-deficient mice expel *T. muris* over a similar time course as to wild-type mice; RT-PCR was used to determine whether these mice mount a concurrent T_H2-type immune response to *T. muris* infection. The levels of *IL-4* and *IL-13* were elevated in the caecum after infection in the NaS1-deficient and wild-type mice compared to naïve mice (**Supplementary Figure 5.3**). Interestingly, up-regulation of *IL-4* and *IL-13* was significantly lower in the NaS1-deficient mice compared to the wild-type mice on day 18 pi. but the levels were similar on day 25 pi. (**Supplementary Figure 5.3A** & **B**). A 2 to 6-fold increase in *IFN-γ* levels was also observed across the time course of infection (**Supplementary Figure 5.3C**) in both NaS1-deficient and wild-type mice although this did not significantly affect worm expulsion. The effect of the elevated IL-

4/IL-13 levels was reflected in the histological analysis, as goblet cell hyperplasia was observed in the NaS1-deficient mice similarly to the wild-type mice (**Supplementary Figure 5.4**). HID-AB staining intensity showed that mucin sulphation in the caecum of the NaS1-deficient naïve mice was significantly depleted as compared to their wild-type littermates (**Figure 5.7B**). However, as the infection advanced, the levels of mucin sulphation recovered in the NaS1-deficient mice, and by day 25 of infection no difference was observed in the sulphation of the goblet cells in the caecal crypts (**Figure 5.7**).

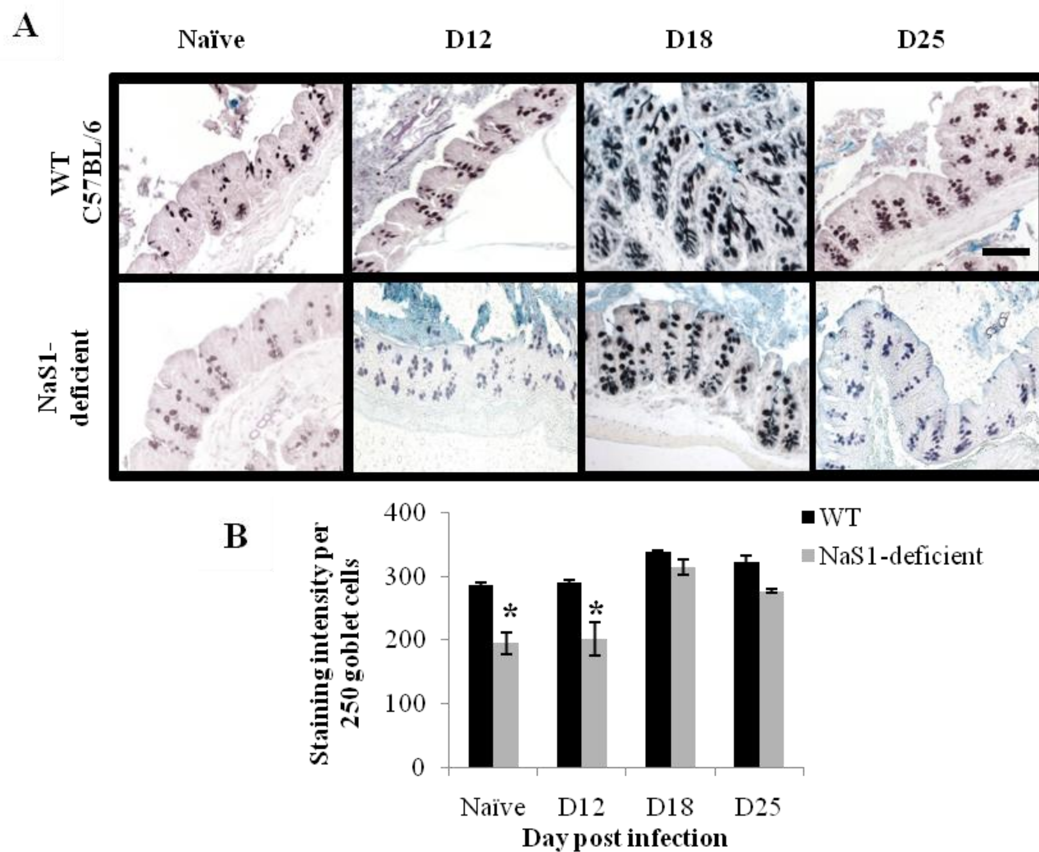


Figure 5.7: HID-AB staining (A) of caecal tissue from wild-type and NAS1-deficient illustrates the changes in glycosylation during infection. HID-AB staining intensity measured per 250 goblet cell (B). Results represent the mean value of 3-5 mice per group ± SEM. Scale bar =10μm.

Up-regulation of Sat1 in the absence of NaS1 after **T. muris** *infection*

As the *T. muris* infection progresses in the NaS1-deficient mice, the depletion in the sulphomucin content within the goblet cells recovered, suggesting that free sulphates were absorbed through another transporter within the caecum. In addition to NaS1, sulphate anion transporter 1 (Sat1) has been shown to be expressed within the caecum (Dawson *et al*, 2010) and may regulate the sulphomucin content within the goblet cells.

Therefore, we used RT-PCR (**Figure 5.8A**) and immunohistochemistry (**Figure 5.8B**), to determine the expression of Sat1 in response to *T. muris* infection. Sat1 expressed was significantly elevated in the NaS1-deficient mice on day 18 pi., however, the up-regulation in the wild-type mice was observed on day 25 pi.

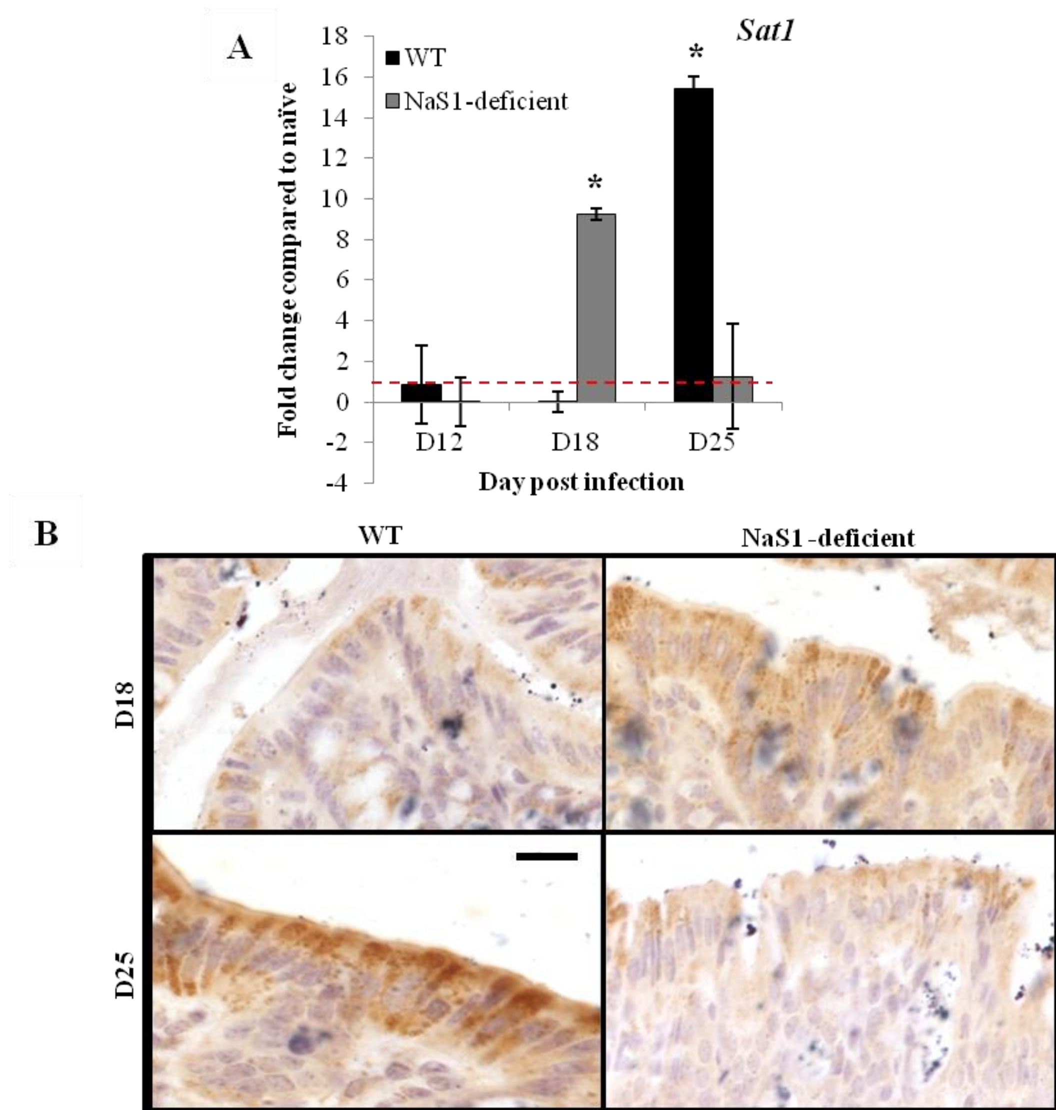


Figure 5.8: Levels of Sat1 were determined using RT-PCR (A) and immunohistochemistry (B) in the WT and NaS1-deficient mice during infection. Results represent the mean value of 3-5 mice per group ± SEM. Scale bar =10μm. * = $P<0.05$.

DISCUSSION

Mucins, the major macromolecular component of the mucus barrier, are heavily glycosylated proteins; glycosylation of mucins is site specific and is thought to be involved in diverse biological processes. Alterations in mucin glycosylation have been reported to

coincide with inflammation in several gastrointestinal diseases such as ulcerative colitis (Corfield *et al*, 1992; Theodoropoulos *et al*, 2001) and during several models of nematode infection; *N. brasiliensis*, *T. spiralis* and *H. polygyrus* (Holmen *et al*, 2002; Karlsson *et al*, 2000; Yamauchi *et al*, 2006). Whether these changes occur as a result of an on-going inflammatory response or as a result of an active change in order to resolve infection is not yet known. Nonetheless, it is thought that these glycans that decorate the mucin protein backbone, contribute to the protective properties of the mucus gel (Thornton *et al*, 2008). Recently we have demonstrated a protective role of mucins, in nematode expulsion (Hasnain *et al*, 2010b); furthermore, we have shown that mucins glycosylation is altered and mucins are more highly charged during resistance (Hasnain *et al*, 2010a).

In this study, we described the changes in glycosylation, in particular sulphation and sialylation, in mouse models that are resistant or susceptible to chronic *T. muris* infection. The strong T_H2 environment, typically seen in resistance, sulphation on the mucins was maintained throughout the course of infection. In contrast, majorly sialylated mucins were found within the goblet cells during chronic *T. muris* infection. To our knowledge the effect of *T. muris* ESPs on mucus has not been previously studied; we demonstrated for the first time that *T. muris* ESPs can degrade mucins and, furthermore that mucins with high sulphation were less prone to degradation. Despite this, depleted mucin sulphation in NaS1-deficient mice did not affect worm establishment. Interestingly though, we found that *T. muris* infection is a strong inducer of mucin sulphation even in the absence of the major apical sulphate transporter, NaS1, in the caecum.

In total, the data presented here suggests that distinct mechanisms regulate glycosyltransferases responsible for mucin sialylation and sulphation, corroborating previously published work (Soga *et al*, 2008). Histological analysis revealed that the changes in glycosylation with chronic *T. muris* infection were gradual and localised within the caecum. Interestingly, similar to goblet cell hyperplasia which is restricted to the caecum (Hasnain *et al*, 2010b), no major changes in sulphation/sialylation of mucins were observed in the colon post *T. muris* infection. This suggested that these changes are a result of a local response to or by the nematode itself. Mirroring the histological changes observed, an increase in the gene expression of sulphotransferases was observed in resistance, and sialotransferases were up-regulated in susceptibility. The loss of sulphation was apparent in naïve IL-4R-deficient mice when compared to the IL-4-deficient mice, suggesting that maintained sulphation is in part due to IL-13. Along with the down-

regulation of goblet cell differentiation transcription factors, during chronic infection, mucin glycosylation is also perturbed: there is evidence of a general loss of glycosylation on mucins in chronic infection (Hasnain *et al*, 2010a). Thus, these alterations in glycosylation could affect the hydration of the mucus gel and consequently affect the viscosity of the barrier. Moreover, the particular changes in glycosylation can also lead to changing the ability of the mucus barrier to sequester host defence factors.

Several studies have shown that parasitic exo-products to have the ability to degrade mucins (Lidell *et al*, 2006). The ESPs of the *T. muris* nematode have been shown to be highly immunogenic (Drake *et al*, 1994a). Although, the adult *T. muris* nematode is closely associated with the mucus barrier, whether ESPs affect the mucus layer has never been investigated before. Therefore, comparable amounts of mucus extracted from naïve NaS1-deficient (less sulphated mucins) and wild-type mice (more sulphated mucins) were treated with *T. muris* ESPs. Interestingly, the less sulphated mucins (from NaS1-deficient mice) were altered in size within 2 hours and thus highly susceptible to degradation. Therefore, this implies that ESPs may be released as part of the nematodes regime to promote its own survival (*addressed in detail in Chapter 6*). The degradation of mucins would therefore aid invasion by diminishing the gel-like consistency of the mucus barrier; as shown previously the mucus barrier is less porous during susceptibility (Hasnain *et al*, 2010b). Furthermore, the data suggests that sulphation plays an important role in preventing degradation, and that highly sulphated mucins protect the mucin protein core from the degradative effects of the nematodes ESPs. How this is achieved requires further investigation, however, a recent study on von Willebrand factor (vWF), a large multimeric glycoprotein homologous to gel-forming mucins, revealed that sialylated glycans increase susceptibility to proteolysis (McGrath *et al*, 2010).

Increased sulphation on intestinal mucins was also shown to affect the establishment of the helminth *Strongyloides venezuelensis* (Ishikawa *et al*, 1995). We, therefore, hypothesised that specific sulphation/sialylation patterns seen during *T. muris* infection, could potentially influence the establishment and/or expulsion of the parasite. The absence of NaS1 results in disturbed sulphate homeostasis leading to depleted intestinal mucin sulphation (Dawson *et al*, 2009). Furthermore, the decrease in mucin sulphation has been shown to increase the susceptibility of these mice to colitis and systemic bacterial infections (Dawson *et al*, 2009). Using the NaS1-deficient mouse model, we attempted to address the role of sulphated mucins in *T. muris* infection. In contrast to

previous observations with *S. venezuelensis* infection (Ishikawa *et al*, 1995), the depleted levels of sulphation had no major effect in the establishment of *T. muris* infection. It is possible that although apparent, the depletion in sulphation was not sufficient to have an effect on worm establishment. Interestingly, the levels of T_H2-type cytokines were lower in the NaS1-deficient mice on day 18 pi. This was reflected in the epithelial cell response, as fewer goblet cells were present in the caecum of these mice. However, these slight differences did not have a significant effect on the rejection of the *T. muris* nematode. Importantly, in addition to the increase in goblet cell numbers, an increase in mucin biosynthesis is also observed during worm expulsion (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b). Therefore, it was surprising to observe that the differences in mucin sulphation did not become more prominent as infection progressed but converged with the T_H2-goblet cell expansion and, by day 18 pi., most of the goblet cell thecae in the NaS1-deficient mice had similar levels of mucin sulphation as compared to the wild-type mice. In the absence of NaS1 (Dawson *et al*, 2003; Dawson *et al*, 2009), an up-regulation of the expression of Sat1 was observed (Dawson *et al*, 2010). Interestingly, Sat1 is elevated with the increasing demand for sulphates during the rejection of *T. muris*, earlier in the NaS1-deficient mice compared to the wild-type mice. Sat1 levels return almost to baseline in the NaS1-deficient mice by day 25 pi., which is when the goblet cell hyperplasia seemed to subside. This indicates that Sat1 stimulates sulphate transport at a time when sulphate requirement has increased.

In summary, maintained mucin sulphation, influenced by the T_H2-type immune response, is clearly a feature of resistance to *T. muris* infection. This may be at least in part a response against *T. muris* as alterations in glycosylation can change the properties of the mucus barrier: highly sulphated mucins (as seen in resistance) are more resistant to degradation. With the high demand of cellular sulphate during *T. muris* infection, Sat1 was up-regulated in the hyposulphataemic NaS1-deficient mice and the wild-type mice. We are currently investigating the role of Sat1 in *T. muris* infection, using the Sat1-deficient mice (Dawson *et al*, 2010). Nevertheless, this study highlights the complex process by which alterations in sulphation occur following *T. muris* infection and indicate that increased sulphomucin levels are a physiological response against this enteric parasite.

REFERENCES

An G, Wei B, Xia B, McDaniel JM, Ju T, Cummings RD, Braun J, Xia L (2007). Increased susceptibility to colitis and colorectal tumors in mice lacking core 3-derived O-glycans. *J Exp Med* **204**(6)**:** 1417-1429.

Bancroft AJ, Artis D, Donaldson DD, Sypek JP, Grencis RK (2000). Gastrointestinal nematode expulsion in IL-4 knockout mice is IL-13 dependent. *Eur J Immunol* **30**(7)**:** 2083-2091.

Bancroft AJ, Else KJ, Humphreys NE, Grencis RK (2001). The effect of challenge and trickle *Trichuris muris* infections on the polarisation of the immune response. *Int J Parasitol* **31**(14)**:** 1627-1637.

Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* **367**(9521)**:** 1521-1532.

Cliffe LJ, Grencis RK (2004). The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol* **57:** 255-307.

Corfield AP, do Amaral Corfield C, Wagner SA, Warren BF, Mountford RA, Bartolo DC, Clamp JR (1992). Loss of sulphate in human colonic mucins during ulcerative colitis. *Biochem Soc Trans* **20**(2)**:** 95S.

Corfield AP, Myerscough N, Bradfield N, Corfield Cdo A, Gough M, Clamp JR, Durdey P, Warren BF, Bartolo DC, King KR, Williams JM (1996). Colonic mucins in ulcerative colitis: evidence for loss of sulfation. *Glycoconj J* **13**(5)**:** 809-822.

Dawson PA, Beck L, Markovich D (2003). Hyposulfatemia, growth retardation, reduced fertility, and seizures in mice lacking a functional NaSi-1 gene. *Proc Natl Acad Sci U S A* **100**(23)**:** 13704-13709.

Dawson PA, Huxley S, Gardiner B, Tran T, McAuley JL, Grimmond S, McGuckin MA, Markovich D (2009). Reduced mucin sulfonation and impaired intestinal barrier function in the hyposulfataemic NaS1 null mouse. *Gut* **58**(7)**:** 910-919.

Dawson PA, Russell CS, Lee S, McLeay SC, van Dongen JM, Cowley DM, Clarke LA, Markovich D (2010). Urolithiasis and hepatotoxicity are linked to the anion transporter Sat1 in mice. *J Clin Invest* **120**(3)**:** 706-712.

Deplancke B, Gaskins HR (2001). Microbial modulation of innate defense: goblet cells and the intestinal mucus layer. *Am J Clin Nutr* **73**(6)**:** 1131S-1141S.

Drake L, Korchev Y, Bashford L, Djamgoz M, Wakelin D, Ashall F, Bundy D (1994a). The major secreted product of the whipworm, *Trichuris*, is a pore-forming protein. *Proc Biol Sci* **257**(1350)**:** 255-261.

Drake LJ, Bianco AE, Bundy DA, Ashall F (1994b). Characterization of peptidases of adult *Trichuris muris*. *Parasitology* **109 (Pt 5):** 623-630.

Else K, Wakelin D (1989). Genetic variation in the humoral immune responses of mice to the nematode *Trichuris muris*. *Parasite Immunol* **11**(1)**:** 77-90.

Hasnain SZ, Thornton DJ, Grencis RK (2010a). Changes in the mucosal barrier during acute and chronic *Trichuris muris* infection. *Parasite Immunology*. *In press.*

Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010b). Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* **138**(5)**:** 1763-1771.

Hill RR, Cowley HM, Andremont A (1990). Influence of colonizing micro-flora on the mucin histochemistry of the neonatal mouse colon. *Histochem J* **22**(2)**:** 102-105.

Holmen JM, Olson FJ, Karlsson H, Hansson GC (2002). Two glycosylation alterations of mouse intestinal mucins due to infection caused by the parasite *Nippostrongylus brasiliensis*. *Glycoconj J* **19**(1)**:** 67-75.

Humphreys NE, Xu D, Hepworth MR, Liew FY, Grencis RK (2008). IL-33, a potent inducer of adaptive immunity to intestinal nematodes. *J Immunol* **180**(4)**:** 2443-2449.

Hurd EA, Holmen JM, Hansson GC, Domino SE (2005). Gastrointestinal mucins of Fut2-null mice lack terminal fucosylation without affecting colonization by Candida albicans. *Glycobiology* **15**(10)**:** 1002-1007.

Ikehara Y, Nishihara S, Yasutomi H, Kitamura T, Matsuo K, Shimizu N, Inada K, Kodera Y, Yamamura Y, Narimatsu H, Hamajima N, Tatematsu M (2001). Polymorphisms of two fucosyltransferase genes (Lewis and Secretor genes) involving type I Lewis antigens are associated with the presence of anti-Helicobacter pylori IgG antibody. *Cancer Epidemiol Biomarkers Prev* **10**(9)**:** 971-977.

Ishikawa N, Shi BB, Khan AI, Nawa Y (1995). Reserpine-induced sulphomucin production by goblet cells in the jejunum of rats and its significance in the establishment of intestinal helminths. *Parasite Immunol* **17**(11)**:** 581-586.

Karlsson NG, Olson FJ, Jovall PA, Andersch Y, Enerback L, Hansson GC (2000). Identification of transient glycosylation alterations of sialylated mucin oligosaccharides during infection by the rat intestinal parasite *Nippostrongylus brasiliensis*. *Biochem J* **350 Pt 3:** 805-814.

Lidell ME, Moncada DM, Chadee K, Hansson GC (2006). Entamoeba histolytica cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. *Proc Natl Acad Sci U S A* **103**(24)**:** 9298-9303.

Linden S, Boren T, Dubois A, Carlstedt I (2004). Rhesus monkey gastric mucins: oligomeric structure, glycoforms and Helicobacter pylori binding. *Biochem J* **379**(Pt 3)**:** 765-775.

Linden SK, Bierne H, Sabet C, Png CW, Florin TH, McGuckin MA, Cossart P (2008a). Listeria monocytogenes internalins bind to the human intestinal mucin MUC2. *Arch Microbiol* **190**(1)**:** 101-104.

Linden SK, Florin TH, McGuckin MA (2008b). Mucin dynamics in intestinal bacterial infection. *PLoS One* **3**(12)**:** e3952.

Longman RJ, Poulsom R, Corfield AP, Warren BF, Wright NA, Thomas MG (2006). Alterations in the composition of the supramucosal defense barrier in relation to disease severity of ulcerative colitis. *J Histochem Cytochem* **54**(12)**:** 1335-1348.

McGrath RT, McKinnon TA, Byrne B, O'Kennedy R, Terraube V, McRae E, Preston RJ, Laffan MA, O'Donnell JS (2010). Expression of terminal alpha2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13. *Blood* **115**(13)**:** 2666-2673.

McKenzie GJ, Emson CL, Bell SE, Anderson S, Fallon P, Zurawski G, Murray R, Grencis R, McKenzie AN (1998). Impaired development of Th2 cells in IL-13-deficient mice. *Immunity* **9**(3)**:** 423-432.

Soga K, Yamauchi J, Kawai Y, Yamada M, Uchikawa R, Tegoshi T, Mitsufuji S, Yoshikawa T, Arizono N (2008). Alteration of the expression profiles of acidic mucin, sialytransferase, and sulfotransferases in the intestinal epithelium of rats infected with the nematode *Nippostrongylus brasiliensis*. *Parasitol Res* **103**(6)**:** 1427-1434.

Spicer SS (1965). Diamine Methods for Differentialing Mucosubstances Histochemically. *J Histochem Cytochem* **13:** 211-234.

Szymanski CM, Wren BW (2005). Protein glycosylation in bacterial mucosal pathogens. *Nat Rev Microbiol* **3**(3)**:** 225-237.

Theodoropoulos G, Hicks SJ, Corfield AP, Miller BG, Carrington SD (2001). The role of mucins in host-parasite interactions: Part II - helminth parasites. *Trends Parasitol* **17**(3)**:** 130-135.

Theodoropoulos G, Hicks SJ, Corfield AP, Miller BG, Kapel CM, Trivizaki M, Balaskas C, Petrakos G, Carrington SD (2005). *Trichinella spiralis*: enteric mucin-related response to experimental infection in conventional and SPF pigs. *Exp Parasitol* **109**(2)**:** 63-71.

Thornton DJ, Carlstedt I, Sheehan JK (1994). Identification of glycoproteins on nitrocellulose membranes and gels. *Methods Mol Biol* **32:** 119-128.

Thornton DJ, Davies JR, Kraayenbrink M, Richardson PS, Sheehan JK, Carlstedt I (1990). Mucus glycoproteins from 'normal' human tracheobronchial secretion. *Biochem J* **265**(1)**:** 179-186.

Thornton DJ, Rousseau K, McGuckin MA (2008). Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* **70:** 459-486.

Thornton DJ, Sheehan JK (2004). From mucins to mucus: toward a more coherent understanding of this essential barrier. *Proc Am Thorac Soc* **1**(1)**:** 54-61.

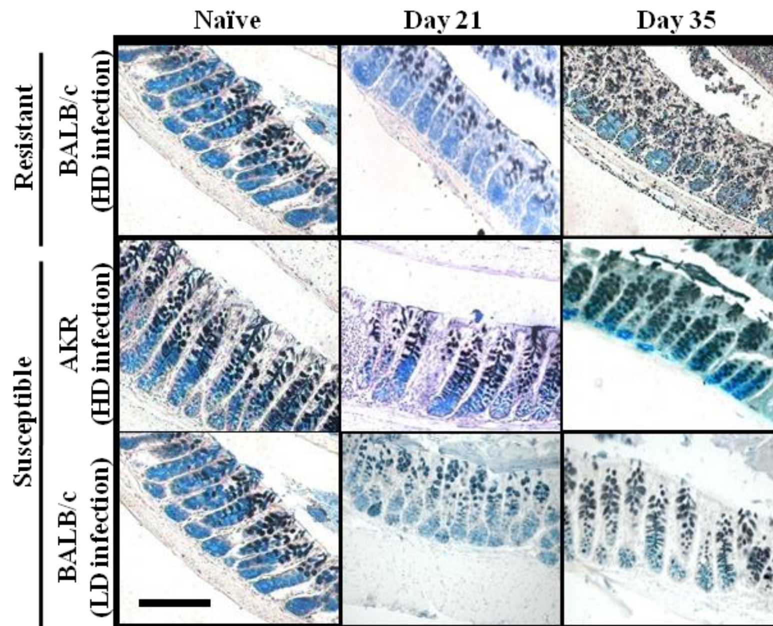
Tobisawa Y, Imai Y, Fukuda M, Kawashima H (2010). Sulfation of colonic mucins by N-acetylglucosamine 6-O-sulfotransferase-2 and its protective function in experimental colitis in mice. *J Biol Chem* **285**(9)**:** 6750-6760.

Tsubokawa D, Nakamura T, Goso Y, Takano Y, Kurihara M, Ishihara K (2009). *Nippostrongylus brasiliensis*: increase of sialomucins reacting with anti-mucin monoclonal antibody HCM31 in rat small intestinal mucosa with primary infection and reinfection. *Exp Parasitol* **123**(4)**:** 319-325.

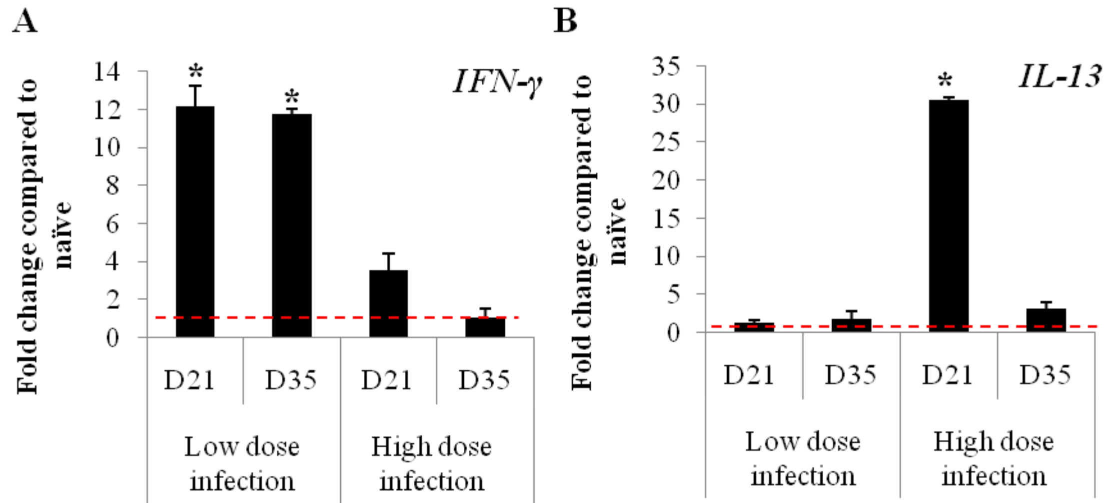
Wakelin D (1967). Acquired immunity to *Trichuris muris* in the albino laboratory mouse. *Parasitology* **57**(3)**:** 515-524.

Yamauchi J, Kawai Y, Yamada M, Uchikawa R, Tegoshi T, Arizono N (2006). Altered expression of goblet cell- and mucin glycosylation-related genes in the intestinal epithelium during infection with the nematode *Nippostrongylus brasiliensis* in rat. *APMIS* **114**(4)**:** 270-278.

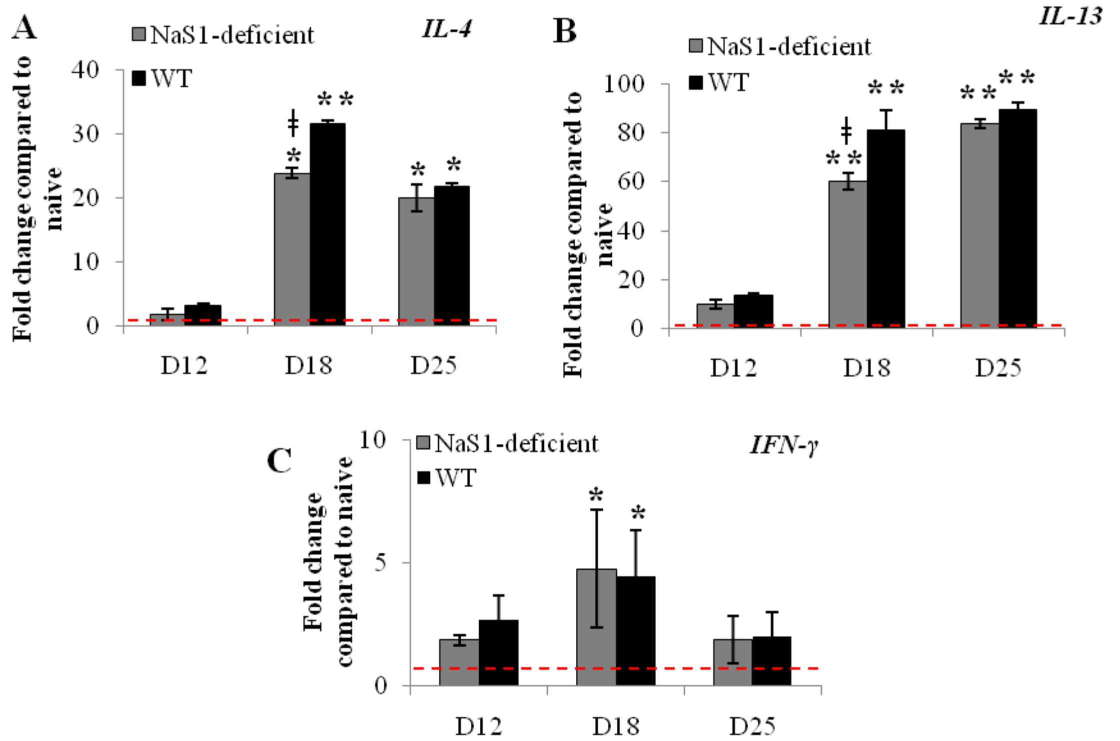
SUPPLEMENTARY FIGURES



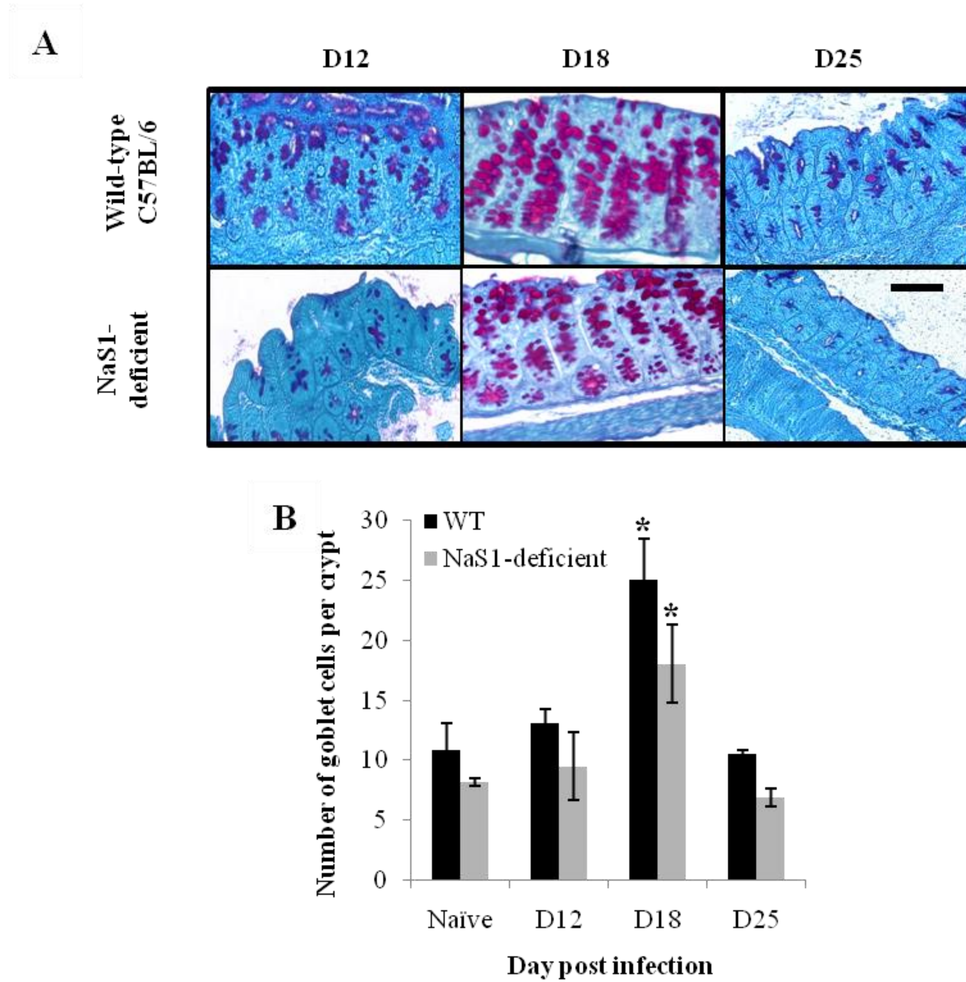
Supplementary Figure 5.1: HID-AB staining of the colon of the models resistant and susceptible to chronic *T. muris* infection. Scale bar =10µm.



Supplementary Figure 5.2: RT-PCR of caecal RNA demonstrated that high levels of *IFN-γ* (A) accompany chronic infection, whereas a subsequent high dose infection leads to inducing high levels of *IL-13* (B). Red dashed line = naïve levels. Results represent the mean value of 5 mice per group ± SEM. * = $P<0.05$.



Supplementary Figure 5.3): RT-PCR was used to determine the levels of T_H2-type cytokines: *IL-4* (A) and *IL-13* (B), and the T_H1-type cytokine; *IFN-γ* (C) in the wild-type and NaS1-deficient mice during infection . Red dashed line = naïve levels. Results represent the mean value of 3-5 mice per group ± SEM. * = P<0.05 and ** = P<0.01, compared to naïve controls. ǂ = P<0.05 compared to wild-type day 18 pi.



Supplementary Figure 5.4: PAS staining was used to determine the changes in goblet cells during infection in NaS1-deficient and WT mice. Quantitation of goblet cell numbers (B) shows the increase in goblet cell numbers after *T. muris* infection. Results represent the mean value of 3-5 mice per group ± SEM. Scale bar =10μm. * = $P<0.05$.

SUPPLEMENTARY TABLE

Gene of interest	5'-Forward primer-3'	5'-Reverse primer-3'
Gal3ST1	CCTGCTCGTCTCTTCGAGTC	GTAGTAGCGATCGGGGTCCT
Gal3ST2	AGGCTGGCTCAGACCTGTTC	CCACTACCTGCTGAGAGGCT
Gal3ST3	GAGGAGGTCTTCTCGCTCGT	GTAGAGCACGTCGTCCAGGT
Gal3ST4	GCTAACTCAGGGGGCAGACT	GGCAGAGAGAGCTGGAGATG
GlcNAc6ST1	TCCATACTAACGCCAGGAACG	TGGTGACTAAGGCTGGAACC
GlcNAc6ST2	TGCTGGTACTGTCCTCGTGG	TGATGTTGCCACGAGCGAAGG
GlcNAc6ST3	TCAACCTAAAGGTGGTGCAACT	GGTTAAGAAGAAATCAGCGCGT
GlcNAc6ST4	AAGCCCTACAACCTGGATGTG	GAGTTGCGCACTGTGCTGTAT
ST3Gal1	CTAGAGGGTGGCTTCACCTG	AGAGGACAGAGGGACAGCTG
ST3Gal2	GGGCCTCACTAGTGTCAGGA	CGCTGAGAAGGTGGTAGGAG
ST6GalNAc	CTGAGGGGAAGGAGAAGAGG	ACTACCCAGAGCTCCAGGAG
ST8GalNac	GCCCTACATACGTCCAGAGG	CCTCTGTGTGGTGGTCCTCT
IL-13	CTCCCTCTGACCCTTAAGGAG	GAAGGGGCCGTGGCGAAACAG
IL-4	GAGCTCGTCTGTAGGGCTTC	GCCCGAAAGAGTCTCTGC
IFN-γ	AGCTCTTCCTCATGGCTGTTTC	ATGTTGTTGCTGATGGCCTGA
β-actin	ATGCTCCCCGGGCTGTAT	CATAGGAGTCCTTCTGACCCATTC

Supplementary Table 5.1: Forward and reverse primer sequences.

SERINE PROTEASES SECRETED BY THE NEMATODE *Trichuris muris* DEGRADES Muc2

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ABSTRACT

The *Trichuris* nematodes secrete immunogenic excretory secretory products (ESPs), which contain proteases. These nematodes survive in the host by tunnelling through the caecal epithelium, while their posterior end remains free in the lumen within the mucus layer. We investigated the effect of ESPs on the mucin components of the intestinal mucus barrier, in particular Muc2 by assessing their effect on the sedimentation behaviour of the mucins on 6-8M guanidinium chloride gradients. The ESPs isolated from adult *T. muris* nematode markedly decreased the sedimentation rate of mucins isolated from naïve mice indicating that they were degraded. Using different doses of infection, susceptibility (low dose) and resistance (high dose) to *T. muris* infection was modelled. During worm expulsion, the mucins isolated from resistant mice were less prone to the degradative effects exerted by the ESPs. Surprisingly, the main intestinal Muc2 mucin was degraded by the ESPs; however ESPs were unable to alter the sedimentation behaviour of the Muc5ac mucin which is induced in the intestine during worm expulsion. To further investigate the effects of the degradative of ESPs we employed MUC2 produced by the human cell line, LS147T. ESPs were shown to have the ability to depolymerise the network formed by the intestinal mucin, MUC2. Interestingly, ESPs contained serine proteases that degraded MUC2 and potentially affect the uncharacterised 'non-reducible' MUC2 linkage. We demonstrate for the first time that during chronic infection, ESPs released by the *T. muris* nematode consist of serine proteases that have the ability to degrade the mucus gel, which may be part of its regime to aid colonisation within the host.

ABBREVIATIONS

PAS, Periodic-Acid Schiff's; pi., Post infection; T_H, T Helper; *T. muris*, *Trichuris muris*; LD, Low dose infection; HD, High dose infection; ESP, Excretory secretory products.

INTRODUCTION

Trichuriasis is a chronic infection caused by the nematode *Trichuris*, which is a ubiquitous gastrointestinal nematode of vertebrates that affects up to a billion people worldwide (Bethony *et al*, 2006; Cliffe and Grencis, 2004). The adult *Trichuris* worm shares a very intimate relationship with the mucus barrier that blankets the intestinal epithelial surface (Artis and Grencis, 2008). Their anterior ends penetrate the intestinal crypts by forming syncytical tunnels within the epithelial cells; while their posterior ends extend into the intestinal lumen (Else, 2005). The extracellular mucus barrier is an essential part of the innate immune system. It provides physical protection and, by sequestering host defence factors within its complex matrix provides a 'chemical' barrier to protect the underlying epithelium (Thornton *et al*, 2008). Goblet cells present in the epithelial cell layer are specialised secretory cells responsible for the production of the major structural components (gel-forming mucins) that form the mucus barrier (Thornton and Sheehan, 2004).

Excretory secretory products (ESPs) released from the *Trichuris* nematode in different species are thought to be very well-conserved and similar in terms of their antigenicity (Roach *et al*, 1988). Drake and co-workers have shown that the major protein secreted by the *Trichuris* nematode has the ability to induce ion-conducting pores in a lipid bilayer, which in turn may aid nematode invasion (Drake *et al*, 1994a). Other studies have attributed the tunnel formation through the intestinal epithelium, to protease activity (zinc metalloproteases, thiol protease and phenol oxidase) detected in the ESPs (Hill *et al*, 1993; Hill and Sakanari, 1997). Although several studies have shown that ESPs released through the course of infection are highly immunogenic, their function *in vivo* remains largely unknown.

Recently, we have demonstrated that gel-forming mucins are an important component of the response involved in the rejection of the *Trichuris muris* (*T. muris*) nematode (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b). In this study, we investigated the effect of the ESPs on the mucus gel and demonstrated that ESPs have the ability to alter the mucus gel, by affecting the polymerisation of the mucin network. Our findings also show that serine proteases within the ESPs degrade Muc2 (the major mucin in the intestine) but not Muc5ac (mucin expressed during worm expulsion). These data suggest that one of the functions of the nematode secreted ESPs is to alter the gel-like consistency of the mucus gel and therefore aid establishment.

MATERIALS AND METHODS

Animals

BALB/c (Harlan Olac) mice were maintained in the Biological Services Unit at The University of Manchester. The protocols employed were in accordance with guidelines by the Home Office Scientific Procedures Act (1986). All mice (6-12wk old) were kept in sterilized, filter-topped cages, and fed autoclaved food.

Parasitological technique

The techniques used for *T. muris* maintenance and infection were described previously (Wakelin, 1967). Mice were orally infected with approximately 150 eggs for a high dose infection and <15 eggs for a low dose infection. Worm burdens were assessed by counting the number of worms present in the caecum. Worms present in the caecum on day 12 confirmed that infection had established in both groups of mice (high dose and low dose infection). Adult worms present in the caecum of the low dose infected group of mice, on day 35 post infection, confirmed chronic infection (**Supplementary Figure 6.1**).

Antibodies

Immunodetection was carried out using a polyclonal antibody raised against a murine Muc2 (mMuc2) (Hasnain *et al*, 2010b; Heazlewood *et al*, 2008) or human MU2 (hMUC2) (Herrmann *et al*, 1999). Commercially available 45M1 antibody was used for the detection of mouse Muc5ac (Lidell *et al*, 2008).

Mucus extraction and treatment with **T. muris** *excretory secretory products*

The caecum was gently flushed with PBS to remove the faecal matter, subsequently scraped into equal volumes of PBS and kept at -80°C until required. The *T. muris* ESPs were isolated using the method previously described (Else and Wakelin, 1989). Aliquots of crude mucus were incubated at 37°C with the ESPs at 50µg/ml for various time points (later specified). Control samples were not treated with the ESPs, but were incubated at 37°C for the maximum time point. ESPs were heat inactivated at 100°C before incubation or incubated at 4°C with mucus as negative controls. ESP activity was quenched using the protease inhibitors: ethylenediaminetetraacetic acid (EDTA), N-ethylmaleimidide (NEM), Lupeptin, Chymostatin and Antipain at 50-150µg/ml concentrations.

Agarose gel electrophoresis

Extracted mucus samples were solubilised in 6M Urea and subsequently reduced using 50mM dithiothreitol (DTT) and carboxylmethylated using 0.125M iodoacetamide prior to electrophoresis on a 1% (w/v) agarose gel for 22 hours. Whole mucus samples were separated by agarose gel electrophoresis before reduction in SSC (0.6M NaCl, 60mM sodium citrate) containing 0.1M DTT for 30 minutes. The fractions were taken from the top of the tubes, analysed by slot blotting or agarose gel electrophoresis followed by western blotting on to a nitrocellulose membrane (Thornton *et al*, 1990). Mucins were detected by using PAS staining or mucin-specific antisera. Staining intensity was measured using the GS-800 calibrated densitometer (Bio-Rad Laboratories, U.K).

Rate zonal centrifugation

6–8M guanidinium chloride (GuCl) gradients were formed in centrifuge tubes using an MSE gradient maker connected to a Gilson Minipuls 2 peristaltic pump. Mucin samples (in 4M GuCl) were loaded onto the tops of the gradients and centrifuged in a Beckman Optima™ L-90K Ultracentrifuge (Beckman SW40 rotor) at 40,000RPM for 2.75 hours for whole mucus extracts and 3.50 hours for purified mucins (15°C). The refractive index of each fraction was measured using a refractometer; all gradients were comparable (data not shown).

Purification of mucins

Mucins were purified using the isopycnic density gradient centrifugation as described by Davies and Carlstedt, 2000. In brief, solubilised mucus was purified using caesium chloride (CsCl)/4M GuCl density gradient at a starting density of 1.4g/ml, centrifuged in a Beckman Optima™ L-90K Ultracentrifuge (Beckman Ti70 rotor) at 40,000RPM for 65 hours at 15°C. Periodic Acid Schiff's (PAS) rich fractions were pooled, dialysed into 0.2M GuCl before being subjected to a second CsCl density gradient centrifugation (at a starting density of 1.5g/ml) (Davies and Carlstedt, 2000).

Anion-Exchange Chromatography

The PAS-rich fraction pooled after the second CsCl density gradient was subjected to anion exchange chromatography as describe previously (Linden *et al*, 2002; Thornton *et al*, 1996), using a Resource™ Q column. Samples were eluted with the starting buffer (20mM Tris-HCl at pH8) for 15 minutes (0.5ml/min), followed by a linear gradient (60 minutes)

up to 0.4M Lithium perchlorate-10mM piperazine at pH5 in 6M Urea containing 0.02% 3-[(chloamidopropyl) dimethylammonio]-1-propanosulphonate (Linden *et al*, 2002; Thornton *et al*, 1996).

RESULTS

T. muris *secretes products that can degrade the mucus gel*

ESPs released from the *T. muris* nematode, thought to be a mixture of enzymes were isolated; SDS-PAGE analysis showed that ESPs contained several components (**Supplementary Figure 6.2**) and a major 43kDa protein (Drake *et al*, 1994a). The mucus collected from uninfected BALB/c mice was treated with 50μg/ml of ESPs for 24 hours. Treated and untreated (control) mucus was subjected to rate zonal centrifugation to assess whether the ESP treatment can alter the size distribution of mucins. Gradients were fractionated from the top, transferred to nitrocellulose membrane and stained with PAS and mMuc2 antibody (**Figure 6.1**).

The typical size distribution of 'whole' intestinal mucins from mice is shown in **Figure 6.1A**. The PAS staining showed that the general carbohydrate response was comparable to the mMuc2 antibody reactivity; therefore, confirming that the major glycoprotein within the whole mucus sample was Muc2. PAS and mMuc2 antibody reacted with a component of the whole mucus which sedimented to the bottom of the gradient. This may be the 'insoluble' component of the gel which was characterised previously by Carlstedt *et al*, 1982 . Due to cross-reactivity of mMuc2 with components in the ESPs, PAS was used to detect mucins (Muc2) after treatment with ESPs (**Supplementary Figure 6.2**). After 24 hours of treatment with the ESP products, the mucins were redistributed to the top of the gradient. The slower sedimentation rate of mucins after ESP treatment indicated that the mucus was altered and mucins had a decreased size. Importantly, the ESPs were unable to have this affect on the mucus gel if heat inactivated or incubated at 4◦C (**Figure 6.1B**), suggesting that enzyme activity was responsible for the degradation of mucins**.**

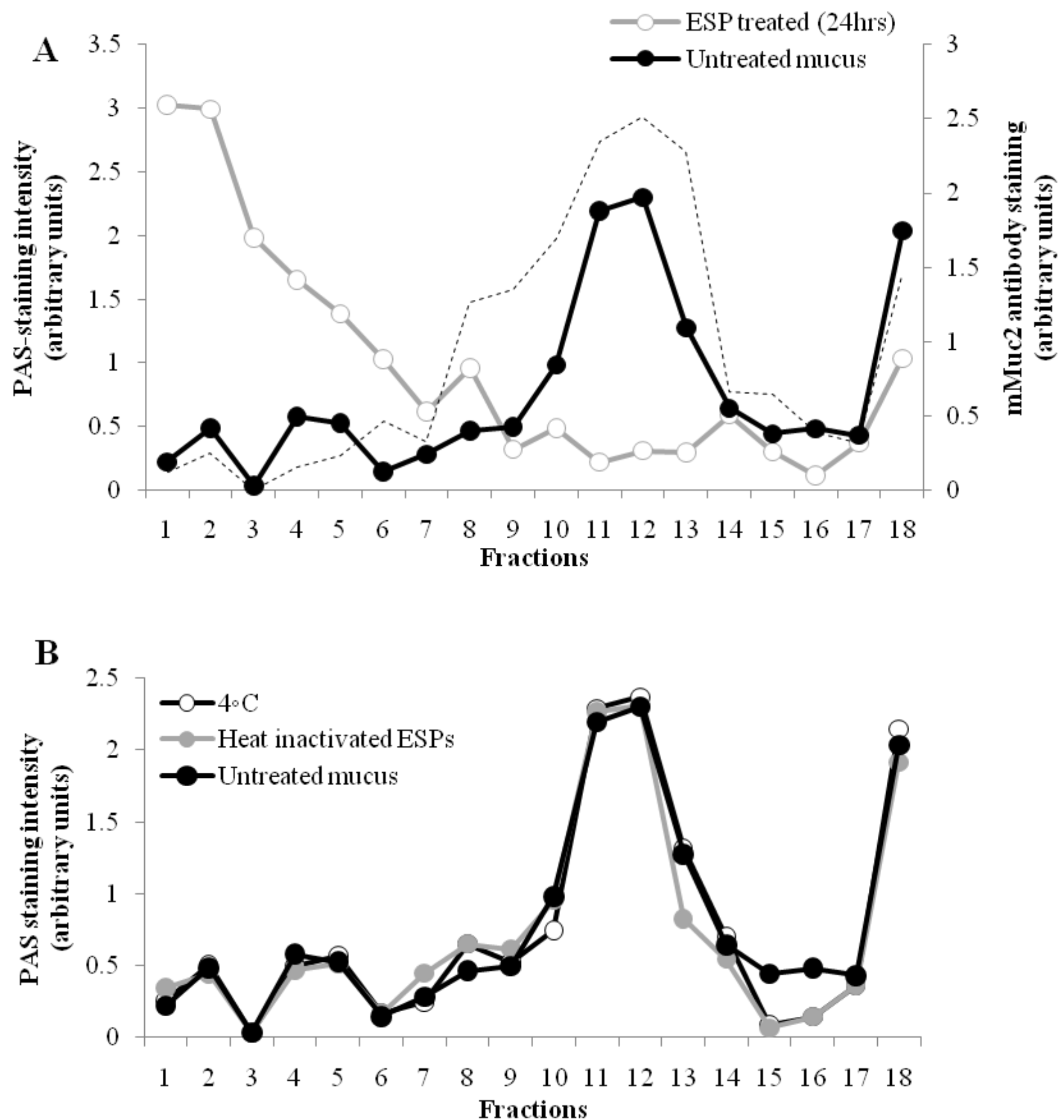


Figure 6.1: Mucus extracted from uninfected BALB/c mice was treated with either 50µg/ml of ESPs for 24 hours (A), heat inactivated 50µg/ml of ESPs at 37°C or treated at 4°C for 24 hours (B) before being subjected to 6-8M GuCl rate zonal gradients. Subsequently fractions were taken from the top of the gradient and analysed by PAS-staining. Fractionated untreated mucus was transferred to nitrocellulose membrane and probed with the mMuc2 antibody (dashed line). Representative of 3-5 mice.

Mucins are less prone to degradation during resistance

Previous studies from our laboratory have shown that the composition of the mucus barrier is altered during infection with *T. muris* (Hasnain *et al*, 2010b): in resistance, the mucus barrier is formed of a less porous network containing a higher ratio of mucins which are differentially glycosylated (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b). Therefore, we sought to determine whether these changes in the mucus barrier during worm rejection could hinder the ability of *T. muris* ESPs to degrade the mucins. To this end, mucus was

extracted from resistant (high dose infection in BALB/c mice) and susceptible (low dose infection in BALB/c mice) mice on day 14 pi., when infection has established in both models, and treated with ESPs (**Supplementary Figure 6.3**). Untreated and treated (50µg/ml of *T. muris* ESPs for 24 hours) mucins were subjected to rate zonal centrifugation prior to fractionation and analysis. The rate zonal profiles of mucins extracted on day 14 pi. in the resistant and susceptible mice after treatment with *T. muris* ESPs (**Supplementary Figure 6.3**) showed that mucins were altered as previously observed with mucin extracts from naïve mice (**Figure 6.1A**).

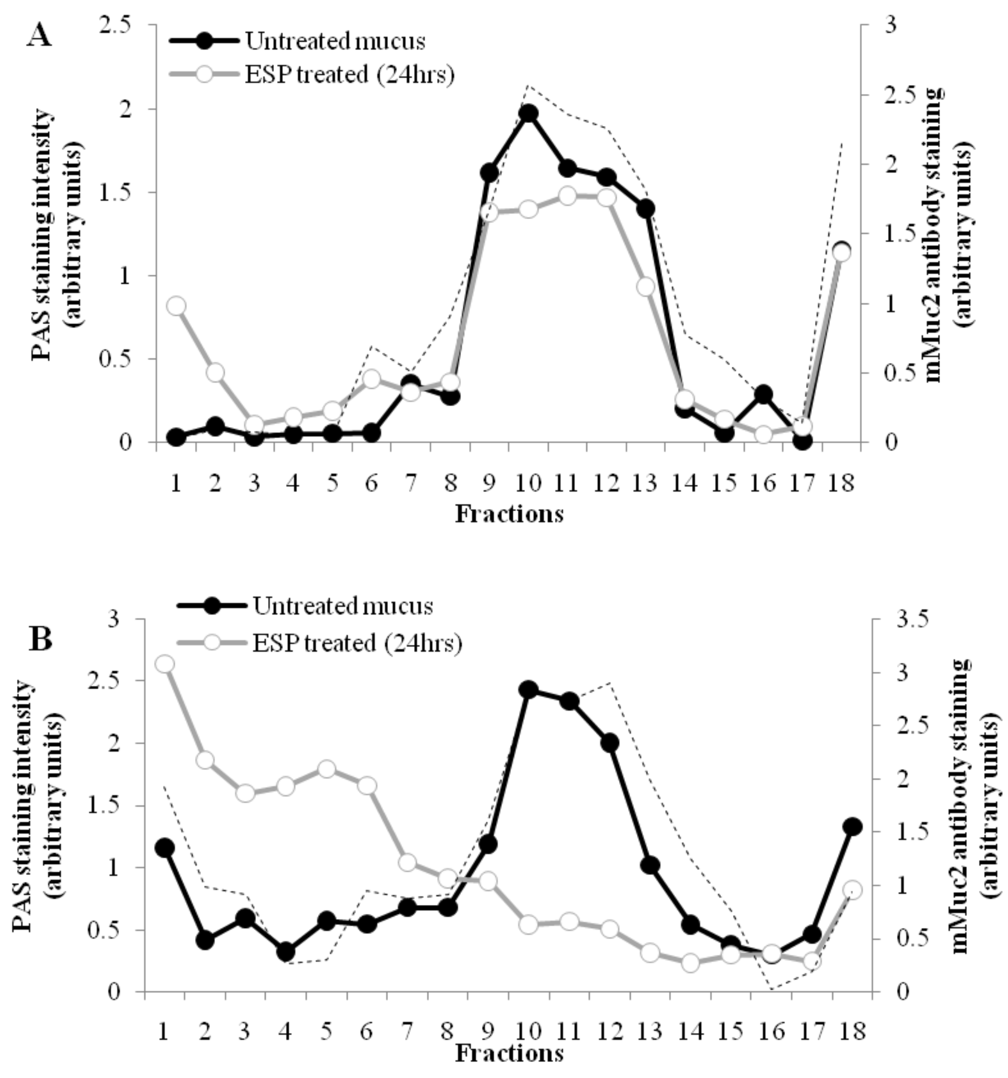


Figure 6.2: Crude mucus extracted on day 21 of infection from resistant (high dose infection) in BALB/c (A) and susceptible (low dose infection) in BALB/c (B) mice was treated with 50µg/ml of ESPs for 24 hours and subjected to 6-8M GuCl rate zonal gradients. Subsequently fractions were taken from the top of the gradient and analysed by PAS-staining. Fractionated untreated mucus was transferred to nitrocellulose membrane and probed with the mMuc2 antibody (dashed line). Representative of 3 mice.

It is well established that worms are expelled in mice resistant to chronic infection by day 21. Interestingly, a difference was observed in the ability of ESPs to alter the mucins extracted from resistant and susceptible mice. The sedimentation profile of the mucins from resistant mice (extracted on day 21 pi.) was not altered when treated with 50μg/ml of *T. muris* ESPs (**Figure 6.2A**). Moreover, higher concentrations of ESP (up to 300μg/ml) did not have an effect on the sedimentation profile of the mucins extracted from resistance mice on day 21 pi. (data not shown). Furthermore, mucins isolated from resistant mice extracted on day 56 pi., 35 days after the expulsion of the nematode were only partially degraded with ESP treatment (**Supplementary Figure 6.4**). It is important to note that some of the mucin content of the mucus samples extracted from the susceptible mice on day 21 appeared degraded before treatment (**Figure 6.2**). In contrast, the main PAS-peak, of mucus from the susceptible mice (on day 21 pi.) did alter when treated with ESPs (**Figure 6.2B**); suggesting mucins were prone to degradation during susceptibility.

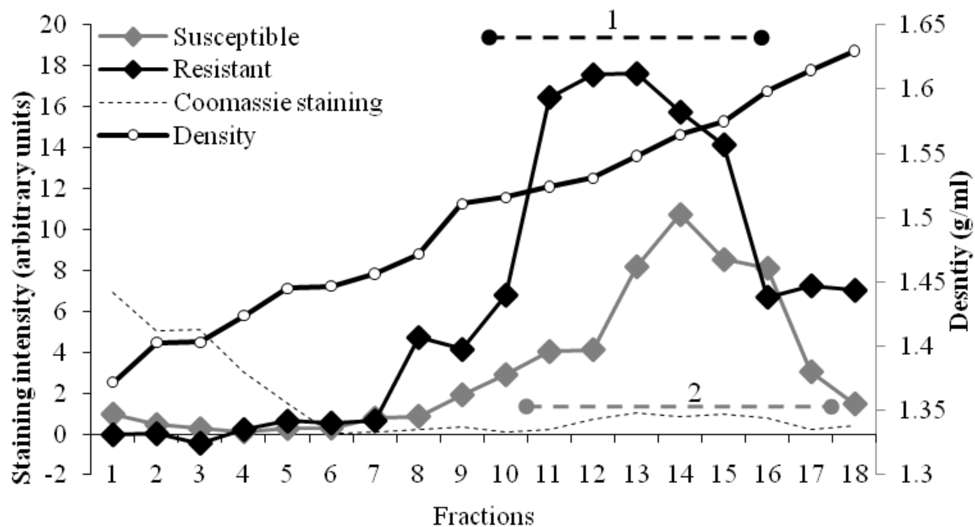


Figure 6.3: CsCl-density gradient centrifugation in 4M GuCl of mucus extracted from resistant (high dose infection in BALB/c) and susceptible (low dose infection in BALB/c) mice on day 21 after infection. Fractions were analysed by PAS and coomassie staining intensity and, density was measured. PAS-rich fractions from resistant (1) and susceptible (2) mice were pooled as shown corresponding dashed lines.

A difference was observed in the ability of ESPs to alter resistant and susceptible mucins on day 21 after infection (**Figure 6.2**). To investigate the effect of ESPs on mucins in more detail, the mucus samples were reduced and carboxymethylated. Subsequently, the mucins from resistant and susceptible mice on day 21 pi., were purified from the mucus by two isopycnic density gradient centrifugation steps: firstly in CsCl/4M GuCl to separate

mucins from lower buoyant density proteins (**Figure 6.3**) (Davies and Carlstedt, 2000). Following the second step in CsCl/0.2M GuCl, used to separate mucins from nucleic acid (**Figure 6.4**).

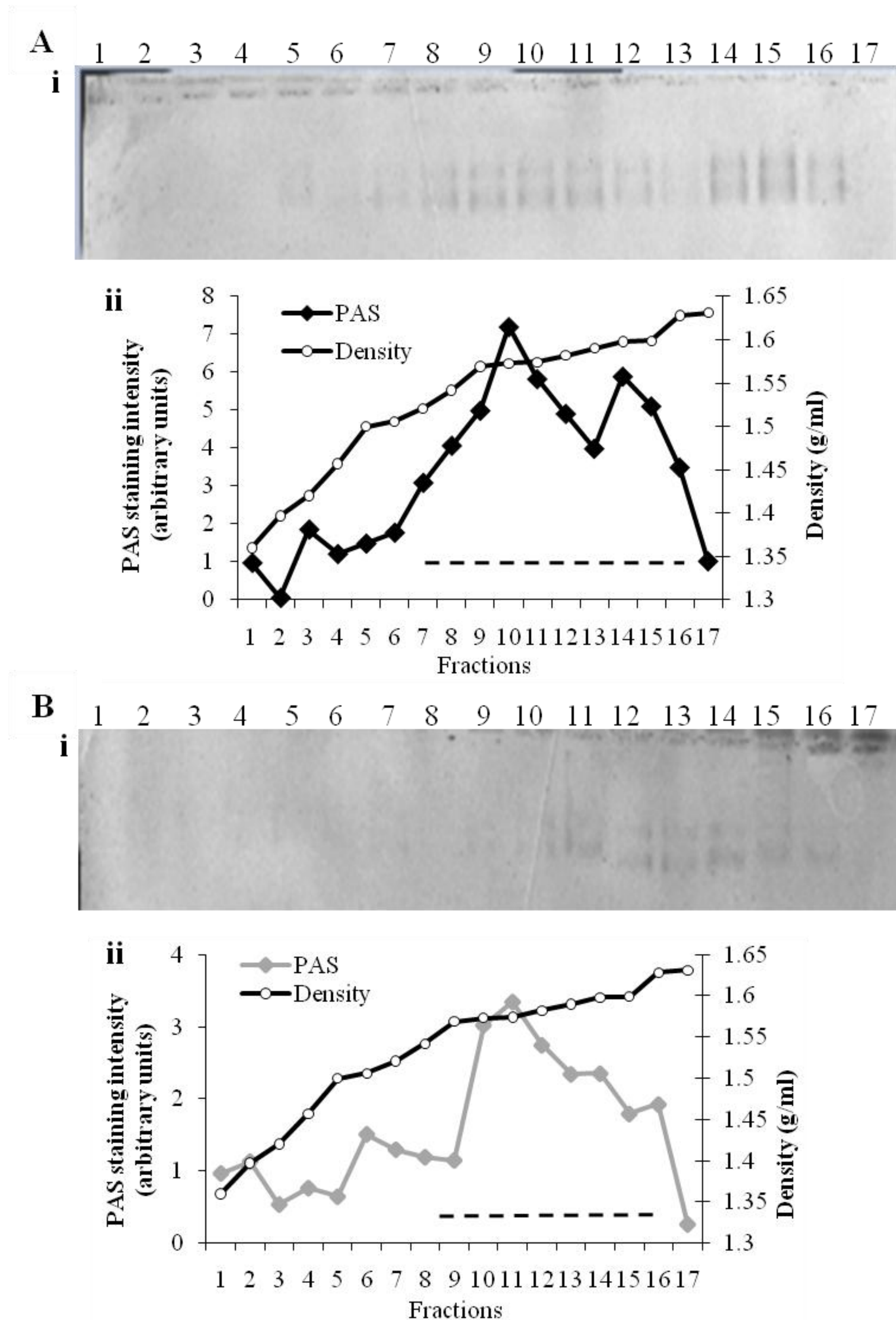


Figure 6.4: PAS-rich fractions from resistant (A; high dose infection in BALB/c) and susceptible (B; low dose infection in BALB/c) mice, on day 21 after infection, (Figure 6.4) were subjected to a CsCl density gradient in 0.2M GuCl. Fractions were analysed by slot blotting onto a nitrocellulose membrane (ii) or separated by agarose gel electrophoresis (i), western blotted onto a nitrocellulose and stained with PAS. PAS-rich fractions (as highlighted by the dashed line) were pooled.

The buoyant density of the mucins purified from resistant and susceptible mouse intestinal mucus was similar, however as expected higher amounts of mucins were present in the mucus of resistant mice (on day 21) (Hasnain *et al*, 2010b) as compared to susceptible mice as seen by agarose gel electrophoresis and slot blotting (**Figure 6.4**). Subsequently, the mucin-rich fractions were further purified by using anion exchange chromatography (**Figure 6.5**). As previously reported, *de novo* expression of Muc5ac is observed during *T. muris* infection in the caecum of resistant mice (Hasnain *et al*, 2010b). Therefore, fractions were analysed by PAS, mMuc2 antibody and Muc5ac staining (45M1 antibody); as expected the PAS-positive material coincided with Muc2 and Muc5ac staining (**Figure 6.5A**). The chromatogram shows that Muc2 was eluted before Muc5ac (**Figure 6.5**); therefore suggesting that within the mucus barrier, Muc5ac is more highly charged as compared to Muc2. No Muc5ac was detected in the mucus from susceptible mice (data not shown).

***T. muris* ESPs are unable to degrade Muc5ac**

The purified mucins from the resistant mice were separated into the Muc2 and Muc5ac-rich fractions and treated with 50μg/ml of ESPs for 24 hours. After treatment, samples were analysed by rate zonal centrifugation; the gradients were centrifuged for a longer period of time as the samples were previously reduced and carboxymethylated. Fractions analysed by slot blotting and PAS staining revealed that ESPs degrade Muc2, considering a significant amount of Muc2 was present in the fractions at the top of the gradient after treatment (**Figure 6.6A**). This suggests that ESPs can affect the protein core, glycosylation of Muc2 and/or polymerisation of mucins. Unexpectedly, the sedimentation profile of the Muc5ac-rich fraction was largely unaltered after treatment with ESPs (**Figure 6.6B**). This implied that ESPs specifically act on the intestinal mucin, Muc2 and not on the Muc5ac mucin which is only induced in the resistant mice with exposure to *T.muris* infection.

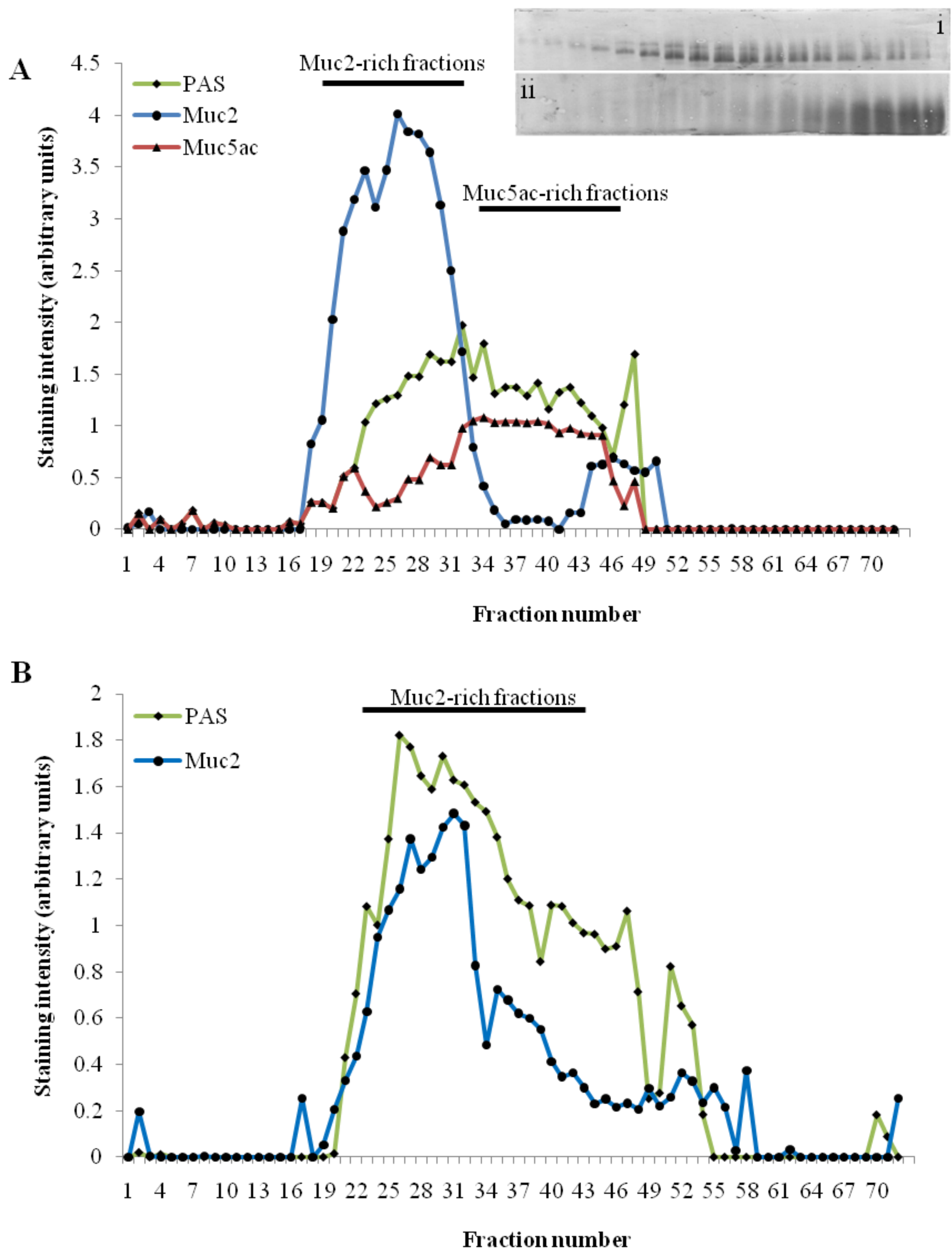


Figure 6.5: Anion-exchange chromatography on PAS-rich fraction from Figure 6.5 (A; high dose infection in BALB/c) and susceptible (B; low dose infection in BALB/c) mice, on day 21 after infection. Fractions were transferred onto a nitrocellulose membrane and stained with Muc2 (alternate fractions 19-37) or Muc5ac antibodies (alternate fractions 28-46). PAS-rich fractions from the resistant (BALB/c) mice was subjected to agarose gel electrophoresis, western blotted and stained with mMuc2 antibody (Ai) or Muc5ac antibody (Aii).

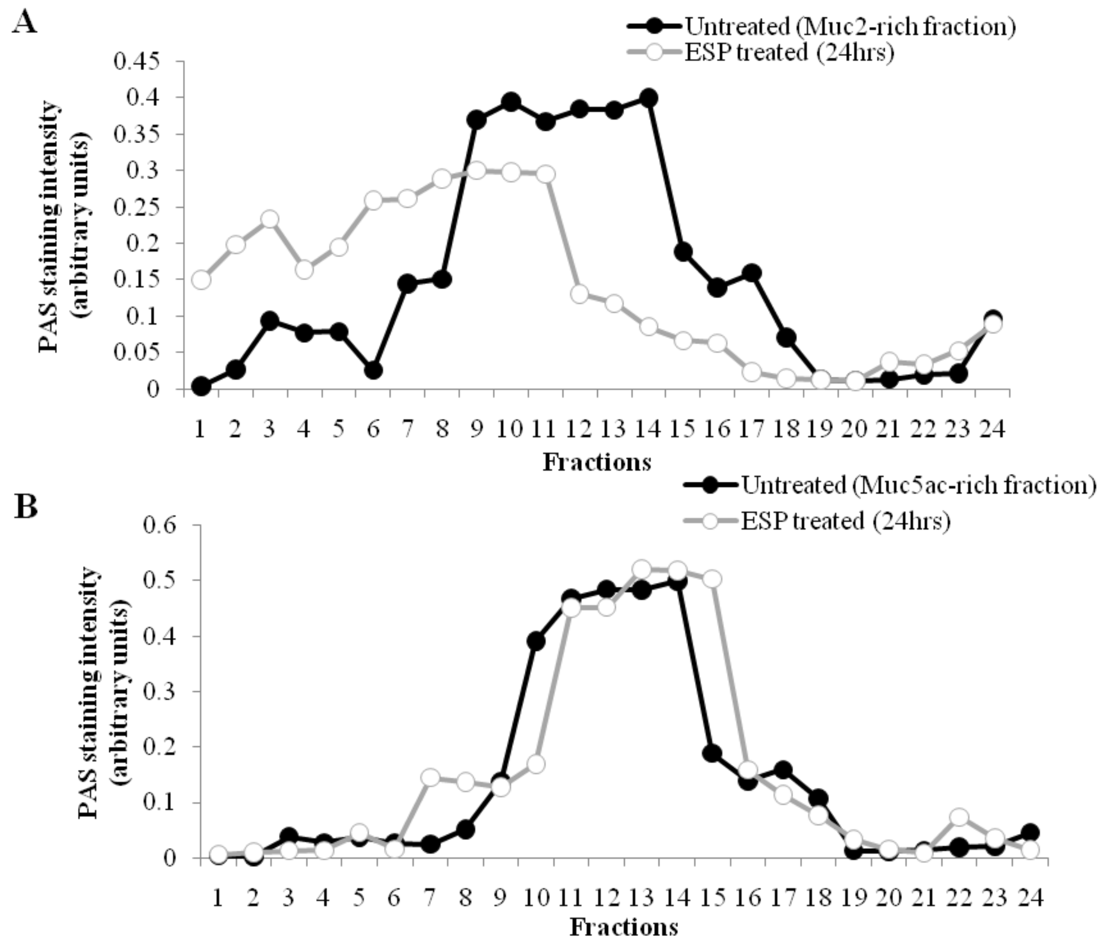


Figure 6.6: Muc2-rich fraction (A) and Muc5ac-rich fraction (B) from resistant BALB/c mice (Figure 6.6) were treated with 50µg/ml of ESPs for 24 hours and subjected to 6-8M GuCl rate zonal gradients. Fractions were taken from the top of the gradient, transferred to a nitrocellulose membrane and stained with PAS.

ESPs depolymerise the intestinal mucin, Muc2

As previously stated, Muc2 isolated from mice was not suitable for analysis after ESP treatment with the mMuc2 antibody (**Supplementary Figure 6.2**). To further explore the affect of ESPs on the MUC2 glycoprotein and in particular its polymerisation, LS174T cell line was utilised as a source of MUC2 (**Figure 6.7**). LS174T cells are a goblet-cell differentiated human colonic adenocarcinoma cell line that produces MUC2. Whole MUC2 isolated from LS174T cells was treated with 50µg/ml of ESPs for 6, 24 or 72 hours, subjected to agarose gel electrophoresis (**Figure 6.7**), transferred onto a nitrocellulose membrane and probed with the hMUC2 antibody. The unreduced MUC2 can be seen as at least three bands in the control samples which likely represent different multimeric forms of MUC2.

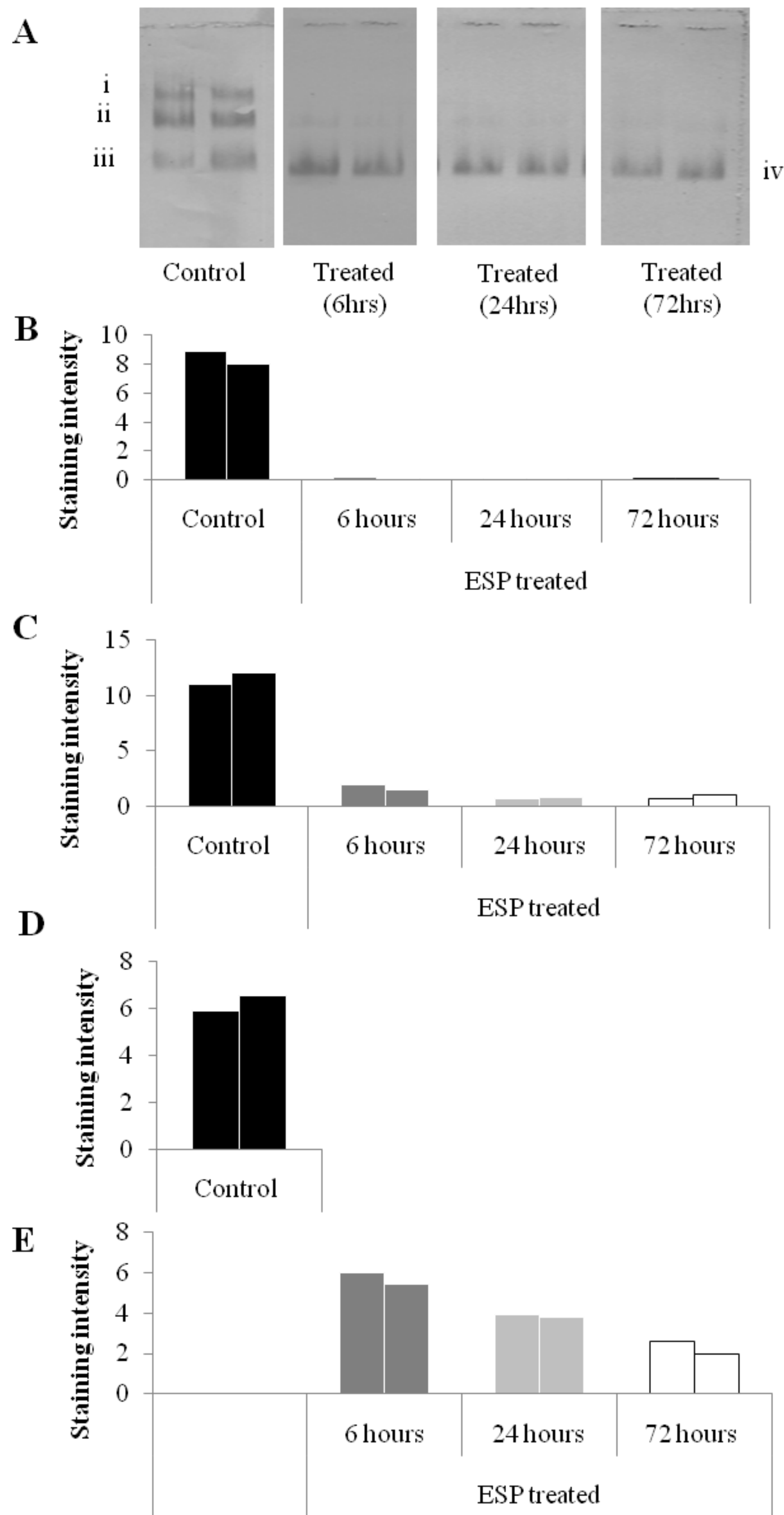


Figure 6.7: MUC2 extracted from LS174T cell lysates was treated with 50µg/ml of ESPs for 6, 24 or 72 hours analysed by agarose gel electrophoresis, western blotted onto a nitrocellulose membrane and probed with the hMUC2 antibody (A). The intensity of staining was measured for the bands in the mucus samples, most likely represent the multimeric forms of MUC2. Staining intensity of the bands was measured: i = B, ii= C, iii=D and iv = E.

Importantly, after ESP treatment the intensity of these bands was markedly decreased (**Figure 6.7A-D**). Whereas the intensity of a slightly faster migrating band was increased, with increasing periods of treatment with ESPs, the staining intensity of this band was also decreased (**Figure 6.7E**). Untreated and treated samples from LS174T cells were subjected to rate zonal centrifugation, fractionated, transferred to a nitrocellulose membrane and probed with the hMUC2 antibody. The rate zonal profiles showed a shift in MUC2 content to the top of the gradients gradually with increasing time of treatment (**Supplementary Figure 6.5**). The alteration of MUC2 was apparent just after 6 hours of treatment (**Supplementary Figure 6.5B**). However, the difference was further pronounced, after 72 hours of treatment most of the MUC2-positive material was present in the top 6 fractions (**Supplementary Figure 6.5D**). It was noted that along with the shift in the MUC2-positive material, the intensity of hMUC2 antibody was lower after treatment with ESPs (**Figure 6.7E**). Moreover, detection of the putative 'insoluble' mucin content, present at the bottom of the tube in the control samples gradually decreased after ESP treatment; no 'insoluble' fraction was observed after the 72 hour treatment.

Trichuris *ESPs have serine protease activity that degrades the mucin network*

ESPs secreted by the *T. muris* nematode are thought to consist of several different proteases such as cysteine, serine and metalloproteases (Drake *et al*, 1994b). To determine whether the ability of ESPs to degrade and alter the polymerisation of MUC2 was due to a specific type of protease, ESPs were incubated with protease inhibitors (**Supplementary Table 1**), prior to treatment of mucus extracted from LS174T cells. Note that the control samples contained a mixture of all the stated protease inhibitors. With N-ethylmaleimidide (NEM), ethylenediaminetetraacetic acid (EDTA) and Aprotinin treatment higher order MUC2 polymers were not observed (**Figure 6.8**). However, it was also noted that after incubation with NEM, the lowest band of MUC2 was still intact and a small reactivity of the MUC2 with an upper band was also observed, whereas, treatment with aproprotinin and EDTA, did not prevent the overall loss of MUC2. Interestingly, after treatment with chymostatin and antipain, the ESPs were unable to depolymerise MUC2: the higher order multimeric forms of MUC2 were still present after treatment (**Figure 6.8A**). Overall the shift in the MUC2 multimeric forms observed after ESP treatment could not be prevented by protease inhibitor treatment. This data implies that proteases secreted by the nematode, degrade Muc2/MUC2 during chronic infection.

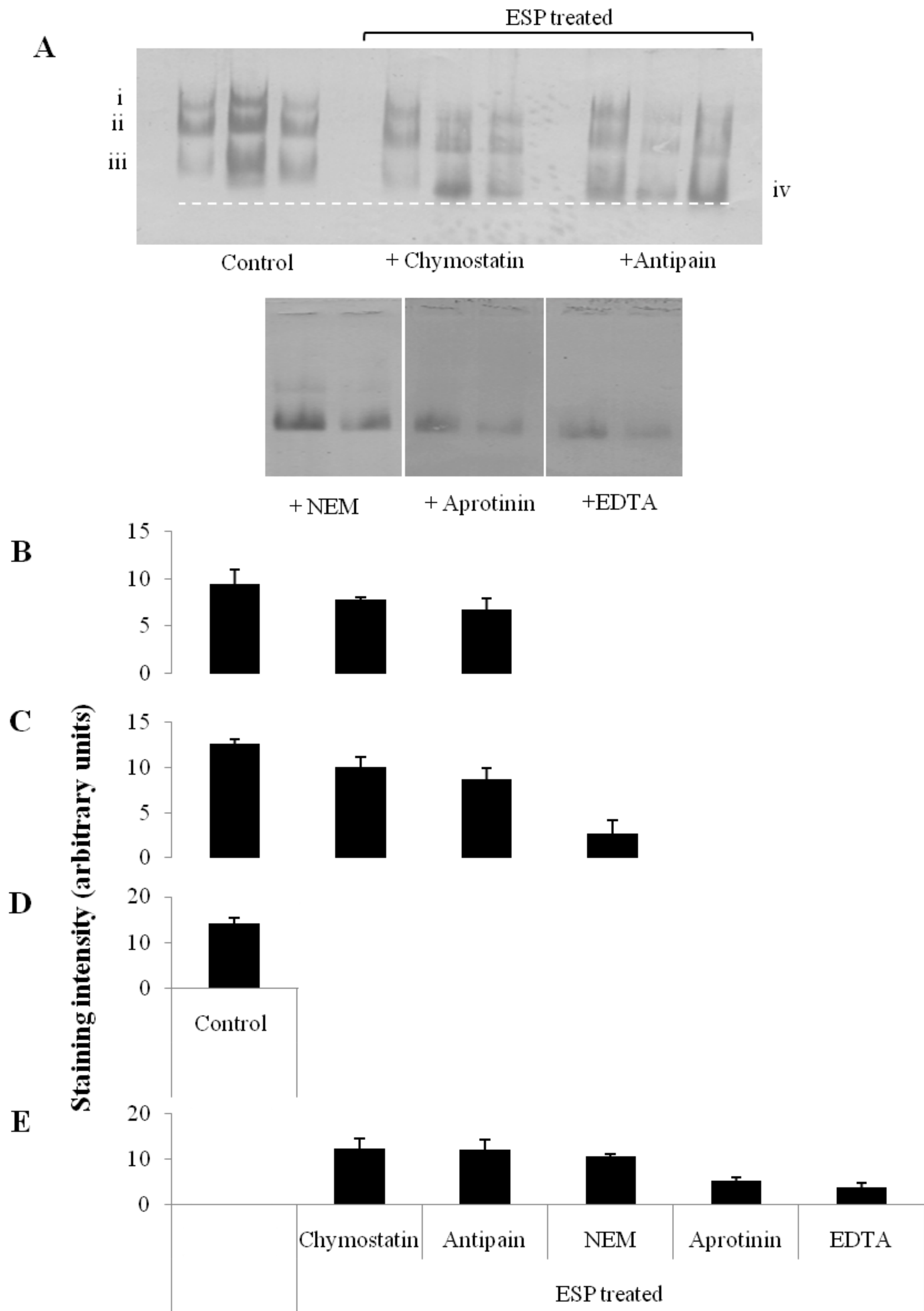


Figure 6.8: MUC2 extracted from LS174T cell lysates was incubated for 72 hours at 37◦C with or without 50µg/ml of ESPs in the presence of the specified protease inhibitors (A). Samples were subsequently analysed by agarose gel electrophoresis, western blotted onto a nitrocellulose membrane and probed with the hMUC2 antibody (A). White dashed line highlights the difference in electrophoretic mobility of MUC with and without ESP treatment. The intensity of staining was measured for the multimeric forms of MUC2: i = B, ii = C, iii = D and iv = E.

DISCUSSION

The *Trichuris* nematodes are extremely successful within the host, because they not only have the ability to evade an immune attack but are thought to actively subvert the immune responses generated by the host by exerting its immunomodulatory effects (Cliffe and Grencis, 2004). These nematodes have been shown to secrete ESPs, which contain proteases and proteins that have immunogenic properties (Drake *et al*, 1994a; Else *et al*, 1990; Hill and Sakanari, 1997). The nematodes moult into adults by day 35 pi., after which the infection becomes chronic in the susceptible mouse strains (Cliffe and Grencis, 2004). However, in the resistant models of *T. muris* the worm is rejected before it moults into an adult (Day 14-21 pi.). The properties of the mucus barrier have been shown to change to facilitate worm expulsion (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b) and it is thought that during chronicity the immunogenic ESPs may be secreted as part of a co-ordinated response to divert the immune response (Cliffe and Grencis, 2004; Grencis and Entwistle, 1997). However, ESPs may also be released by the nematode to alter the properties of the mucus barrier and thus facilitate its own survival and/or improve the conditions within its niche. To investigate the effect of ESPs on the mucins, the main components responsible for the physical properties of the mucus barrier, we treated intestinal mucus with secretions collected from adult *T. muris* nematodes. Biochemical analysis of the extracted mucins showed that the ESPs affect the polymerisation of the mucin network.

Mucin polymerisation, which is mediated disulphide bond formation involving the cysteine-rich domains at the N and C-termini of the mucin polypeptide, is essential for the protective function of the mucus barrier (Thornton *et al*, 2008). However, there is an added level of unique complexity in the assembly of the intestinal MUC2: the uncharacterised ‘non-reducible linkage’ which results in an ‘insoluble’ gel enabling MUC2 to form a barrier resistant to the harsh environment of the intestine (Carlstedt *et al*, 1995). In addition, other associated proteins such as Fc Ig binding protein (Fcgbp) was shown to be covalently attach to Muc2/MUC2 and could potentially act as a cross-linker (Johansson *et al*, 2009). Interestingly, for the first time we have demonstrated ESPs degrade the Muc2/MUC2 polymers into smaller subunits and potentially alter the ‘non-reducible’ linkage of Muc2/MUC2. The ‘insoluble’ mucin content within the mucus sample and the Muc2MUC2 was also degraded, when treated with ESPs, supporting the evidence that ESPs may be capable of altering the ‘non-reducible’ linkage of Muc2/MUC2.

During *T. muris* infection the properties of the mucus barrier change in the resistant mice to facilitate worm expulsion (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b).

Interestingly, the ESPs were unable to degrade the mucins in “whole” mucus collected from resistant mice on day 21 after infection. Along with an increase in the amount of mucins present in the mucus barrier, during the rejection of the nematode (day 21 pi.), the physical properties of the barrier change (Hasnain *et al*, 2010b). Therefore, the changes to the properties and composition of the mucus barrier can hinder ESP activity and in turn may affect the vitality of the nematode (Hasnain *et al*, 2010b). In contrast, in the susceptible mice the diminished mucus barrier is formed of mucins that are readily degradaded by the nematodes ESPs that results in more porous mucin network than in the resistant mice. It is possible, therefore, that the ESPs secreted by the nematode contribute the colonisation of the nematode and during chronicity aid movement through the epithelial cell layer.

Recently, it was demonstrated using von Willebrand factor (vWF), which is a large multimeric glycoprotein homologous to gel-forming mucins, that sialylated glycans render the protein core more susceptible to proteolysis (McGrath *et al*, 2010). This supports our hypothesis of mucins in susceptibility being more prone to degradation because mucins had reduced apparent glycosylation during chronic infection (Hasnain *et al*, 2010a). Therefore, the differences in glycosylation of the mucins in resistance and susceptibility could facilitate the “degradative” activity of ESPs. Another possibility for the lack of degradation of mucus isolated from resistant mice could be due to the increased concentration of mucins and other proteins within the mucus barrier (Hasnain *et al*, 2010b). The increased levels of proteins could result in more competition for ESPs to cleave sites and therefore make the mucins less susceptible to degradation (Innes *et al*, 2009). Data suggests that the mucus barrier and its mucin components remains altered in resistance, even after the expulsion of the nematode. We showed that even 21 days after the worms have been expelled from the resistant mice the mucins were only partially altered by ESPs. This raises an interesting possibility of the mucus barrier remaining altered which could, therefore, potentially limit re-infection as reported previously (Bancroft *et al*, 2001). Surprisingly, the ESPs have the ability to degrade mouse and human Muc2/MUC2, but not the stomach/airway mucin Muc5ac, suggesting that ESPs are relatively specific. This may be because the *Trichuris* nematodes have evolved in the intestine, where Muc2 is the major mucin expressed. Therefore, Muc5ac up-regulated in the intestine during worm expulsion could result in the inability of the worm to degrade mucins and subsequently be detrimental for the nematode.

Data described in this study revealed that polymeric mucins could be degraded into smaller polymers by the ESPs released by the *T. muris* nematode. This is not the first time parasite exoproducts have been shown to degrade mucins, previous studies have shown that protozoan parasites such as *Entamoeba histolytica* (Lidell *et al*, 2006)*, Trichomonas vaginalis* (Lehker and Sweeney, 1999) and *Naegleria fowleri* (Cervantes-Sandoval *et al*, 2008) all release cysteine proteases which have the ability to degrade mucins. However, unlike these other studies it is unlikely that a cysteine protease is solely responsible for degrading the mucins in the present study. NEM and aprotinin (cysteine protease inhibitors) only very partially limited the activity of ESPs to degrade MUC2. Treatment with chymostatin and antipain inhibited the depolymerisation of MUC2 implicating trypsin and/or serine protease activity. However, since degradation was not inhibited by aprotinin (cysteine and trypsin protease inhibitor) it is most likely that serine protease activity is responsible for degrading Muc2/MUC2. Interestingly, serine proteases have been reported to be isolated with *T. muris* ESPs previously (Drake *et al*, 1994b). Data implies that cysteine proteases present in the ESPs were in part responsible for the affects observed on the insoluble MUC2-gel. Serine and cysteine proteases, therefore, may act in concert to disrupt the polymeric mucin network (Corfield and Myerscough, 2000). Adding to the complexity, the ESPs had a further affect on the MUC2 protein core as there was a change in the electrophorectic migration and gradual loss of MUC2 with treatment. This suggests that the ESPs not only depolymerise MUC2, they may also affect the glycosylation, the MUC2 protein core or possibly the associated proteins such as the cross-linking Fcgbp protein. Therefore, ESPs may be released as part of the nematodes regime, to break down the mucin network by depolymerising, deglycosylating and affecting the MUC2 core itself.

Overall, the stability and turnover of the ESPs is not known *in vivo*, however data suggests that the proteases present in ESPs may be released within the nematodes proximity to open-up the mucus layer to aid its movement through the mucus layer. The release of ESPs could result in the altering the viscoelastic properties of the mucus barrier (Thornton *et al*, 2008; Thornton and Sheehan, 2004), which could explain the porous mucus network observed during susceptibility (Hasnain *et al*, 2010b). Furthermore, it would improve the niche of the nematode allowing its continuous establishment within the caecal crypts of host and therefore result in chronicity.

REFERENCES

Artis D, Grencis RK (2008). The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunol* **1**(4)**:** 252-264.

Bancroft AJ, Else KJ, Humphreys NE, Grencis RK (2001). The effect of challenge and trickle *Trichuris muris* infections on the polarisation of the immune response. *Int J Parasitol* **31**(14)**:** 1627-1637.

Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* **367**(9521)**:** 1521-1532.

Carlstedt I, Herrmann A, Hovenberg H, Lindell G, Nordman H, Wickstrom C, Davies JR (1995). 'Soluble' and 'insoluble' mucins--identification of distinct populations. *Biochem Soc Trans* **23**(4)**:** 845-851.

Carlstedt I, Karlsson H, Sundler F, Fransson LA (1982). An insoluble mucin complex from rat small intestine. *Adv Exp Med Biol* **144:** 155-157.

Cervantes-Sandoval I, Serrano-Luna Jde J, Garcia-Latorre E, Tsutsumi V, Shibayama M (2008). Mucins in the host defence against Naegleria fowleri and mucinolytic activity as a possible means of evasion. *Microbiology* **154**(Pt 12)**:** 3895-3904.

Cliffe LJ, Grencis RK (2004). The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol* **57:** 255-307.

Corfield AP, Myerscough N (2000). Glycosidase activity. *Methods Mol Biol* **125:** 403-416.

Davies JR, Carlstedt I (2000). Isolation of large gel-forming mucins. *Methods Mol Biol* **125:** 3-13.

Drake L, Korchev Y, Bashford L, Djamgoz M, Wakelin D, Ashall F, Bundy D (1994a). The major secreted product of the whipworm, Trichuris, is a pore-forming protein. *Proc Biol Sci* **257**(1350)**:** 255-261.

Drake LJ, Bianco AE, Bundy DA, Ashall F (1994b). Characterization of peptidases of adult *Trichuris muris*. *Parasitology* **109 (Pt 5):** 623-630.

Else K, Wakelin D (1989). Genetic variation in the humoral immune responses of mice to the nematode *Trichuris muris*. *Parasite Immunol* **11**(1)**:** 77-90.

Else KJ (2005). Have gastrointestinal nematodes outwitted the immune system? *Parasite Immunol* **27**(10-11)**:** 407-415.

Else KJ, Wakelin D, Wassom DL, Hauda KM (1990). MHC-restricted antibody responses to *Trichuris muris* excretory/secretory (E/S) antigen. *Parasite Immunol* **12**(5)**:** 509-527.

Grencis RK, Entwistle GM (1997). Production of an interferon-gamma homologue by an intestinal nematode: functionally significant or interesting artefact? *Parasitology* **115 Suppl:** S101-106.

Hasnain SZ, Thornton DJ, Grencis RK (2010a). Changes in the mucosal barrier during acute and chronic *Trichuris muris* infection. *Parasite Immunology*. *In press.*

Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010b). Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* **138**(5)**:** 1763-1771.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA (2008). Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* **5**(3)**:** e54.

Herrmann A, Davies JR, Lindell G, Martensson S, Packer NH, Swallow DM, Carlstedt I (1999). Studies on the "insoluble" glycoprotein complex from human colon. Identification of reduction-insensitive MUC2 oligomers and C-terminal cleavage. *J Biol Chem* **274**(22)**:** 15828-15836.

Hill DE, Gamble HR, Rhoads ML, Fetterer RH, Urban JF, Jr. (1993). Trichuris suis: a zinc metalloprotease from culture fluids of adult parasites. *Exp Parasitol* **77**(2)**:** 170-178.

Hill DE, Sakanari JA (1997). Trichuris suis: thiol protease activity from adult worms. *Exp Parasitol* **85**(1)**:** 55-62.

Innes AL, Carrington SD, Thornton DJ, Kirkham S, Rousseau K, Dougherty RH, Raymond WW, Caughey GH, Muller SJ, Fahy JV (2009). Ex vivo sputum analysis reveals impairment of protease-dependent mucus degradation by plasma proteins in acute asthma. *Am J Respir Crit Care Med* **180**(3)**:** 203-210.

Johansson ME, Thomsson KA, Hansson GC (2009). Proteomic analyses of the two mucus layers of the colon barrier reveal that their main component, the Muc2 mucin, is strongly bound to the Fcgbp protein. *J Proteome Res* **8**(7)**:** 3549-3557.

Lehker MW, Sweeney D (1999). Trichomonad invasion of the mucous layer requires adhesins, mucinases, and motility. *Sex Transm Infect* **75**(4)**:** 231-238.

Lidell ME, Bara J, Hansson GC (2008). Mapping of the 45M1 epitope to the C-terminal cysteine-rich part of the human MUC5AC mucin. *FEBS J* **275**(3)**:** 481-489.

Lidell ME, Moncada DM, Chadee K, Hansson GC (2006). Entamoeba histolytica cysteine proteases cleave the MUC2 mucin in its C-terminal domain and dissolve the protective colonic mucus gel. *Proc Natl Acad Sci U S A* **103**(24)**:** 9298-9303.

Linden S, Nordman H, Hedenbro J, Hurtig M, Boren T, Carlstedt I (2002). Strain- and blood group-dependent binding of Helicobacter pylori to human gastric MUC5AC glycoforms. *Gastroenterology* **123**(6)**:** 1923-1930.

McGrath RT, McKinnon TA, Byrne B, O'Kennedy R, Terraube V, McRae E, Preston RJ, Laffan MA, O'Donnell JS (2010). Expression of terminal alpha2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13. *Blood* **115**(13)**:** 2666-2673.

Roach TI, Wakelin D, Else KJ, Bundy DA (1988). Antigenic cross-reactivity between the human whipworm, *Trichuris trichiura*, and the mouse trichuroids *Trichuris muris* and *Trichinella spiralis*. *Parasite Immunol* **10**(3)**:** 279-291.

Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK (1996). Respiratory mucins: identification of core proteins and glycoforms. *Biochem J* **316 (Pt 3):** 967-975.

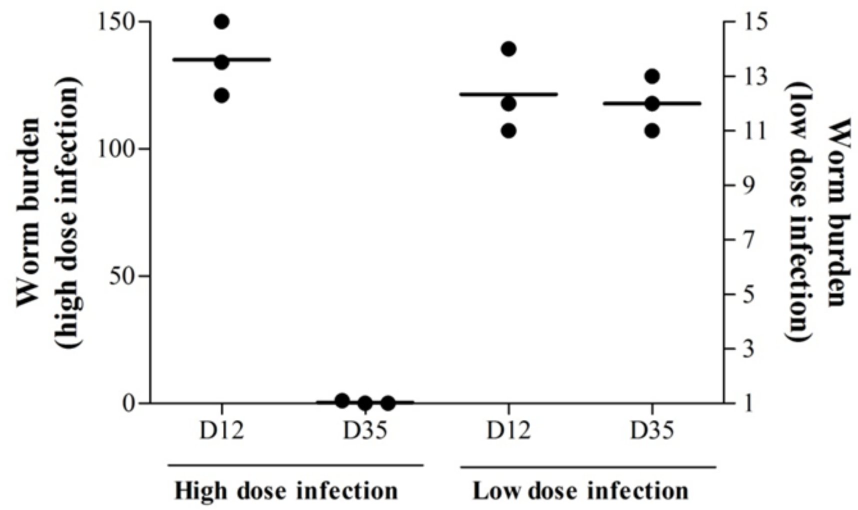
Thornton DJ, Davies JR, Kraayenbrink M, Richardson PS, Sheehan JK, Carlstedt I (1990). Mucus glycoproteins from 'normal' human tracheobronchial secretion. *Biochem J* **265**(1)**:** 179-186.

Thornton DJ, Rousseau K, McGuckin MA (2008). Structure and function of the polymeric mucins in airways mucus. *Annu Rev Physiol* **70:** 459-486.

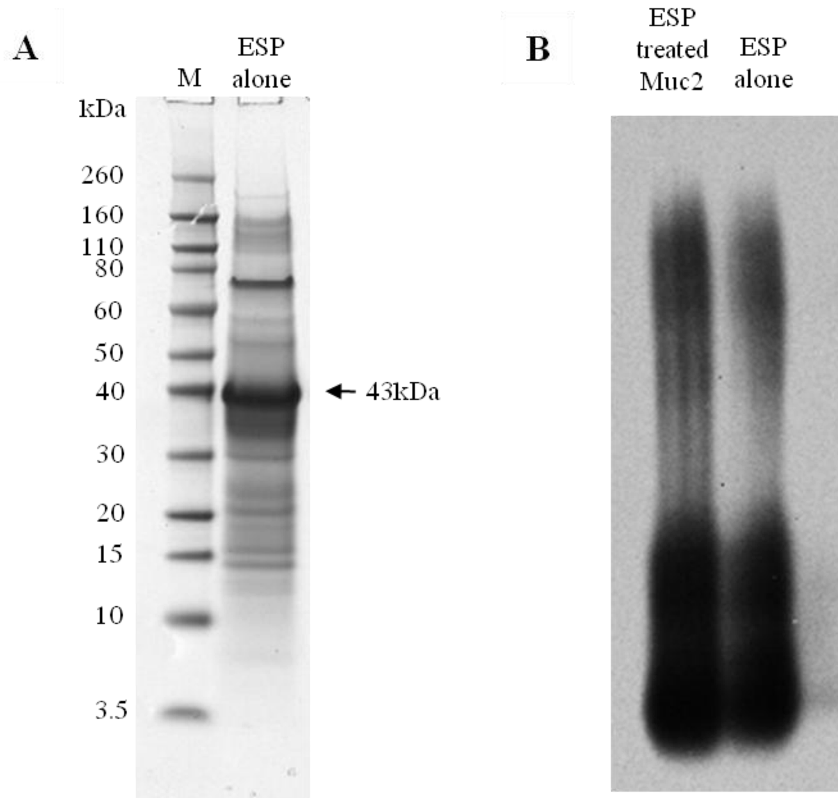
Thornton DJ, Sheehan JK (2004). From mucins to mucus: toward a more coherent understanding of this essential barrier. *Proc Am Thorac Soc* **1**(1)**:** 54-61.

Wakelin D (1967). Acquired immunity to *Trichuris muris* in the albino laboratory mouse. *Parasitology* **57**(3)**:** 515-524.

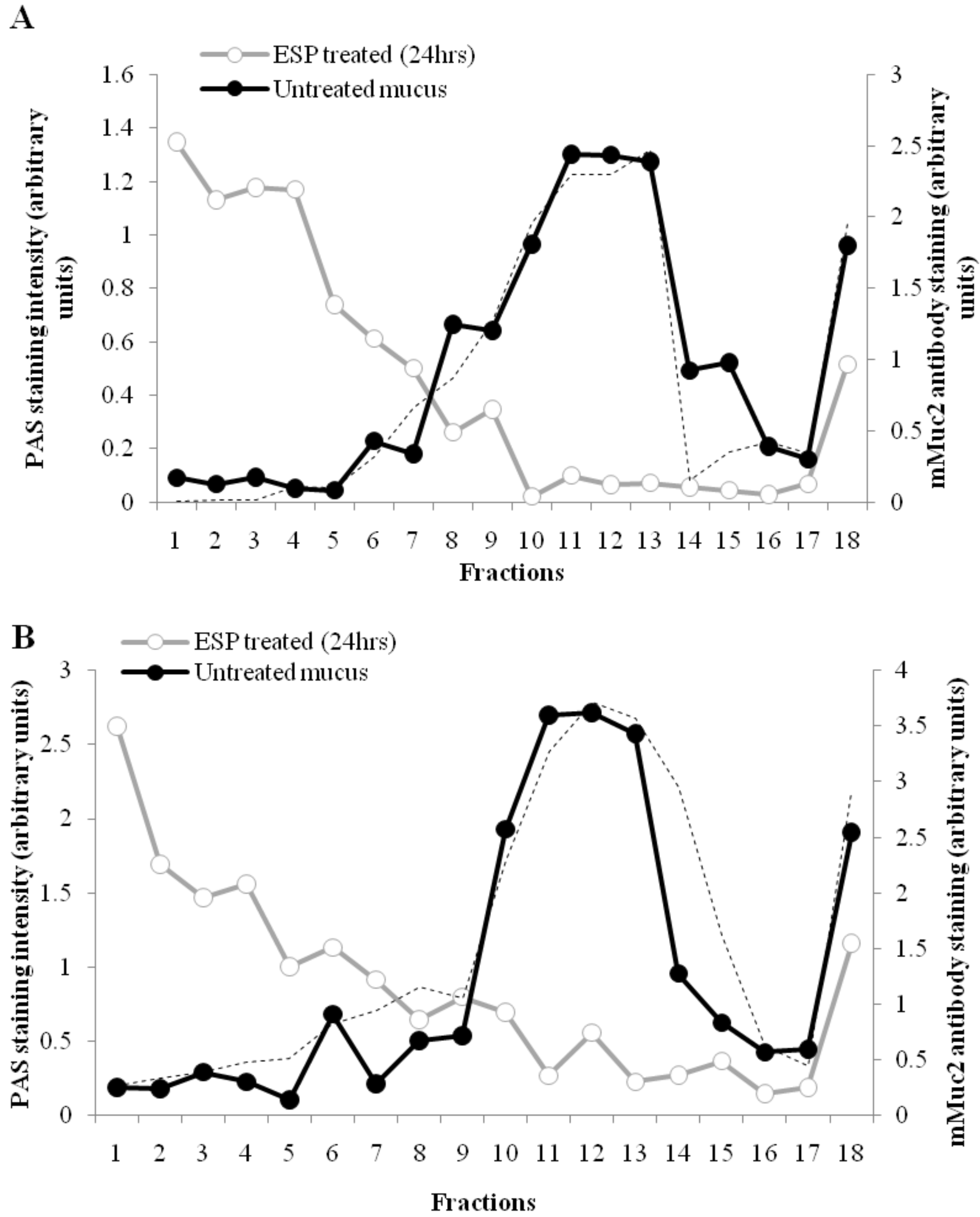
SUPPLEMENTARY FIGURES



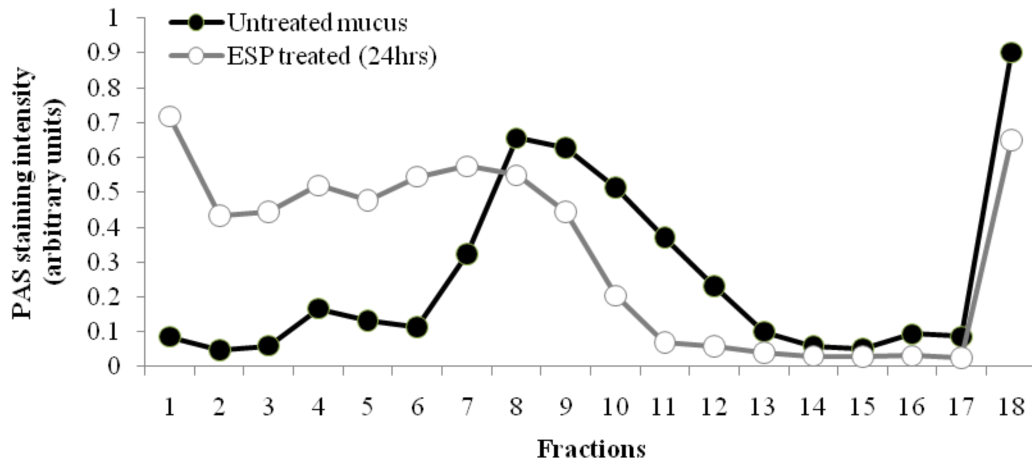
Supplementary Figure 6.1: BALB/c mice were infected with a high dose (150) or a low dose (<15) *T. muris* eggs. Worm burdens were assessed on day 12 and 35 pi.



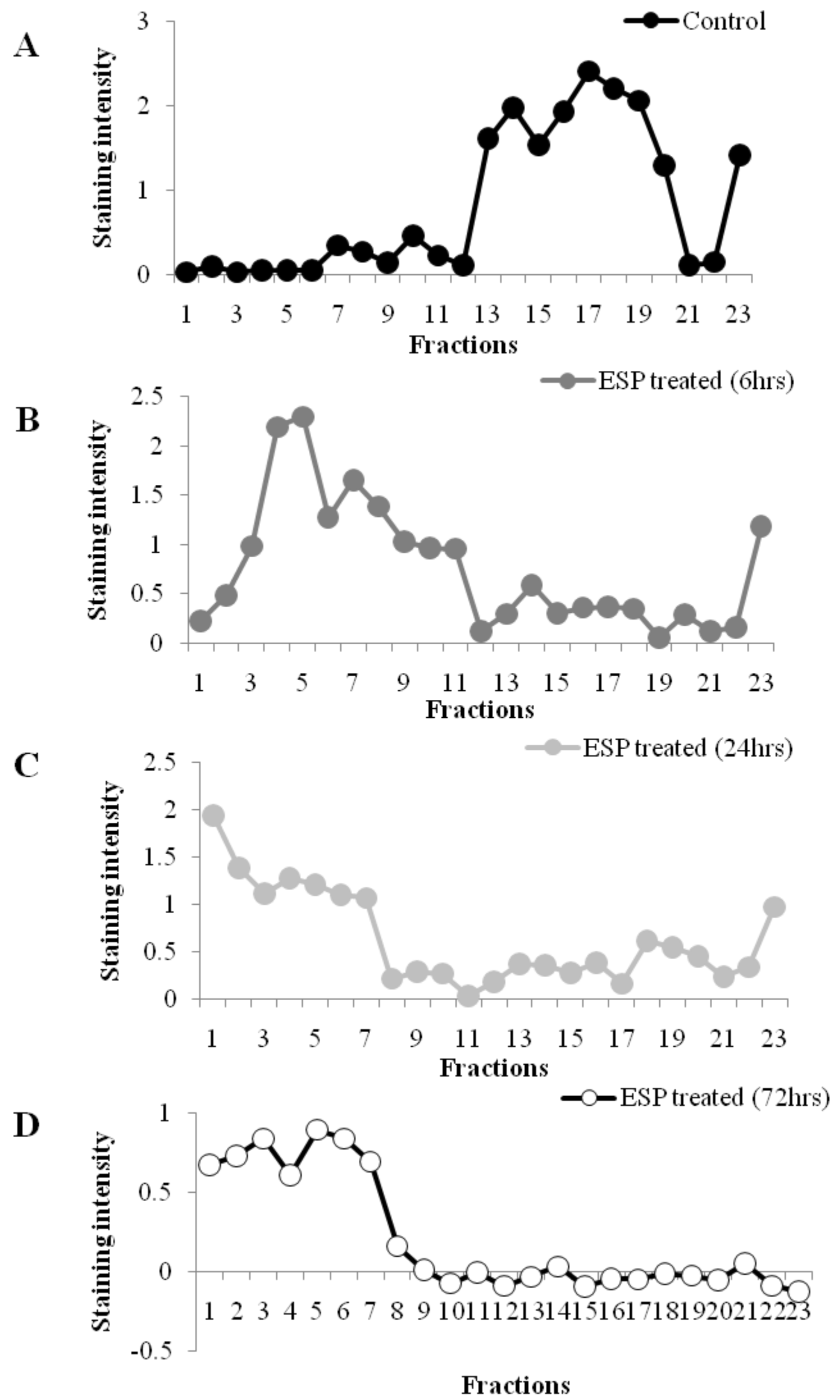
Supplementary Figure 6.2: (A) 50µg/ml of *T. muris* ESPs were analysed by SDS-PAGE and stained with coomassie blue. ESPs contain a major 43kDa product as resported before (Drake *et. al.* 1994). (B) Whole mucus sample treated with ESP and ESP alone was separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane and probed with the mMuc2 antibody.



Supplementary Figure 6.3: Crude mucus extracted on day 14 of infection from resistant (high dose infection) in BALB/c (A) and susceptible (low dose infection) in BALB/c (B) mice was treated with 50μg/ml of ESPs for 24 hours and subjected to 6-8M GuCl rate zonal gradients. Subsequently fractions were taken from the top of the gradient and analysed by PAS-staining. Representative of 3 mice.



Supplementary Figure 6.4: Whole mucus extracted from resistant BALB/c mice (on day 56 pi.) was treated with 50µg/ml of ESPs at 37°C for 24 hours. Samples were subjected to 6-8M GuCl rate zonal gradients and subsequently fractions were taken from the top of the gradient and analysed by PAS-staining. Representative of 4 mice.



Supplementary Figure 6.5: MUC2 extracted from LS174T cell lysates was treated with 50µg/ml of ESPs for 6 (B), 24 (C) or 72 (D) hours subjected to rate zonal centrifugation, fractions were taken from the top of the gradient, transferred to a nitrocellulose membrane and analysed by hMUC2 antibody staining. Control samples were incubated at 37◦C for 72 hours (A).

SUPPLEMENTARY TABLE

Protease inhibitors	Activity against
Chymostatin	Serine/Cysteine proteases
Antipain	Trypsin/Serine/Cysteine proteases
N-ethylmaleimidide (NEM)	Cysteine proteases
Aprotinin	Chymotrypsin/Trypsin/Plasmin
Ethylenediaminetetraacetic acid (EDTA)	Metalloproteases

Supplementary Table 1: Protease inhibitors and their activity against proteases

GENERAL DISCUSSION

The immune mediated elimination of gastrointestinal nematodes has been the subject of considerable investigation (Artis and Grencis, 2008). In general, however, the protective effector mechanisms by which worm expulsion is achieved remain poorly defined. Interestingly, about 25 years ago, several groups observed that gastrointestinal nematodes were contained within globules of mucus when expelled from the host (Bell *et al*, 1984; Miller *et al*, 1981; Miller and Nawa, 1979). This led to the "Mucus-Trap" hypothesis, which suggested that the mucus barrier covering the gastrointestinal epithelium, was an active effector component involved in the rejection of nematodes (Miller *et al*, 1981). Despite this, the role of the mucus barrier in this setting had not been thoroughly investigated.

The goal of my PhD was to investigate the role of the mucus barrier as part of the immune defence against gastrointestinal nematodes. I utilised the *Trichuris muris* (*T.muris*) whipworm which infects mice naturally (Cliffe and Grencis, 2004). It is closely related at the morphological, physiological and antigenic levels to the human whipworm *Trichuris trichiura*, the causative agent of Trichuriasis which affects up to a billion people worldwide (Bethony *et al*, 2006). By combining approaches from two specialist fields of glycoprotein biochemistry and parasite immunology, I aimed to answer two major questions:

- Is the mucus barrier and its mucin component an integral part of the immunomodulated resistance mechanism?
- During chronicity, does the nematode exert any effects on the mucus gel to promote its own survival within the host?

It has been well-established that during resistance, T_H2-type immune response results in worm expulsion (Cliffe and Grencis, 2004; Grencis, 2001). The T_H2-type cytokines have been shown to drive effector mechanisms such as the 'epithelial cell escalator' (Cliffe *et al*, 2005) and smooth muscle hypercontractility (Khan and Collins, 2004) which displace the worm out of its niche and therefore facilitate worm expulsion (**Figure 7.1**). Under the control of IL-13 an increase in proliferation of cells is observed within the caecal crypts during resistance (Artis *et al*, 1999). We observed an increase in transcription factors involved in goblet cell differentiation, which leads to goblet cell hyperplasia and increased mucin secretion into the mucus barrier during worm expulsion (Hasnain *et al*, 2010a) (**Chapter 2**). In the intestine, the dominant mucin Muc2 produced

by goblet cells is proposed to form a barrier composed of two distinct layers; a porous outer layer and an adherent inner layer protecting the mucosal surface (Johansson *et al*, 2008). At the site of infection, a significant increase in Muc2 containing goblet cells and increased Muc2 secretion was observed in immunocompetent mice resistant to chronic infection (**Chapter 3**). Critically, worm expulsion was significantly delayed in Muc2-deficient mice without an alteration to the goblet cell lineage (Hasnain *et al*, 2010b).

A more startling observation from this work was that Muc5ac, a mucin normally exclusively located at other mucosal sites, notably the lung and stomach, was expressed in the intestine during worm expulsion (Hasnain *et al*, 2010b). It is important to note that no goblet cell hyperplasia or induction of Muc5ac was observed in uninfected mice and mice susceptible to chronic infections that were unable to expel *T. muris*. Strikingly, in the absence of Muc5ac the mice (on a resistant background) were susceptible to chronic infection, despite a strong underlying T_H2-type cytokine response (**Chapter 4**). This data implies that Muc5ac is acting as an effector. Moreover, changes were not only observed in the quantity of the mucins, but also the quality of the mucins: mucins were more highly charged and sulphated during worm expulsion (**Chapter 5**). Overall, these changes within the barrier result in altering the rheological properties of the mucus layer; mucus was less porous during worm expulsion. The data supported a novel role for mucins which directly 'damaged' the nematodes, reflected by a significant reduction in their ATP levels (Hasnain *et al*, 2010b). Furthermore, during chronicity the nematode itself is capable of depolymerising the mucin network possibly to aid its movement in the intestine and/or facilitate mating (**Chapter 6**).

Therefore, data generated from my PhD studies has demonstrated a pivotal role for the mucin components of the mucus barrier as effectors that facilitate worm expulsion and, demonstrated how the worm exerts its effects on the mucin environment to promote its own survival within the host.

Similar to allergic responses, eosinophilia, mastocytosis and a strong peripheral IgE antibody response are a prominent feature of nematode infection (Maizels *et al*, 2009). These 'classical' arms of the immune response, however, have been shown not to be critical to *T. muris* expulsion (Artis and Grencis, 2008; Cliffe and Grencis, 2004). What influences the balance between the T_H1 –T_H2 type immune response to result in chronic or acute infection is still undecided. However, it is known that the T_H2-type cytokine responses are integral to the protective immunity against *T. muris* infection; multiple

physiological mechanisms mediated through the T_H2-type immune response enforce intestinal expulsion of the nematode (Artis and Grencis, 2008). The changes in the mucus barrier and mucins during chronic *T. muris* infection are very similar to those observed during inflammatory bowel disease (IBD). For instance, the depleted mucus barrier (McGuckin *et al*, 2009), lower number of goblet cells with smaller thecae (Van der Sluis *et al*, 2006), the loss of mucin sulphation (Corfield *et al*, 1992) and mature glycosylation (Heazlewood *et al*, 2008) are all a feature of IBD. Corroborating this observation, a recent transcriptional profiling study by Pennock and colleagues has highlighted the phenotypic and transcriptional similarities of *T. muris* chronic infection to models of IBD and human IBD (Levison *et al*, 2010). In chronic infection the goblet cells appear depleted, with reduced mucin synthesis and storage within the goblet cells; furthermore, the mucins are lowly charged and apparent immature glycosylation (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b).

The altered mucin glycosylation and down-regulation of goblet cell differentiation transcription factors suggests that inflammation during chronic infection induces cell stress. Supporting this hypothesis, are a recent set of studies that highlight the overarching hypothesis of intestinal secretory cells being susceptible to endoplasmic reticulum (ER) stress due to the high rates of biosynthesis of secretory proteins (Heazlewood *et al*, 2008; Podolsky *et al*, 2009; Zhao *et al*, 2010). During ulcerative colitis the mucins within the goblet cells were shown to have apparent immature glycosylation (similar to that observed during chronic *T. muris* infection) (Heazlewood *et al*, 2008). Interestingly, these partially synthesised mucins accumulate within the goblet cell endoplasmic reticulum (ER) resulting in the activation of the ER stress response which consequently leads to a diminished mucus barrier and development of intestinal inflammation (Heazlewood *et al*, 2008). Therefore, during chronicity the reduced glycosylation may result in the accumulation of Muc2 within the goblet cells, eventually reaching a point where Muc2 production ceases which can, in turn, result in the diminished mucus barrier. Therefore, an underlying ER stress response within the intestinal epithelia may be the link that ties these molecular changes observed in the mucins and the induction of inflammation observed during chronicity.

Interestingly, nematodes including *Trichuris suis* have been used to treat IBD in mice and in humans (Summers *et al*, 2003; Summers *et al*, 2005a). A caveat to this is the fact that in susceptible individuals, nematodes can induce IBD-type symptoms. However, *T. suis* is believed to only transiently infects humans and has been shown to be successful in controlling IBD (Summers *et al*, 2005b). Perhaps as the helminth-induced T_H2-type

immune response counteracts the T_H1-type IBD, this results in the hypersecretion of mucins and limits inflammation by replenishing the mucus barrier. The stimulus that results in exocytosis of mucins is not known. However, we have for the first time identified an intestinal receptor (gamma amino-butyric acid α-3) that may be involved in the secretion of mucins during nematode infection. Studies on lung models have shown that GABA receptors are directly responsive to IL-13 (Xiang *et al*, 2007). Therefore, this raises the interesting possibility that the receptor could be manipulated to increase the thickness of the mucus barrier and potentially reduce inflammation during chronic nematode infection or even in IBD.

The diminished mucus barrier could result in exacerbating inflammation, not only due to increased foreign toxin and antigen exposure but also because of the commensal flora coming into contact with epithelium. Commensal flora in the intestine, however, plays an important biological role in *T. muris* infection. It has been shown that commensal flora can induce *T. muris* egg hatching and therefore effect establishment of the nematode within the host (Hayes *et al*, 2010). The loose outer layer of the mucus barrier is the home of the commensals and therefore the changes in the mucus barrier could dictate the changes in the commensal flora (Johansson *et al*, 2008). The 'breaks' in the mucus barrier during chronic infection may result in bacterial-epithelial cell contact consequently exacerbating inflammation. In fact, recently it was clearly demonstrated that Muc2 protects against enteric bacterial infections and its absence makes mice more susceptible to infection (Bergstrom *et al*, 2010). Moreover, commensal flora has been shown to be in direct contact with the intestinal mucosa in the Muc2-deficient mice, subsequently resulting in bacterial translocation due to the barrier disruption (Johansson *et al*, 2008). It is clear that secondary bacterial infections during *T. muris* infection result in increased mortality (*R. K. Grencis, personal communication*). Indeed, the survival rates of IL-10-deficient mice infected with *T. muris*, which have a diminished mucus barrier, improve when treated with antibiotics (Schopf *et al*, 2002).

The nematode could modulate the decrease in the mucus barrier observed during chronicity, by skewing the immune response towards T_H1-type response as it secretes an IFN-γ like molecule (Grencis and Entwistle, 1997). Moreover, the 'breaks' in the mucus layer maybe as a result of the nematodes ability to disrupt the mucin network by secreting serine and cysteine proteases. Excretory secretory products (ESPs) isolated from the adult *T. muris* E isolate (which was used during these studies) specifically degraded Muc2 and were unable to degrade Muc5ac. However, it is possible that other isolates of *T. muris*, in

particular the S isolate (D'Elia and Else, 2009) which is able to evade the immune response resulting in chronic infection, may be able to secrete proteases capable of degrading Muc5ac as well as Muc2. In addition, it is also possible that *T. muris* at the larval stage secretes different molecules that have specialised effects on mucins. Whether the ESPs secreted from *T. muris* larvae are also capable of disrupting the mucus barrier remains to be seen; however, considering the nematode is embedded at the base of the intestinal crypts during the larval stages and is not directly associated with the mucus barrier it seems like an unlikely possibility. Several other parasites have also adopted similar mechanisms to survive within the intestine. For instance, *Entameoba histolytica* secretes cysteine proteases that degrade the mucus barrier (Moncada *et al*, 2003). *Helicobacter pylori* has also been shown to secrete 'mucinases' which alter the rheological properties of the mucus gel (containing Muc5ac and Muc6) and this allows its corkscrew like motion through the mucus layer (Celli *et al*, 2009). Therefore, the difference in the increased porosity of the mucus layer during chronic infection may be as a direct result of the ESPs secreted by *T.muris in vivo*. Inhibiting the ability of parasites to secrete proteases may result in gradually re-creating a mucin network that can affect the vitality of the nematode or at least result in reducing inflammation by repairing the mucus layer.

Glycans can influence the extension and expansion of the protein and hydration of the mucus barrier. Furthermore, glycans could bind to and aid the *T. muris* worm expulsion process as glycans have been shown to act as decoy receptors that bind and remove pathogens (Linden *et al*, 2008). Another possibility is that mucin glycosylation is involved in sequestering factors that have the detrimental effect on the nematode, as seen during resistance (Hasnain *et al*, 2010b). Moreover, the changes in mucin glycosylation that occurred during resistance remained after the expulsion of the nematode was complete (Hasnain *et al*, 2010a); in fact, ESPs were unable to completely degrade mucins even after 21 day post expulsion. This raises an interesting possibility of glycans being important in *T. muris* establishment, considering re-infection results in accelerated expulsion (Bancroft *et al*, 2001). Furthermore, it is important to acknowledge that in endemic areas, people are usually infected with low doses of infection repeatedly (Bethony *et al*, 2006); therefore, the gradual change in glycosylation that occurs as a result of infection may eventually contribute to 'acquired immunity'. Although, lowered mucin sulphation in the intestine did not have an effect on the establishment of *T. muris*, further studies are required to determine whether abolishing mucin sulphation completely would have an effect on establishment of infection. Interestingly, an up-regulation in sulphate transporters is

observed during resistance, resulting in the active uptake of sulphates (IL-13-mediated), which may be directly or indirectly important during *T. muris* infection. Certainly, reduced sulphation and increased sialylation of mucins results in making the mucins more prone to degradation, as previously observed with von Willebrand factor (McGrath *et al*, 2010). Muc5ac, present in the mucus barrier during worm expulsion, is more highly charged compared to Muc2 which is a property conferred by glycans. More importantly, Muc5ac was directly shown to damage the nematode; how this occurs is still unknown. Nevertheless, it may be mediated by the Muc5ac protein core or its glycan chains. Recent studies have also reported an up-regulation of *Muc5ac* expression *Nippostrongylus brasiliensis* (Takeda *et al*, 2010) and *T. suis* (Kringel *et al*, 2006) with nematode exposure. Furthermore, we examined the expression of Muc5ac in two other murine models of nematode infection that inhabit the small intestine: *Trichinella spiralis* and *Heligmosomoides bakeri/polygyrus*. *T.spiralis* is expelled from the intestine via a strong T_H2-type immune response (Lee, 2002), whereas a primary infection with *H.bakeri/polygyrus* results in chronicity (Maizels *et al*, 2009). Interestingly, we observed an up-regulation of Muc5ac mRNA and protein only in the *T. spiralis* infected mice (data not shown). This supports the hypothesis that Muc5ac is a mucin expressed in the intestines during protective immunity to intestinal nematodes. Previously, it has been demonstrated that specifically targeting an enteric response against one nematode can result in promoting concomitant expulsion of unrelated nematodes, via a non-specific protective mechanism (Dineen *et al*, 1977; Miller and Nawa, 1979). Therefore, we suggest that Muc5ac may be part of the IL-13 induced 'universal response' to clear intestinal enteric parasitic infections.

In conclusion, the findings from this thesis clearly demonstrate that the mucus barrier is a critical effector during enteric infection (Hasnain *et al*, 2010a; Hasnain *et al*, 2010b). During susceptibility to *T. muris*, under a T_H1-type immune response no major changes in the number of goblet cells are observed. Resulting in a depleted mucus barrier, which consists of the mucin (Muc2) that is highly sialylated but more prone to degradation by ESPs. The diminished mucus barrier, along with the ability of ESPs released from the nematode to disrupt the polymeric mucin network results in a more porous mucus network (Hasnain *et al*, 2010b). Therefore, this in turn could result in damage to the epithelial tissue and potential translocation of commensal flora across the epithelium, thus exacerbating inflammation during chronicity (**Figure 7.2**). In contrast, during resistance, under the immunological control of a T_H2-type immune response, goblet cell hyperplasia is

observed. This is accompanied by an increase in the synthesis and secretion of Muc2 and *de novo* expression of the Muc5ac mucin in the intestine. The mucins within the mucus barrier are highly charged and sulphated, and less prone to degradation by ESPs. These changes result in a less porous mucin network which is detrimental to the nematodes vitality (**Figure 7.2**). Overall, the mucin components are critical to worm expulsion and in the absence of Muc5ac mice are susceptible to chronic infection. We hypothesise that the mucus barrier, therefore, is an integral part of the co-ordinated worm expulsion response. The epithelial cell escalator (Cliffe *et al*, 2005) operating within the intestine can dislodge the worm out of its niche. Whereas, changes in the rheological properties of the mucus barrier can trap and its bioactive components damage the nematode (Hasnain *et al*, 2010b). This along with smooth muscle hypercontractility (Khan and Collins, 2004) can physically facilitate worm expulsion.

The work presented here provides compelling evidence showing that mucins play a major role in protection against gastrointestinal nematode infection. In addition, we define potential mechanisms by which mucins may mediate protection. This will not only have application to other types of intestinal pathogens and mucosal protection in general, but may open up new avenues for developing anti-parasitic intervention.

FUTURE PROSPECTIVES

In these studies we have expanded on the previously described 'Mucus-Trap' hypothesis during worm expulsion and demonstrated that the mucus barrier and its components are much more than just a 'trap'. The qualitative and quantitative changes in the mucus barrier during worm expulsion can result in directly damaging the nematode. It is important to investigate whether inducing mucus secretion and increasing mucus turnover specifically in the intestine promote worm expulsion? It is also essential to investigate whether the changes that occur during resistance, with regards to mucin glycosylation, contribute in the accelerated expulsion that has been reported with secondary *T. muris* infection. Finally, the crucial step would be to dissect the exact mechanism by which mucins, in particular Muc5ac protects against nematode infection, i.e. directly or via an associated molecule. The potential function of Muc5ac as a 'universal' effector molecule during nematode expulsion should be addressed by using different models of different intestinal-dwelling nematodes. It would be interesting to investigate whether these changes in the mucus barrier are also observed during human Trichuriasis and can these be manipulated to reduce inflammation and limit re-infection.

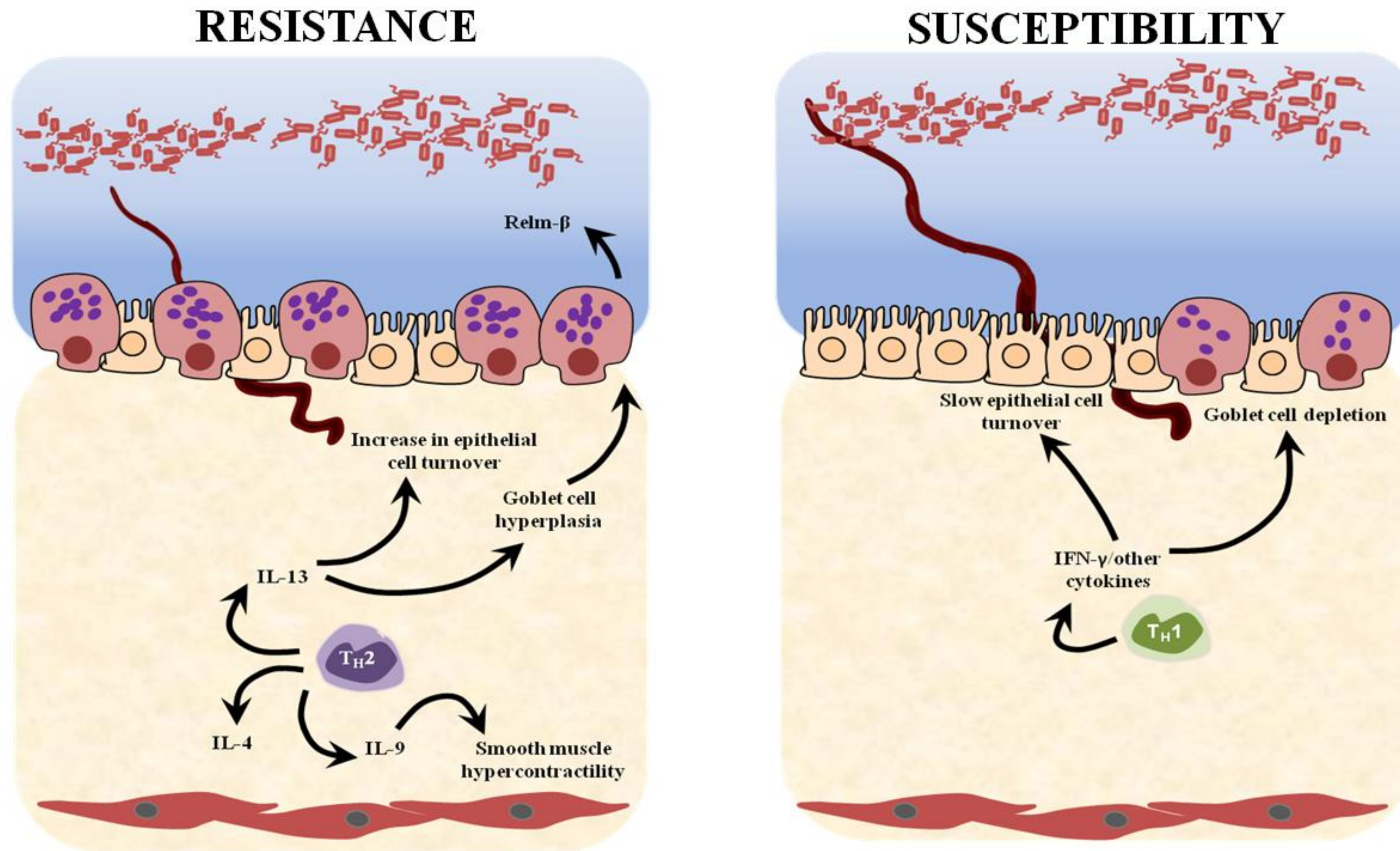


Figure 7.1: A simplified schematic overview of mechanisms previously understood to be involved in resistance and susceptibility to chronic *Trichuris muris* infection. In resistance, a T_H2-type immune response, characterised by the increased levels in IL-4, IL-9 and IL-13, is activated. IL-13 mediated intestinal epithelial cell escalator (increased cell turnover) results in dislodging the worms from their optimum niche. IL-9 results in an increase in smooth muscle contractility, which may facilitate worm expulsion. An increase in goblet cell numbers and the levels of the goblet cell product resistin-like molecule-β (Relm-β) correlate with worm expulsion. Susceptibility to chronic infection, which is characterised by a T_H1-type immune response (IFN-γ), is accompanied by slow epithelial cell turnover and no major changes in goblet cell numbers.

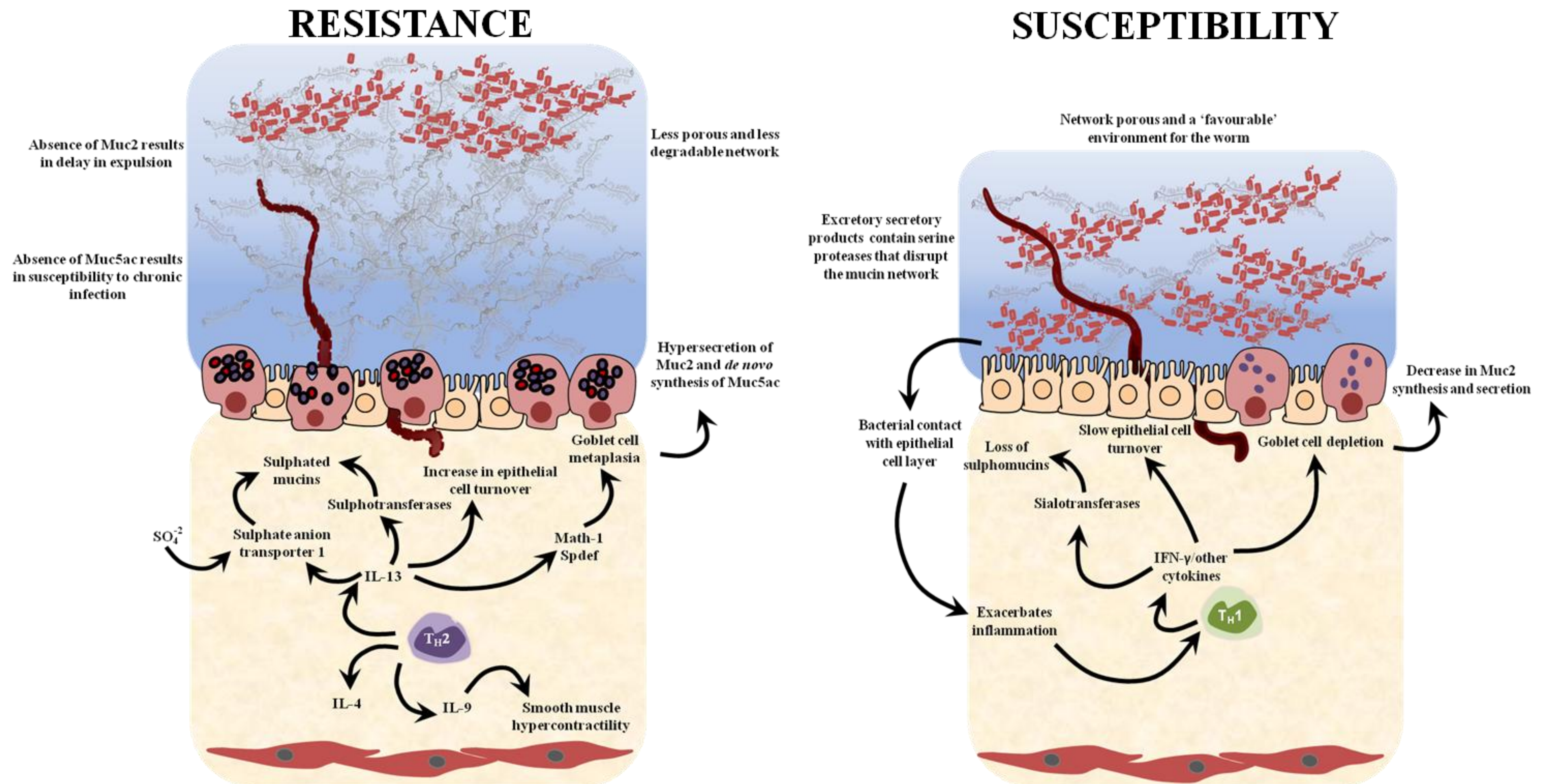


Figure 7.2: Our current understanding of the mechanisms involved in resistance and susceptibility to chronic *Trichuris muris* infection. In resistance, a T_H2-type immune response, characterised by the increased levels in IL-4, IL-9 and IL-13, is activated. IL-13 mediates the up-regulation of transcription factors (Math-1/Spdef) resulting in goblet cell hyperplasia. Increase synthesis and secretion of the mucins, Muc2 and Muc5ac, is observed and the mucins are highly sulphated. Overall, resulting in a less porous mucus network that is resistant to degradation by the nematodes excretory secretory products which has an affect on the worm's vitality. Acting along with the epithelial cell escalator and smooth muscle hypercontractility the nematodes are rejected by the host. During susceptibility, which is characterised by a T_H1-type immune response (IFN-γ), goblet cell depletion and mucins with low sulphation content which are more prone to degradation. The excretory secretory products released by the nematode disrupt the polymeric mucin network, resulting in a diminished mucus barrier which results in bacterial contact with the epithelial cell layer and exacerbated inflammation.

REFERENCES

Artis D, Grencis RK (2008). The intestinal epithelium: sensors to effectors in nematode infection. *Mucosal Immunol* **1**(4)**:** 252-264.

Artis D, Potten CS, Else KJ, Finkelman FD, Grencis RK (1999). *Trichuris muris*: host intestinal epithelial cell hyperproliferation during chronic infection is regulated by interferon-gamma. *Exp Parasitol* **92**(2)**:** 144-153.

Bancroft AJ, Else KJ, Humphreys NE, Grencis RK (2001). The effect of challenge and trickle *Trichuris muris* infections on the polarisation of the immune response. *Int J Parasitol* **31**(14)**:** 1627-1637.

Bell RG, Adams LS, Ogden RW (1984). Intestinal mucus trapping in the rapid expulsion of *Trichinella spiralis* by rats: induction and expression analyzed by quantitative worm recovery. *Infect Immun* **45**(1)**:** 267-272.

Bergstrom KS, Kissoon-Singh V, Gibson DL, Ma C, Montero M, Sham HP, Ryz N, Huang T, Velcich A, Finlay BB, Chadee K, Vallance BA (2010). Muc2 protects against lethal infectious colitis by disassociating pathogenic and commensal bacteria from the colonic mucosa. *PLoS Pathog* **6**(5)**:** e1000902.

Bethony J, Brooker S, Albonico M, Geiger SM, Loukas A, Diemert D, Hotez PJ (2006). Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. *Lancet* **367**(9521)**:** 1521-1532.

Celli JP, Turner BS, Afdhal NH, Keates S, Ghiran I, Kelly CP, Ewoldt RH, McKinley GH, So P, Erramilli S, Bansil R (2009). Helicobacter pylori moves through mucus by reducing mucin viscoelasticity. *Proc Natl Acad Sci U S A* **106**(34)**:** 14321-14326.

Cliffe LJ, Grencis RK (2004). The *Trichuris muris* system: a paradigm of resistance and susceptibility to intestinal nematode infection. *Adv Parasitol* **57:** 255-307.

Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* **308**(5727)**:** 1463-1465.

Corfield AP, do Amaral Corfield C, Wagner SA, Warren BF, Mountford RA, Bartolo DC, Clamp JR (1992). Loss of sulphate in human colonic mucins during ulcerative colitis. *Biochem Soc Trans* **20**(2)**:** 95S.

D'Elia R, Else KJ (2009). In vitro antigen presenting cell-derived IL-10 and IL-6 correlate with *Trichuris muris* isolate-specific survival. *Parasite Immunol* **31**(3)**:** 123-131.

Dineen JK, Gregg P, Windon RG, Donald AD, Kelly JD (1977). The role of immunologically specific and non-specific components of resistance in cross-protection to intestinal nematodes. *Int J Parasitol* **7**(3)**:** 211-215.

Grencis RK (2001). Cytokine regulation of resistance and susceptibility to intestinal nematode infection - from host to parasite. *Vet Parasitol* **100**(1-2)**:** 45-50.

Grencis RK, Entwistle GM (1997). Production of an interferon-gamma homologue by an intestinal nematode: functionally significant or interesting artefact? *Parasitology* **115 Suppl:** S101-106.

Hasnain SZ, Thornton DJ, Grencis RK (2010a). Changes in the mucosal barrier during acute and chronic *Trichuris muris* infection. *Parasite Immunology*. *In press*.

Hasnain SZ, Wang H, Ghia JE, Haq N, Deng Y, Velcich A, Grencis RK, Thornton DJ, Khan WI (2010b). Mucin gene deficiency in mice impairs host resistance to an enteric parasitic infection. *Gastroenterology* **138**(5)**:** 1763-1771.

Hayes KS, Bancroft AJ, Goldrick M, Portsmouth C, Roberts IS, Grencis RK (2010). Exploitation of the intestinal microflora by the parasitic nematode *Trichuris muris*. *Science* **328**(5984)**:** 1391-1394.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA (2008). Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* **5**(3)**:** e54.

Johansson ME, Phillipson M, Petersson J, Velcich A, Holm L, Hansson GC (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci U S A* **105**(39)**:** 15064-15069.

Khan WI, Collins SM (2004). Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. *Parasite Immunol* **26**(8-9)**:** 319-326.

Kringel H, Iburg T, Dawson H, Aasted B, Roepstorff A (2006). A time course study of immunological responses in *Trichuris suis* infected pigs demonstrates induction of a local type 2 response associated with worm burden. *Int J Parasitol* **36**(8)**:** 915-924.

Lee DL (2002). *The Biology of Nematodes*. Taylor and Francis London.

Levison SE, McLaughlin JT, Zeef LA, Fisher P, Grencis RK, Pennock JL (2010). Colonic transcriptional profiling in resistance and susceptibility to trichuriasis: Phenotyping a chronic colitis and lessons for iatrogenic helminthosis. *Inflamm Bowel Dis*.

Linden SK, Florin TH, McGuckin MA (2008). Mucin dynamics in intestinal bacterial infection. *PLoS One* **3**(12)**:** e3952.

Maizels RM, Pearce EJ, Artis D, Yazdanbakhsh M, Wynn TA (2009). Regulation of pathogenesis and immunity in helminth infections. *J Exp Med* **206**(10)**:** 2059-2066.

McGrath RT, McKinnon TA, Byrne B, O'Kennedy R, Terraube V, McRae E, Preston RJ, Laffan MA, O'Donnell JS (2010). Expression of terminal alpha2-6-linked sialic acid on von Willebrand factor specifically enhances proteolysis by ADAMTS13. *Blood* **115**(13)**:** 2666-2673.

McGuckin MA, Eri R, Simms LA, Florin TH, Radford-Smith G (2009). Intestinal barrier dysfunction in inflammatory bowel diseases. *Inflamm Bowel Dis* **15**(1)**:** 100-113.

Miller HR, Huntley JF, Wallace GR (1981). Immune exclusion and mucus trapping during the rapid expulsion of *Nippostrongylus brasiliensis* from primed rats. *Immunology* **44**(2)**:** 419-429.

Miller HR, Nawa Y (1979). *Nippostrongylus brasiliensis*: intestinal goblet-cell response in adoptively immunized rats. *Exp Parasitol* **47**(1)**:** 81-90.

Moncada D, Keller K, Chadee K (2003). *Entamoeba histolytica* cysteine proteinases disrupt the polymeric structure of colonic mucin and alter its protective function. *Infect Immun* **71**(2)**:** 838-844.

Podolsky DK, Gerken G, Eyking A, Cario E (2009). Colitis-associated variant of TLR2 causes impaired mucosal repair because of TFF3 deficiency. *Gastroenterology* **137**(1)**:** 209-220.

Schopf LR, Hoffmann KF, Cheever AW, Urban JF, Jr., Wynn TA (2002). IL-10 is critical for host resistance and survival during gastrointestinal helminth infection. *J Immunol* **168**(5)**:** 2383-2392.

Summers RW, Elliott DE, Qadir K, Urban JF, Jr., Thompson R, Weinstock JV (2003). *Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory bowel disease. *Am J Gastroenterol* **98**(9)**:** 2034-2041.

Summers RW, Elliott DE, Urban JF, Jr., Thompson R, Weinstock JV (2005a). *Trichuris suis* therapy in Crohn's disease. *Gut* **54**(1)**:** 87-90.

Summers RW, Elliott DE, Weinstock JV (2005b). Why *Trichuris suis* should prove safe for use in inflammatory bowel diseases. *Inflamm Bowel Dis* **11**(8)**:** 783-784.

Takeda K, Hashimoto K, Uchikawa R, Tegoshi T, Yamada M, Arizono N (2010). Direct effects of IL-4/IL-13 and the nematode *Nippostrongylus brasiliensis* on intestinal epithelial cells in vitro. *Parasite Immunol* **32**(6)**:** 420-429.

Van der Sluis M, De Koning BA, De Bruijn AC, Velcich A, Meijerink JP, Van Goudoever JB, Buller HA, Dekker J, Van Seuningen I, Renes IB, Einerhand AW (2006). Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology* **131**(1)**:** 117-129.

Xiang YY, Wang S, Liu M, Hirota JA, Li J, Ju W, Fan Y, Kelly MM, Ye B, Orser B, O'Byrne PM, Inman MD, Yang X, Lu WY (2007). A GABAergic system in airway epithelium is essential for mucus overproduction in asthma. *Nat Med* **13**(7)**:** 862-867.

Zhao F, Edwards R, Dizon D, Afrasiabi K, Mastroianni JR, Geyfman M, Ouellette AJ, Andersen B, Lipkin SM (2010). Disruption of Paneth and goblet cell homeostasis and increased endoplasmic reticulum stress in Agr2-/- mice. *Dev Biol* **338**(2)**:** 270-279.

MATERIALS AND METHODS

MATERIALS

All reagents were obtained from Sigma-Aldrich, Poole, U.K unless stated otherwise.

ABgene UK (ThermoScientific), Epsom, UK: Absolute QPCR SYBR green used for quantitative PCR.

Axiovision, CarlZeiss Ltd., UK: Images acquired.

BDH Ltd., Poole, Dorset, UK: EDTA-disodium salt, glacial acetic acid, periodic acid, sodium chloride, sodium dodecyl sulphate (SDS), Trizma Base, Urea.

Beckman Coulter, High Wycombe, UK: Quick-Seal® and ultracentrifuge tubes.

Dukes Scientific, Brookhaven Instruments Ltd., Worcestershire, UK: Monodisperse fluorescent polymer microspheres

Harlan Ltd., Oxon, UK: Provided the BALB/c, AKR and C57BL/6 mice.

Invitrogen, San Diego, USA: Dulbecco's modified eagle media (DMEM) with essential amino acids, RPMI-1640 media, foetal calf serum, L-glutamate, penicillin-streptomycin solution, sterile phosphate buffered saline without magnesium and calcium and trypsin, Superscript III for reverse transcription.

Leica Microsystems, Milton Keynes, UK: Dissecting microscope.

Melford Labs Ltd., Ipswich, UK: Dithiothrethiol (DTT).

Eurofins MWG Operon, Ebersberg, Germany: Primer synthesis.

PerkinElmer, Waltham, USA: Enhanced chemiluminescence (ECL) Western blot detection reagent.

Nikon C1 Upright confocal microscope, Melville, USA: Analysis of mucus layer.

Olympus widefield microscopes, Essex, UK: Immunofluorescent images acquired.

Promega Biosciences, Southampton, UK: IMPROM-reverse transcription kit and CellTiter-Glo® Luminescent cell viability assay.

METHODS

Animals

The protocols used at the University of Manchester were in accordance with the guidelines set out by the Home Office Scientific Procedures Act (1986). Guidelines by the McMaster University Animal Care Committee and the Canadian Council on the Use of Laboratory Animals were implemented at the McMaster University for the Muc2-deficient mouse studies. NaS1-deficient mouse studies, carried out at the Mater Medical Research Institute, were approved by the University of Queensland Animal Experimentation Ethics Committee. The mice were kept in sterilised, filter topped cages at all the animal facilities. The *T. muris* strain was maintained at the University of Manchester as previously described (Wakelin, 1967). The animals (6-12weeks) were infected with either a high dose (approximately 100-300 eggs) or a low dose infection (approximately <15 eggs) by oral gavage. At various time points during infection (as shown below), the mice were sacrificed using the standard schedule 1 method.

Naïve mice	*Uninfected, control mice*
D12-D13 pi.	*L2 Stage, (worm burden to ensure the establishment of infection)*
D14-D15 pi.	*L2 Stage*
D18-D21 pi.	*L3 moults to L4 Stage (expulsion in resistant mice)*
D35-D42 pi.	*Adult worms*

The worm burden was assessed at various time points, using the method previously described (Wakelin, 1967). In brief, the caecum from infected mice was isolated, scraped thoroughly, and the number of worms were counted using a Leica dissecting microscope.

Antibodies

Primary Antibodies

mMuc2 antibody: The mouse Muc2 (mMuc2) polyclonal antibody was raised in rabbits against a murine Muc2 peptide sequence (NGLKPVRVPDADNC) located in the N-terminus of Muc2 (Heazlewood *et al*, 2008). The peptide purified antibody was used at a dilution of 1:1000 for western blots and 1:200 dilution for immunohistochemistry (IHC).

mITF antibody: The affinity purified goat polyclonal α-ITF antibody, raised against an undisclosed peptide mapping to an internal region of mouse ITF, was obtained from

SantaCruz Biotechnology, Inc (California, USA) and used according manufacturer's instructions.

mRelm-β antibody: Rabbit polyclonal to Relm-β, raised against an undisclosed mouse Relm-β peptide, was obtained from Abcam, Cambridge, UK (Artis *et al*, 2004).

mSpdef antibody: Goat polyclonal antibody, against the N-terminus of human PDEF (known to have reactivity against mouse Spdef (Noah *et al*, 2009)) was obtained from SantaCruz Biotechnology, Inc (California, USA) and used according manufacturer's instructions.

45M1 antibody: Commercially available 45M1 antibody (Sigma-aldrich, UK) was used for the detection of mouse Muc5ac (Lidell *et al*, 2008) at a dilution of 1:1000 for western blots and 1:200 for IHC.

mMuc5b antibody: The mouse Muc5b-specific antibody was a kind gift from Dr. Camille Ehre (University of North Carolina, Chapel Hill, USA). The rabbit polyclonal antibody was raised against the repetitive sequence CQPQCQWTKWIDVDY, in the Cys-rich domains of Muc5b (Zhu *et al*, 2008).

α-IFN-γ and α-IgG antibodies: The rat immunoglobulin G1 monoclonal antibody XMG1.6 and GL113 antibody (for isotype control) were purified from supernantant by cell culture passage and protein G-Sepharose column and concentrated using a Centricon Centriprep tube. Antibody was administered at 0.5mg per 200μl of phosphate buffered saline (PBS) by intraperitoneal injection. Injections were given every 4 days starting from day -2 to day 26 post infection

mSat1 antibody: The mouse specific Sat1antibody was a kind gift from Dr. Paul Dawson (Mater Medical Institute, Brisbane, Australia) and Dr. Daniel Markovich (University of Queensland, Brisbane, Australia) (Dawson *et al*, 2010).

BrdU antibody: The rate of intestinal epithelial cell turnover was assessed by visualising bromodeoxyuridine (BrdU) incorporated into nuclei after mice were injected with 10mg of

BrdU 12-16 hours before sacrifice. The monoclonal anti-BrdU antibody was purchased from AbD Serotect, Oxford, UK to detect the BrdU by using IHC (Cliffe *et al*, 2005).

Biotinylated lectins: Biotinylated lectins (*Sambucus nigra*, *Mackia amuresis*, Peanut agglutinin, *Maclura pomifera*, *Dolichos biflorus*, *Triticum vulgaris* and *Ulex europeaus*), purchased from Sigma-Aldrich, Poole, UK, were used at 1:500 dilutions for immunofluorescent microscopy (Hasnain *et al*, 2010).

Secondary Antibodies

Anti-rabbit peroxidase labelled (1:12000) or alkaline phosphatase-conjugated (1:10000) secondary antibodies purchased from Jackson Immunoresearch, West Grove, PA were used to detect the primary antibodies on nitrocellulose membrane. Anti-rabbit and anti-goat biotin conjugated secondary antibodies were used at 1:250 for immunohistochemical microscopy. Immunofluorescent detection of primary antibodies was carried out using Alexa Fluor® anti-rabbit, anti-mouse and anti-goat dyes (1:800 dilution), obtained from Invitrogen, Paisley, UK.

Histochemical analysis

"Swiss Roll" technique

The intestine was gently cleaned using PBS, the tissue cut longitudinally, pinned straight and fixed in 95% ethanol for 1 hour. The intestine was rolled around tweezers, pinned into 'rolls' and fixed for a further 24 hours in 95% ethanol before being processed.

Tissue processing

The specimens (intestine rolled or snipped) were transversally processed (Table A.1) using the Microm Spin Tissue processor (STP) 120 (Microm UK Ltd.) and subsequently embedded in wax (Microm EC 3501). Paraffin cell blocks were stored at room temperature until cut to a thickness of 5μm using a Micron Cool Cut HM355S microtome. Sections were floated in a warm water bath (37.4°C) before being positioned on microscope slides, dried at 37°C overnight and stored at room temperature until used.

Treatment	Time
50% Ethanol	2 minutes
70% Ethanol	7 hours
90% Ethanol	45 minutes
95% Ethanol	3 x 30 minutes
100% Ethanol	3 x 30 minutes
Xylene	3 x 30 minutes
Paraffin wax	2 hours

Table A.1: Tissue processing protocol

Staining

Prior to staining, wax was removed from all slides using xylene (Genta medical, York, U.K). The tissue was placed in industrial methylated spirit (IMS) for 10 minutes, followed by a brief wash in ddH_2O or PBS.

Alcian blue and Periodic Acid Schiff's staining: For alcian blue (AB) staining the slides were placed in AB (0.6% glacial acetic acid, 19.4% dH_2O and 2g of alcian blue at pH 2.5) for 5 minutes and washed in ddH_2O briefly. For Periodic Acid Schiff's (PAS) staining the slides were treated with 0.1M potassium hydroxide for 30 minutes, placed in 1% periodic acid for 5 minutes and washed in ddH_2O. The slides were then placed in Schiff's reagent for 10 minutes and washed again in ddH_2O. All slides were washed under running dH_2O for 10 minutes and counterstained. Slides were washed in hot ddH_2O for 15 to 30 seconds and placed in IMS for dehydration, mounted using Crystal Mount™ aqueous mounting medium and sealed with cover slips.

High Iron Diamine-Alcian blue staining: Solution was prepared fresh every time as follows: 120mg of N, N-dimethly-M-phenylenediamine $(HCl)_2$ and 20mg of N, N-dimethyl-P-phenylenediamine (HCl) were dissolved in 50ml of ddH_2O and 1.4ml of 10% $FeCl_3$ added immediately. Slides were stained for 18 hours, washed with ddH_2O and stained with AB for 30 minutes (Spicer, 1965).

Immunohistochemical staining: Dewaxed tissue was rehydrated for 2 minutes in 100%, 95% and 70% IMS and washed with PBS (3 x 5 minutes). Antigen was retrieved by

incubating the slides in 10mM sodium citrate (pH6) in microwave (at full power) for 5 minutes. For mucin staining, the slides were reduced at room temperature for 30 minutes using 10mM DTT in 0.1M Tris-HCl (pH8) followed by alkylation using 25mM iodoacetamide in 0.1M Tris-HCl (pH8) in the dark at room temperature for 30 minutes. All the slides were rinsed in PBS again before the endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. Non-specific binding sites were blocked for 1 hour in 10% donkey serum and 1% BSA in PBS. Slides were incubated overnight at 4°C with primary antibody in block. Slides were washed with PBS and incubated with secondary antibody in block for 1 hour and subsequently washed with PBS once more. The slides were incubated with Dako-Strep-avidin Complex Kit horseradish peroxidase (DakoCytomation, Denmark) for 1 hour, washed with PBS and incubated for 30 minutes with Dako-DAB (di-aminobenzadine) detection solution. Slides were washed with PBS as before and counterstained with hematoxylin. The slides were dehydrated in 70%, 95% and 100% IMS before being mounted. All images were captured using the AxioVision imaging system version 4.2 (CarlZeiss Ltd, U.K).

BrdU staining: Slides were incubated in xylene overnight, washed in absolute ethanol and peroxidise activity was blocked as described above. Slides were hydrolysed with 1M HCl for 8 minutes at 60°C and subsequently neutralised for 6 minutes using boric acid buffer (0.2M othoboric acid/0.05M sodium tetraborate solution at pH8.4). Non-specific binding was blocked using 5% rabbit serum for 30 minutes at room temperature. Slides were washed with PBS and incubated with anti-BrdU antibody for 1 hour. Thereafter, the slides were developed as described above (immunohistochemical staining).

Immunofluorescent staining: Dewaxed tissue was rehydrated for 2 minutes in 100%, 95% and 70% IMS and washed with PBS (3 x 5 minutes). Antigen was retrieved by incubating the slides in 10mM sodium citrate (pH6) in microwave (at full power) for 5 minutes. The slides were reduced at room temperature for 30 minutes using 10mM DTT in 0.1M Tris-HCl (pH8) followed by alkylation using 25mM iodoacetamide in 0.1M Tris-HCl (pH8) in the dark at room temperature for 30 minutes. The slides were rinsed in PBS again before the endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol. Non-specific binding sites were blocked for 1 hour in 10% goat serum and 1% BSA in PBS. Slides were then incubated at room temperature for 2 hours with primary antibody in block. Slides were washed with PBS and incubated with the corresponding secondary antibody in PBS

for 1 hour, and subsequently washed with PBS once more. Slides were counterstained with DAPI and mounted using the Prolong®-antifade mounting medium (Invitrogen, Paisley, UK). All images were captured using the MetaVue, Olympus imaging system (Olympus, Essex, UK).

Counterstaining

Hematoxylin staining

Slides were stained with haematoxylin (diluted 1:5) for 1 minute, washed in ddH_2O, stained with Eosin for 1 minute, washed with ddH_2O and subsequently dehydrated 50% to 100% ethanol. The slides were then mounted and sealed with cover slips.

Fast-green staining

Slides were stained with 1% fast-green in acetic acid for 1 minute, washed in ddH_2O and subsequently incubated with 1% acetic acid for 5 minutes. The slides were then dehydrated using 50% to 100% IMS, mounted and sealed with cover slips.

Controls

A tissue section on each slide was incubated with secondary antibody only to measure the background staining and autofluorescence. In addition, primary antibody was saturated with the immunising peptide, prior to staining to confirm antibody specificity.

Biochemical analysis of mucins and mucus

Total mucus extraction

The intestine (caecum and colon kept separate) was gently flushed with PBS to remove the faecal matter and then flushed extensively using a 19.5G" needle and syringe with a total volume of 7ml of 6M guanidinium chloride (GuCl) reduction buffer (containing 0.1M Tris-HCl and 5mM EDTA at pH8). Residual mucus was scraped using a cell scraper and the samples were solubilised by rotating at 4°C overnight. For crude mucus analysis, the intestine was scraped, lyophilised and stored at -80°C until required.

Secreted mucus extraction

The intestine was gently flushed with PBS and subsequently flushed 5 times with 3M urea to obtain the secreted mucus from the lumen of the intestine. The tissue was processed for

histology before and after treatment to ensure that only the secreted mucus was isolated and the intestinal crypts remained intact.

Separation of mucins by agarose gel electrophoresis

Before gel electrophoresis the samples were reduced with 50mM dithiothreitol (DTT) for 2 hours at 37°C and subsequently carboxymethylated using 0.125M iodoacetamide for 30 minutes, at room temperature, in the dark to prevent reformation of the disulphide bonds. The samples were then dialysed into 6M Urea and 10% (v/v) loading buffer was added before electrophoresis (Table A.2).

Solution	Contains
TAE	40mM Tris acetate and 1mM EDTA, pH8 made up in 1 litre of dH_2O
10% Loading buffer	50% (v/v) glycerol, 10 x TAE, bromophenol blue, 1% (w/v) SDS
TBST	10mM Tris-HCl, 150mM NaCl, 0.05% Tween-20, pH8

Table A.2: Solutions for agarose gel electrophoresis and immunodetection

Samples were electrophoresed on 0.7-1% (w/v) agarose gels in TAE buffer and 0.1% SDS at 30 volts for 15 hours (Sheehan *et al*, 2000). After electrophoresis, mucins were transferred to a nitrocellulose membrane by vacuum blotting in 0.6M sodium chloride and 60mM sodium citrate at a pressure of 45-50 mbar using a Vacugene XL (Pharmacia Biotech, Amersham, UK) for 2 hours (Sheehan *et al*, 2000).

Slot blot Analysis

Fractions taken from the tubes were blotted onto nitrocellulose membrane using a 72-well slot blot manifold (Schleicher & Schuell, Dassel, Germany) attached to a vacuum pump. Membranes were then assayed for glycoprotein using PAS reagent or probed with the relevant antibody (as described previously). Staining intensity was measured using a Bio-Rad GS-800 Calibrated densitometer (Thornton *et al*, 1994).

Glycoprotein detection using PAS staining: Nitrocellulose membrane was washed with dH_2O, incubated at room temperature for 30 minutes with 1% (v/v) periodic acid and 3%

(v/v) acetic acid, washed with dH_2O and incubated with 0.1% (w/v) sodium metabisulphite/0.01M HCl for 2 x 5 minutes. Schiff's reagent was subsequently added to the membrane for approximately 10-15 minutes, this was rinsed with sodium metabisulphite and dH_20 to stop the reaction and dried (Thornton *et al*, 1994).

HID assay: Nitrocellulose membranes, following a wash with dH_2O were incubated with HID stain (described previously) for 18 hours at room temperature. The membrane was subsequently washed with dH_2O and dried.

Immunodetection: Nitrocellulose membrane was briefly washed in TBST and then blocked using 1% (w/v) skimmed milk powder in TBST (Table A.2) for at least 30 minutes, followed by 2 x 15 minute TBST washes and subsequent incubation of the primary antibody overnight. The membrane was washed 3 x 5 minutes with TBST, before and after the incubation with the appropriate secondary antibody for 30 minutes (refer to antibodies section). The membrane was incubated with enhanced chemiluminescence western detection reagent (Perkin-Elmer, USA) for approximately 2 minutes and then washed to remove excess and subsequently exposed to BIOMAX™BML film (Kodak, UK). For the phosphatase conjugated secondary antibody the membrane was washed with TBST (2 x 15 minutes) followed by approximately 4-5 minute incubation with Nitro-blue tetrazolium/5-bromo-4-chloroindol-3yl phosphate.

Purification of mucins

Saliva was collected and solubilised in 4M GuCl at 4°C. Purification was performed by isopycnic density gradient centrifugation as described by Davies & Carlstedt: caesium chloride (CsCl) (Melford, UK) was added to the solubilised mucus to achieve a starting density of 1.4g/ml. This suspension was then centrifuged in a Beckman Optima™ L-90K Ultracentrifuge (Beckman Ti 70 rotor) at 40,000RPM for 65 hours at 15°C. Following centrifugation, fractions were taken from the top of the tubes, densities were determined, and fractions were then analysed by slot blotting. The mucin-containing fractions (as specified in chapters) were then pooled and dialysed into 0.2 M GuCl before being subjected to a second CsCl density gradient centrifugation step at a starting density of 1.5mg/ml in a Beckman Optima™ L-90K Ultracentrifuge (Beckman Ti 90 rotor) at 40,000RPM for 65 hours at 15°C. Following centrifugation, fractions were taken from the

top of the tubes, densities were determined, and fractions were then analysed by slot blotting and by agarose gel electrophoresis followed by western blotting.

Anion Exchange Chromatography

The PAS-rich fractions were pooled after the second CsCl density gradient and subjected to anion exchange chromatography as described previously (Linden *et al*, 2002; Thornton *et al*, 1996), using a Resource™ Q column. Samples were eluted with the starting buffer (10mM piperazine at pH5 in 6M Urea containing 0.02% 3-[(chloamidopropyl) dimethylammonio]-1-propanosulphonate) for 15 minnutes (0.5ml/min), followed by a linear gradient (60 minutes) up to 0.4M Lithium perchlorate-10mM piperazine at pH 5 in 6M Urea containing 0.02% 3-[(chloamidopropyl) dimethylammonio]-1-propanosulphonate (Linden *et al*, 2002; Thornton *et al*, 1996).

Rate Zonal Centrifugation

6–8M GuCl gradients were formed in centrifuge tubes using an MSE gradient maker connected to a Gilson Minipuls-2 peristaltic pump. Mucin samples were adjusted to 4M GuCl and loaded onto the tops of the gradients and centrifuged in a Beckman Optima™ L-90K Ultracentrifuge (Beckman SW40 rotor) at 40,000RPM for 2.75 or 3.50 hours (as stated in chapters) at 15°C. Fractions were taken from the top of the tubes, analysed by slot blotting and PAS-staining (Thornton *et al*, 1990). The refractive index of each fraction was measured using a refractometer to ensure gradients were comparable.

Analysis of mucus network properties

Caecal tissue isolated from BALB/c and AKR mice was cut longitudinally, washed with PBS and kept hydrated with PBS in a 6 well plate. 0.1µm blue fluorescently labelled polymer microspheres (Dukes Scientific, UK) were placed on top of the luminal surface of the caecum (set as a reference) and their position analysed using the Nikon C1 Upright confocal microscope. 3D optical stacks were taken every 5µm and combined to obtain a Z-stack at the time points stated. All images were analysed using the EZ-C1 freeviewer software (*version 3.9*).

Gene Expression Analysis

Epithelial cell isolation

The intestine was flushed with Hank's Balanced Salt Solution (HBSS) without Ca^{2+} and Mg^{2+} (Invitrogen, Paisley, UK) using a 19.5G" needle. The intestine was cut longitudinally and then cut into 5mm lengths. To remove the mucus the tissue was incubated at 37°C with 10mM DTT in HBSS for 30 minutes with samples being shaken gently every 10 minutes. The supernatant was removed and 0.8M EDTA in HBSS was added to the tissue, which was incubated at 37°C for 30 minutes and shaken gently every 10 minutes to detach the epithelial cells (Humphreys and Grencis, 2009). The supernatant was filtered through a 100µm cell strainer to separate the tissue from the epithelial cells and centrifuged at 109.5 x g for 5 minutes. The supernatant was removed and 1ml of TRIZOL® (Invitrogen) was added to the cells (stored at -80°C).

RNA extraction

RNA was separated from the high molecular weight DNA and cell debris by centrifugation at 12470 x g for 10 minutes at 4°C (Biofuge Fresco, Heraeus Instruments). The supernatant was recovered and incubated at room temperature for 5 minutes. To separate the phases 0.2ml of chloroform was added and samples were mixed vigorously for 15 seconds, followed by centrifugation at 12470 x g for 10 minutes at 4°C. The upper aqueous layer was withdrawn and precipitated using 0.5ml of isopropyl alcohol. The samples were stored overnight at -80°C. Samples were centrifuged at 12470 x g at 4°C for 10 minutes; the supernatants were withdrawn, the pellet was washed with 1ml of 75% ethanol and centrifuged at 12470 x g for 5 minutes at 4°C twice. The supernatant was withdrawn and the RNA pellet was air-dried at RT for 15-20 minutes; RNA was resuspended in 50µL of DEPC-treated water. The concentration of RNA was determined using a ND-1000 Spectrophotometer (NanoDrop technology) and ND-1000 version 3.2.1 software. The quality of RNA was verified using the Agilent 2100 Bioanalyzer (Agilent Technologies Inc., USA).

Reverse Transcription

1µg of RNA was denatured at 95°C for 5 minutes and subsequently chilled to 4°C for 1 minute before the addition of 2.5µl of Random primer mix, 1.5µl of 10mM dNTP, 6µl of 5x buffer, 0.6µL of RNAsin®, 1.5µl of reverse transcriptase, 3.6µl of $MgCl_2$ (from

IMPROM-RT or Superscript III kits). Nuclease free water was added to make up the final volume to 24µl, the samples were heated to 25°C for 5 minutes followed by incubation at 38°C for 1 hour to synthesise the cDNA strand. The enzymes were inactivated by heating the samples to 70°C for 15 minutes and finally chilling the samples to 4°C. The cDNA was stored at -20°C until analysed by polymerase chain reaction (PCR). The negative controls lacked either the extracted RNA or the reverse transcriptase as stated in chapters.

Quantitative PCR

PCR was performed to measure the gene expression of several intestinal genes. Primer sequences (specified within chapters) were designed using the Jellyfish programme and synthesised by Eurofins MWG Operon, U.K. The housekeeping gene β-actin allowed normalisation of the genes of interest. Efficiencies were determined for all the individual primer sets by using primer dilutions and cDNA dilutions. PCR amplifications were performed in samples consisting of 1µl of cDNA sample, 1pmol of primers (sense and antisense), master mix (Absolute qPCR SYBR Green Kit, ABgene, UK) and DEPC-treated water to make up the final volume (25µl). The amplification and analysis was carried out under the following conditions using DNA Engine OPTICON and OPTICON Monitor software *v2.03* respectively (Real-Time systems; MJ Research): after denaturation for 10 minutes at 95°C, 40 cycles of primer annealing for 20 seconds at 60°C, elongation step for 20 seconds at 72°C, and denaturation at 94°C for 20 seconds. SYBR Green fluorescence was monitored after each cycle at 72°C to monitor the melting curves.

Sequencing

In gel digestion: The amplified PCR product was visualised on a 2% agarose gel using 2µL ethidium bromide and a UV transluminator and consequently excised from the gel. The products were purified using the GENECLEAN® TURBO KIT (Qbiogen, Cambridge, U.K) using the following process. 100µL/0.1g of GENECLEAN® turbo salt solution was added to the excised gel slice to extract the DNA and incubated at 55°C for 5 minutes to melt the gel. The solution was placed in the GENECLEAN® turbo cartridge which binds the DNA and centrifuged for 5 seconds to eliminate the unbound liquid. GENECLEAN® turbo wash solution was used to wash the bound DNA twice before eluting the DNA using 30µL of GENECLEAN® turbo elution solution.

In solution digestion: The PCR products were digested with exonuclease I and calf intestinal phosphatise at 37°C for 1 hour. Enzymes were denatured at 75°C for 15 minutes and subsequently sequenced.

Sequencing: The sequencing reaction consisted of 3μl Big Dye® terminator (v3.1 Applied Biosystems, USA), 2.5% (v/v) DMSO, 5-7μl of isolated DNA and 1pmol of sense and anti-sense primers of the gene of interest. Sequencing reaction involved denaturation for 4 minutes at 96°C, 30 cycles of denaturation at 98°C for 30 seconds, primer annealing for 30 seconds at 50°C, elongation step for 60°C for 4 minutes and 2:1 (v/v) of 100% ethanol and 3M sodium acetate was added to the sample to precipitate the DNA, which was incubated at -80°C overnight. The sample was centrifuged at 12470 x g for 15 minutes, the supernatant was withdrawn, the pellet was washed with 100μl of 70% ethanol and the pellet was dried at 60°C for 20 minutes. The samples were subsequently sequenced using the ABI sequencing machine by the sequencing Facility, Faculty of Life Sciences, University of Manchester. The data was analysed using ChromasPro® v1.34 (www.technelysium.com.au/ChromasPro.html) and the sequences obtained were compared against a database (GenBank at http://www.ncbi.nlm.nih.gov/BLAST).

In vitro analysis

Worm isolation for ATP analysis

The caecum was longitudinally cut and segmented before incubation with 0.1M NaCl for 2 hours at 37°C with frequent shaking. Worms were counted after separation from debris and epithelial cells using a 0.7μm filter and kept in RPMI-1640 supplemented with 10% foetal bovine serum (FBS). Alive worms were subsequently homogenised using the FastPrep® homogeniser (MP Biomedicals, Inc.).

Energy status of worms

The CellTiter-Glo® luminescent cell viability assay was carried out according to manufacturer's instructions (Promega Corp., USA). Relative light units (RLUs) were calculated per worm: RLU = (sample light units − blank light units)/number of worms. Substrate only was used as a blank control; worms were boiled before homogenisation for negative controls. To determine recovery of energy status, worms were washed extensively in DMEM, added to 6 well plates with LS174T cells for 24 hours prior to measuring ATP levels.

LS174T cells

LS174T cells (European Collection of Cell Culture, Salisbury, UK) were cultured with complete medium containing DMEM, 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 10% heat inactivated FBS (all from Invitrogen, Paisley, U.K) at 37°C in a humidified incubator (95% air with 5% CO_2). When confluent, cells were gently scraped until dislodged from the flasks surface and rinsed with approximately 5ml of complete media. The suspended cells were dispersed using a 25G" needle, resuspended at approximately 1 x 10^6 cells/ml (Hayes et al, 2007).

Treatment of mucins with *T. muris* ESPs

Adult worms were harvested and cultured *in vitro,* after 24 hours the released excretory secretory products (ESPs) were collected (Else and Wakelin, 1989). Aliquots of crude mucus (in equal volumes of PBS) were incubated at 37°C with 50-300µg/ml of ESPs for various time points (as specified in figure legends). Control samples were not treated with the ESPs, but were incubated at 37°C for the maximum time point. All samples were analysed by rate zonal centrifugation.

Cytokine analysis

Mesenteric lymph nodes (mLNs) were removed, cells were isolated and resuspended at 5 x 10^6 cells/ml in RPMI 1640 with 10% FBS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cultures were stimulated with 50µg/ml of ESPs for 24 hours at 37°C and 5% CO_2. Cell free supernatants were stored at -80°C; cytokine analysis was carried out by Cytometric Bead Analysis (CBA) provided by BD Biosciences, using the manufacturer's instructions.

<u>Analysis</u>

Goblet cell number analysis

Stained goblet cells (number stated) similar sized crypts were counted per mouse and the mean number of goblet cells per 50-100 crypts was determined in three individual mice.

Goblet cell size and staining intensity analysis

Images taken at x20 magnification were calibrated to the same scale in pixels using the Image J software. The area of goblet cells contained in 50-100 crypts was determined as

the area/mm^2 (pixels). The staining intensity was measured using the Image J software, data represented as arbitrary units.

Statistical analysis

Statistical analysis was performed using the one-way ANOVA when comparing more than two samples and paired t-tests were used to compare two samples with the GraphPad PRISM version 3.2 (GraphPad software, Inc.) or with SPSS Version 16.0 (SPSS Inc, Chicago, USA). All results are presented as the mean ± standard error of the mean. $P<0.05$ was considered statistically significant.

REFERENCES

Artis D, Wang ML, Keilbaugh SA, He W, Brenes M, Swain GP, Knight PA, Donaldson DD, Lazar MA, Miller HR, Schad GA, Scott P, Wu GD (2004). RELMbeta/FIZZ2 is a goblet cell-specific immune-effector molecule in the gastrointestinal tract. *Proc Natl Acad Sci U S A* **101**(37)**:** 13596-13600.

Cliffe LJ, Humphreys NE, Lane TE, Potten CS, Booth C, Grencis RK (2005). Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion. *Science* **308**(5727)**:** 1463-1465.

Dawson PA, Russell CS, Lee S, McLeay SC, van Dongen JM, Cowley DM, Clarke LA, Markovich D (2010). Urolithiasis and hepatotoxicity are linked to the anion transporter Sat1 in mice. *J Clin Invest* **120**(3)**:** 706-712.

Else K, Wakelin D (1989). Genetic variation in the humoral immune responses of mice to the nematode *Trichuris muris*. *Parasite Immunol* **11**(1)**:** 77-90.

Hasnain SZ, Thornton DJ, Grencis RK (2010). Changes in the mucosal barrier during acute and chronic *Trichuris muris* infection. *Parasite Immunology*. *In press.*

Hayes KS, Bancroft AJ, Grencis RK (2007). The role of TNF-alpha in *Trichuris muris* infection II: global enhancement of ongoing Th1 or Th2 responses. **29**(11)**:** 583-594.

Heazlewood CK, Cook MC, Eri R, Price GR, Tauro SB, Taupin D, Thornton DJ, Png CW, Crockford TL, Cornall RJ, Adams R, Kato M, Nelms KA, Hong NA, Florin TH, Goodnow CC, McGuckin MA (2008). Aberrant mucin assembly in mice causes endoplasmic reticulum stress and spontaneous inflammation resembling ulcerative colitis. *PLoS Med* **5**(3)**:** e54.

Humphreys NE, Grencis RK (2009). IL-1-dependent, IL-1R1-independent resistance to gastrointestinal nematodes. *Eur J Immunol* **39**(4)**:** 1036-1045.

Lidell ME, Bara J, Hansson GC (2008). Mapping of the 45M1 epitope to the C-terminal cysteine-rich part of the human MUC5AC mucin. *FEBS J* **275**(3)**:** 481-489.

Linden S, Nordman H, Hedenbro J, Hurtig M, Boren T, Carlstedt I (2002). Strain- and blood group-dependent binding of Helicobacter pylori to human gastric MUC5AC glycoforms. *Gastroenterology* **123**(6)**:** 1923-1930.

Noah TK, Kazanjian A, Whitsett J, Shroyer NF (2009). SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells. *Exp Cell Res* **316**(3)**:** 452-465.

Sheehan JK, Brazeau C, Kutay S, Pigeon H, Kirkham S, Howard M, Thornton DJ (2000). Physical characterization of the MUC5AC mucin: a highly oligomeric glycoprotein whether isolated from cell culture or in vivo from respiratory mucous secretions. *Biochem J* **347 Pt 1:** 37-44.

Spicer SS (1965). Diamine Methods for Differentialing Mucosubstances Histochemically. *J Histochem Cytochem* **13:** 211-234.

Thornton DJ, Carlstedt I, Howard M, Devine PL, Price MR, Sheehan JK (1996). Respiratory mucins: identification of core proteins and glycoforms. *Biochem J* **316 (Pt 3):** 967-975.

Thornton DJ, Carlstedt I, Sheehan JK (1994). Identification of glycoproteins on nitrocellulose membranes and gels. *Methods Mol Biol* **32:** 119-128.

Thornton DJ, Davies JR, Kraayenbrink M, Richardson PS, Sheehan JK, Carlstedt I (1990). Mucus glycoproteins from 'normal' human tracheobronchial secretion. *Biochem J* **265**(1)**:** 179-186.

Wakelin D (1967). Acquired immunity to *Trichuris muris* in the albino laboratory mouse. *Parasitology* **57**(3)**:** 515-524.

Zhu Y, Ehre C, Abdullah LH, Sheehan JK, Roy M, Evans CM, Dickey BF, Davis CW (2008). Munc13-2-/- baseline secretion defect reveals source of oligomeric mucins in mouse airways. *J Physiol* **586**(7)**:** 1977-1992.