Investigating the role of the intestinal barrier in regulation of immune homeostasis in the gut

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

FELIPE ANDRES MELO GONZALEZ

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Abstract

The University of Manchester

Felipe Andres Melo Gonzalez

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Investigating the role of the intestinal barrier in regulation of immune homeostasis in the gut

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The intestinal barrier represents a complex environment, composed of different physical barriers and immune cells, which act to prevent the entrance of potentially harmful enteric pathogens and to maintain gut tolerance to food antigens and commensal bacteria. Thus, cross-talk between the different components of the intestinal barrier such as the mucus layer, dendritic cells (DC) and intestinal intraepithelial lymphocytes (IELs) may be important in maintenance of gut homeostasis.

This thesis investigates how different components of the intestinal barrier regulate immune responses in the gut. Thus, expression of the transmembrane receptor integrin $\alpha v\beta 8$ on DCs is shown to be required for the development of a specific IEL subset marked by expression of CD4 and CD8$\alpha\alpha$, suggesting that intestinal DC play important roles in regulating the IEL compartment. Moreover, considering that intestinal DCs are likely in close contact with intestinal mucus, it was hypothesized that interactions between DCs and mucins, the predominant proteins that form the mucus layer, may modulate DC function. To test this hypothesis, intestinal mucin was purified and used to treat human monocyte-derived DCs. It was found that that expression of the chemokine IL-8 and co-stimulatory DC markers CD86 and CD83 are significantly upregulated on human DCs in the presence of intestinal mucins. Additionally, IL-8 produced by mucin-treated DCs is able to recruit neutrophil-like cells in transmigration assays. These effects were not due to mucin sample contaminants such as LPS, DNA or contaminant proteins. Instead, mucin glycans seem to be important for the induction of these effects on moDCs. Thus, in contrast to recent published results, intestinal mucins appear capable of inducing important pro-inflammatory functions in DC.

To investigate whether mucins modulated DCs found in the intestinal environment, intestinal mucins were used to treat murine intestinal DCs, and gene changes explored using microarray analysis. It was found that, amongst several genes modulated in intestinal DC, up-regulation of the mucosal cytokine IL-22 was induced by intestinal mucin.

Therefore, interactions between different components of the intestinal barrier might be crucial for maintaining gut homeostasis. Understanding how different components of the intestinal barrier system work together to maintain homoeostasis may identify pathways that can be targeted to restore this balance in inflammatory disorders such as inflammatory bowel disease.

DECLARATION

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ABBREVIATIONS

DC: dendritic cell

DTT: dithiothreitol

EDTA: Ethylenediaminetetraacetic acid

FCS: foetal calf serum

FOXP3: forkhead box P3

GP: glycopeptide

IEC: intestinal epithelial cell

IEL: intraepithelial lymphocyte

IFN: interferon

IL: interleukin

LI: large intestine

LP: lamina propria

LPS: lipopolysaccharide

MACS: magnetic-activated cell sort

MALLS: multi-angle laser light scattering

MLN: mesenteric lymph node

moDC: monocyte-derived dendritic cell

PBMC: peripheral blood mononuclear cell

PBS: phosphate buffered saline

RA: retinoic acid

RT: room temperature

SEC: size exclusion chromatography

SI: small intestine

Th: helper t cell

TGF-β: transforming growth factor beta

T_{reg}: regulatory T cell

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1 CHAPTER 1: Introduction

1.1 Introduction

The intestinal tract constitutes a complex environment, populated by a high number of commensal bacteria and exposed daily to a diverse range of food antigens and potentially pathogenic agents ingested orally. A protective mucus barrier covers the intestinal tract, and its differential organisation between the small intestine and colon protects the intestine from pathogens but also allows the absorption of food nutrients. Moreover, lined by mucus, a layer of specialised epithelial cells separates the host from the intestinal luminal environment, constituting another physical barrier. These cells include absorptive enterocytes, specialised for metabolic and digestive functions, and secretory intestinal epithelial cells (IECs) such as enteroendocrine cells, Paneth cells and goblet cells (Peterson and Artis, 2014). Goblet cells secrete the main intestinal mucin MUC2 whereas Paneth cells are the main source of antimicrobial peptides such as defensins, lysozyme C, C-type lectins and phospholipases (Bevins and Salzman, 2011). Additionally, IECs express different pattern-recognition receptors (PRRs) such as toll-like receptors (TLR), NOD-like receptors (NLR) and RIG-I-like receptors (RLR), sensing the gut microbiota and regulating homeostasis, by secreting different factors and cytokines (Peterson and Artis, 2014). Thus, IECs may regulate the function of different immune cells residing in the epithelium, such as intraepithelial lymphocytes (IELs), and other immune cells residing underneath the epithelium, in the LP (LP).

LP mononuclear phagocytes, including macrophages and dendritic cells (DC) are particularly important in gut homeostasis, as they are antigen-presenting cells that can promote tolerance or effector functions against enteric pathogens. DCs can migrate to the mesenteric lymph nodes (MLN), where they can prime naïve $CD4^+$ T cells and promote their differentiation into T helper (Th) subsets or regulatory T cells (T_{regs}). Th subsets constitute effector cells including Th1, Th2 and Th17, which secrete characteristic cytokines that induce different types of immune responses against intracellular or extracellular pathogens (Coombes and Powrie, 2008, Rescigno and Di Sabatino, 2009). On the other hand, T_{regs} constitute a tolerogenic $CD4^+$ T-cell subset that can supress immune responses against food antigens or antigens from commensal bacteria, preventing inappropriate inflammatory responses and leading to tolerance. The balance between T_{regs} cells and Th1, Th2 and Th17 responses is crucial in gut homeostasis and abnormal inflammation is related with the development of inflammatory bowel disease (IBD), characterised as two separate diseases; ulcerative colitis and Crohn's disease (Coombes and Powrie, 2008, Rescigno and Di Sabatino, 2009). This PhD thesis

researches different aspects of intestinal barrier immunity, including intestinal factors regulating the development of gut intraepithelial lymphocytes and components of the intestinal mucus layer regulating DC function. These different aspects of intestinal immunity are introduced below.

1.2 Role of the intestinal mucus barrier in gut homeostasis

1.2.1 Intestinal mucus organisation

Mucus, a dynamic, viscoelastic fluid covering mucosal epithelia, plays a defensive role against different environmental agents, including pathogens. The mucus layer is especially relevant in the intestine, a place highly exposed to bacteria and food antigens (Linden et al., 2008, Thornton et al., 2008, Atuma et al., 2001). The mucus layer is present along the entire length of the intestine, although it shows a different organisation between the small and large intestine (Atuma et al., 2001, Johansson et al., 2011).

Two different intestinal mucus layers have been identified in rodents: an inner layer, firmly attached to the epithelia and an outer layer, easily removed by suction (Atuma et al., 2001). Both layers are thinner and less well defined in the small intestine, especially in the jejunum, whereas colonic layers are thicker, reaching 116 μm and 716 μm in the inner and outer layer, respectively (Figure 1.1)(Atuma et al., 2001, McGuckin et al., 2011). These findings are consistent with the function of the different portions of the intestine; small intestinal mucus must allow macromolecule diffusion and nutrient absorption, whereas large intestinal mucus must protect the intestinal epithelia from bacterial invasion. Thus, the inner mucus layer is proposed to be completely sterile in a healthy human colon (Johansson et al., 2011, McGuckin et al., 2011).

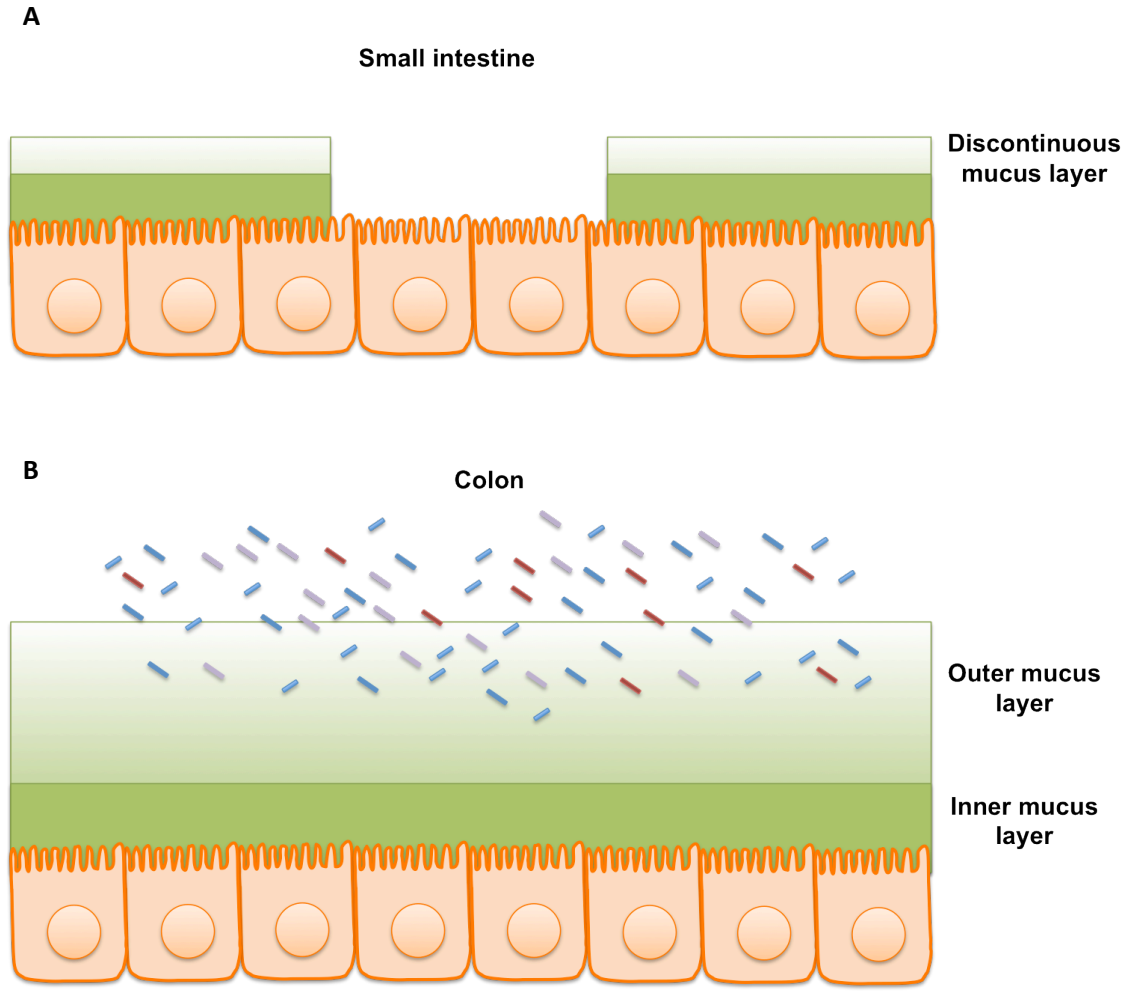

Figure 1.1 Organisation of the mucus layer in the intestinal tissue.

Schematic representation of the mucus layer throughout the gastrointestinal tract. (A) The small intestine has a thin and discontinuous mucus layer, which facilitates the absorption of nutrients. Although two layers can be identified, they are less defined compared to the colon. (B) The colon is covered by a thick mucus layer, which can be subdivided in two different layers; an inner and firmly attached mucus layer, which is proposed to be completely sterile during steady-state conditions, and an outer loose layer, which populated by commensal microbiota (represented as coloured small lines). Adapted from Johansson et al., 2011.

1.2.2 Mucins are extensively glycosylated

Mucins are the main macromolecular component of mucus and are responsible for its viscoelastic properties. They constitute a family of heavily glycosylated proteins (MUC proteins), which exhibit high molecular weights between 2 and 20 MDa (Harrop et al., 2012). The mucin core protein is characterised by the presence of a variable number of tandem repeat (TR) sequences, containing up to 500 TR, and each TR can provide 5-100 glycosylation sites (Hollingsworth and Swanson, 2004). These TR domains are rich in proline, serine and threonine, allowing the binding of glycans to hydroxyl groups on serine and threonine, via the linking sugar N-acetylgalactosamine (Figure 1.2A) (Hasnain et al., 2013a, Hattrup and Gendler, 2008). All mucin glycans are initiated by a primary core structure composed by a N-acetylgalactosamine α-linked to a serine or threonine (GalNAcα1Ser/Thr) also known as Tn antigen (Van den Steen et al., 1998). Thus, additional glycans are added and 8 different types of linear or branched core structures can be generated (Van den Steen et al., 1998). Core 1 and core 3 O-derived glycans are required for Muc2 protective effects in the intestine. Core 1-O derived glycans are synthesised by Core 1 β1,3-galactosyltransferase (C1galt1), which transfers galactose (Gal) to the Tn antigen, whereas core 3 O-derived glycans and synthesised by Core 3 β1,3-N-acetylglucosaminyltransferase (C3GnT), which transfers N-acetylglucosamine (GlcNAc) from UDP-GlcNAc to the Tn antigen (Bergstrom and Xia, 2013). Interestingly, core 1 O-derived O-glycans are expressed in most tissues whereas expression of core 3 O-derived glycans is mainly restricted to the colon (Bergstrom and Xia, 2013). Glycosylation diversity is increased by additional modification such as sulphation of galactose and N-acetylglucosamine and addition of fucose and sialic acid, which give a negative charge to mucins (Hattrup and Gendler, 2008). Mucin glycans can be recognised by lectins, adhesion molecules and bacteria (Hollingsworth and Swanson, 2004). The relevance of these interactions in immune cells, especially between sialic acid and some specific lectins, will be discussed later.

Mucins can be secreted extracellularly by goblet cells or expressed on the surface of epithelial cells. Secreted mucins form a highly hydrated gel that can immobilise pathogens and protect the epithelia against chemical or mechanical damage. There are 5 members of the secreted mucin subfamily: MUC2/Muc2, MUC5AC/Muc5ac, MUC5B/Muc5b, MUC6/Muc6 and MUC19/Muc19 (herein human mucins will be referred with capital letters and mouse mucins with lower case). These mucins are usually characterised by amino- and carboxyl-terminal cysteine-rich regions, allowing formation of polymers by intermolecular disulphide bonds and organisation as polymers (Figure 1.2B) (Linden et al., 2008). Membrane-associated mucins are

the major constituent of the glycocalyx**,** located underneath the mucus layer, which is a zone found in the surface of the cells composed by glycolipids and glycoproteins with a variable thickness (Linden et al., 2008). Usually membrane-associated mucins are composed of two subunits: one heavily glycosylated extracellularly and an intracellular domain, which comprises a short extracellular region, a transmembrane domain and a cytoplasmic tail (Harrop et al., 2012). Examples of cell surface mucins are MUC1, MUC3A/B, MUC4, MUC13 and MUC17.

The main intestinal secreted mucin is MUC2, whereas stomach mucus is characterised by MUC5AC and MUC6 secretion (McGuckin et al., 2011). Muc2 is found in both the outer and inner intestinal mucus layers, although the structure of this mucin in both layers is different. Muc2 is structured as sheets of polymers, firmly attached to the inner layer, and acting as an impenetrable barrier to enteric bacteria. In contrast, it forms an expanded structure in the outer layer due to partial proteolytic cleavage, and is soluble in the noncovalent bond-breaking agent guanidinium chloride (Johansson et al., 2011).

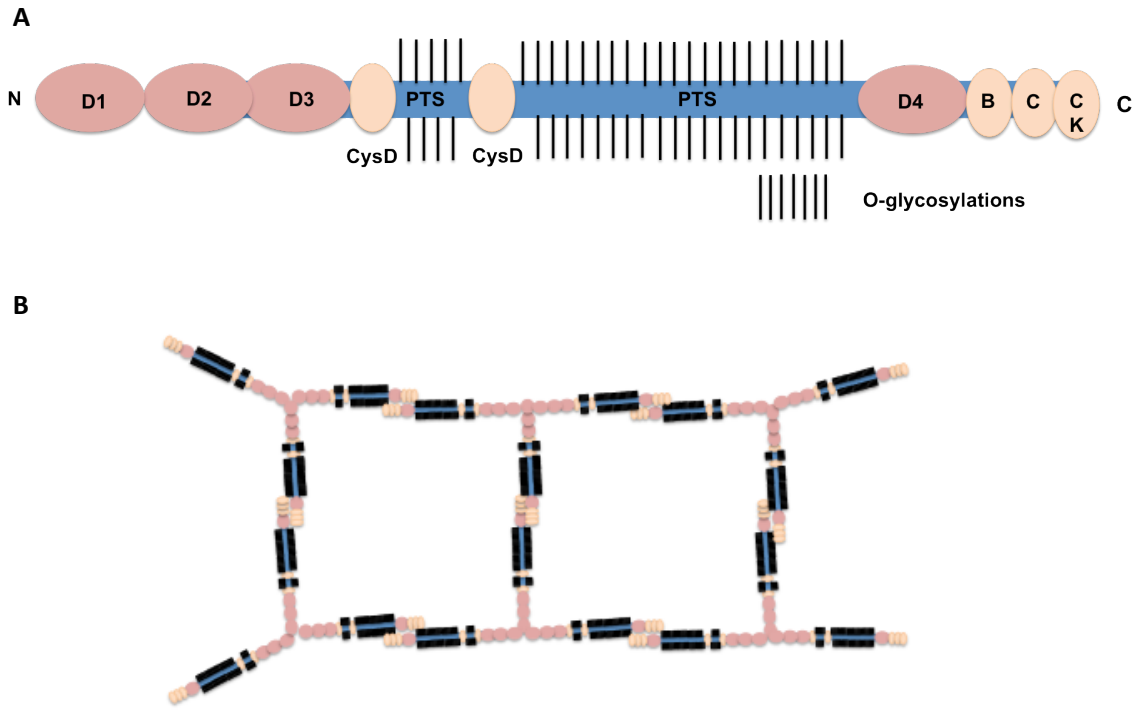

Figure 1.2 Structure of the intestinal secreted mucin MUC2.

Illustration of the different domains in the secreted mucin MUC2. (A) At the N-terminal region are D domains, which allow homo-oligomerisation in secreted mucins, followed by the central mucin domain, which contains variable number of tandem repeats, which are rich in proline, threonine and serine (PTS), allowing extensive O-glycosylation. This central region is interrupted by Cys domains, which are not glycosylated. (B) Mucin polymers covalently linked at N- and C-termini by disulphide bonds to form polymers. Proposed trimerisation at the N-termini produces a cross-linked network in mucus. Adapted from Johansson et al., 2011 and McGuckin et al., 2011.

1.2.3 MUC2 can prevent intestinal inflammation

Several studies support the idea that mucins may be involved in immunoregulation, with Muc2-deficient mice being a useful tool in understanding the role of this mucin during health and infection. Muc2-/- mice develop spontaneous inflammation as the lack of mucus barrier facilitates contact between the gut microbiota and the epithelium (Velcich et al., 2002, Van der Sluis et al., 2006). Furthermore, Muc2-deficient mice challenged with a colitis-inducing agent suffered increased colonic inflammation, which suggest that Muc2 may be important preventing intestinal inflammation (Petersson et al., 2011, Van der Sluis et al., 2006).

Mutation in Muc2 oligomerisation domains in mice leads to less stored mucin in goblet cells and a reduced mucus barrier, generating an animal model resembling ulcerative colitis (Heazlewood et al., 2008). Accumulation of a non-glycosylated Muc2 precursor in the endoplasmic reticulum and endoplasmic reticulum stress were observed, which has also been reported in human goblet cells from ulcerative colitis patients(Heazlewood et al., 2008). Also, decreased Muc2 production has been observed in IL-10 deficient mice, which develop spontaneous colitis, and double knockout IL-10/Muc2 mice exhibited more severe inflammation (Schwerbrock et al., 2004, van der Sluis et al., 2008). In addition, IL-22 has been linked with amelioration of ulcerative colitis by increasing the production of secreted mucins and membrane-bound mucins (Sugimoto et al., 2008). Recently, it has been demonstrated that IL-10 can promote mucin production by goblet cells, improving protein folding and reducing endoplasmic reticulum stress (Hasnain et al., 2013b). This finding is consistent with the existence of IL-10 polymorphisms linked to IBD, which may help explain decreased mucus production in this disease (Hasnain et al., 2013b).

Additionally, it has been suggested that intestinal mucin may exhibit altered glycosylation patterns during inflammation. In humans, MUC1, MUC2, MUC13 and MUC19 polymorphisms have been linked with increased susceptibility to inflammatory bowel disease (IBD) (Franke et al., 2010, Moehle et al., 2006). In addition, decreased mucus thickness, reduced mucin sulphation and goblet cell number have been described in ulcerative colitis, whereas increased mucin sialylation has been reported in both ulcerative colitis and Crohn's disease (Boltin et al., 2013).

Furthermore, there is evidence from mice lacking enzymes required for core 1 and core 3 O-derived glycans synthesis suggesting that glycans are important preventing inflammation. Mice lacking the core 1 O-glycan enzyme C1galt1 in intestinal epithelial cells exhibit spontaneous

colitis, characterised by early recruitment of granulocytes and monocytes to the colonic mucosa and impaired barrier integrity, whereas mice lacking the core 3 O-glycan enzyme C3Gnt exhibit decreased expression of Muc2, impaired mucosal integrity and increased susceptibility to DSS-colitis and experimental colorectal tumours (An et al., 2007, Fu et al., 2011). Additionally, C3Gnt-/- mice exhibit increased epithelial barrier disruption during *Salmonella* infection, which suggests that core 3 O-derived glycans can control barrier disruption(Zarepour et al., 2013). Defective expression of core 1 O-derived glycans has been reported in some patients with ulcerative colitis, whereas patients with colorectal cancer exhibit decreased expression of C3Gnt, suggesting that defective glycosylation may be involved with the pathogenesis of both diseases (Fu et al., 2011, Iwai et al., 2005). Thus, mucin glycans are responsible for protective effects in the intestine, which may be attributed to structural mucus properties and/or important interactions with intestinal cells. Taken together, this evidence indicates that Muc2 may prevent exacerbate intestinal inflammation observed in IBD pathogenesis.

1.2.4 MUC2 can induce immunosuppression

Additionally, Muc2-/- mice can also develop tumours after several months, exhibiting increased proliferation of intestinal cells and development of invasive adenocarcinoma in the small intestine and colorectal cancer (Velcich et al., 2002). Thus, Muc2 may play a relevant role as a tumour suppressor, which is supported by the abnormal upregulation and glycosylation of Muc2 detected in different cancer types. Indeed, some aberrant O-glycans, such as sialyl Lewis A antigen and sialyl Tn antigen have been classified as tumour-associated carbohydrate antigens (Ishida et al., 2008, Monti et al., 2004). Aberrant O-glycans may be recognised by sialic acid Ig-like lectins (Siglecs), which can bind sialic acid by an amino-terminal V-set Ig domain, and their interaction has been linked with cancer development (Crocker et al., 2007). Siglecs are expressed on different immune cells (but not the majority of T-cells) and may regulate the recognition of self and non-self molecules, dampening the immune response against self-tissues and avoiding autoimmune responses (Paulson et al., 2012).

Studies using MUC2 secreted by the colon cancer cell line LS180 have proposed that interaction between MUC2 and DCs, via Siglec ligation, has immunosuppressive effects. Ishida et al. reported that mature DCs expressing the activation marker CD83 are decreased when treated with MUC2 and apoptosis is increased in a dose-dependent manner, which may be mediated by interaction with Siglec3 (Ishida et al., 2008). A similar assay showed that

immature DCs treated with LPS in the presence of MUC2 or artificial glycopolymers exhibited decreased IL-12 production, without changes in IL-10 production (Ohta et al., 2010). These downregulatory effects seem to be dependent on interaction with Siglec-3 and Siglec-9 (Ohta et al., 2010). Similarly, it has been recently reported that MUC2 may modulate DC function by supressing production of pro-inflammatory cytokines such as IL-12 but promote anti-inflammatory cytokines such IL-10 and TGF-β in the presence of LPS (Shan et al., 2013). These effects could be mediated by the interaction of MUC2 glycans to the soluble receptor Galectin 3, which anchors to the surface of DCs, and then forms a complex with the CLR Dectin-1 and FcγRIIB, mediating anti-inflammatory effects via β-catenin induction (Shan et al., 2013). Taken together, this evidence indicates that mucins may play an immunomodulatory role, interaction between glycosylation sites on mucins and Siglecs implicated as important.

1.2.5 Mucins can prevent intestinal infection

The intestinal mucus barrier is highly dynamic, undergoing continuous removal and replacement in response to environmental changes (Linden et al., 2008). Infections can induce increased mucus production and release in order to replace the mucus degraded by enteric pathogens (McGuckin et al., 2011). Th2-associated cytokines, IL-4 and IL-13, have been strongly associated with goblet cell hyperplasia and increased mucus production (Khan et al., 2001, McKenzie et al., 1998). In addition, changes in mucin glycosylation have been reported in infection, which may be interpreted as a strategy to alter the binding of pathogens to mucins and facilitate pathogen clearance (Takeda et al., 2010).

Nematode resistance is commonly associated with the induction of Th2 responses, which correlates with increased mucin production induced by Th2 cytokines (Else and Grencis, 1991). There is extensive research about the role of mucins in the helminth infection model *Trichuris muris*. Acute *Trichuris muris* infection is characterised by a Th2 cytokine profile and IL-13 controls goblet cell hyperplasia and mucin hypersecretion (Hasnain et al., 2010, Hasnain et al., 2011b). In addition, IL-13 induces Muc5ac up-regulation, which is predominant in the gastric mucosa but not normally present in the intestine (Hasnain et al., 2010, Hasnain et al., 2011b). This increased Muc5ac expression correlates with decreased worm burden during acute infection and Muc5ac deficiency lead to defective *T. muris* expulsion as well as delayed *Trichinella spiralis* and *Nippostrongylus brasiliensis* expulsion, supporting a protective role during helminth infection (Hasnain et al., 2010, Hasnain et al., 2011a). Worm viability is

affected by direct interaction with Muc5ac, although modulation of DC function may also be a potential explanation (Hasnain et al., 2011a).

Additionally, Muc2 can also play a protective role during *T. muris* infection, as shown in Muc2-KO mice which exhibit delayed nematode expulsion (Hasnain et al., 2010). However, increased Muc5ac expression has been reported in these mice and it seems to compensate for Muc2 deficiency, avoiding susceptibility to chronic infection (Hasnain et al., 2010). Importantly, serine proteases secreted by *T. muris* are able to degrade Muc2, leading to depolymerisation but Muc5ac is not degraded, highlighting the critical protective role of Muc5ac during *T. muris* infection (Hasnain et al., 2012).

Muc2-/- mice display increased susceptibility to different enteric pathogens such as *Citrobacter rodentium* and *Salmonella typhimurium*, suggesting that MUC2/Muc2 plays important protective roles during infection (Bergstrom et al., 2010, Zarepour et al., 2013). Recently, it has been shown that Muc2/MUC2 may promote host response against microbes in the colon by stimulating the production of β-defensin 2 in intestinal epithelial cells and this induction is higher in the presence of the pro-inflammatory cytokine IL-1β and ATP (Cobo et al., 2015). Additionally, Muc2-/- mice show deficient production of β-defensin 2, which could be associated with the deficient anti-microbial response seen in this model. Interestingly, MUC2 may play a dual role promoting anti-microbial peptides but also protecting β-defensin 2-susceptible bacteria such as non-pathogenic and pathogenic *E. coli*, which may prevent harmful inflammatory responses (Cobo et al., 2015). These effects seem also be mediated by mucin glycans, as glycan chemical modification using sodium metaperiodate abrogated production of β-defensin 2 *in vitro* and the protective effect of Muc2 on bacterial growth (Cobo et al., 2015).

Taken together, there is clear evidence for a protective role of mucins in intestinal immunity. One potential explanation for the role of mucins during infection may be related to the potential interaction between secreted mucins and DCs, and the immunomodulatory roles mucins are proposed to play in other diseases. The role of DCs regulating intestinal immunity will be discussed in section 1.4. The next section will explore the importance of other important innate cells residing in the intestinal barrier, intraepithelial lymphocytes.

1.3 Intraepithelial lymphocytes

Gut intraepithelial lymphocytes (IELs) reside within the intestinal epithelial layer and play important roles regulating barrier immunity and initiating the early immune response (van Wijk and Cheroutre, 2009). These IELs are mainly antigen-experienced T cells, unlike conventional T cells, and are distributed differently along the gut epithelium. Frequency of murine small intestinal IELs (1 IEL per 5-10 intestinal epithelial cell) is higher than colonic IELs (1 IEL per 40 intestinal epithelial cell) (Beagley et al., 1995). TCR$\gamma\delta^+$ T cells constitute up to 60% of the small intestine IELs, in contrast to the predominant TCR$\alpha\beta^+$ population found in the spleen or lymph nodes (Bonneville et al., 1988, Goodman and Lefrancois, 1988). Additionally, gut IELs comprise TCR$\alpha\beta^+$ conventional (or induced) T cells, expressing $CD4^+$ and CD8$\alpha\beta^+$, and both TCR$\alpha\beta^+$ or TCR$\gamma\delta^+$ unconventional (or natural) T cells expressing CD8$\alpha\alpha^+$ homodimers (Cheroutre et al., 2011). Natural IEL subsets are present at birth whereas induced IEL subsets are induced in the periphery and increase with age (Cheroutre et al., 2011). IEL phenotype has been defined as "activated yet resting" immune cells, as they express both effector and regulatory molecules (Shires et al., 2001). Thus, these cells represent a unique type of T cell, displaying unconventional phenotype and functions.

1.3.1 Origin of gut IELs

Both types of IELs originate in the thymus from bone marrow precursors, but follow different maturation pathways (Gangadharan et al., 2006). TCR lineage decision occurs during the development of CD4 CD8 double negative (DN) thymic precursors, specifically during stage 3 (DN3) (Carpenter and Bosselut, 2010). Lineage choice is modulated by Notch signal strength and stronger Notch signals promote TCR$\gamma\delta$ T cell development. The Notch ligand Jagged2 induces the strongest signal through interactions with Notch3 promoting $\gamma\delta$ differentiation, whereas the ligand delta-like 4 would induce TCR$\alpha\beta$ differentiation through Notch1 interactions (Van de Walle et al., 2013).

In the case of $\alpha\beta$-committed T cells, after β-selection, thymic precursors differentiate into $CD4^+$ CD8$\alpha\beta^+$ double positive (DP) precursors and receive survival signals through TCR$\alpha\beta$-MHC interactions (Carpenter and Bosselut, 2010, Gangadharan et al., 2006). Thus, DP thymocytes undergo conventional thymic selection, eliminating self-reactive T cells and expressing either CD4 or CD8$\alpha\beta$, and then migrate to the periphery. These naïve T cells can mature after peripheral antigen exposure in GALTs, acquiring expression of gut homing receptors and

entering to the gut tissue. In contrast, TCR$\alpha\beta^+$ CD8$\alpha\alpha^+$ IELs are originated from triple positive precursors (CD4$^+$ CD8$\alpha\beta^+$ CD8$\alpha\alpha^+$), which undergo alternative agonist selection and recognise self-antigens with high avidity (Leishman et al., 2002, Gangadharan et al., 2006). At this stage, these cells become double negative CD4- CD8- TCR$\alpha\beta^+$ and express gut homing receptors, migrating to the intestinal epithelium, where they upregulate CD8$\alpha\alpha$ and CD103 in presence of local signals (Figure 1.3) (Andrew et al., 1996, Gangadharan et al., 2006).

TCR$\gamma\delta$ T cells do not undergo positive selection, as they can develop without encountering cognate ligand in the thymus, and their antigen specificity is generated by gene rearrangements (Chien et al., 2014, Jensen et al., 2008). Additionally, these antigen naïve $\gamma\delta$+ T cells preferentially express the gut homing receptor CCR9 and thus can exit the thymus and migrate to the gut epithelium (Jensen et al., 2009). TCR$\gamma\delta^+$ T cells do not express either CD4 or CD8$\alpha\beta$ but they upregulate CD8$\alpha\alpha$ expression in the gut, similar to natural TCR$\alpha\beta^+$ IELs.

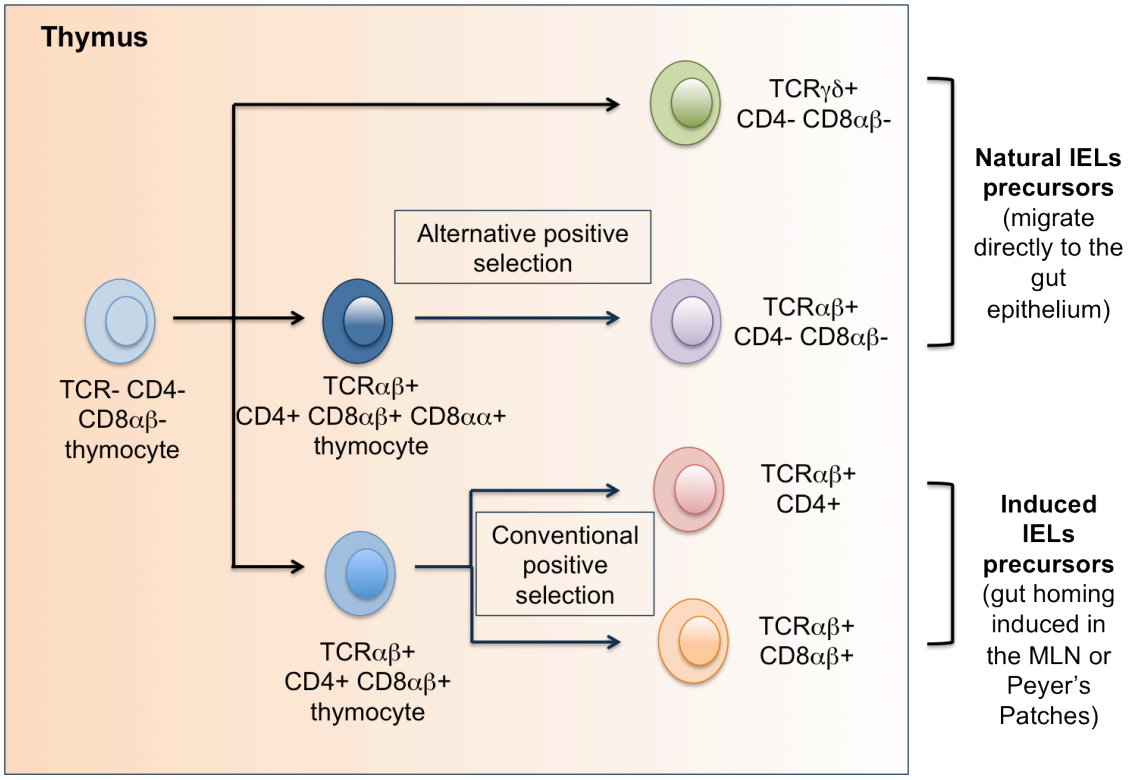

Figure 1.3 Origin of thymic IEL precursors.

Natural IEL precursors (TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$, which do not express CD4 or CD8αβ) originate in the thymus and acquire expression of gut homing receptors during their maturation process. In the case of natural TCR$\alpha\beta^+$ IEL precursors, these cells originate from triple positive CD4$^+$ CD8$\alpha\alpha^+$ CD8$\alpha\beta^+$ precursors, which undergo agonist positive selection (or alternative positive selection). In contrast, induced IEL precursors originate from double positive CD4$^+$ CD8$\alpha\beta^+$ precursors, which undergo conventional positive selection. Thus, they leave the thymus and can migrate to gut lymphoid tissue (MLN or Peyer's patches), where they acquire gut homing potential and differentiate into effector T cells. They differentiate into IELs in the presence of the gut microenvironment. Adapted from Cheroutre et al., 2011

1.3.2 Induction of CD8αα expression in the periphery

Differentiation and expansion of CD8αα on TCRαβ$^+$ and TCRγδ$^+$ IELs requires the transcription factors T-bet and runt-related transcription factor 3 (Runx3). T-bet is induced in the thymus after agonist selection and then further increased by intestinal epithelial cell derived-IL-15 stimulation (Klose et al., 2014). T-bet expression supresses transcription factors related with T-helper programs, such as T helper–inducing POZ/Krüppel factor (ThPOK), FoxP3 and RORγt, and promotes the expression of Runx3, which induces important genes associated with IELs, such as CD103 and CD8αα (Figure 1.4) (Reis et al., 2014). Additionally, retinoic acid and T-bet-inducing cytokines promote differentiation of distinct IEL populations. IFN-γ stimulation may play a role in the T-bet-mediated induction of TCRαβ$^+$ CD4$^+$ CD8αα$^+$ and single positive TCRαβ$^+$ CD8αα$^+$ IELs, whereas IL-27 may be required for T-bet mediated induction of TCRαβ$^+$ CD8αβ$^+$ CD8αα$^+$ and TCRγδ$^+$ CD8αα$^+$ IELs (Reis et al., 2014). Additionally, TGF-β plays important roles during early development in the thymus and also in the induction of CD8αα$^+$ expression in the periphery, together with a T-bet inducing environment (RA, IL-27 and IFN-γ) (Reis et al., 2014, Reis et al., 2013, Konkel et al., 2011). Roles of TGF-β in the regulation of intestinal immunity will be discussed in later sections.

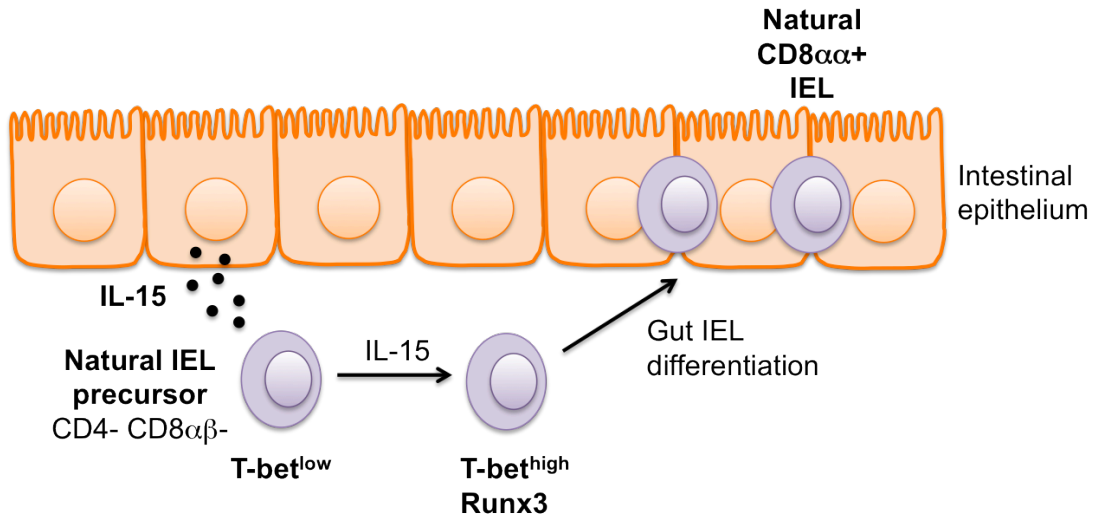

Figure 1.4 Differentiation of natural IEL precursors in the gut.

Induction of CD8αα expression and IEL differentiation occurs in the intestine and is dependent on IEC-derived IL15, which promotes increased T-bet expression and Runx3 expression. These two transcription factors collaborate to induce IEL differentiation: T-bet inhibits Th transcription factors whereas Runx3 promotes CD8αα and CD103 expression. Adapted from Klose et al. 2014.

1.3.3 Functions of CD8αα$^+$ IELs

CD8αα homodimers are markers of activated T cells but do not function as conventional CD4 or CD8αβ co-receptors (Cheroutre and Lambolez, 2008). CD8αα does not associate with TCR-CD3 activation complexes and acts as a co-repressor of T cell activation by sequestering TCR activation signalling components such as Lck and Lat (Cheroutre et al., 2011). Additionally, CD8αα can interact with thymic leukemia ligand (TL), a non-classical MHC I molecule abundantly expressed by intestinal epithelial cells, and this interaction may stabilise CD8αα expression and promote long-term IEL survival (Leishman et al., 2001, Madakamutil et al., 2004). As CD8αα expression on IELs might prevent self-reactivity, it has been proposed that CD8αα$^+$ IELs may play some regulatory roles but their specific function is not clear. Although TCRαβ$^+$ CD8αα$^+$ IELs express high levels of molecules related with cytotoxic activity such as granzyme and FASL, they express increased levels of TGFβ and low levels of IFNγ, consistent with the "activated yet resting" IEL phenotype (Denning et al., 2007, Shires et al., 2001). Importantly, TCRαβ$^+$ CD8αα$^+$ IELs ameliorate DSS-induced colitis and T cell-mediated colitis,

emphasising their contribution to gut homeostasis (Denning et al., 2007, Klose et al., 2014, Poussier et al., 2002).

1.3.4 Functions of induced TCR$\alpha\beta^+$ IEL

Similar to natural TCR$\alpha\beta^+$ IELs, it has been reported that $CD4^+$ IELs expressing CD8$\alpha\alpha^+$ display a cytotoxic but regulatory phenotype and may play roles in antiviral response and tolerance (Cheroutre et al., 2011). Additionally, different *in vitro* differentiation models have shown that $CD4^+$ T cells co-expressing CD8αα may express TGFβ, IL-10 and IFN-γ and may prevent colitis in the T-cell transfer model of colitis (Das et al., 2003, Van Kaer et al., 2013). As mentioned above, these cells are characterised by upregulation of Runx3 and loss of ThPOK. Recent studies have associated the expression of Runx3 with loss of Th17 function and potential regulatory functions of $CD4^+$ CD8αα^+ IELs, whereas loss of ThPOX has been associated with gain of cytotoxic activity, which could become pathogenic in response to an excess of IL-15 (Mucida et al., 2013, Reis et al., 2013). These findings are consistent with pathogenic roles reported for induced $CD4^+$ IELs in coeliac disease and IBD by production of pro-inflammatory cytokines and/or promotion of cytotoxic responses (Cheroutre et al., 2011). Therefore, most of evidence suggests that during steady state conditions $CD4^+$ CD8$\alpha\alpha^+$ may play regulatory roles but under a pro-inflammatory environment they could become pathogenic.

On the other hand, most of the induced IELs are single positive TCR$\alpha\beta^+$ CD8$\alpha\beta^+$, which have cytotoxic properties that may be protective against viruses and intracellular pathogens. Additionally, due to the unique characteristics of the gut environment, memory CD8$\alpha\beta^+$ IEL display stronger and longer effector responses compared to memory $CD8^+$ T cells from spleen or blood (Masopust et al., 2006). However, an exacerbated response of these cells may also play some pathogenic roles during chronic inflammatory conditions such as coeliac disease and IBD (Cheroutre et al., 2011). Further investigation is required to address the contribution of induced IEL during intestinal inflammatory conditions.

1.3.5 Functions of natural TCR$\gamma\delta^+$ IELs

TCR$\gamma\delta^+$ IELs display important roles protecting barrier immunity and integrity. TCR$\gamma\delta$-deficient mice display increased susceptibility to DSS-induced colitis and *Listeria monocytogenes* infection, which has been associated with the secretion of keratinocyte growth factor and contribution to tissue repair (Cheroutre et al., 2011). Additionally, these cells represent a first line of defence, protecting the intestinal barrier from different pathogens. TCR$\gamma\delta^+$ IELs can be

protective against invasion of resident bacteria by producing the antimicrobial factor RegIIIγ and chemotactic factors (Ismail et al., 2009, Ismail et al., 2011). Protection against parasitic infections such as *Toxoplasma gondii* and bacterial infections such as *Salmonella typhimurium* can be mediated by rapid migration to infection sites, production of antimicrobial molecules and cytotoxic activity (Edelblum et al., 2015, Lepage et al., 1998). Also, both TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ IEL can produce type I and type III interferons and induce antiviral response in intestinal epithelial cells (Swamy et al., 2015). Moreover, it has been postulated that TCR$\gamma\delta^+$ IELs can play pathogenic roles during intestinal inflammatory diseases as increased numbers of TCR$\gamma\delta^+$ IELs are observed in IBD and coeliac disease and promote a pro-inflammatory environment (Kanazawa et al., 2001, Tang et al., 2009).

As mentioned above, IEL differentiation can be modulated by the latent cytokine TGF-β, which activation is modulated by intestinal DC. The next section will explore the importance of DCs in the regulation of barrier immunity.

1.4 Dendritic cells

DCs are crucial in the sampling of intestinal antigens and regulating gut immune responses. DCs are antigen-presenting cells (APCs), together with B cells and macrophages, and display antigens on major histocompatibility complex (MHC) molecules for presentation to T-cells. Compared to other APCs, DCs are unique in their ability to stimulate naïve T cells and induce different Th subsets. Recognition of inflammatory signals, such as pathogen-associated molecular patterns (PAMPs), leads to DC activation and increased MHC and co-stimulatory molecule expression. Under steady state conditions, in the absence of inflammatory signals, innocuous or self-antigens are displayed in the absence of co-stimulatory signals, promoting tolerogenic T-cell responses (Dresch et al., 2012, Turnbull et al., 2005). Intestinal DCs can be found in the lamina propria (LP) of small and large intestine, in the MLN and in gut-associated lymphoid tissues (GALT) such as Peyer's patches and isolated lymphoid follicles (ILFs) (Persson et al., 2013a). DCs constitute a small percentage of the total cells in these tissues, being 0.2% of lymphoid tissue cells after collagenase digestion (Steinman and Cohn, 1973). To regulate T-cell responses, intestinal DC can migrate to the MLN and control differentiation of T cells into effector or regulatory cells (Coombes and Powrie, 2008).

Intestinal DCs have unique properties compared to other DC populations. Different studies have shown that certain intestinal DCs have a high tolerogenic potential, which is consistent with the tolerogenic environment observed in the gut. For example, the anti-inflammatory

cytokine IL10 is more abundantly expressed by intestinal DCs from Peyer's patches and LP compared to splenic DCs (Iwasaki and Kelsall, 1999, Chirdo et al., 2005). Intestinal DCs can also mediate gut tolerance by promoting anergy in antigen-specific T cells and by activating the latent cytokine TGF-β, which promotes FoxP3 expression in $CD4^+$ T-cells, a transcription factor essential for T_{reg} differentiation (Rutella and Locatelli, 2011). On the other hand, tolerogenic intestinal DC are also important in inducing secretory IgA production via T cell dependent and independent mechanisms, which regulates the abundance of commensal bacteria and the clearance of enteric pathogens (Endt et al., 2010, Strugnell and Wijburg, 2010). These unique properties are associated with specific intestinal DC subsets, which will be analysed in further sections.

1.4.1 Intestinal DC markers

Different subsets of intestinal DCs have been identified based on the expression of characteristic cell markers. Initially, the traditional lymphoid organ DC markers CD11c, CD11b, MHC II and CD8α were proposed to identify intestinal DCs (Iwasaki and Kelsall, 1999, Chirdo et al., 2005). However, CD11c, CD11b and MHC II are also expressed by macrophages in peripheral tissues, including the intestine, and other specific DC and macrophages markers have been identified. In the last decade, expression of the αE integrin, CD103, a receptor for E-cadherin, has been useful to distinguish DCs from macrophages and also to identify different intestinal DC subsets. A high proportion of small and large intestinal LP $CD11c^+$ MHC II^+ DCs express CD103, which is not expressed by intestinal macrophages (Cerovic et al., 2014). Based on the expression of CD103 and CD11b, it is possible to define 4 subsets of intestinal DCs: $CD103^+$ $CD11b^+$, $CD103^+$ $CD11b^-$, $CD103^-$ $CD11b^+$ and $CD103^-$ $CD11b^-$. $CD103^+$ $CD11b^+$ DCs are predominantly observed in the small intestine (70% of total $CD103^+$ DC), whereas $CD103^+$ $CD11b^-$ DCs are more abundant in the colon (75% of total $CD103^+$ DC) (Denning et al., 2011). In contrast, macrophages, but not DCs, express F4/80 and the high affinity IgG receptor FcgR1, CD64 (Bain et al., 2013). Other developmental, phenotypic and functional differences between macrophages and DCs will be discussed in later sections.

1.4.2 Origin and development of intestinal DCs

1.4.2.1 Precursors and growth factors

All murine conventional DCs, including all subsets of conventional intestinal DC, are originated from common pre-conventional DCs precursors, which develop in the bone marrow and circulate in the blood (Ginhoux et al., 2009, Schraml et al., 2013). $CD103^+$ DC develop from

these precursors in response to fms-like tyrosine kinase 3 (Flt3) ligand and granulocyte macrophage colony-stimulating factor (GM-CSF), similar to lymphoid organ DCs (Ginhoux et al., 2009, Varol et al., 2009). $CD103^+$ DC subsets are absent in mice lacking Flt3 receptor or ligand and administration of exogenous Flt3 ligand promotes expansion of $CD103^+$ DC subsets, particularly $CD103^+$ $CD11b^-$ (Bogunovic et al., 2009, Kinnebrew et al., 2012, Cerovic et al., 2013, Persson et al., 2013b). Furthermore, mice lacking expression of GM-CSF display impaired development of $CD103^+$ $CD11b^+$ DC in non-lymphoid tissue, including the intestine, and administration of exogenous GM-CSF promotes expansion of this subset (Greter et al., 2012). These findings suggest that different growth factor requirements control the development of the different $CD103^+$ subsets.

On the other hand, development of $CD103^-$ cells is still under investigation, due to the initial lack of precise strategies to discriminate between DCs and macrophages. Initially, it was proposed that $CD103^-$ DCs derived from $Ly6C^{hi}$ monocytes under the control of M-CSF, but these studies did not use the appropriate markers to distinguish $CD103^-$ DC from macrophages (Bogunovic et al., 2009, Ginhoux et al., 2009). Another recent study supported the idea that blood monocytes may give rise to $CD103^-$ DC. Expression of the chemokine receptor CCR2 is required for the recruitment of monocytes, and administration of anti-CCR2 antibody *in vivo* depleted $CD11b^+$ $Ly6C^{low}$ cells during inflammation, including $CD103^-$ DC (Zigmond et al., 2012). However, more recent studies suggest that $CD103^-$ DC origin is more likely to be related to DC precursors, as $Ly6C^{hi}$ monocytes seem to give rise exclusively to $F4/80^+$ $CD64^+$ macrophages but not $CD103^-$DC, at steady state in the intestinal LP (Bain et al., 2013, Scott et al., 2015). Furthermore, it has been shown that $CD103^-$ $CD11b^+$ DC can originate from DC-committed precursors, and that this DC subset is severely reduced in mice lacking Flt3 ligand, suggesting similar requirements to intestinal $CD103^+$ DC (Scott et al., 2015). Scott et al. (2015) addressed the discrepancies with Zigmond et al. (2012) by evaluating CCR2 expression on $CD103^-$ DCs, and found a novel $CD103^-$ population, expressing both $CD11b^+$ and CCR2, with pro-inflammatory properties. Therefore, this evidence seems to support that $CD103^-$ DC share similar precursors and cytokine requirements with $CD103^+$ DC, although further investigation is required.

$CD103^-$ $CD11b^-$ DCs are the smallest intestinal DC subset and have only recently been characterised (Cerovic et al., 2013). Mice lacking RORγt expression and therefore lacking intestinal secondary lymphoid tissue (Peyer's patches or ILFs) display normal numbers of $CD103^+$ DC subsets and $CD103^-$ $CD11b^+$ DC but reduced $CD103^-$ $CD11b^-$ DC, suggesting that they

derived from lymphoid tissues (Cerovic et al., 2013). Both $CD103^-$ DC subsets expand *in vivo* in response to exogenous Flt3 ligand, suggesting similar ontogeny, but differentiation of $CD103^-$ $CD11b^-$ from DC precursors has not been directly confirmed.

1.4.2.2 Transcription factors required for differentiation of DC subsets

Development of $CD103^+$ ($CD11b^-$ and $CD11b^+$) subsets depends on different transcription factors. Evidence from different knockout mouse models indicates that $CD103^+$ $CD11b^-$ DCs are dependent on the transcription factors basic leucine zipper transcription factor ATP-like 3 (Batf3), IFN-regulatory factor 8 (IRF8) and inhibitor of DNA-binding 2 (Id2) (Edelson et al., 2010, Ginhoux et al., 2009, Hildner et al., 2008, Jackson et al., 2011). Batf3 and IRF8 are related to the development of tissue-resident DCs expressing CD8α, similar to conventional lymphoid tissue DC. DCs expressing this molecule are able to cross-present antigens on MHC I to CD8 T cells, activating cytotoxic response against viruses and tumours.

In contrast, $CD103^+$ $CD11b^+$ DCs are independent of the above-mentioned transcription factors and are dependent of IRF4 and Notch2 (Edelson et al., 2010, Ginhoux et al., 2009, Lewis et al., 2011, Persson et al., 2013b, Satpathy et al., 2013, Schlitzer et al., 2013). Mice lacking expression of IRF4 and Notch2 display reduced numbers of intestinal $CD103^+$ $CD11b^+$ DC and splenic $CD4^+$ $CD11b^+$, which suggests a developmental link between these DC populations (Lewis et al., 2011, Satpathy et al., 2013, Schlitzer et al., 2013, Persson et al., 2013b). Less is known about the transcription factors expressed by $CD103^-$ DC subsets but recently it has been shown that $CD103^-$ $CD11b^+$ express IRF4 but not IRF8, similar to $CD103^+$ $CD11b^+$ DCs (Scott et al., 2015). Specific transcription factors expressed by $CD103^-$ $CD11b^-$ DC are still unknown.

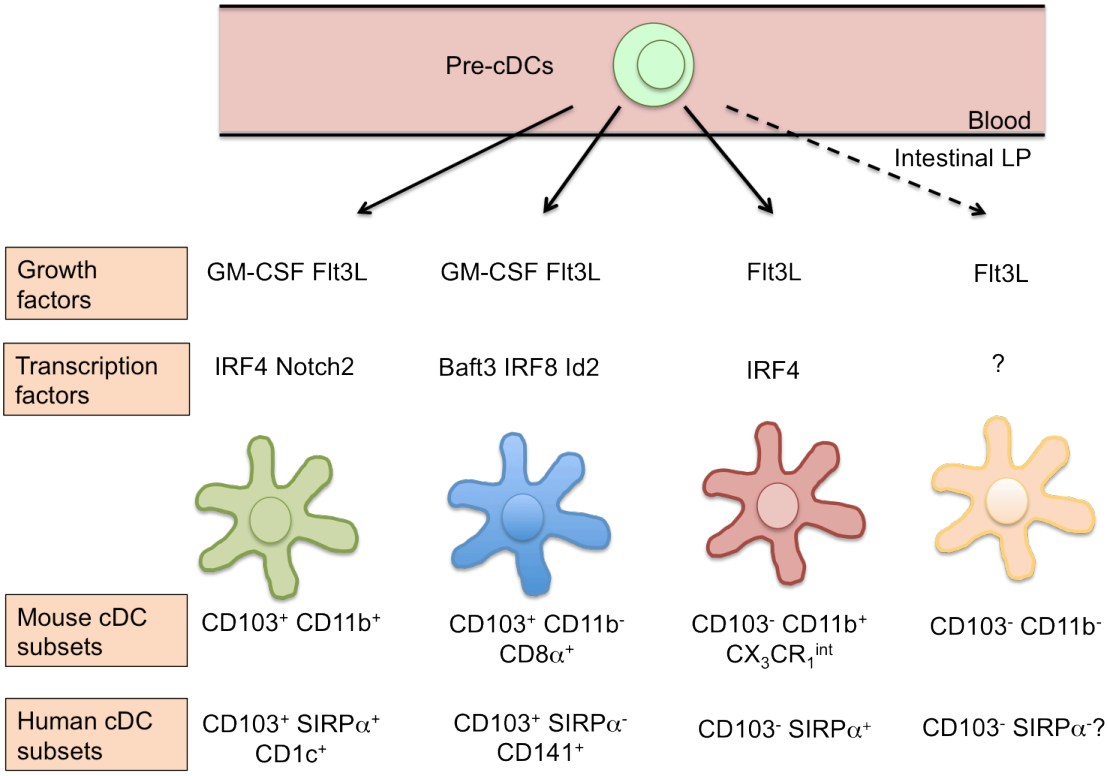

Figure 1.5 Intestinal DC subsets identified in mouse and human.

Intestinal cDCs subsets are originated from pre-cDCs blood precursors that seed the intestine. These intestinal cDCs subsets are identified based on the expression of CD103 and CD11b. This diagram summarises the growth factors and transcriptions factors known to be development in each intestinal cDCs subsets (Cerovic et al., 2014, Persson et al., 2013; Scott et al., 2015). Additionally, markers for human intestinal cDCs counterparts are shown (Watchmaker et al., 2014). Adapted from Persson et al. 2013.

1.4.3 Antigen sampling

1.4.3.1 Extension of transepithelial dendrites

The mechanisms used by DCs to sample soluble and particulate antigens from the intestinal lumen are still not completely understood. One of the most studied ideas in the last decade is the extension of transepithelial dendrites to sample luminal content. Extension of transepithelial dendrites appears to be dependent on the expression of the chemokine receptor CX3CR1, and may contribute to the antigen sampling in the small intestine, specifically in the terminal ileum (Niess et al., 2005, Chieppa et al., 2006). Furthermore, CX3CR1-deficient mice exhibit decreased T_{reg} proliferation in the gut, suggesting that $CX3CR1^+$ cells may be required for the development of oral tolerance (Hadis et al., 2011).

However, $CX3CR1^+$ cells represent a heterologous population of cells, which include DC, macrophages, T cells, intestinal epithelial cells and NK cells (Niess and Adler, 2010). However, based on expression levels, CX3CR1 has been proposed as another marker to differentiate DCs and macrophages. Thus, intestinal $CD103^-$ $CD11b^+$ DCs exhibit intermediate expression levels of CX3CR1, compared to the high levels observed in macrophages (Schulz et al., 2009). There was controversy about the nature of these $CX3CR1^+$ cells extending dendrites, due to the phenotypic and functional similarities between intestinal DCs and macrophages.

Currently, it is more accepted that $CX3CR1^+$ cells extending transepithelial dendrites in the intestine are macrophages. Most of $CX3CR1^+$ LP cells in the small intestine and colon are macrophages and a significant reduction in frequency and number of macrophages is observed in two strains of CX3CR1-deficient mice, and also in mice deficient in the CX3CR1 ligand, CX3CL1 (Medina-Contreras et al., 2011, Tamoutounour et al., 2012). These $CX3CR1^+$ macrophages display little mobility *in vitro* and *in vivo*, in contrast to highly motile $CD103^+$ DCs (Farache et al., 2013). Most of the evidence suggests that $CX3CR1^{hi}$ macrophages are sessile and have not been found in the intestinal draining lymph as reported by Cerovic et al. (2013) and Schultz et al. (2009). It has been reported that during certain challenges $CX3CR1^+$ macrophages can migrate to the MLN but these findings require further confirmation as it is unclear whether this study took into account the expression of CX3CR1 by some DC subsets (Diehl et al., 2013). $CD103^-$ $CD11b^+$ CX3CR1int DC subsets can migrate to the MLN under steady state and stimulate T-cells efficiently, in contrast to macrophages (Cerovic et al., 2013). However, there is no direct evidence of the CX3CR1int DC subset extending transepithelial dendrites.

Alternatively, recent evidence indicates that $CD103^+$ intestinal DCs can directly sample luminal bacteria during steady state and infection, extending dendrites to the lumen and efficiently acquiring bacterial antigens by phagocytosis (Farache et al., 2013). Salmonella challenge seems to recruit these cells within the epithelia through chemokine and TLR-dependent mechanisms. However, $CD103^+$ DCs are less efficient sampling soluble proteins and therefore alternative mechanisms are required for soluble antigen uptake (Figure 1.4) (Farache et al., 2013).

1.4.3.2 Antigen transfer to intestinal DCs

Conventionally, it has been described that intestinal DCs receive luminal antigens through other cell types. Specialised epithelial cells called microfold (M) cells, observed predominantly in the Peyer's patch, can incorporate luminal bacteria and macromolecules and deliver them via transcytosis to LP-residing APCs (Neutra et al., 1999). In recent years, new mechanisms describing selective antigen transfer from other cell types to $CD103^+$ DC in the intestinal LP have been reported. Soluble luminal antigens can be acquired and delivered by goblet cells to intestinal DC, preferentially $CD103^+$ cells and, similarly, $CX3CR1^+$ macrophages can capture soluble food antigens and transfer them to $CD103^+$ DCs (especially $CD103^+$ $CD11b^+$) through gap junctions, specifically via Connexin-43 (Figure 1.6) (Mazzini et al., 2014, McDole et al., 2012). This latter idea is consistent with the fact that $CX3CR1^+$ macrophages can extend transepithelial dendrites, as discussed above. These recent findings represent novel mechanisms of induction of oral tolerance and maintenance of gut homeostasis.

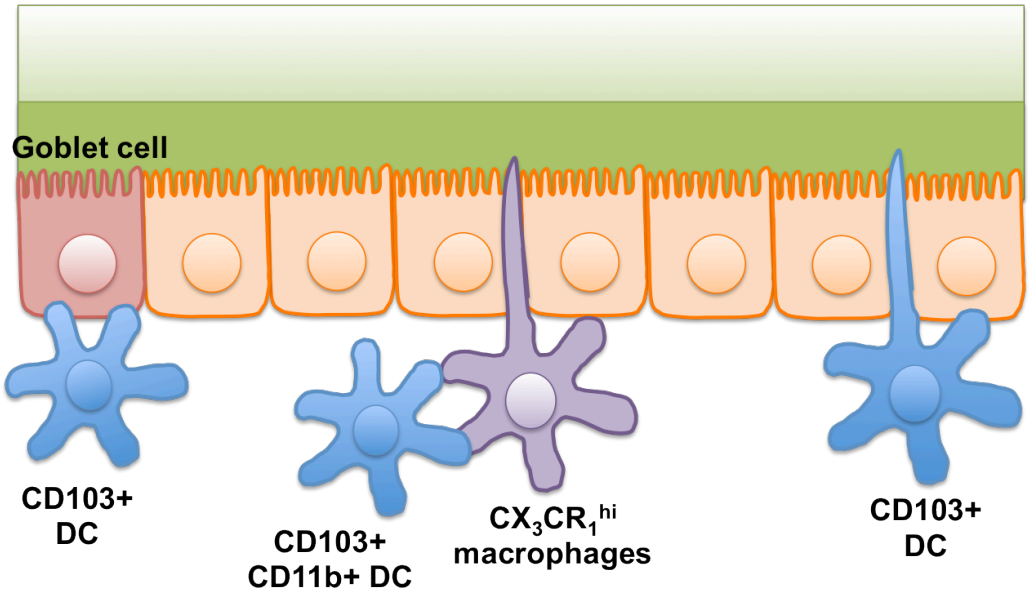

Figure 1.6 Antigen uptake by intestinal DCs.

Schematic representation of different mechanisms of acquisition of soluble antigens by intestinal DCs. Specialised epithelial cells such as goblet cells or M cells may transfer antigens to $CD103^+$ DCs (McDole et al., 2012; Neutra *et al.*, 1999). Furthermore, CX3CR1hi macrophages, able to extend transepithelial dendrites can uptake luminal antigens and may transfer them to $CD103^+$ $CD11b^+$ DC (Mazzini et al., 2014). $CD103^+$ are not efficient at acquiring soluble antigens, but they may be recruited to the epithelium by bacterial antigens and extend transpepithelial dendrites (Farache et al., 2013).

1.4.4 Intestinal DC roles regulating intestinal immunity

1.4.4.1 Antigen presentation in the MLN

After antigen sampling, LP DC can migrate to the MLNs in a CCR7-dependent manner, where they can prime T cells and promote effector or tolerogenic responses (Milling et al., 2010). A recent study has demonstrated that the small intestine and colon drain to different nodes within the MLN and thus DC populations from small intestine and colon migrate to distinct lymph nodes (Houston et al., 2015). Small intestinal MLN (sMLN) and colonic MLN (cMLN) are also functionally distinct, as food antigens are preferentially presented by sMLN DC but not cMLN DC. Furthermore, sMLN can specifically promote the gut homing markers CCR9 and integrin $\alpha_4\beta_7$ on T-cells, allowing homing of these cells to the small intestine (Coombes and Powrie, 2008, Johansson-Lindbom and Agace, 2007, Houston et al., 2015).

1.4.4.2 Induction of gut homing and tolerogenic responses

$CD103^+$ DC are required for inducing expression of gut homing receptors on T cells, and this ability seems to be dependent of the generation of retinoic acid (RA). $CD103^+$ intestinal DCs display an enhanced capacity to metabolise retinal (vitamin A) into RA, due to the high expression of retinal dehydrogenase (RALDH) *aldh1a2* (Coombes et al., 2007, Sun et al., 2007). RA is required for the *in vitro* and *in vivo* induction of gut-tropic T cells, but also is relevant for the induction of tolerogenic responses. Recent evidence indicates that sMLN DC but not cMLN DC are able to generate RA, and also enhanced levels of gut homing markers CCR9 and $\alpha4\beta7$ integrin are found on T cells in the sMLN (Houston et al., 2015). Homing to the colon, particularly of tolerogenic $Foxp3^+$ T_{regs}, may be controlled by the expression of GPR15, which is modulated by TGF-β and gut microbiota but not by RA (Kim et al., 2013). Frequency of $Foxp3^+$ T_{regs} is higher in the mouse colon but no differences are observed between sMLN and cMLN, which suggests that both small intestinal and colonic DC can induce tolerance (Houston et al., 2015).

On the other hand, both $CD103^+$ DC subsets, CD11b- and CD11b+ are able to induce gut homing. Mice selectively lacking either $CD103^+$ $CD11b^-$ or $CD103^+$ $CD11b^+$ MLN DC are still able to induce gut tropic $CD4^+$ T cells *in vivo*, but defective homing is observed in mice lacking both $CD103^+$ DC subsets (Edelson et al., 2010, Persson et al., 2013b, Welty et al., 2013). Similarly, both $CD103^+$ DC subsets are required for induction of tolerance in the intestinal LP. Mice lacking either $CD103^+$ $CD11b^-$ or $CD103^+$ $CD11b^+$ intestinal DC display normal number of $FoxP3^+$ T_{regs} but depletion of both $CD103^+$ subsets resulted in reduced numbers of gut tropic $FoxP3^+$ T

cells (Edelson et al., 2010, Persson et al., 2013b, Welty et al., 2013). These findings suggest that $CD103^+$ DC subsets have overlapping mechanisms to control T_{reg} differentiation, similar to the induction of gut homing, and therefore for the induction of tolerance.

1.4.4.3 Mechanism of induction of tolerogenic T_{regs}

It was initially proposed that MLN $CD103^+$ DC promoted differentiation of T_{regs} in a TGF-β and RA-dependent manner (Coombes et al., 2007, Sun et al., 2007). TGF-β is secreted as a latent cytokine and intestinal DCs mediate TGF-β activation by αvβ8 integrin-dependent mechanisms, which will be discussed in later sections. αvβ8 integrin is upregulated in $CD103^+$ intestinal DCs and results in enhanced activation of latent TGF-β by these cells (Paidassi et al., 2011, Worthington et al., 2011a). This elevated integrin-mediated TGF-β activation is wholly required for enhanced induction of $Foxp3^+$ T_{regs} by $CD103^+$ intestinal DCs, with RA only able to enhance T_{reg} induction in the presence of αvβ8-mediated TGFβ activation by $CD103^+$ DCs (Worthington et al., 2011a). RA can also supress Th17 differentiation mediated by TGF-β in presence of pro-inflammatory cytokines (Mucida et al., 2007, Yang et al., 2008). Moreover, expression of αvβ8 integrin has been investigated in different $CD103^+$ DC subsets, with unpublished observations suggesting that only $CD103^+$ $CD11b^-$ subsets express αvβ8 integrin (Persson et al., 2013a). However, as mentioned above, selective deletion of $CD103^+$ DCs subsets does not alter normal T_{reg} generation and thus further characterisation of αvβ8 expression in DC subsets is required.

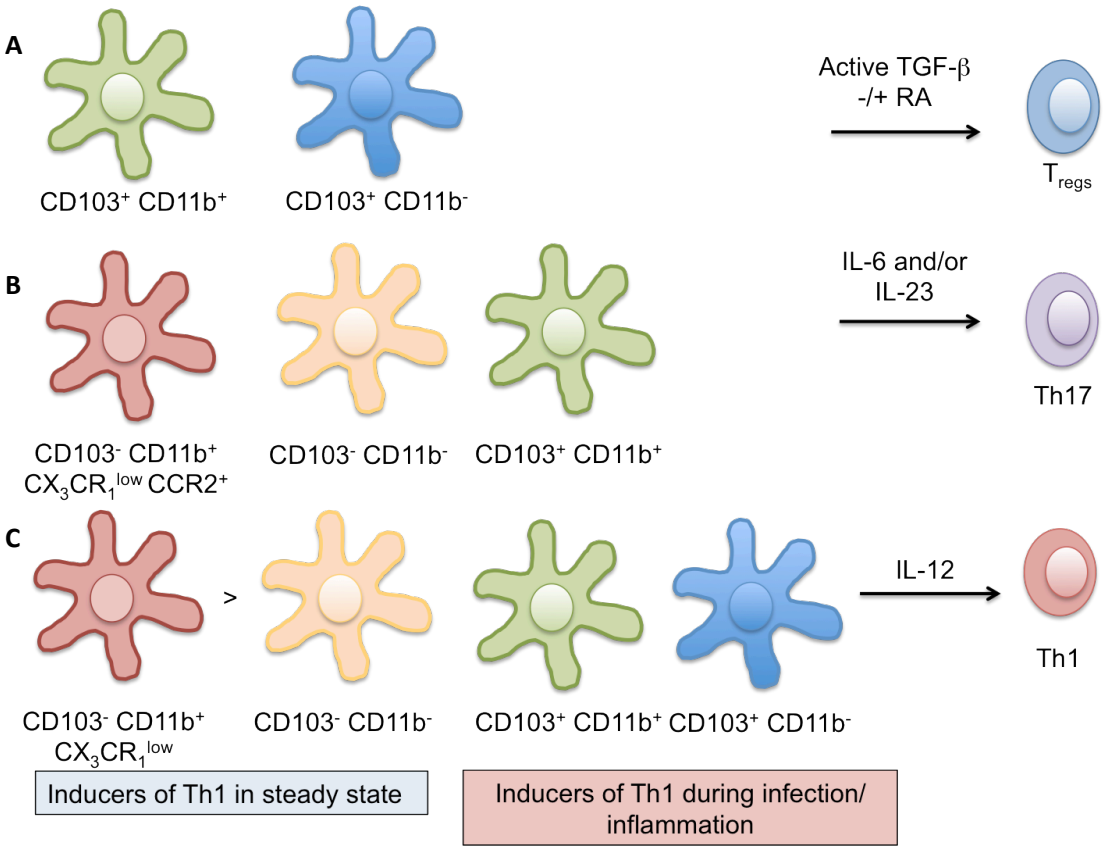

Figure 1.7 Mouse intestinal DC subsets induce different tolerogenic and effector T cells.

A. CD103$^+$ DCs have tolerogenic properties, activating TGFβ and converting retinal into RA. Up-regulation of integrin αvβ8 has been associated with this subset. Both TGFβ and RA have been associated with the generation of Foxp3$^+$ T_{regs} in the intestine, although RA can be dispensable (Worthington et al., 2011a). **B.** CD103$^+$ CD11b$^+$ DC and both CD103- DC subsets are able to drive th17 differentiation by producing IL6 or IL23 (Cerovic et al., 2013, Persson et al., 2013; Scott et al., 2015; Uematsu et al., 2008). **C.** CD103- DC subsets can promote Th1 differentiation in absence of pro-inflammatory stimuli whereas CD103$^+$ DC subsets can promote Th1 response during infection or inflammation (Cerovic et al., 2013; Laffont et al., 2010; Uematsu et al., 2008).

1.4.4.4 Induction of effector responses

Increasing evidence suggests that intestinal DCs are not completely tolerogenic but also important promoting effector Th1/Th17 T cells. $CD103^+$ $CD11b^+$ DC seem to be especially relevant in the promotion of Th17 T cells, as mice with reduced numbers of this subset display reduced Th17 T cells but normal total $CD4^+$ T cells (Persson et al., 2013b, Welty et al., 2013). This induction of Th17 cells seems to be IL-6 mediated *in vivo* and IL-6 production has been reported after stimulation with the TLR5 agonist flagellin in this subset (Persson et al., 2013b, Uematsu et al., 2008). Uematsu et al. reported that after flagellin stimulation, $CD103^+$ $CD11b^+$ DCs were able to induce Th1 and Th17 cells and also promote IgA production. Another similar study reported that, after TLR5 stimulation, $CD103^+$ $CD11b^-$ intestinal DCs produced IL-6 but also IL-23, and proposed that $CD103^+$ $CD11b^+$ DC may promote IL-22 production, which plays important roles in regulating mucosal defence (Kinnebrew et al., 2012). However, genetic ablation of $CD103^+$ $CD11b^+$ DC (huLangerin-DTA mice) did not affect IL22 production or increase susceptibility to the pathogen *Citrobacter rodentium*, which suggest that other DC subsets may play a compensatory role (Welty et al., 2013). Both $CD103^-$ DC subsets, $CD11b^+$ and $CD11b^-$, are also able to prime IL-17-producing T cells even in the absence of TLR stimulation (Cerovic et al., 2013). Interestingly, Th17 polarising properties have been also observed in the novel $CD103^-$ $CD11b^+$ $CCR2^+$ DC subset and it has been proposed that this function could be associated with IL-23 production (Scott et al., 2015). Taken together, these data suggest that $CD103^+$ $CD11b^+$ DC and $CD103^-$ DCs may play an important role promoting Th17 and anti-microbial response but further investigation is required to understand which signals modulate these processes.

Both $CD103^+$ and $CD103^-$ DC subsets have also been associated with promoting Th1 responses. Cerovic et al. reported that all DC subsets have Th1-polarising properties, although $CD103^-$ $CD11b^+$ DCs are more efficient and can promote Th1 response in the absence of TLR stimulation (Cerovic et al., 2013). On the other hand, MLN $CD103^+$ DCs may lose their tolerogenic properties during inflammation, associated with decreased expression of *tgfb2* and *aldh1a2* (Laffont et al., 2010). Thus, $CD103^+$ DCs prime IFNγ-producing and IL17- producing $CD4^+$ T cells instead of promoting T_{reg} in colitic mice, suggesting that the $CD103^+$ DCs may adapt to environmental conditions (Laffont et al., 2010). On the other hand, $CD103^+$ $CD11b^-$ DC expressing $CD8\alpha^+$ cross-present antigens to $CD8^+$ T cells and promote their cytotoxic activity after stimulation with a TLR7/8 agonist *in vitro* (Fujimoto et al., 2011). Similarly, cross-presentation of $CD103^+$ $CD11b^-$ $CD8\alpha^+$ DCs has also been shown *in vivo*, inducing proliferation

of gut tropic $CD8^+$ T cells in response to antigens derived from intestinal epithelial cells and driving IFNγ production in $CD8^+$ T cells during inflammation (Cerovic et al., 2015). Therefore, $CD103^+$ $CD11b^-$ $CD8\alpha^+$ DCs may play important roles promoting effector T cell responses during inflammation.

1.4.5 Human intestinal DCs phenotype and function

1.4.5.1 Human intestinal DC phenotype

Recent studies have identified human intestinal DC subsets based on the expression of similar markers to their mouse counterparts. CD103 is similarly highly expressed in both human small intestine and colon and different subsets can be identified based on the expression of SIRPα, equivalent to CD11b mouse subsets (Mann et al., 2015, Persson et al., 2013b, Watchmaker et al., 2014). Additionally, IRF4 and Blimp1 have been identified as transcription factors controlling the development of $CD103^+$ $SIRP\alpha^+$ DC, whereas IRF8 and Bcl6 control are required for the development of $CD103^+$ $SIRP\alpha^-$ DC (Watchmaker et al., 2014). However, there seem to be differences in the distribution of DC subsets between mice and humans as the most predominant subset in both human small intestine and colon are $CD103^+$ $SIRP\alpha^+$ DCs, the human equivalent of $CD103^+$ $CD11b^+$ (Mann et al., 2015). There is an increased proportion of $CD103^+$ $SIRP\alpha^-$ DC in the human colon compared to the ileum, reflecting a similar pattern to mouse DC subsets, but the significance of this reduced abundance of $CD103^+$ single positive DC in the human colon remains unknown.

On the other hand, there are similarities in the intestinal DC subsets able to cross present antigens in the human intestine. Equivalent $CD8\alpha^+$ and $CD8\alpha^-$ DC subsets have been defined in human based on the expression of CD141 and CD1c, respectively (Robbins et al., 2008, Poulin et al., 2010). It has been shown that human CD141 DCs are able to cross-present antigens form necrotic cells and activate cytotoxic activity, similar to their mouse counterparts (Jongbloed et al., 2010). Transcriptomic and phenotypic analysis have aligned the human intestinal $CD103^+$ $SIRP\alpha^-$ DC with the human $CD141^+$ DC and mouse cross-presenting $CD103^+$ $CD11b^-$ $CD8\alpha^+$ DC, whereas the human $CD103^+$ $SIRP\alpha^+$ DC aligned with the human $CD1c^+$ DC lineage and mouse $CD103^+$ $CD11b^+$ $CD8\alpha^-$ DC (Watchmaker et al., 2014). Similar transcriptomic analysis clustered human $CD103^-$ $SIRP\alpha^+$ DC with mouse $Ly6C^+$ monocytes, suggesting that this DC subset may derived from blood monocytes (Watchmaker et al., 2014). However, data from mouse $CD103^-$ $CD11b^+$ DC was not available for this study and thus further analyses are required to confirm

whether CD103$^-$ SIRPα^+ DC align with their mouse counterparts and whether they share similar precursors with macrophages or conventional DC.

1.4.5.2 Human intestinal DCs function

Human intestinal DCs seem to display similar functions compared with their mouse counterparts. All human intestinal DC subsets identified by Watchmaker et al. can express CCR7, associated with migratory properties as described above. Furthermore, human CD103$^+$ intestinal DCs from the small intestine and MLN can induce gut homing receptors (CCR9) and increased retinoic acid receptor signalling on T cells (Jaensson et al., 2008, Watchmaker et al., 2014). According to Watchmaker et al., CD103$^+$ SIRPα^+ DCs are the stronger inducers of CCR9 expression on T cells, which is consistent with their higher RALDH activity and with higher induction of Foxp3$^+$ cells (Watchmaker et al., 2014). Furthermore, it has been recently reported that colonic DCs display enhanced tolerogenic properties compared to human ileal DCs (Mann et al., 2015). A lower proportion of colonic DCs express the pro-inflammatory cytokines TNF-α and IL1β, and these cells exhibit enhanced ability to induce Foxp3$^+$ T_{regs}. Additionally, higher levels of the inhibitory receptor ILT3, associated with increased tolerogenic properties in APCs, is observed in colonic DCs and this increase expression is restricted to CD103$^-$ DC (Mann et al., 2015). Additionally, human colonic DCs express higher levels of CCR7 compared to ileal DC and also display enhanced endocytic capacity (Mann et al., 2015). Interestingly, colonic DCs selectively induce the skin homing receptor CCR4, compared to the CCR9 induction by ileal DCs, indicating that human colonic and small intestinal DCs have differential abilities to induce gut homing but the significance of this difference remains unknown (Mann et al., 2015).

Different studies have reported that human DCs also display the ability to promote T cell effector responses. Intestinal LP CD1c$^+$ DCs, which comprise CD103$^+$ and CD103$^-$ DC express higher levels of activation markers and increased levels of IL-6, IL-10 and TNF-α than blood CD1c$^+$ DC (Dillon et al., 2010). Also, intestinal CD1c$^+$ DCs are hyporesponsive to TLR4 ligands but produce IL23 in response to viral ligands, consistent with the idea that human intestinal DCs can promote effector pro-inflammatory responses (Dillon et al., 2010). Furthermore, Watchmaker et al. identified the CD103$^+$ SIRPα^+ subset as the most efficient inducer of Th17 responses, whereas CD103$^-$ SIRPα^+ DC induced IFNγ-producing T cells more efficiently, similar to their mouse counterparts (Watchmaker et al., 2014).

Moreover, human intestinal DCs from patients with IBD exhibit abnormal and pro-inflammatory properties. Intestinal LP DCs from patients with IBD express higher levels of TLR2 and TLR4 and increased levels of pro-inflammatory cytokines (Hart et al., 2005). Similarly, MLN DCs from patients with Crohn's disease preferentially promote a Th1 response through increase IL23 and reduced IL10-production (Sakuraba et al., 2009). Also, DC composition in the MLN from inflamed patients exhibited an increased proportion of DCs expressing intermediate levels of HLADR, compared with the predominant DR high population observed in healthy patients (Magnusson et al., 2015). On the other hand, colonic $CD103^+$ DCs from patients with IBD, expressing either CD1c or CD141, were decreased in inflamed tissue (Magnusson et al., 2015). Additionally, colon tissue from ulcerative colitis patients but not Crohn's disease exhibited reduced numbers of DCs and macrophages expressing aldehyde dehydrogenase and reduced ALDH activity in DCs, which was found regardless of the inflammation (Magnusson et al., 2015). Thus, it is suggested that the colonic environment in UC patients causes reduced ALDH expression by mononuclear phagocytes, as circulating monocytes and DCs exhibited normal ALDH expression (Magnusson et al., 2015). Additionally, $CD103^-$ $SIRP\alpha^+$ DCs are increased in the gut from inflamed patients, which suggests that they may play a role during intestinal inflammation (Watchmaker et al., 2014). Taken together, this evidence supports the idea that mechanisms for induction of gut homing, tolerance and effector T cells induction are conserved between mice and humans. Due to the critical role of DCs activating TGF-β, the next section will explore the mechanisms by which TGF-β is activated in the gut.

1.5 TGFβ, a crucial cytokine in gut homeostasis

1.5.1 TGFβ structure and isoforms

TGFβ is a critical cytokine in gut homeostasis, and extensive research about its function has taken place in the last few years. It is considered a pleiotropic cytokine, which induces diverse effects on multiple cell types, and can have anti-inflammatory or pro-inflammatory effects in the immune system depending on the context (Worthington et al., 2011b). TGF-β belongs to the TGFβ superfamily, which also includes activins and growth differentiation factors (Li et al., 2006). There are three different isoforms of TGFβ, TGFβ1, 2 and 3, with TGFβ1 proposed to be the most highly expressed in the immune system. TGFβ1 knockout mice die around 3 weeks after birth due to a multifocal inflammatory disease (Shull et al., 1992). These mice exhibit excessive inflammation with increased lymphocyte and macrophage infiltration in several organs, especially heart and lungs, suggesting a prominent role of this isoform in immune regulation (Kulkarni et al., 1993).

All three TGF-β isoforms are secreted as latent cytokines (Annes et al., 2003). Once activated, TGF-β is recognised by the type II TGF-β receptor (TGF-βRII) and a conformational change causes the dimerization of this receptor with the type I TGF-β receptor (TGF-βRI). Both subunits form the TGF-β receptor complex and initiate downstream signalling pathways such as Smad-, MAP kinase-, PI3-kinase-, and Wnt –signalling pathways (Guo and Wang, 2009, Shi and Massague, 2003). These pathways all result in the modulation of gene transcription.

Latent TGF-β consists of a N-terminal latency associated peptide (LAP) and a C-terminal growth-factor which are associated by non-covalent interactions (Shi et al., 2011). LAP-TGFβ then associates as a dimer (Shi et al., 2011). Additionally, LAP-TGF-β binds to the latent TGFβ binding protein (LTBP), aiding localisation in the extracellular matrix and subsequent activation (Worthington et al., 2011b). Moreover, TGF-β1 and TGF-β3 prodomains have a RGD motif, which is recognized by αv integrins (Worthington et al., 2011b). This interaction is important for activation of TGF-β and will be discussed in the next section.

1.5.2 αv integrins are important activators of TGFβ

Several processes have been implicated in TGF-β activation including heat, acidic pH and reactive oxygen species, which may destabilise the TGF-β latent complex (Annes et al., 2003). Other proposed mechanisms involve the participation of other proteins such as proteolytic cleavage of LAP by plasmin, matrix metalloproteases MMP2 and MMP9, or disruption of the LAP/TGF-β complex by thrombospondin-1 (TSP-1) (Worthington et al., 2011b). However, integrins has been demonstrated to be crucial activators of TGF-β1 and TGF-β3 in the immune system (Annes et al., 2003, Yang et al., 2007).

Integrins are cell-surface heterodimers composed of α and β subunits. The αv subunit can associate with five different β subunits (β1, β3, β5, β6 and β8) and these integrins modulate different cellular processes such as cell adhesion to extracellular matrix proteins and migration (Hynes, 2002). The role of integrins αvβ3, αvβ5, αvβ6 and αvβ8 in activating the TGF-β latent complex has been demonstrated (Asano et al., 2005a, Asano et al., 2005b, Munger et al., 1999, Mu et al., 2002). The integrins αvβ6 and αvβ8 are particularly important for TGF-β1 and TGFβ3 activation *in vivo*, although αvβ6 is not expressed in immune cells and is restricted to epithelial cells (Aluwihare et al., 2009, Busk et al., 1992).

Studies using conditional KO mice have shown the critical role of αv integrins expression on immune cells (Lacy-Hulbert et al., 2007, Travis et al., 2007). Loss of αv integrin expression in the immune system, specifically in myeloid cells, leads to the development of ulcerative colitis and autoimmunity in mice (Lacy-Hulbert et al., 2007). Reduced number of T_{regs} and decreased ability of DCs to generate T_{regs} was reported in these mice, which suggested that the 5 αv integrins are important in regulating intestinal immunity (Lacy-Hulbert et al., 2007). A similar study reported that specific loss of αvβ8 integrin expression on DCs lead to the development of autoimmunity and colitis in mice, which was associated with a decreased induction of T_{regs} due to reduced TGF-β activation by DCs (Travis et al., 2007). Importantly, αvβ8 is upregulated in $CD103^+$ intestinal DCs, highlighting the critical role of this integrin in TGF-β activation and its relevance in gut homeostasis, as discussed in section 1.4.4.3 (Worthington et al., 2011a).

1.6 Aims

The overall aim of this thesis was to increase our understanding of the regulation of immunity at the intestinal barrier, specifically looking at the interactions between intestinal mucins, DCs and the IEL compartment. As discussed, regulation of intestinal DC function is not fully understood and the intestinal environment may play a crucial role in gut homeostasis and modulation of DC function. Based on the potential immunomodulatory properties of mucins and the potential close contact between DCs and the mucus layer, the intestinal mucus layer may play key roles regulating intestinal immunity. DCs may be able to sense changes in mucin expression and glycosylation patterns, and thus play an important role in gut homeostasis. Moreover, both the mucus layer and DC may be important regulators of the gut IEL compartment, which are important immune cells residing in the gut epithelium. IEL development may be regulated by the latent cytokine TGF-β, which is activated by integrin $\alpha v\beta 8$ expressed on intestinal DC. Therefore, we investigated three specific aims:

1.- Is the gut IEL comparment regulated by the mucus layer and $\alpha v\beta 8$- expressing DCs? To investigate whether expression of integrin $\alpha v\beta 8$ may be functionally relevant for the development of gut IEL subsets, we explored whether expression of this integrin on DCs is required for the regulation of the gut IEL compartment, analysing IEL subsets in different $\alpha v\beta 8$-deficient mice. Additionally, we explored whether the mucus layer plays a role in the regulation of the IEL compartment analysing IEL subsets on Muc2-/- mice.

2.- Do the intestinal mucin MUC2/Muc2 regulate human DC function? To investigate this idea, we purified mucin from both human and murine sources and analysed changes in DC function in human monocyte-derived DCs incubated with purified intestinal mucin. Furthermore, we explored whether these effects were specific to mucins, but not sample contaminants, and investigate the role of mucin glycans.

3.- Do the intestinal mucus layer modulate DC subset phenotype and DC function? To address this aim, we explored whether intestinal DC subsets (expressing CD11b and CD103) are altered in mice lacking the intestinal mucin Muc2. Moreover, we investigated whether purified murine Muc2 modulated global gene expression on mouse MLN DCs, using microarrays and qPCR.

2 CHAPTER 2 Materials and methods

2.1 Animals

C57BL/6 mice lacking integrin αvβ8 on DCs or T cells via expression of a conditional floxed allele of β8 integrin in combination with CD11c-cre (*CD11c-cre.Itgβ8*) mice) and CD4-cre (*CD4-cre.Itgβ8* mice), respectively, have been previously described (Travis et al., 2007). Additionally, C57BL/6 mice lacking expression of the intestinal mucin Muc2 (Muc2-/-)(Velcich et al., 2002) were obtained by breeding heterozygous $Muc2^{+}/^{-}$ mice, a kind gift from Professor Richard Grencis (University of Manchester). All these mice strains together with wild-type C57BL/6 mice were maintained under specific pathogen-free conditions in the Biological Unit Services Unit at The University of Manchester and used at 6-12 weeks old, unless stated. All procedures were performed in accordance with the Home Office Scientific Procedures Act (1986) and under the DERFA license.

2.2 Cell culture

2.2.1 LS174T cells

The human colon adenocarcinoma cell line LS174T (European Collection of Cell Culture, UK) was used as a source of glycosylated MUC2 (Bu et al., 2011). This cell line was cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FCS, 1% L-glutamine and 1% penicillin/ streptomycin (all from Invitrogen, UK). Cells were maintained at 37° C and 5% CO_2 in a humidified incubator. When confluent, cells were trypsinised and resuspended in fresh media. For further cytokine analysis, supernatants were collected and stored at -20°C, adding 10X protease inhibitor (1 M aminohexanoic acid, 0.05 M benzamidine hydrochloride, 0.1 M NEM, leupeptin, trypsin inhibitor, aprotinin, chymostatin, pepstatin A and antipain) to avoid protein degradation.

2.2.2 HL60 and EaHy926 cells

The promyelocytic leukemia cell line HL60 (ATCC; provided by Dr. Caroline Milner, University of Manchester) was cultured using Iscove's modified Dulbecco's Medium (IMDM) (Gibco, Life Technologies, UK) supplemented with 20% (v/v) FCS, 2% L-glutamine and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were maintained at 37° C and 5% CO_2 in a humidified incubator. Cell were kept in suspension below 1 million cells/ml, diluting cells with fresh media when required. To differentiate these cells into neutrophil-like cells, 300,000 cells were incubated in culture medium containing with 1.5% (v/V) DMSO for 5 days (Jacob et al., 2002), with differentiation evaluated by measuring CD11b up-regulation by flow cytometry.

EA.hy 926 endothelial cells were cultured using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FCS, 1% L-glutamine and 1% penicillin/ streptomycin (all from Invitrogen, UK). Cells were maintained at 37° C and 5% CO_2 in a humidified incubator. When confluent, cells were trypsinised and resuspended in fresh media.

2.3 Purification of mucins by isopycnic density gradient centrifugation

Isopycnic density gradient centrifugation was performed to purify mucins from cell culture supernatants as described (Davies and Carlstedt, 2000). Cell culture supernatants were adjusted to 4M GuHCl and measured using a refractometer to calculate the GuCl concentration (based on its refractive index). Then, caesium chloride (CsCl) (Melford, UK) was added to achieve a starting density of 1.4 g/ml. This weight was calculated considering the following equation:

$$X=v(1.347\rho-0.0318M-1.347)$$

Where X is CsCl weight (g), v is the final volume (ml), ρ is the density (1.4 g/ml or 1.5 g/ml) and M is the molarity of GuHCl (4M or 0.2M depending on the density gradient).

This solution was then centrifuged at 40000 g, 15°C for 66 hrs in a Beckman Optima^{TM} L-90K Ultracentrifuge (Beckman Ti70 rotor for 40 ml or Ti45 for 100ml capacity centrifuge tubes, respectively). After ultracentrifugation, tubes were pierced at the bottom, and 20 fractions of equal volume were collected using a fraction collector. Density was estimated weighing 1 ml of every fraction, and absorbance at 280 nm was measured using a S-22UV/VIS spectrophotometer (Boeco, Germany) to determine fractions enriched in other proteins and /or nucleic acids. Additionally, to detect nucleic acids, samples were loaded in 0.7% agarose gels and stained with SafeView dye (NBS-SV1). The presence of glycoproteins and mucins was analysed by immunodetection after slot blotting (see below).

A second density gradient, using mucin-rich fractions pooled from the first density gradient and dialysed into 0.2 M GuHCl, was performed, calculating a starting CsCl density of 1.5 g/ml and 0.2M GuHCl, repeating the same procedure as described above.

Mucin-rich samples were finally pooled and dialysed in 1X PBS. To increase mucin concentration, samples were transferred into a 10 kDa molecular weight cut off Vivaspin centrifugal concentrating column and spun at 3000 rpm until sample volume was reduced.

2.4 Slot blotting

Samples for mucin detection were loaded onto 0.2 pore size nitrocellulose membrane (Whatman, Germany) using a 72-well slot blot manifold (Schleicher & Schuell, Germany) by vacuum (attached to a vacuum pump). MUC2 detection was then performed by immunodetection (see below). To estimate band intensity, a Bio-Rad GS-800 Calibrated densitometer was used (Thornton et al., 1994).

For detection of glycoprotein by periodic acid Schiff (PAS) staining, nitrocellulose membrane was washed with dH_2O after slot blotting vacuum transfer and incubated with 1% (v/v) periodic acid and 3% (v/v) acetic acid for 30 minutes at room temperature. Following this, the membrane was washed 4 x 2 minutes in dH_2O and incubated with 0.1% (w/v) sodium metabisulphite/0.01 HCl for 2 x 5 minutes. Schiff's reagent was then added for 10-15 minutes, until the coloured reaction was observed. Subsequently, the membrane was rinsed with sodium metabisulphite and the reaction was stopped by addition of excess dH2O (Thornton *et al*., 1994).

For antibody-mediated detection, nitrocellulose membranes were reduced in 6 M guanidinium chloride reduction buffer (containing 0.1 M Tris-HCl and 5 mM EDTA at pH8) and 0.01 M dithiotreitol (DTT) for 10-15 minutes and then washed for 4 x 5 minutes with TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH8). Subsequently, the membrane was blocked with 4% skimmed milk powder in TBST for at least 30 minutes. Following this, the membrane was incubated overnight with a rabbit anti-mouse Muc2 antiserum (Hasnain et al., 2010, Heazlewood et al., 2008) in 4% milk/TBST, at dilutions 1:1000 and 1:2000, respectively. After 4 x 5 minute washes with TBST, the membrane was incubated with an alkaline phosphatase (AP) conjugated anti-rabbit secondary antibody for 1 h. After 4 x 2 minutes washes, the membrane was incubated with Nitro-blue tetrazolium/5-bromo-4-chloroindol-3yl phosphate diluted in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5mM $MgCl_2$, pH 9.5), until colour developed. The reaction was stopped by addition of excess dH_2O.

2.5 Characterisation of mucin preparations

2.5.1 Multi-angle laser light scattering (MALLS) analysis

To determine the molecular weight distribution and concentration of mucin preparations, samples were dialysed in 1x PBS and analysed using multi-angle laser light scattering after chromatography on a Shodex (for intact mucins) or Superose 6 (for reduced mucin and glycopeptides) column. A solution containing 0.2 M sodium chloride, 1 mM EDTA and 0.05% sodium azide was used as running buffer. The column effluent was passed through an in-line Dawn HELEOS II laser photometer (laser wavelength 658 nm) and an Optilab rEX refractometer, at a flow rate of 0.5 ml/min. Continuous measurements of light scattering were obtained at multiple angles and refractive index (to determine mucin concentration) was also measured. These data was analysed using Astra software provided with the Dawn, obtaining the molar mass and the root mean square radius of the samples.

2.5.2 Tandem mass spectrometry

Previously trypsin-digested sample (see section 2.6.4) was acidified to pH2.0 using 10% formic acid. Once acidified, digested peptides were recovered using C18 ZipTips. Tips were previously wetted with 10 μl of 50% acetonitrile and subsequently washed with 10 μl 0.1% formic acid. The sample was then pipetted into the ZipTip and transferred to a fresh tube, until 100 μl of sample had been transferred. The ZipTip was then washed with 10 μl 0.1% formic acid and pipetted 5 x with 50% acetonitrile and 0.1% formic acid to elute the tryptic peptides retained in the tip. Recovered peptides were dried using a vacuum centrifuge and resuspended in 10 μl of 50% acetonitrile and 0.1% formic acid.

Samples were analysed by LC-MS/MS using an UltiMate 3000 Rapid Separation LC coupled to a LTQ Velos Pro mass spectrometer. Peptides were concentrated on a pre-column (20 mm x 180 μm) and separated by a gradient from 99% solution A (0.1% formic acid in water) and 1% solution B (0.1% formic acid in acetonitrile), to 25% solution B, in 45 minutes at 200 ml min^{-1}, using a 75 mm x 250 mm 1.7 mM BEH C18, analytical column. Peptides were chosen for fragmentation automatically by data dependent analysis.

To analyse MS data, the in-house MASCOT (Matrix Science) search engine was used. Fragmentation data were searched against the Uni-prot or mouse human database, selecting the following parameters: 1.2 Da mass for peptide mass tolerance (accuracy for parent ions) and 0.6 Da for fragment mass tolerance (accuracy for fragment ions), allowance for one missed

cleavage and selecting 2^+ and 3^+ ions (Rousseau et al., 2007). MASCOT search data was analysed using Scaffold 3.0 (Proteome software) and number of exclusive unique peptides was obtained, with a peptide probability > 95%.

2.6 Treatment of mucins

2.6.1 DNAse digestion

Mucin was treated with 10 U/ml DNAse I (grade II, Roche), 5 mM $MgCl_2$ (Promega), and 50 mM Tris-HCl (United Biochemicals, USA). Then, reaction mixture was incubated for 1 h at 37°C and the reaction stopped by adding 25 mM EDTA and heating for 10 minutes at 75°C. To wash the sample and remove DNAse, sample was spun in a 10 KDa MWCO Vivaspin column, replacing the digestion media for 1X PBS. The degree of DNA digestion was checked by running the sample in a 0.7% agarose gel, stained with SafeView dye. Mucin concentration was confirmed by SEC-MALLS (section 2.5.1).

2.6.2 Sodium metaperiodate

Mucins were treated with different concentrations of sodium metaperiodate (Thermo Scientific, UK) on ice for 1 h, diluting the sodium metaperiodate and mucin in a ratio 1:1. Oxidation was verified by slot blotting followed by Schiff's staining, but omitting the first oxidation step with periodic acid/acetic acid. The buffer was exchanged with 1X PBS using 10 MWCO VivaSpin filters.

2.6.3 Neuraminidase treatment

Mucins were treated overnight with different concentrations of neuraminidase (Sigma or New England BioLabs) including 5X Sigma reaction buffer or 10X NEB reaction buffer, at 37°C. The reaction was stopped by heating the sample at 100°C for 5 minutes.

2.6.4 Trypsin digestion

In order to prepare mucin glycopeptides or samples for tandem mass spectrometry, mucins were reduced, alkylated and digested with trypsin. A volume of mucin preparation was transferred into a 10 kDa molecular weight cut off Vivaspin centrifugal concentrating column, reduced with 10 mM DTT for 3 hours at 37°C and alkylated with 25 mM iodoacetamide (protected from light) for 45 min at room temperature. The solution was then centrifuged and reduced and alkylated proteins were retained above the filter. The filter was washed twice

with 0.1 M ammonium bicarbonate, leaving a final volume of 200μl. Subsequently, proteomic grade trypsin (Promega, USA) was added to the retained sample in a final protease:protein ratio of 1:100, as recommended by the manufacturer, and incubated at 37°C overnight. To ensure complete digestion, fresh trypsin was added the following day and incubated for 3-4 hours. The retained volume was then centrifuged through the column and recovered for either mucin glycopeptide analysis or mass spectrometry.

2.7 Lectin staining

To confirm that neuraminidase digestion was effective, samples were slot blotted onto nitrocellulose, blocked with TBST/Tween 0.2% and incubated with 1 μg/ml of biotinylated Sambucus nigra lectin (SNA), or Maackia amurensis lectin II (MAL II) (Vector Labs, UK), followed by incubation with avidin alkaline phosphatase (Sigma-Aldrich, UK). Each step was followed by several washes with TBST. Membranes were developed by enhanced chemiluminescence (ECL), using Clarity Western ECL blotting substrate (BioRad, UK), mixing equal volumes of the peroxide reagent and the luminol/enhancer reagent. Membranes were imaged using a Bio-Rad ChemiDoc MP Imaging System and band intensities were measured using Bio-Rad Image Lab software (version 4.1).

2.8 Endotoxin analysis

Endotoxin concentration was measured using a Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation Kit (Thermo-Scientific, UK), following manufacturers' protocol. Samples were diluted 1/50 or 1/100 in endotoxin-free 1x PBS and added into a 96-well plate. Subsequently, they were incubated at 37°C for 5 minutes, followed by incubation with LAL for 10 minutes and the chromogenic substrate for 6 minutes. The reaction was stopped using 25% acetic acid and absorbance was measured at 410 nm on a plate reader (Infinite M1000 Pro, Tecan). Endotoxin concentration was calculated based on a standard curve performed with dilutions of endotoxin standard provided by the manufacturer.

2.9 Isolation and purification of mouse intestinal cells

2.9.1 Intestinal intraepithelial lymphocytes isolation

Intestinal intraepithelial lymphocytes (IELs) were isolated as described by Konkel et al. (2011). Small intestine and colon were isolated, opened longitudinally, Peyer's patches excised from the small intestines, and intestinal tissue incubated for 20 min at 37°C in RPMI-1640 medium

supplemented with 3% (v/v) FBS, 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, Sigma-Aldrich, UK), 5 mM EDTA (Sigma, UK) and 0.145 mg/ml of dithiothreitol (Sigma-Aldrich, UK). Then, IELs were separated from gut tissues by vigorous shaking in RPMI-1640 supplemented with 2 mM EDTA and 20 mM HEPES and passed through 70 μm cell strainers. Following this, cells were separated from remaining mucus in 30% (v/v) Percoll (GE, Healthcare Life Sciences), after centrifugation for 20 min at 1600 rpm and room temperature, with no brake on the centrifuge. Cells were recovered from the pellet, washed in RPMI-1640 supplemented with 3% FCS, and further characterised by flow cytometry.

2.9.2 Intestinal LP cells isolation

Intestinal LP cells were isolated as described in Cerovic et al. (2013) and Houston et al (2015). Small intestine and colon were isolated as for IEL isolation, cut into small segments and washed thoroughly in Hank's Balanced Salt Solution (HBSS) supplemented with 2% FCS. Then, tissue was incubated for 20 min at 37°C in HBSS supplemented with 2% FCS and 2mM EDTA. Gut tissue was recovered using a 70 μm cell strainer, washed in HBSS and incubated for a further 20 min at 37°C as above. Then, small intestine was digested at 37°C in RPMI-1640 supplemented with 10% FCS and 1mg/ml collagenase VIII (Sigma-Aldrich) whereas colon was digested in RPMI-1640 supplemented with 10% FCS, 0.85 mg/ml collagenase V (Sigma-Aldrich), 1.25 mg/ml collagenase D (Roche, Germany) and 1 mg/ml dispase (Gibco, UK). Digestion was performed until most of the tissue was digested (30-40 min). Cell suspensions were passed through a 40 μm cell strainer, washed in RPMI-1640 supplemented with 10% FCS and characterised by flow cytometry.

2.9.3 Mesenteric lymph node (MLN) DC purification

MLN DCs were prepared as described by Worthington et al. (2011a). mLNs were isolated from C57BL/6 mice, cut in smaller pieces and digested in RPMI-1640 containing 10% FCS, 50U/ml deoxyribonuclease I (Roche, Germany) and 0.08U/ml liberase TM (Roche, Germany) at 37°C for 20-30 minutes. Then, digested tissue was poured through a 40 μm nylon sieve, and remaining tissue disrupted with the plunger of a sterile 2 ml syringe and the sieve washed with full media. Cells were then centrifuged at 300g for 5 minutes and a MACS separation protocol followed. To separate MLN DC from the rest of the cells, anti-CD11c magnetic beads (25μl per 100 million cells) (Miltenyi Biotech, Surrey, UK) were added to cells and incubated for 30 minutes at 4°C. Centrifugation was repeated as above, and the pellet resuspended in MACS buffer (PBS pH 7.2, 0.5% (w/v) BSA and 2 mM EDTA). The cell solution was then passed through an LS

column (Miltenyi Biotech, UK) according to manufacturers' protocol. Cells were passed through just one LS column to prevent DC activation and further enriched by flow cytometry sorting, using anti-mouse CD11c antibody, anti-mouse MHC II antibody and 7AAD as a live/dead marker. FACS staining was performed using phenol red-free DMEM, containing 2% (v/v) FCS, 1% (v/v) P/S and 1% (v/v) glutamine and stained cells were sorted in a FACSAria III cell sorter (BD Bioscience, Oxford, UK). Live $CD11c^+$ cells were collected in FCS and isolated by centrifugation for further assays. All isolation and staining steps were performed in a biosafety cabinet.

2.10 Purification of monocytes from PBMC and generation of monocyte-derived DC

Peripheral blood mononuclear cells (PBMCs) were obtained from the buffy coat or apheresis cones of healthy donors, provided by the NHS blood bank (Manchester, UK). Buffy coats were diluted 1:1 in 1x PBS, and layered on top of 12 ml Ficoll-Paque Plus (GE-Healthcare, UK), adding 25 ml diluted blood. When apheresis cones were used, 10 ml blood were diluted 1:2 in 1X PBS and layered on top of 10 ml Ficoll-Paque Plus (GE-Healthcare, UK). Samples were centrifuged for 40 min at 400 g at room temperature, with no brake on the centrifuge. The layer formed between plasma and Ficoll-plaque, containing PMBC, was removed using a Pasteur pipette and washed twice with 1x PBS. PBMC were resuspended in MACS buffer and monocyte separation performed. To separate human monocytes, 4 μl per 100 million cells of magnetic beads coated with anti-human CD14 antibody (Miltenyi Biotech, Surrey, UK) were added and MACS separation was performed as recommended by the manufacturer. $CD14^+$ monocytes were incubated in RPMI-1640-1640 media containing 10% FCS, 1% penicillin/streptomycin and 1% L-glutamine, at a concentration of 2 million cells/ml, with 50 ng/ml IL-4 and 50 ng/ml GM-CSF (PeproTech, London, UK) for 6-7 days. At day 3, half of the media was removed and replaced with fresh media, containing GM-CSF and IL-4 and maintaining the initial 50 ng/ml concentration of each. Cells were collected at day 6 and resuspended in a dilution 1:1 of old and fresh media, at a concentration of 1 million cells/ml, putting 100000 or 250000 cells/well. In some experiments, cells were then treated with 10 ng/ml LPS (Sigma-Aldrich, UK), mouse small or large intestinal mucin (50 μg/ml), or human MUC2 (50 μg/ml) isolated from LS174T cells as described above. In some experiments, cells were pre-incubated with 1 μg/ml of the TLR4 inhibitor CLI-095 (InVivoGen, UK) for 1 hour at 37^oC to block the TLR4 signalling pathway and prevent LPS-mediated effects. Cells were incubated for 22-24 h before staining for flow cytometric analysis.

2.11 Flow cytometry

Cells were washed with PBS and incubated with a live/dead stain (as indicated in Table 2.3) for 10 minutes, protected from light. Then, cells were washed with FACS buffer (1% (w/v) BSA, 0,5% (w/v) sodium azyde in 1x PBS) and then incubated with FcR binding inhibitor (eBioscience, UK) diluted to 5 μg/ml in FACS buffer for 10 minutes at 4°C. Subsequently, fluorophore-labelled antibodies targeting cell surface markers, diluted in FACS buffer (see concentrations and antibody specifications in Table 2.1), were added and cells incubated for 30 minutes at 4°C. Cells were washed, resuspended in fixation buffer (1 % formaldehyde in FACS buffer) and stored at 4°C or analysed immediately using a LSRII flow cytometer or Fortessa (BD Bioscience, Oxford, UK). Data was analysed using FACSDiva (BD bioscience, UK) or FlowJo software (Tree Star, Ashland, Oregon).

Antibody target	Fluorochrome	Concentration used	Specifications
B220	FITC, APC	1 μg/ml	Clone RA3-6B2, Biolegend UK
CD4	FITC	1 μg/ml	Clone RM4-5, eBioscience, UK
CD45	BV510, BV650, APC-Cy7	1 μg/ml	Clone 30F11, BD and Biolegend, UK
CD8α	PE, E450	1 μg/ml	Clone 53-6.7, Biolegend, UK
CD8β	APC-efluor780	1 μg/ml	Clone H35-17.2, ebioscience
CD11b	PE-Cy7, BV605	1 μg/ml	Clone M1/70, eBioscience or Biolegend
CD11c	PE, PerCP- Cy5.5, APC	3 μg/ml	Clone N418, BioLegend, UK
CD103	PE, eFluor450	1 μg/ml	Clone 2E7, eBioscience, UK
F4/80	eFluor 450	1 μg/ml	Clone BM8 ,

			eBioscience, UK
FoxP3	APC	3 μg/ml	Clone FJK-165, eBioscience, UK
Ki67	BV421	3 μg/ml	Clone 16A8, Biolegend, UK
Ly6C	FITC, BV510	1 μg/ml	Clone HK1.4, Biolegend UK
Ly6G and Ly6C (Gr-1)	BV510, FITC	1 μg/ml	Clone RB6-8C5, BD or Biolegend, UK
MHC II	E450, AF700	3 μg/ml	Clone AF6-120.1, eBioScience, UK
MHC II	AF700	1 μg/ml	Clone M5/114.15.2, Biolegend, UK
ROR-γt	PerCP-eFluor710	3 μg/ml	Clone B2D, eBioscience, UK
TCRβ	APC, PerCP- Cy5.5, AF700	1 μg/ml	Clone H57-597, Biolegend, UK
TCRγδ	PE-Cy7	1 μg/ml	Clone eBioGL3 (GL-3, GL3), eBioscience, UK

Table 2.1 Concentration and specifications of the fluorochrome-conjugated anti-mouse antibodies.

Antibody target	Fluorochrome	Concentration used	Specifications
Integrin αvβ8	PE	5 μg/ml	Clone ADWA16
CD14	PerCP- Cy5.5	2 μg/ml	Clone M5E2, BioLegend, UK
CD86	APC	2 μg/ml	Clone IT2.2, eBioscience and BioLegend, UK
CD83	PE-CF594	2 μg/ml	Clone HB15e, BD Bioscience, UK
CD11b	FITC	1 μg/ml	Clone ICRF44, eBioscience, UK

Table 2.2 Concentration and specifications of the fluorochrome-conjugated anti-human antibodies.

Viability dye	Dilution	Manufacturer
Fixable viability dye eFluor 780	1/1000	eBioScience, UK
Fixable viability dye Zombie UV	1/500	Biolegend, UK
7AAD Viability staining solution	1/100	eBioScience, UK

Table 2.3.Concentration and specifications of the different viability dyes used.

2.12 Genotyping of mouse strains

Ear punches were obtained from mice and digested overnight at 55°C in a digestion buffer containing 1 mM EDTA, 50 mM Tris-HCl (United Biochemicals, USA), 20 mM NaCl, 1% Tween and proteinase K at 0.3 mg/ml (Sigma-Aldrich). Subsequently, proteinase K was inactivated by heating at 95°C for 10 min followed by incubation for 10 min on ice. Thus, resulting digestion solution was analysed by PCR, using the primers listed in Table 2.4. Each PCR reaction contained 1x PCR reaction buffer, 1.5 mM MgCl2, 10 mM dNTPs, 0.25 μl Taq polymerase (all from Bioline) and 20 μM of each primer for 25 μl reaction. For the genotyping of floxed

integrin αvβ8 and Cre genes, each PCR reaction was performed using a PCR machine and the following cycles: 1) 95°C for 5 minutes, 2) 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for seconds, 3) a final extension of 72°C for 10 minutes. For the genotyping of Muc2-/- mice, two similar PCR reactions were performed, amplifying the wild type allele (primers AV83 and AV85, annealing temperature: 56°C) or the knock out allele (primers AV83 and AV84, annealing temperature: 52°C). PCR products were run in a 1% or 1.5% agarose gel, stained with SafeView dye.

Gene	Sequence
Muc2	Forward (AV83): 5' TCCACATTATCACCTTGAC 3'
	Reverse 1 (AV84): 5' GGATTGGGAAGACAATAG 3'
	Reverse 2 (AV85): 5' AGGGAATCGGTAGACATC 3'
Integrin αvβ8	Forward: 5' GAGATGCAAGAGTGTTTACC 3'
	Reverse: 5' CACTTTAGTATGCTAATGATGG 3'
Cre	Forward: 5' ACGACCAAGTGACAGCAATG 3'
	Reverse: 5' CTCGACCAGTTTAGTTACCC 3'

Table 2.4 List of primers used for genotyping of mouse knock out strains.
Sequences of the forward and reverse primer are indicated for each gene.

2.13 RNA extraction

To measure gene expression on monocyte-derived DCs, RNA was purified using the RNeasy Mini Kit (Qiagen, UK) for qPCR or RNeasy plus kit (Qiagen) for PCR arrays, following manufacturers' instructions. RNA concentration was measured using a NanoDrop (2000c, Thermo Scientific) and RNA purity was assessed by the ratio of absorbance at 260/280nm.

2.14 Preparation of complementary DNA

Complementary DNA (cDNA) was prepared using the GoScript reverse transcription system (Promega). Each reaction mixture contained 100ng RNA, which were incubated with OligodT for 5 minutes at 70°C and immediately chilled on ice for 5 minutes. Subsequently, the reverse transcription reaction mixture was added, including GoScript 5X reaction buffer, recombinant RNAsin ribonuclease inhibitor, $MgCl_2$, PCR nucleotide mix and reverse transcriptase. Reverse transcription was performed using a PCR machine and the following cycles: 25°C for 5 minutes, 42°C for 60 minutes and 70°C for 15 minutes. Typically cDNA was used immediately for real-time qPCR or stored at -20°C.

cDNA for PCR array was prepared using the RT2 First Strand kit (Qiagen) using 500ng of total human moDC RNA and following manufacturers' protocol. cDNA was finally diluted to a total of 110 μl with RNAse-free water.

2.15 PCR array

Gene expression of moDC untreated and mucin-treated was compared using a human dendritic cell PCR array (Qiagen), which analyses 84 different DC relevant genes. cDNA from untreated and treated cells were added to 2 different PCR array plates. For each PCR array plate, 92 μL cDNA were mixed with 1350 μL 2X RT2 SYBR green master mix and 1248 μL RNAse-free water. 25 μl of this mixture was added to each well of a 96-well plate pre-coated with different primers (Qiagen). The PCR array was performed using a Chromo4 PCR machine (Bio-Rad), with cycling conditions of 95°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds, for 40 cycles, followed by a melting point determination. Gene expression levels are indicated by the number of cycles needed for the cDNA amplification (Ct value) to reach a determined threshold (set at 0.005 for each experiment). Ct values were analysed using RT2 Profiler PCR array data analysis software version 3.5 (Qiagen) and normalized based on a set of 5 housekeeping genes provided in the PCR array. Normalized Ct values for treated-DC cDNA were compared with respect to untreated-DC cDNA and changes in gene expression are expressed as fold-regulation.

2.16 Real-time qPCR

Real-time qPCR was performed using an AB Biosystems real-time PCR system (Life technologies, UK). Each reaction mixture contained 10 ng of cDNA, 2X Fast SYBR green master mix (AB Biosystems, USA) and 0.6 μM of primers (see table 2.5 for human primer sequences and table 2.6 for mouse primer sequences). Samples were incubated for 95°C for 5min, followed by denaturation for 5s at 95°C and combined annealing/extension for 30 s at 60°C for a total of 40 cycles. Values of target mRNA were corrected relative to the housekeeping gene coding for human beta-microglobulin (B2M) or mouse hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data were analyzed using the $2^{-\Delta\Delta CT}$ method and expressed as fold changes.

Gene	Sequence
IL-22	Forward: 5’ TACATGCAGGAGGTGGTGCCTT 3’
	Reverse: 5’ TGTCTCCTTCAGCCTTCTGACA 3’
HPRT	Forward: 5’ GCGTCGTGATTTAGCGATGATGAAC 3’
	Reverse: 5’ GAGCAAGTCTTTCAGTCCTGTCCA 3’

Table 2.5 List of primers used for detection of mouse genes by qPCR.

Sequences of the forward and reverse primers are indicated for each gene.

Gene	Sequence
IL-8	Forward: 5’ TCCTGATTTCTGCAGCTCTGTG 3’
	Reverse: 5’ TGGTCCACTCTCAATCACTCTC 3’
B2M	Forward: 5’ CTCCGTGGCCTTAGCTGTG 3’
	Reverse: 5’ TTTGGAGTACGCTGGATAGCC 3’
CXCR1	Forward: 5’ CAGTCCAGTTTGCTATGAGGTCC 3’
	Reverse: 5’ CGCAGGGTGAATCCATAGCAGAA 3’
TAPBP	Forward: 5’ CTGGAGCTTGCTGTGTACAAAC 3’
	Reverse: 5’ AGAAGGGTAGAAGTGGGACACA 3’

Table 2.6 List of primers used for detection of human genes by qPCR.

Sequences of the forward and reverse primer are indicated for each gene.

2.17 RNA quality analysis

Total RNA integrity was analysed using the Agilent RNA 6000 Nano kit following manufacturers’ protocol. RNA was denatured at 70°C for 2 minutes and then loaded in a RNA Nano chip, which was inserted and analysed in an Agilent 2100 Bioanalyzer (Agilent Technologies).

2.18 Microarray

Gene expression of mLN DCs treated and untreated with mouse intestinal mucin was analysed using a Mouse genome 430 2.0 GeneChip microarray (Affymetrix), which was performed by the Genomic Facility at the University of Manchester. Briefly, 100ng RNA was used to synthesise cDNA by two-cycle cDNA synthesis and was subsequently biotin-labelled. Thus, labelled cDNA was hybridized to the microarray (mouse genome, Affymetrix) and array data was analysed and normalised by dChip (DNA-Chip) Analyser. Normalised data were analysed

using Robust Multichip Average (RMA), as described by Bolstad et al. (2003). Fold changes and gene ontology were analysed using Ingenuity Pathways Analysis software.

2.19 ELISA

IL-8 was quantified from the supernatant of moDCs by the human IL-8 ELISA Ready-SET-Go! (2nd generation, eBioscience, UK) and IL22 was quantified from the supernatant of MLN DCs by the mouse IL-22 ELISA Ready-SET-Go! (2nd generation, eBioscience, UK) as per the manufacturer's specifications. 96-well ELISA plates were coated with capture antibody diluted in coating buffer (100 μl/well) overnight at 4°C and blocked with 1x assay diluent (200 μl/well), which contains foetal bovine serum, for 1 h at room temperature. Plates were incubated with dilutions 1/100 and 1/1000 of each sample (100 μl/well) overnight at 4°C, followed by several washes with 1x PBS-0.05% Tween-20. A standard curve was made with 2-fold dilutions (100 μl/well) of human IL-8 recombinant protein for a total of 8 dilutions, the top standard concentration was 250 pg/ml. Samples and standard were diluted in assay diluent. Detection antibody (biotinylated anti human IL-8) diluted in assay diluent was then incubated in wells (100μl/well) for 1 h at RT. After incubation with 1x avidin peroxidase, diluted in assay diluent, for 30 min at RT, the colour was developed by adding 3,30,5,50-tetramethylbenzidine (TMB) (100 μl/well) for 10 min at RT and substrate stopped by the addition of 1M H_2SO_4. (50 μl/well) Absorbance was read at 450nm on an ELISA microplate reader (Infinite M1000 Pro, Tecan). IL-8 concentration in each sample was calculated based on the standard curve determined for each plate.

2.20 Transmigration assays

For transmigration assays, 50,000 EaHy926 cells in 100 μl DMEM supplemented with 10% (v/v) FCS, 1% L-glutamine and 1% penicillin/ streptomycin were added onto the top of 6.5 mm Corning transwell inserts (3.0 μm polyester membrane, Sigma, UK) in a 24 well plate, adding 600 μl of culture medium below the membrane. Cells were incubated overnight at 37°C and 5% CO_2, and endothelial monolayer formation was visually confirmed. Media was removed from the inserts and washed with PBS to remove dead and non-adherent cells. Transwells were transferred to fresh 24 well plates containing 600 μl serum-free X-Vivo 15 medium with or without recombinant IL-8 (10-100 ng/ml) and serum-free supernatants from moDCs treated and untreated with mucins. Differentiated HL60 cells were added to the top of each insert (250,000 cells in 250 μl X-vivo15 medium) and plates incubated at 37° C and 5% CO_2 for 24 h to

allow transmigration. Media below the membrane, containing migrated HL-60 cells, was recovered and centrifuged (5 min, 400g). Cell pellets were re-suspended in 50 ul of the remaining media and cells counted using a haemocytometer. Transmigration was calculated as a percentage of the number of cells that had migrated in the presence of supernatants from untreated moDCs.

To evaluate whether transmigration was IL-8-dependent, a neutralising IL-8 monoclonal antibody (0.4 μg/ml, monoclonal mouse IgG1, clone #6217, R&D Systems, Minneapolis, USA) was added to moDC supernatants. As a neutralisation control, 0.4 μg/ml of the anti-IL-8 antibody was added to wells containing 20 ng/ml recombinant IL-8.

2.21 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 for Mac, version 5.01. All data were expressed as the mean plus standard error (SEM). For all flow cytometry analysis, data was compared using repeated measures one-way ANOVA followed by Dunnett's multiple comparison test or unpaired t test. A p value of < 0.05 was considered significant. For all ELISA experiments, data were expressed as the mean plus the standard error (SEM) and compared using the Kruskal Wallis test followed by Dunn's multiple comparisons test.

3 CHAPTER 3 Determining the role of the intestinal environment in the regulation of the intestinal IEL compartment

3.1 Introduction

The intestinal barrier is composed of different components that play crucial roles in preventing the invasion of enteric pathogens, and initiating early immune responses against them. Cross-talk between different parts of the intestinal barrier, composed primarily of the single epithelial cell layer (and associated lymphocytes) and the mucus layer, may play key roles in their development and regulation of their functions. Intraepithelial lymphocytes (IELs), residing in the gut epithelium, play both regulatory and cytotoxic effects that can regulate barrier integrity and initiate early immune responses against pathogens (van Widj & Cheroutre, 2009). IELs display unique properties compared to conventional T cells, as most gut natural IELs are antigen experienced T cells expressing both activating and inhibitory types of receptors. These natural IELs, expressing TCR$\gamma\delta^+$ and TCR$\alpha\beta^+$ but no CD4 or CD8$\alpha\beta^+$, express gut homing receptors during their development in the thymus resulting in migration directly to the gut epithelium, where they differentiate in the presence of local factors (Cheroutre et al., 2011). Intestinal epithelial cells play an important role promoting the development of certain intestinal intraepithelial lymphocyte populations by secreting IL-15, which promotes the expression of CD8$\alpha\alpha$ on natural IELs (TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$) and IEL differentiation (Klose et al., 2014). This effect depends on the expression of the transcription factor T-bet (which supresses Th programs) and Runx3 (which promotes CD103 and CD8$\alpha\alpha$ expression) (Klose et al., 2014; Reis et al., 2014).

It is important to understand how the intestinal environment controls IEL differentiation and which intestinal factors regulate IEL subset differentiation. TGF-β has been postulated as a key regulator of IEL development and intestinal DCs are important contributors to the activation of latent TGF-β. Activation of TGF-β is $\alpha v\beta 8$ integrin-dependent, as shown in mice lacking expression of $\alpha v\beta 8$ integrin on dendritic cells (*CD11c-cre.Itgβ8$^{fl/fl}$* mice), which develop spontaneous colitis (Travis et al. 2007). This integrin is highly expressed in CD103$^+$ intestinal DCs and promotes high levels of TGF-β activation, which drives induction of FoxP3$^+$ T_{regs} (Worthington et al., 2011a). Furthermore, recent research suggests that intestinal DC function can be relevant in IEL homeostasis. Mice lacking expression of IRF8 on CD11c$^+$ cells (*CD11c-cre.Irf8$^{fl/fl}$*), which lack CD103$^+$ CD11b- DC, exhibit dramatically reduced numbers of TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ and TCR$\alpha\beta^+$ CD4$^+$ CD8$\alpha\alpha^+$ IELs in the small intestine, suggesting that intestinal CD103$^+$ CD11b- DCs are crucial regulators of IEL homeostasis (Luda et al., 2015, submitted manuscript). Therefore, we hypothesised that integrin $\alpha v\beta 8$ expression by intestinal DCs may play a functional role in the development of IELs subsets, including CD4 CD8$\alpha\alpha^+$ IELs.

Additionally, IELs are in close proximity to the intestinal mucus barrier. The goblet cell-secreted mucin Muc2 is the main component of the intestinal tract, constituting a protective barrier of the gut (McGuckin et al., 2011). Different studies have described the development of spontaneous colitis in mouse lacking the expression of the intestinal mucin, Muc2 (Muc2-/- mice), indicating the importance of the colonic mucus barrier preventing infection and inflammation (Van der Sluis et al. 2006; Velcich et al. 2002). It is possible that the lack of mucin barrier may affect the phenotype and/or development of IELs due to their proximity with the mucus barrier. It has been shown that mucin glycans may have some immunomodulatory properties and thus the mucus barrier may either regulate IEL function/differentiation by direct contact with IELs or by regulating the function of other intestinal barrier cells such as intestinal epithelial cells, DCs or macrophages. Thus, we hypothesised that Muc2 will play an important role in the development/maintenance of IEL populations in the intestine, and that Muc2-/- mice will show differences in IEL populations

This chapter will explore the contribution of two specific pathways to IEL homeostasis; the role for DC-activated TGF-β, and that of the mucus barrier. To address these aims we will characterise the IEL subsets in mice lacking expression of integrin αvβ8 on $CD11c^+$ cells and lacking the intestinal mucin Muc2 by flow cytometry.

3.2 Results

3.2.1 Characterisation of the gut IEL compartment in mice lacking expression of the integrin αvβ8 on CD11c$^+$ cells

To determine the contribution of αvβ8 expression in the regulation of the IEL compartment, we analysed mice lacking expression of integrin αvβ8 on DC (*CD11c-cre.Itgβ8*) compared with *Itgβ8$^{fl/fl}$* cre-ve control mice. Intestinal IELs were isolated from small intestine and colon as described by Konkel et al. (2011) and further characterised by flow cytometry. As mentioned earlier, gut IELs can be classified as natural IELs (TCRγδ$^+$ and TCRαβ$^+$ CD8αα$^+$ IELs) and induced IELs (TCRαβ$^+$ CD4$^+$ and TCRαβ$^+$ CD8αβ$^+$). To characterise these subsets, live CD45$^+$ cells were gated and characterised by the expression of TCRαβ (using an anti-TCRβ antibody) and TCRγδ (Figure 3.1). TCRαβ$^+$ IELs were then characterised by the expression of CD8α and CD8β, excluding CD4$^+$ IELs, and identifying natural IELs, expressing just CD8α (as are CD8αα+), and conventional/ induced IELs expressing both CD8α and β (CD8αβ$^+$). To analyse TCRαβ$^+$ CD4$^+$ IELs, CD8β$^+$ IEL were excluded and then CD8αα$^+$ expression in the CD4$^+$ subset was evaluated (Figure 3.1). Furthermore, intracellular staining was performed to analyse expression of the transcription factors RORγt and FoxP3 on CD4$^+$ IELs. On the other hand, natural TCRγδ+ IELs, which mainly express CD8αα and do not express CD4, were gated based on the expression of CD8α, excluding CD8α$^+$ IELs.

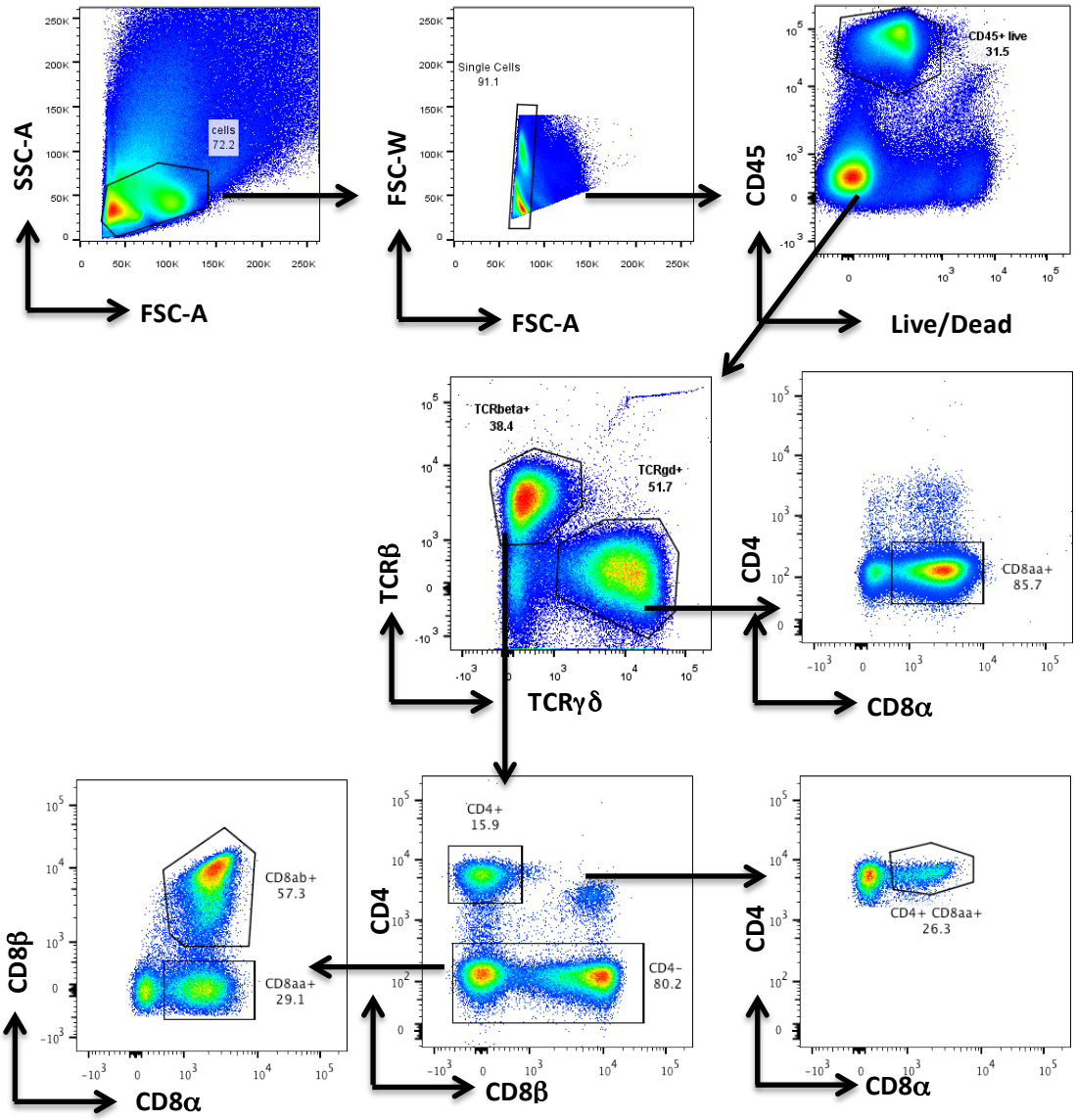

Figure 3.1 Gating strategy for intestinal IELs.

Cells were gated based on forward and side scatter, followed by single cell gating and live CD45$^+$ cell gating. Cells were then subdivided based on the expression of TCRβ and TCRγδ. TCRγδ IEL gate was analysed based on the expression of CD8α, excluding CD4$^+$ IELs. TCRβ$^+$ IEL gate was analysed based on the expression of different subunits of CD8 (CD8α and CD8β, excluding CD4$^+$ IEL) and CD4. CD4$^+$ CD8β$^-$ IEL were gated and then analysed for expression of CD8αα$^+$.

First, we analysed the IEL compartment of 6 to 7 week-old *CD11c-cre.Itgβ8$^{fl/fl}$* mice compared to *Itgβ8$^{fl/fl}$* control mice. Small intestinal IELs did not show significant differences in either TCRγδ$^+$ IELs (CD4$^-$ CD8αα$^+$) (Figure 3.2A) or TCRαβ$^+$ IEL subsets (CD4$^-$ CD8αα$^+$, CD4$^-$ CD8αβ$^+$,

$CD4^+$ $CD8\alpha\beta^-$or $CD4^+$ $CD8\alpha\alpha^+$ subsets compared to age-matched *Itgβ8$^{fl/fl}$* control mice (Figure 3.2B). No significant differences were observed in the colonic IEL compartment (data not shown). These results suggested that the IEL compartment is not altered in *CD11c-cre.Itgβ8* mice. However, these mice develop an age-related T cell inflammation (Travis et al., 2007) and some mice showed overt signs of inflammation, which may have affected results. Thus, we decided to repeat this analysis on 4-week-old *CD11c-cre.Itgβ8$^{fl/fl}$* mice, before mice display signs of inflammation. TCR$\gamma\delta^+$IELs ($CD4^-$ $CD8\alpha\alpha$+) did not show significant differences in *CD11c-cre.Itgβ8$^{fl/fl}$* mice (Figure 3.3A). Analysing TCR$\alpha\beta^+$ IEL subsets, we found no differences in small intestinal $CD4^-$ $CD8\alpha\alpha^+$, $CD4^-$ $CD8\alpha\beta^+$ and $CD4^+$ $CD8\alpha\beta^-$ IEL (Figure 3.3B) but we found significant differences in the $CD4^+$ $CD8\alpha\alpha^+$ IEL subset compared to age-matched control mice (Figure 3.3C and D). No significant differences were observed in the colonic IEL compartment (data not shown). These findings suggest that expression of integrin $\alpha v\beta 8$ on DC may be involved in the correct development of $CD4^+$ $CD8\alpha\alpha^+$ IELs in the small intestine.

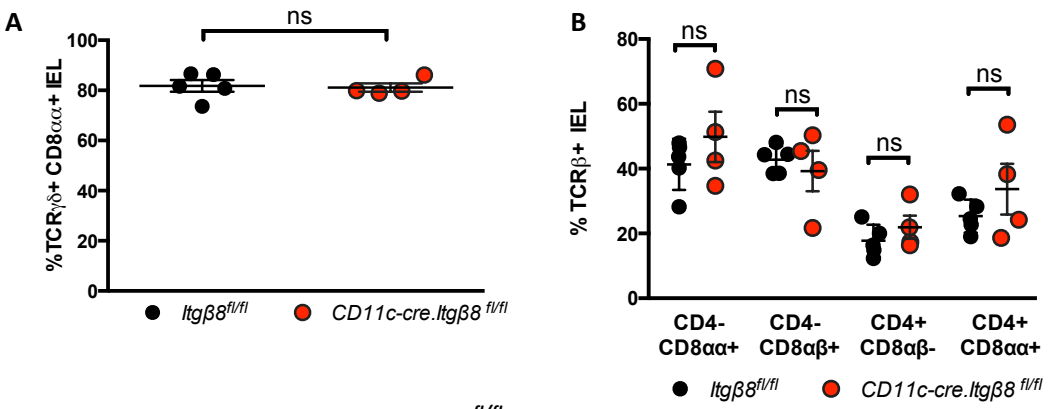

Figure 3.2 6-7 week old *CD11c-cre.Itgβ8$^{fl/fl}$* mice do not show differences in IEL subsets in the small intestine.

IELs were isolated from the small intestine of 6-to-7 week-old *CD11c-cre.Itgβ8$^{fl/fl}$* and *Itgβ8$^{fl/fl}$* control mice and analysed by flow cytometry. IELs were gated as shown in Figure 3.1. (A-B) TCR$\gamma\delta^+$IELs ($CD4^-CD8\alpha\alpha^+$, shown in A) and TCRβ^+ IEL subsets ($CD4^-$ $CD8\alpha\alpha^+$, $CD4^-$ $CD8\alpha\beta^+$, $CD4^+$ $CD8\alpha\beta^-$ and $CD4^+$ $CD8\alpha\alpha^+$, shown in B) were analysed. Results are from 2 independent experiments (n=5 for control mice, n=4 for CD11c-cre mice) and statistical significance was assessed using an unpaired t test.

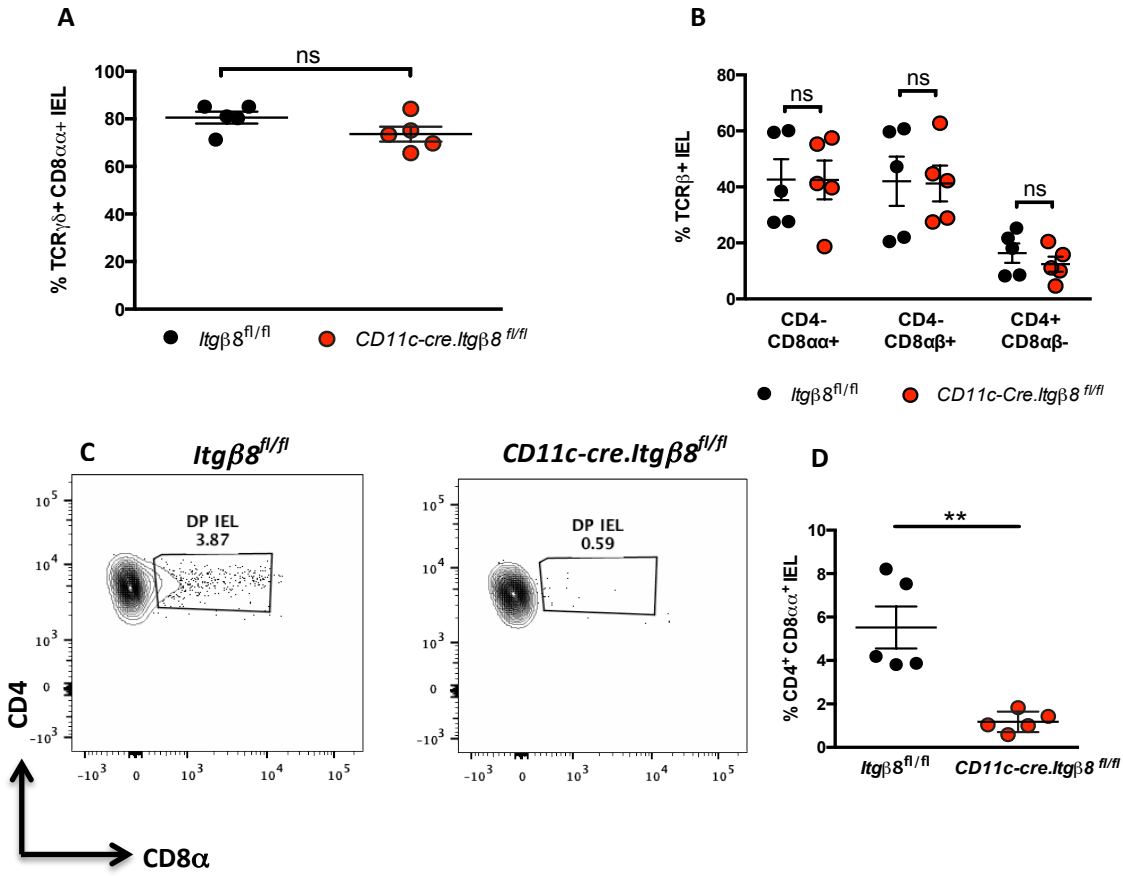

Figure 3.3 *CD11c-cre.Itgβ8$^{fl/fl}$* mice show significantly reduced percentages of CD4$^+$ CD8αα$^+$ IEL in the small intestine at early age.

IEL were isolated from the small intestine of *CD11c-cre.Itgβ8$^{fl/fl}$* and *Itgβ8$^{fl/fl}$* control mice and analysed by flow cytometry. IELs were gated as shown in Figure 3.1. (A-B) TCRγδ$^+$IELs (CD4$^-$ CD8αα$^+$, shown in A) and TCRβ$^+$ IEL subsets (CD4$^-$ CD8αα$^+$, CD4$^-$ CD8αβ$^+$, CD4$^+$ CD8αβ$^-$ and CD4$^+$ CD8αα$^+$, shown in B) were analysed. (C) Representative plots of CD4$^+$ CD8αα$^+$ IEL are shown for control and *CD11c-cre.Itgβ8$^{fl/fl}$* mice. (D) Frequency of CD4$^+$ CD8αα$^+$ IELs on *CD11c-cre.Itgβ8$^{fl/fl}$* mice was compared with age-matched *Itgβ8$^{fl/fl}$* control mice. Results are from 2 independent experiments (n=5 for each group) and statistical significance was assessed using an unpaired t test. **p<0.01

Induced CD4$^+$ CD8αα- IELs also express the transcription factors RORγt and express IL-17 . Defective expression of RORγt has been previously reported in LP T cells from *CD11c-cre.Itgβ8$^{fl/fl}$*, therefore a similar defective induction of RORγt may be observed in the IEL compartment of these mice. Thus, we analysed expression of the transcription factors FoxP3 and RORγt on TCRβ$^+$ CD4$^+$ IEL on CD11c-cre mice compared to age-matched control mice. Interestingly, small intestinal TCRαβ$^+$ CD4$^+$ IEL had significantly reduced expression of the transcription factor RORγt (Figure 3.4A and B). These findings are consistent with the impaired generation of Th17 cells observed in *CD11c-cre.Itgβ8$^{fl/fl}$* mice (Melton et al., 2010).

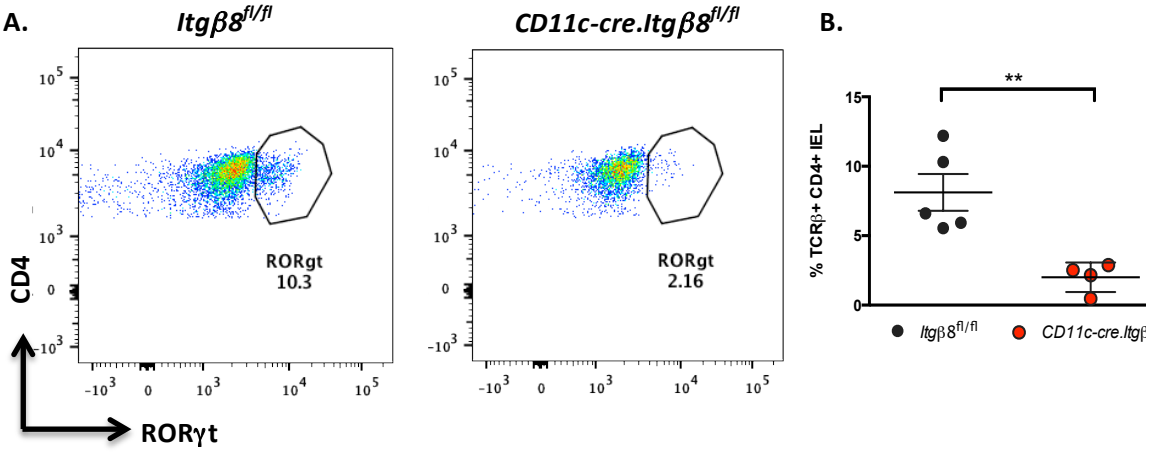

Figure 3.4 RORγt expression is significantly lower in small intestinal TCRβ$^+$ CD4$^+$ IELs from *CD11c-cre.Itgβ8$^{fl/fl}$* mice.

IELs were isolated from the small intestine of 6-to-7 week-old *CD11c-cre.Itgβ8$^{fl/fl}$* and *Itgβ8$^{fl/fl}$* control mice and analysed by flow cytometry. IELs were gated as shown in Figure 3.1. (A) Representative plots of TCRβ$^+$ CD4$^+$ RORγt$^+$ IELs are shown for control and *CD11c-cre.Itgβ8$^{fl/fl}$* mice. (B) Frequency of TCRβ$^+$ CD4$^+$ RORγt$^+$ IELs on *CD11c-cre.Itgβ8$^{fl/fl}$* compared with age-matched control *Itgβ8$^{fl/fl}$* cre-ve mice. Results are from 2 independent experiments (n=5 for each group) and statistical significance was assessed using an unpaired t test. **p<0.01

3.2.2 Characterisation of the gut IEL compartment in mice lacking expression of the integrin αvβ8 on T cells

Next, we analysed whether that modulation of the $CD4^+$ $CD8\alpha\alpha^+$ was specifically associated with the expression of αvβ8 integrin on DC or may regulated by the expression of αvβ8 on other immune cells. It has been recently shown that effector T_{regs} show upregulated levels of αvβ8 and TGF-β activation (Worthington et al., 2015). Thus, we analysed whether mice lacking expression of integrin αvβ8 on T cells (*CD4-cre.Itgβ8$^{fl/fl}$* mice), which do not develop spontaneous colitis and show normal gut homeostasis, exhibited differences in the gut IEL compartment compared to *Itgβ8$^{fl/fl}$* control mice. No significant differences were found in any IEL compartment analysed ($TCR\gamma\delta^+$ $CD4^-$ $CD8\alpha\alpha^+$ or $TCR\alpha\beta^+$ $CD4^-$ $CD8\alpha\alpha^+$, $CD4^-$ $CD8\alpha\beta^+$, $CD4^+$ $CD8\alpha\beta^-$ or $CD4^+$ $CD8\alpha\alpha^+$ IELs) in the small intestine (Figure 3.5A and B) or colon (data not shown). Additionally, no difference was found on the expression of transcription factor RORγt (data not shown). These results suggest that lack of expression of integrin αvβ8 on T cells does not alter the normal development of IEL in the gut. Therefore, expression of αvβ8 integrin on DCs but not T cells seems to be required for the normal development of the small intestinal $CD4^+$ $CD8\alpha\alpha^+$ IEL subset.

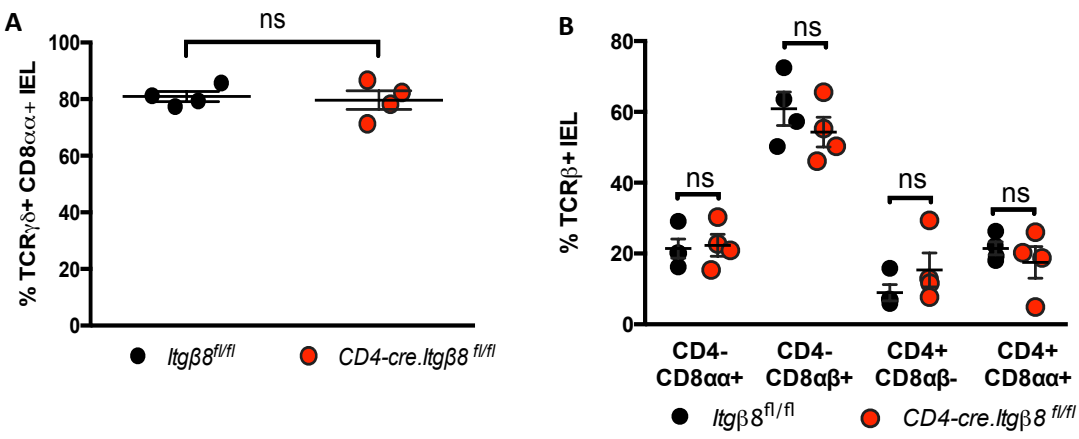

Figure 3.5 *CD4-cre.Itgβ8$^{fl/fl}$* mice do not show differences in IEL subsets in the small intestine.

IEL were isolated from the small intestine of 12 week-old *CD4-cre.Itgβ8$^{fl/fl}$* and *Itgβ8$^{fl/fl}$* control mice and analysed by flow cytometry. IELs were gated as shown in Figure 3.1. TCRγδ$^+$IELs (CD4$^-$ CD8αα$^+$, shown in A) and TCRβ$^+$ IEL subsets (CD4$^-$ CD8αα$^+$, CD4$^-$ CD8αβ$^+$, CD4$^+$ CD8αβ$^-$ and CD4$^+$ CD8αα$^+$, shown in B) were analysed. Results are from 2 independent experiments (n=4 for each group) and statistical significance was assessed using an unpaired t test.

3.2.3 Characterisation of the gut IEL compartment in mice lacking expression of the intestinal mucin Muc2

Lack of a normal mucin layer in the intestine may alter the development and/or function of the different immune cells residing in the intestinal LP or within the epithelial layer. Due to its close proximity to the mucus barrier, we hypothesized that IEL development may be altered in mice lacking Muc2 mucin (Muc2-/-). Similar to our analysis of mice lacking expression of integrin αvβ8 on CD11c cells, we isolated gut IELs from Muc2-/- mice before mice showed any signs of colitis (6 to 7 weeks old). Muc2-/- mice were compared to age-matched littermates, Muc2$^+$/$^+$ (WT). IELs were isolated as described before and subsets analysed by flow cytometry. No significant differences were found in TCR γδ$^+$ IELs (CD4$^-$ CD8αα$^+$) (Figure 3.6A) or TCRαβ$^+$ IEL subsets (CD4$^-$ CD8αα$^+$, CD4$^-$ CD8αβ$^+$, CD4$^+$ CD8αβ$^-$ and CD4$^+$ CD8αα$^+$), in the small intestine (Figure 3.6B) or colon (data not shown). These results suggest that lack of the intestinal mucin Muc2 does not affect the normal development of the intestinal IEL compartment.

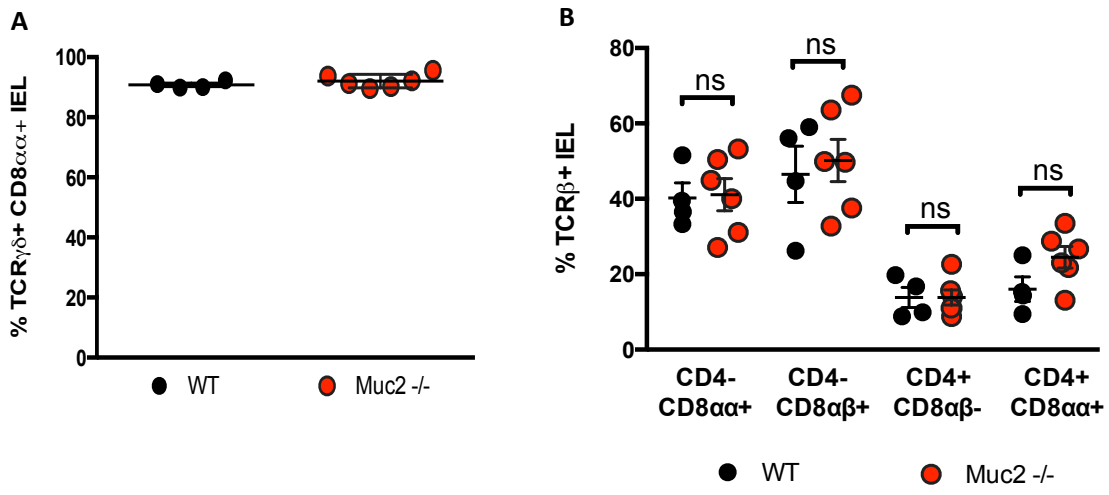

Figure 3.6 Muc2-/- mice do not show differences in IEL subsets in the small intestine.

IEL were isolated from the small intestine of Muc2-/- and WT littermate control mice and analysed by flow cytometry. IELs were gated as shown in Figure 3.1. TCR$\gamma\delta^+$IELs ($CD4^-$ $CD8\alpha\alpha^+$, shown in A) and TCRβ^+ IEL subsets ($CD4^-$ $CD8\alpha\alpha^+$, $CD4^-$ $CD8\alpha\beta^+$, $CD4^+$ $CD8\alpha\beta^-$ and $CD4^+$ $CD8\alpha\alpha^+$, shown in B) were analysed. Results are from 2 independent experiments (n=4 for control mice, n=5-6 for Muc2-/- mice) and statistical significance was assessed using an unpaired t test.

3.3 Discussion

3.3.1 Reduction of CD4$^+$ CD8$\alpha\alpha^+$ IEL in *CD11c-cre.Itgβ8* mice

Our analysis of the IEL compartment of *CD11c-cre.Itgβ8* mice showed that CD4$^+$ CD8$\alpha\alpha^+$ IELs are reduced at early age (4 week-old mice). This finding suggests that expression of αvβ8 integrin on CD11c cells is important for the regulation of the IEL homeostasis. Previous data indicates that this integrin is expressed on intestinal DCs but not macrophages (Travis et al., 2007) and thus expression of αvβ8 integrin on intestinal DCs would be crucial for induction of this IEL subset. We did not observed a reduction of this subset on *CD4-cre.Itgβ8* mice, suggesting that expression of integrin αvβ8 is required only on DCs but not T cells to regulate CD4$^+$ CD8$\alpha\alpha^+$ induction.

Our work is consistent with, and supports the findings from Prof. William Agace's group (Lund University, Sweden). This group have recently developed *CD11c-cre.Irf8$^{fl/fl}$* mice, which do not express the transcription factor IRF8, crucial for differentiation of the CD103$^+$ CD11b- DC subset (Luda *et al*., 2015, submitted manuscript). Thus, these mice lack this DC subset and showed altered T cell homeostasis, with a reduced number of small intestinal CD4$^+$ T cells and altered IEL homeostasis. The mice have an almost complete absence of small intestinal TCR$\alpha\beta^+$ CD8$\alpha\beta^+$ IEL and a dramatic reduction in TCR$\alpha\beta^+$ CD4$^+$ CD8$\alpha\alpha^+$ IEL, which also exhibited decreased expression of CD103$^+$, important for IEL retention in the gut. Of note, this mouse model also lacks pDCs and has reduced numbers of CD11c$^+$ intestinal macrophages but experiments using other KO models and mixed bone marrow chimera suggest that these changes in the IEL compartments are not due to macrophages or pDCs (Luda et al., 2015, submitted manuscript). In contrast, mice lacking expression of IRF4 on CD11c do not display changes in IEL homeostasis, suggesting that CD103$^+$ CD11b$^+$ DC are dispensable for IEL homeostasis (Persson et al., 2013).

Moreover, Luda and colleagues and our own group have found that αvβ8 expression is restricted to the CD103$^+$ CD11b- DC subset and thus lack of this integrin may explain the changes in IEL homeostasis observed in the *CD11c-cre.Irf8$^{fl/fl}$* mice (Luda et al. 2015, submitted manuscript; Dr. Stephanie Houston, University of Manchester personal communication). Generation of CD4$^+$ CD8$\alpha\alpha^+$ IEL is rescued in mixed bone marrow chimera experiments when bone marrow from *CD11c-cre.Irf8$^{fl/fl}$* and control mice are mixed, but not bone marrow from

CD11c-cre.Irf8$^{fl/fl}$ and *CD11c-cre.Itgβ8* (Luda et al 2015, submitted manuscript). Thus, these results suggest a crucial role for αvβ8 integrin in the regulation of CD4$^+$ CD8αα$^+$ IEL generation, and is consistent with our finding that *CD11c-cre.Itgβ8* mice have reduced small intestinal CD4$^+$ CD8αα$^+$ IELs.

3.3.2 Role of TGF-β in the induction of CD4$^+$ CD8αα$^+$ IELs

It is likely that this effect is due to the reduced activation of TGF-β in the gut, which is known to play important roles in the induction of CD8αα$^+$ expression. Different groups have shown that TGF-β can induce *in vivo* and *in vitro* expression of CD8αα$^+$ on peripheral CD4$^+$ T cells. Thus, TGF-β induces CD8αα expression on CD4$^+$ T cells in vitro (Konkel et al 2011)., TGF-β also promotes Runx3 expression, but supresses expression of the CD8αα repressor ThPOK, suggesting that TGF-β can modulate the balance of these two transcription factors (Konkel et al., 2011). Another study suggested that splenic CD4$^+$ T cells can be differentiated into CD4$^+$ CD8αα$^+$ T cells in vitro in the presence of TGF-β, IL-7 and IFN-γ, and that induced cells share some features with gut IELs (Van Kaer et al., 2013). Thus, these cells showed up-regulation of Runx3 and down-regulation of ThPOX, consistent with Konkel et al. (Van Kaer et al., 2013). Furthermore, evidence from knock in reporter mice has confirmed the requirement of Runx3 expression and loss of ThPOK in the development of CD4$^+$ CD8αα$^+$ IELs in vivo, in a TGF-β and retinoic acid- dependent manner (Mucida et al., 2013, Reis et al., 2013). Therefore, TGF-β can play a crucial role regulating IEL homeostasis and defective TGF-β activation is likely to affect the development of CD4$^+$ CD8αα$^+$ IELs, as our data suggests.

3.3.3 Induction of the CD4$^+$ CD8αα$^+$ IEL in the *CD11c-cre-Itgβ8* mice at 6-7 week-old

Interestingly, we observed that numbers of CD4$^+$ CD8αα$^+$ IEL are not totally impaired in *CD11c-cre.Itgβ8* mice, as frequency of this population was not significantly different to control mice at 6-7 week old. This may be attributed to several reasons. First, it is likely that other molecules can compensate the loss of active of TGF-β, as it seems that several intestinal factors may induce the differentiation of CD8αα$^+$ IELs such as the T-bet inducing factors IFN-γ, IL-27 and RA (Klose et al., 2014). Intestinal DC in *CD11c-cre.Itgβ8* mice should have an intact ability to generate RA in the gut and thus promote normal IEL development (Worthington et al., 2011a). The increased inflammatory environment observed in *CD11c-cre.Itgβ8* mice, which might include increased levels of IL-15 and IFN-γ, could promote the expansion of this subset. We stained IELs for the proliferation marker Ki67, with preliminary data suggesting enhanced

proliferation by $CD4^+$ IELs isolated from *CD11c-cre.Itgβ8* mice. Unfortunately, our staining was not consistent and we did not obtain significant differences (data not shown). Although inflammation in *CD11c-cre.Itgβ8* mice seems to be restricted to the colon, it is possible that some of inflammation may affect the small intestinal homeostasis, as these mice develop systemic T cell activation (Travis et al., 2007). One possibility is that TGF-β might regulate $CD4^+$ $CD8\alpha\alpha^+$ IEL differentiation under steady-state conditions but other cytokines or intestinal factors might promote expansion of $CD4^+$ $CD8\alpha\alpha^+$ IEL during inflammation but this idea requires further validation. Moreover, there may be compensatory mechanisms for TGF-β activation, αvβ8 integrin-dependent and independent. We cannot rule out that expression of αvβ8 integrin on other cell types may compensate some of the loss of αvβ8 on DC, such as expression on intestinal epithelial cells, although this idea has not been confirmed in the literature.

3.3.4 Regulation of IEL gut homing by intestinal DCs

Additionally, reduction of $CD4^+$ $CD8\alpha\alpha^+$ IELs in the *CD11c-cre.Irf8$^{fl/fl}$* mice was explained not only by lack of active TGF-β but also to the lack of RA, important for gut homing (Luda *et al*., 2015, submitted manuscript). A recent study has shown that interaction between intestinal DCs and IELs may regulate IEL gut homing. Thus, interaction between class I-restricted T cell-associated molecule (Crtam), expressed on IELs, and cell adhesion molecule 1 (Cadm1), expressed in different intestinal cells including $CD103^+$ CD11b- DCs, is important for homing and retention of $CD4^+$ $CD8\alpha\alpha^+$ IELs under steady-state conditions (Cortez et al., 2014). Mice lacking either Crtam or Camd1 exhibit reduced numbers of $CD4^+$ $CD8\alpha\alpha^+$ small intestinal IELs, as do mice lacking $CD103^+$ $CD11b^-$ DC (Batf3-/-) (Cortez et al., 2014). Mice lacking vitamin D receptor also display reduced $CD4^+$ $CD8\alpha\alpha^+$ IEL, attributed to the lack of CCR9 expression and reduced homing to the small intestine (Yu et al., 2008). As our mice only have intestinal DC with impaired capacity to activate TGF-β but intact ability to generate RA, this could explain why IEL retention was not altered.

3.3.5 Differences in the small intestine versus colon IELs

We observed a specific reduction in the small intestinal $CD4^+$ $CD8\alpha\alpha^+$ IEL but not in the colon. Most of studies characterising the IEL compartment analyse the composition of the small intestinal IEL subsets as they are at least 10 times more abundant than colonic IEL (Beagley et al, 1995). It has described that $TCR\alpha\beta^+$ IELs, which express more $CD8^+$ than $CD4^+$, are more

abundant than TCR$\gamma\delta^+$ IEL in the colonic IEL compartment from C57/BL6 mice, which suggests some potential functional differences between the small intestinal and colonic IELs (Beagley et al., 1995). In our study, we found lower percentages of $CD4^+$ IELs in the colon compared with the small intestine (5-10% vs 10-20%, respectively). We still were able to detect $CD4^+$ $CD8\alpha\alpha^+$ IELs in the colon but we did not find differences in the $CD4^+$ $CD8\alpha\alpha^+$ IEL *CD11c-cre.Itgβ8* mice versus controls. There is not extensive characterisation of colonic IELs in the literature so this may be attributed to either different cytokine requirements for $CD8\alpha\alpha^+$ differentiation, related to a potential different function of colonic IELs and/or the early onset of colonic inflammation in *CD11c-cre.Itgβ8* mice that might increase the expansion of this subset.

Additionally, Luda and colleagues have also described differences in the small intestinal IEL compartment, and in numbers of small intestinal LP $CD4^+$ T cells, but no differences in colon from *CD11c-cre.Irf8$^{fl/fl}$* mice versus controls, which are attributed to the induction of small intestinal homing and the increased ability of $CD103^+$ CD11b- to generate RA (Luda et al., 2015, submitted manuscript). Thus, there may be different mechanisms regulating small intestinal and colonic IELs maintenance and differentiation and further investigation is required to elucidate these mechanisms.

3.3.6 Reduced expression of RORγt in TCR$\alpha\beta^+$ $CD4^+$ CD8αβ- IEL

We additionally found decreased expression of RORγt in small intestinal TCR$\alpha\beta^+$ $CD4^+$ CD8αβ- IELs. This subset has not been extensively studied in the literature but there is evidence that some $CD4^+$ IEL can produce IL-17, and $CD4^+$ CD8αα- IELs may express RORγt (Cortez et al., 2014, Mucida et al., 2013). Mice lacking Crtam exhibited reduced IL-17 production in both $CD4^+$ IELs and $CD4^+$ LP T cells, suggesting that homing and maintenance of $CD4^+$ IL-17-producing IELs is controlled by T cell-DC interactions. Furthermore, previous work on *CD11c-cre.Itgβ8* mice has shown that they exhibit an impaired Th17 response and decreased expression of RORγt in colonic LP $CD4^+$ T cells, due to the lack of active TGF-β, which promotes RORγt expression (Melton et al., 2010). Here, we have found similar results in the small intestinal IEL compartment of *CD11c-cre.Itgβ8* mice, suggesting that expression of αvβ8 integrin on DCs may be required to control expression of IL-17 in the IEL compartment, although further evidence will be required to confirm this idea.

3.3.7 IEL subsets in Muc2-/- mice

Moreover, we did not observe differences in the IEL compartment of the Muc2-/- mice. These data suggest that lack of the mucin Muc2 does not affect the maintenance or development of gut IELs. Of note, although we used relative young Muc2-/- mice (6-7 week-old) and these mice did not have macroscopic signs of colitis, we detected increased neutrophil and monocyte infiltration in the colonic LP, which suggest that these mice were developing inflammation. This neutrophil infiltration was restricted to the colonic mucosa and we did not detect changes in other immune populations from the small intestine even after the mice show signs of colitis. Although we should repeat these experiments with younger mice to confirm that the $CD4^+$ $CD8\alpha\alpha^+$ is not expanded due to an increased inflammatory environment, Muc2-/- mice do not lose factors required for the development or maintenance of the IEL compartment including natural and induced IEL subsets such as TGF-β and T-bet inducing factors. Also, frequency of IEL subsets is not increased even during the course of inflammation in Muc2-/- mice, suggesting that IELs do not play a major role during the development of spontaneous colitis of Muc2-/- mice. Although we did not find major interactions between the mucus layer and the IEL compartment, it is likely that APCs such as DCs may be in close contact with the mucus layer. Thus, the following chapter will explore mucin-DC interactions.

4 CHAPTER 4 Regulation of human monocyte-derived DC function by intestinal mucins

4.1 Introduction

The intestinal mucus barrier separates the epithelium from the microbiota and contents of the lumen, and this barrier is likely to be in close contact with DCs. DCs are proposed to continuously sample luminal antigens and are essential in maintaining gut homeostasis (Coombes & Powrie, 2008). However, whether the interaction between intestinal DCs and the mucus layer is functionally important is a matter of current interest.

Here, we look specifically at the effects of the intestinal mucin MUC2/Muc2 from human and murine sources on DC function. MUC2/Muc2 is the main secreted mucin found in the intestinal mucus barrier. Mucins are highly glycosylated molecules, with glycans comprising up to 80% of the molar mas of mucins (Johansson *et al*., 2011). Thus, it is likely that mucin glycans can interact with different cells expressing lectins and glycan receptors such as DC. Additionally, several reports have shown that MUC/Muc2 may play important immunomodulatory roles by inducing anti-inflammatory cytokines such as IL-10 and preventing the expression of pro-inflammatory cytokines such as IL-12 in the presence of bacterial endotoxin (LPS) (Shan *et al*., 2013). However, most of current studies lack strict molecular characterisation of mucin preparations, which is important due to potential presence of contaminants. Therefore, we aimed to investigate the modulation of DC function by intestinal mucins, using human monocyte-derived DC as a model of DCs.

To this end, density gradient centrifugation was employed to purify mucins from supernatants of the human colonic cell line LS174T and from both mouse small and large intestine. Following detailed biochemical characterisation of mucin preparations, we examined the effect of intestinal mucin on human monocyte-derived DC, initially analysing global changes in gene expression using PCR arrays, followed by validation of gene changes for further analysis. We mainly focussed on modulation of expression of the pro-inflammatory chemokine IL-8; exploring whether mucin glycosylation plays a role in the modulation of expression and mucin-functional effects of mucin-induced changes on DCs.

4.2 Results

4.2.1 Isolation of mucins by GuCl density gradient-centrifugation

In order to study the interaction between intestinal mucins and DC, we first investigated the role of purified human mucins on modulation of DC function. Thus, we purified mucin from the LS174T cell line, which has previously been shown to express high levels of the mucin MUC2, the major secreted mucin found in intestinal mucus (Bu et al., 2011; McGuckin et al., 2011). MUC2 was purified from conditioned cell culture supernatants by CsCl/GuCl isopycnic density gradient centrifugation, which separates mucins from other macromolecules present. Thus, large glycoproteins such as mucins can be successfully separated from other proteins due to their relatively high buoyant density, and from other cellular macromolecules such as DNA, which possess higher buoyant density than mucins (Figure 4.1). To ensure an adequate purity of mucins, two density gradients are required, the first being performed at a density of 1.4 g/ml CsCl dissolved in 4 M GuCl to eliminate proteins and the second at a density of 1.5 g/ml CsCl dissolved in 0.2 M GuCl to remove nucleic acids.

Samples were subjected to ultracentrifugation and 20 fractions collected (from the bottom (fraction 1) to the top (fraction 20) of the tube). Fractions were analysed by slot blotting, transferring a determined sample volume onto a nitrocellulose membrane followed by immunodetection using an anti-MUC2 antiserum. In addition, membranes were stained with periodic acid Schiff (PAS) reagent, which is a general stain for glycoproteins, which usually correlates with the presence of mucins. Band intensity was estimated using a scanning densitometer and illustrated to identify MUC2-rich fractions (Figure 4.1A). Also, density (data not shown) was determined using a densitometer and absorbance (OD_{280}) was measured using a spectrophotometer (Figure 4.1A) in order to check for the potential presence of other contaminating proteins in MUC2-rich fractions. MUC2 appeared to be enriched in fractions 5 to 14 (Figure 4.1A) of the first density gradient, which were then pooled and subjected to a CsCl/0.2M GuCl density gradient at 1.5 g/ml density to further purify the mucin (Figure 4.1B). After this second purification step, MUC2-rich fractions 7-15 (Figure 4.1B) were pooled and dialysed in 1x PBS.

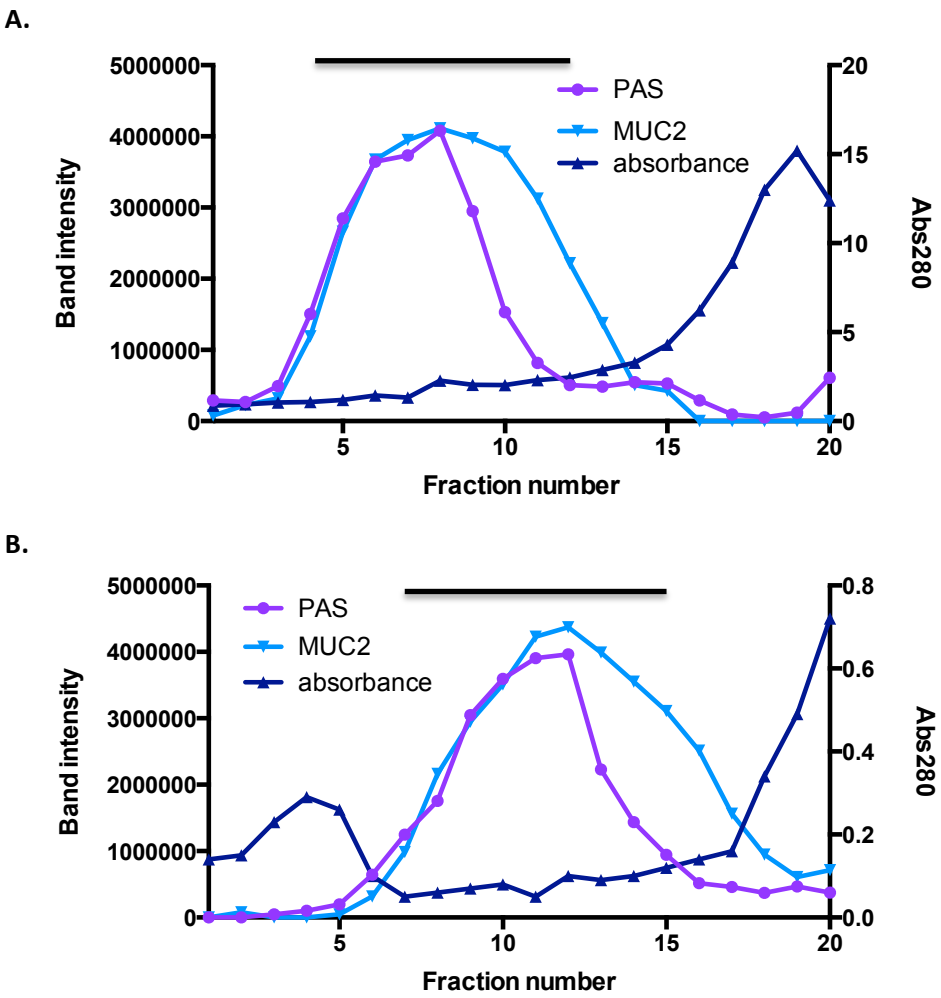

Figure 4.1 Purification of human secreted mucin from LS174T cells supernatant by CsCl/GuCl density gradient centrifugation.

(A) 20 fractions were obtained after a first density gradient centrifugation (CsCl/4M GuCl at 1.4g/ml density), with lower numbered fractions corresponding to those of highest density. Slot blotting was used to measure levels of MUC2 using an anti-MUC2 antiserum, and levels of glycoproteins via PAS staining. MUC2 and PAS levels are displayed as band intensities obtained from slot blotting (left axis). Absorbance readings at 280 nm were also measured (right axis). Pooled fractions are represented by the bold line. (B) A second density gradient centrifugation (CsCl/0.2M GuCl, at 1.5 g/ml density) was performed on pooled fractions from (A), and MUC2, PAS and A280 measurements performed as in (A).

4.2.2 Characterisation of human secreted mucin preparations

To determine the concentration, molecular weight and composition of the mucin preparations, different qualitative and quantitative assays were performed. First, size exclusion chromatography and subsequent multi-angle laser light scattering (SEC-MALLS) were performed to determine concentration and molecular weight of the mucin preparation components (a representative light scattering plot is shown in Figure 4.2). The purified human secreted mucin preparation exhibited a broad degree of polymerisation, characteristic of mucins, showing three high molecular weight components between 1.75 and 10.35 MDa, present in different concentrations (Table 4.1). The total concentration of this sample was 15 μg/ml, which is relatively low but was obtained in a high volume of preparation (40 ml). These different peaks may be attributed to different polymerised forms of MUC2 and/or other purification of other mucins secreted by LS174T cells (Figure 4.2 shows a representative SEC-MALLS plot). This latter idea is supported by mass spectrometry data, which suggests the presence of MUC2, MUC5AC, MUC5B and MUC6 in this preparation. However, as expected from previous reports (Rousseau et al., 2011), the number of unique peptides for each mucin found in this sample suggests that MUC2 is relatively more abundant compared to the other mucins (Table 4.2).

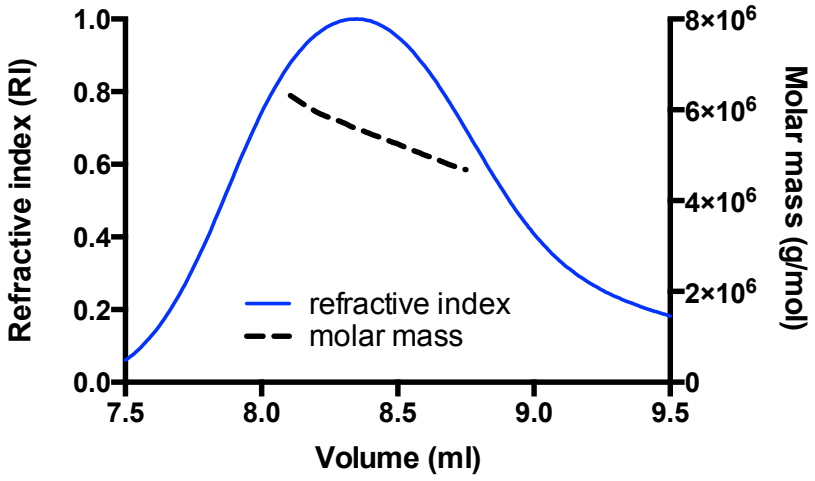

Figure 4.2 Determination of concentration and molar mass by SEC-MALLS analysis.
Representative figure showing SEC-MALLS data. Samples were run in size exclusion chromatography columns (Shodex or Superose 6) and the column effluent was monitored to measure refractive index (RI, solid blue line) and light scattering (not shown). Molar mass (dashed black line) is calculated considering the light scattering measurements and concentration (not shown) is determined using the refractive index values, using the Astra software.

Preparation	Molar mass (MDa)	Concentration (μg/ml)
MUC2	10.4	7.6
	6.9	5.6
	1.8	2.1

Table 4.1 Concentration and molecular weight of components found in mucin preparations by SEC-MALLS.

Molar mass was calculated based on the amount of light scattered and concentration is measured based on the refractive index, using Astra software. Different peaks were identified and molar mass and concentration were calculated.

Identified mucin	Unique peptides
MUC2	24
MUC6	14
MUC5B	12
MUC5AC	8

Table 4.2 Identified mucins in human secreted mucin preparation from LS174T cells by tandem mass spectrometry.

Peptides identified by mass spectrometry were analysed using the Mascot search engine against the human Uniprot database. Identified mucins and number of unique peptides obtained using the Scaffold analysis software for each mucin are indicated (setting a peptide threshold of 95%).

4.2.3 Human secreted mucin can modulate gene expression on moDCs

To determine a potential role for human secreted mucins in the modulation of DC function, human moDCs were treated with purified mucin preparations described above. To this end, monocytes were obtained from human peripheral blood and differentiation to DCs was induced by treatment with GM-CSF and IL-4. Incubation with mucins was performed at day 6 or 7 of DC differentiation. To confirm that GM-CSF/IL4 stimulated moDCs differentiation, cells were also stained with an anti-CD14 antibody and live/dead stain (UV-zombie) and analysed by flow cytometry. Thus, live $CD14^{low}$ cells were gated and considered as moDCs (Figure 4.3).

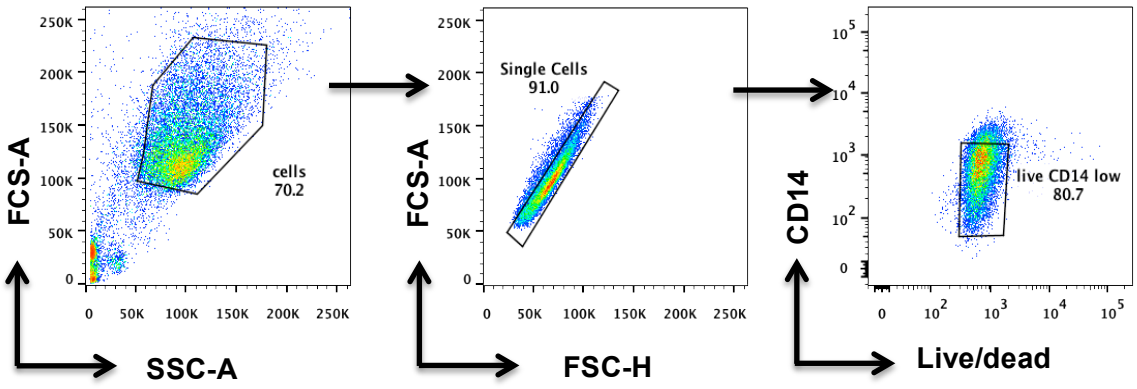

Figure 4.3 Human moDC characterization by flow cytometry.

$CD14^+$ monocytes were isolated from human peripheral blood and incubated in the presence of IL-4 and GM-CSF for 6 days, in order to differentiate them into monocyte-derived DC (moDC). Cells were characterised by flow cytometry and representative plots for moDC gating strategy are shown. Cells were gated from forward vs side scatter, followed by gating of single cells. Thus, live cells expressing low levels of CD14 were considered as moDCs.

To explore potential expression changes induced by human secreted mucin in a wider variety of DC genes, we utilised an RT^2 profiler PCR array (Qiagen), which is designed to analyse several genes relevant to DC function simultaneously. These arrays contain pre-coated primers for 84 different genes related with different DC functions, including cytokine production, antigen presentation, antigen uptake, DC chemotaxis and DC differentiation. To utilise the arrays, RNA from human secreted mucin-treated and untreated DCs was isolated, converted into cDNA and used in the PCR array. Ct values were normalized using housekeeping genes provided for the array and fold changes in expression levels seen in mucin-treated DCs calculated with respect to control DC.

Data suggested that several genes are potentially modulated in human moDC after treatment with human mucin, showing fold-regulation higher than 2 (Table 4.3). Thus, this preliminary assay highlights several potential genes in DCs that may be modulated by human secreted mucins that were taken further for validation.

Gene	Fold regulation
CCR2	**2,5108**
CD1B	**2,691**
CD8A	**2,0823**
CD80	**2,2786**
CXCR1	**3,6254**
FLT3LG	**-2,2066**
ICAM1	**2,359**
ICAM2	**2,2163**
IL-8	**2,0113**
ITGAM	**2,5636**
TAPBP	**-3,3215**

Table 4.3 Human secreted mucin-induced changes on gene expression of human monocyte-derived DC.

PCR arrays using RNA from untreated and human mucin-treated (2 μg/ml) moDC were performed. CT values were normalised with respect to housekeeping genes and fold change between treated vs untreated DC was calculated using Qiagen software. Upregulated genes are highlighted in red and downregulated genes are highlighted in blue (1 independent experiment).

4.2.4 Human secreted mucin upregulates the pro-inflammatory chemokine IL-8

Several genes were evaluated by qPCR in order to validate data from the PCR array. Initially, expression of the most upregulated and downregulated genes CXCR1 (the receptor for the chemokine IL-8) and TABP (a molecule involved in antigen presentation) were evaluated. CXCR1 and TAPBP showed different trends compared to the PCR array after treatment with human secreted mucin and these slight changes were not significant (Figure 4.4). Interestingly, expression of IL-8, an important chemokine in the recruitment of innate cells to infection sites, was significantly increased in moDCs treated with human secreted mucin both at the RNA level (Figure 4.5A) and protein level (Figure 4.5B). Therefore, these results suggest that IL-8 production by human moDCs may be induced by interactions with human secreted mucin.

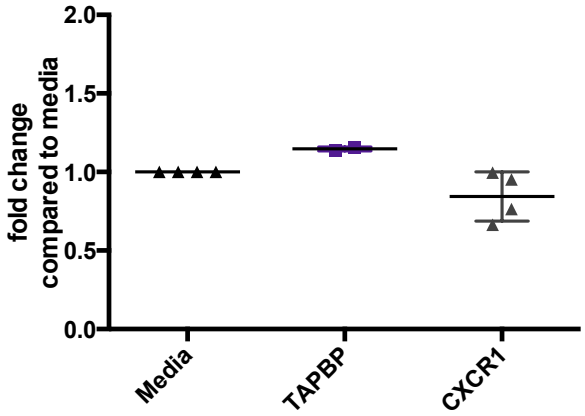

Figure 4.4 Human secreted mucin does not modulate TAPBP and CXCR1 expression by moDCs.

qPCR was performed to detect RNA levels of TAPBP and CXCR1 expressed by human moDCs treated with human secreted mucin (2 μg/ml). Fold change was calculated in each individual experiment compared to untreated moDCs, using β2-microglobulin as a housekeeping gene (4 independent experiments).

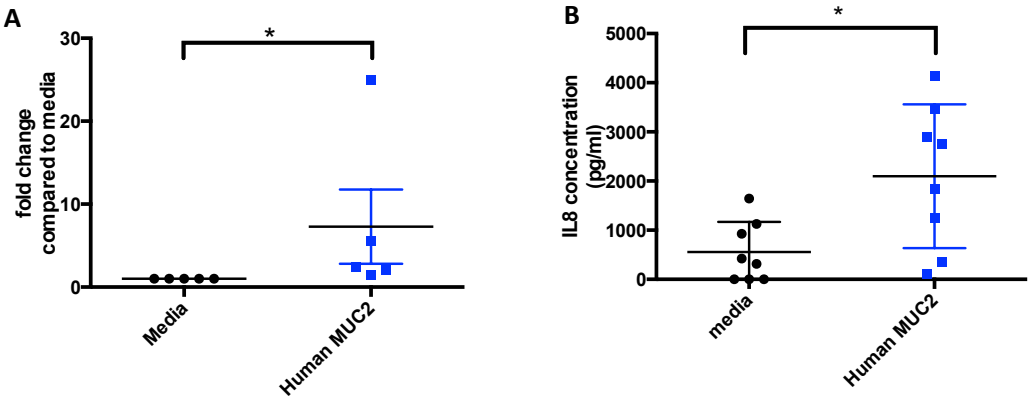

Figure 4.5 Human secreted mucin induced up-regulation of the pro-inflammatory chemokine IL-8.

moDCs were treated with human secreted mucin (2 μg/ml) and IL-8 expression measured by qPCR and ELISA A. qPCR was performed to detect levels of IL-8 gene expression by moDCs treated with human secreted mucin. Fold change was calculated in each individual experiment compared to untreated moDCs, using β2-microglobulin as a housekeeping gene (5 independent experiments) B. ELISA was performed to detect IL-8 protein levels in supernatants from untreated and MUC2- treated moDCs (9 independent experiments). *$p<0.05$

4.2.5 Isolation and characterization of mouse intestinal mucins

As LS174T cells are a transformed cell line, mucins produced by these cells display glycosylation associated with cancer or tumours, which may not represent patterns observed in the steady state. Furthermore, our mucin preparations from LS174T cells contained another secreted mucins and not only MUC2. Thus, intestinal preparations should be further enriched in Muc2, which is the predominant secreted mucin in the intestine with very low levels of other secreted mucin such as Muc5ac or Muc6. Due to these reasons, to better study steady-state conditions and increase Muc2 purity, and given the high percentage of homology between mouse and human mucins (van Klinken et al., 1999, Escande et al., 2004), we repeated experiments using mucin purified from mouse intestine. To this end, mucus was scrapped from mouse small and large intestine (groups of 10-12 C57/BL6 mice) and mucins purified by CsCl/GuCl isopycnic density gradient centrifugation as described above. Representative plots showing 2 consecutive density gradients to purify mouse large intestinal mucins are shown (Figure 4.6). Fractions were stained for Muc2 and PAS to identify mucin-rich fractions and absorbance was measured to ensure separation from proteins and nucleic acids. Mucin-rich fractions were pooled, dialysed in 1X PBS, and further concentrated using 10 KDa molecular weight cut off Vivaspin filters.

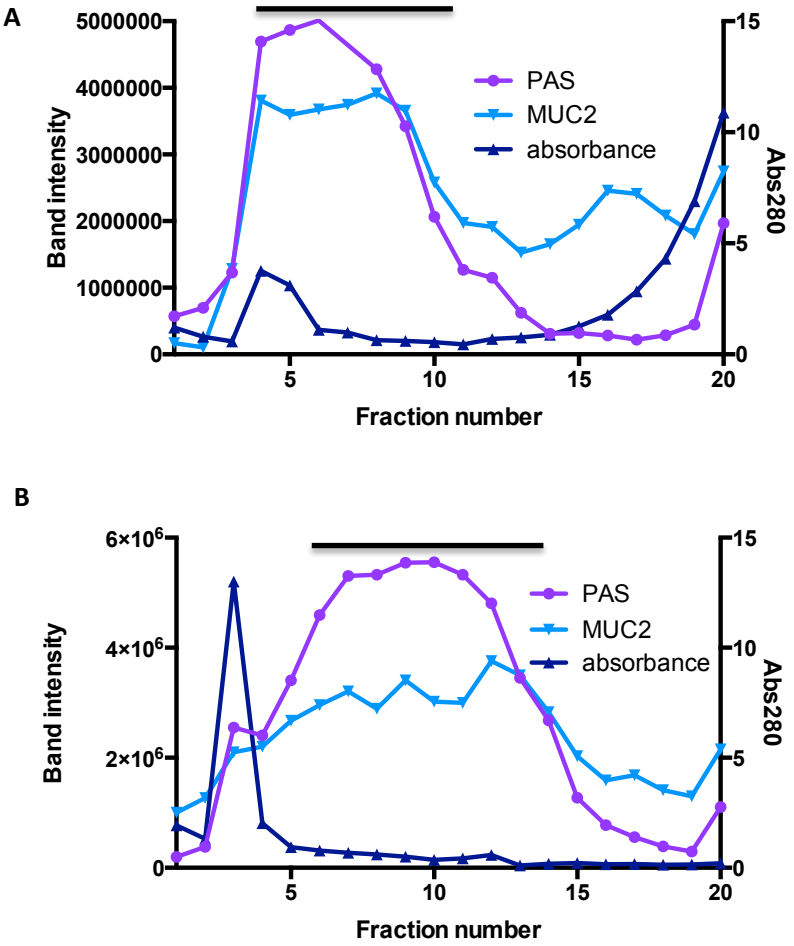

Figure 4.6 Purification of intestinal mucins from mouse intestine by CsCl/GuCl density gradient centrifugation.

Representative figure showing mucin purification from mouse large intestine. (A) 20 fractions were obtained after a first density gradient centrifugation (CsCl/4 M GuCl at 1.4 g/ml density), with lower numbered fractions collected corresponding to the highest density fractions. Slot blotting was used to measure levels of Muc2 using an anti-Muc2 antibody, and levels of glycoproteins via PAS staining. Muc2 and PAS levels are displayed as band intensities obtained from slot blotting (left axis). Absorbance readings at 280 nm were also measured (right axis). Pooled fractions are represented by the bold line. (B) A second density gradient centrifugation (CsCl/0.2M GuCl, at 1.5g/ml density) was performed on pooled fractions from (A), and Muc2, PAS and A280 measurements performed as in (A).

Similar to the previous human cell line mucin preparation, SEC-MALLS was performed to determine mucin concentration and tandem mass spectrometry to determine sample composition. Before performing SEC-MALLS analysis, it should be noted that samples were reduced and alkylated to prevent column blockage, although unreduced mucin were used in further cellular assays. Typically, large intestinal mucin preparations exhibited a higher mucin concentration compared to small intestinal preparations but both reduced mucins showed similar molar masses (Table 4.4). Mass spectrometry analysis also showed similar composition, with Muc2 as the predominant component and the main mucin identified in both preparations (Table 4.5). The gastric mucin Muc5ac was lowly detected in the small intestinal mucin preparation but not found in the large intestinal mucin preparation (Table 4.5). The second main component of each sample is the protein Fcgbp (data not shown, 30 peptides found in small intestinal preparation and 67 found in the large intestinal preparation), which is covalently attached to mouse and human intestinal mucin (Johansson et al., 2009). Similar results were obtained in different independent mucin purifications from mouse intestine.

Sample	Molar mass	Mucin concentration
Small Intestine	4.5 MDa	198.6 μg/ml
Large intestine	5.9 MDa	661.5 μg/ml

Table 4.4 Determination of the concentration of mucin preparations from mouse intestine by SEC-MALLS.

Small and large intestine preparations were reduced with DTT and further analysed by SEC-MALLS. Molar mass was calculated based on the amount of light scattered and concentration is measured based on the refractive index, using Astra software. Data is representative of one mucin purification from 12 mouse intestines.

Mucin preparation	Identified mucin	Unique peptides
Small intestine	Muc2	40
	Muc5ac	4
	Muc6	2
Large intestine	Muc2	188
	Muc13	3

Table 4.5 Identified mucins in mouse intestinal mucin preparations by tandem mass spectrometry.

Peptides identified by mass spectrometry were analysed using the Mascot search engine against the mouse Uniprot database. Identified mucins and number of unique peptides obtained using the Scaffold analysis software for each mucin are indicated (setting a peptide threshold of 95%).

4.2.6 Mouse intestinal mucins induce IL-8 expression on moDCs

To determine whether mouse intestinal mucins were able to induce IL-8 expression, moDCs were stimulated overnight with increasing concentrations of large intestinal mucin, evaluating IL-8 expression by qPCR and ELISA. At both the RNA (Figure 4.7A) and protein level (Figure 4.7B), IL-8 was significantly upregulated by human moDC in response to murine large intestinal mucin. A dose-dependent enhancement was observed, with and a dose of 50 μg/ml inducing a significant increase in IL-8 expression (Figure 4.7).

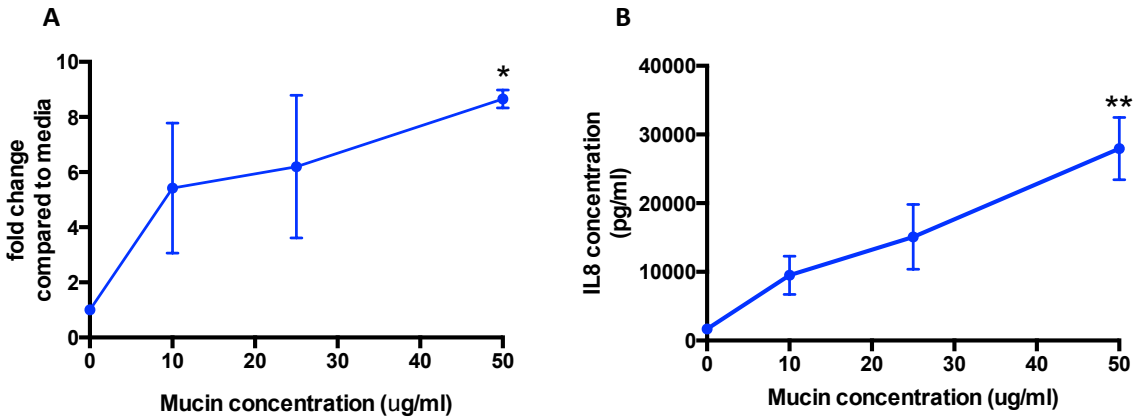

Figure 4.7 Mouse large intestinal mucin induces IL-8 expression by moDCs in a dose-dependent manner.

moDCs were treated overnight with different concentrations of mouse large intestinal mucin (10, 25 and 50 μg/ml) and IL-8 expression measured by qPCR and ELISA A. qPCR was performed to detect gene expression changes in IL-8 on moDCs treated with Muc2. Fold change was calculated in each individual experiment compared to untreated moDCs. Results are from 3 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. B. ELISA was performed to detect IL-8 in supernatants from untreated and Muc2-treated moDCs. Results are from 5 independent experiments and statistical significance was assessed using Kruskal Wallis test followed by Dunn's multiple comparison test. *$p<0.05$ and **$p<0.01$

Next, the capacity of large intestinal mucins to promote IL-8 expression was compared to small intestinal mucins, to determine any location-specific properties of intestinal mucins. moDCs were incubated with small or large intestinal mucins (50 μg/ml) and IL-8 expression evaluated by ELISA on moDCs supernatants after 24 h. Significant up-regulation of IL-8 expression was observed with both small and large intestinal mucins (Figure 4.8), suggesting that this property is apparent for mucin obtained from different intestinal location and not restricted to the large intestine. A higher induction of IL-8 was observed in the presence of large intestinal mucins versus small intestinal mucins, but this difference was not significant. Together, these data suggest that intestinal mucins isolated from healthy mice show similar properties to human cancer cell-derived mucins with respect to up-regulation of IL-8 production by moDCs.

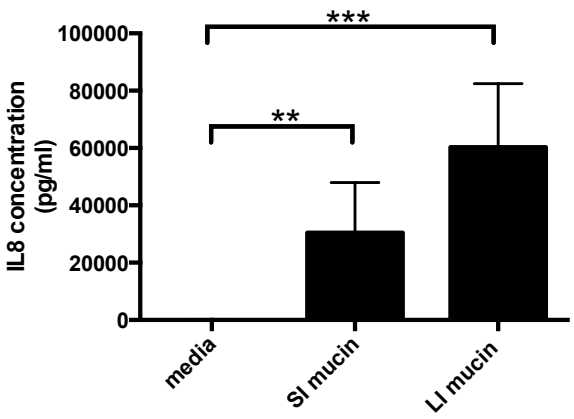

Figure 4.8 Mouse small and large intestinal mucins promote IL-8 production by moDCs.
moDCs were stimulated overnight with mouse small intestinal (SI) and large intestinal (LI) mucins (50 μg/ml both). ELISA was performed to detect IL-8 on supernatants from untreated and mucin-treated moDCs. Results are from 8 independent experiments and statistical significance was assessed using Kruskal Wallis test followed by Dunn's multiple comparison test. **p<0.01 and ***p<0.001

4.2.7 Mucin preparations contain bacterial endotoxin

The bacterial endotoxin LPS is a potent inducer of IL-8 expression (Matsushima et al., 1988, Yoshimura et al., 1987) so one possibility for the results described above was that mucin samples were contaminated with LPS. To test for the presence of LPS in samples, mucin preparations were used in the chromogenic endotoxin quantitation assay, which measures the amount of endotoxin able to catalyse the activation of a pro-enzyme in Limulus Amebocyte Lysate (LAL). The activated proenzyme converts a colourless substrate into the yellow product p-nitroaniline, which can be measured at 410nm in a plate reader. The results of the LAL assay indicated a relatively low concentration of endotoxin in mucin samples isolated from human LS174T cells (1 ng/ml) and murine small intestinal mucins (0.3-4 ng/ml), but a much higher concentration of LPS in large intestinal mucin (12-16 ng/ml), consistent with the enhanced bacterial content of the large intestine (Table 4.6). After dilution of mucins in experiments, levels of LPS in samples were estimated to be 0.075 ng/ml for small intestinal mucin and 0.96 ng/ml for large intestinal mucin. In contrast, LPS concentration used as controls in all experiments below are at least 10 times higher (10 ng/ml).

Sample	Endotoxin concentration
Small Intestine	0.3 ng/ml
Large intestine	12.2 ng/ml

Table 4.6 Endotoxin concentration of mucin preparations from mouse intestine determined by LAL assay.

Endotoxin concentration (ng/ml) was determined by the LAL assay in small and large intestinal mucin preparations. Endotoxin concentration was calculated based on a standard curve, using a control substrate. Data is representative of one mucin purification from 12 mouse intestines

4.2.8 Mouse intestinal mucins induce IL-8 expression by human moDC in a TLR4-independent manner

To test whether LPS contamination of intestinal mucin samples was responsible for their induction of IL-8 production by human moDC, we used a TLR4 inhibitor (CLI-095), which binds to the intracellular domain of TLR4 preventing downstream pro-inflammatory effects (Ii et al., 2006, Kawamoto et al., 2008). The TLR4 inhibitor completely prevented LPS-induced IL-8 production using 10 ng/ml LPS (which is considerably higher than LPS levels in diluted mucin samples) as a control (Figure 4.9). Although the TLR4 inhibitor caused a reduction in IL-8 levels induced in the presence of mucins, both small and large intestinal mucins still significantly induced IL-8 expression by moDC when TLR4 signalling was blocked (Figure 4.9). These results suggest that, although contaminating LPS causes some induction of IL-8 by human moDCs, murine intestinal mucin can enhance IL-8 production in a TLR4-independent.

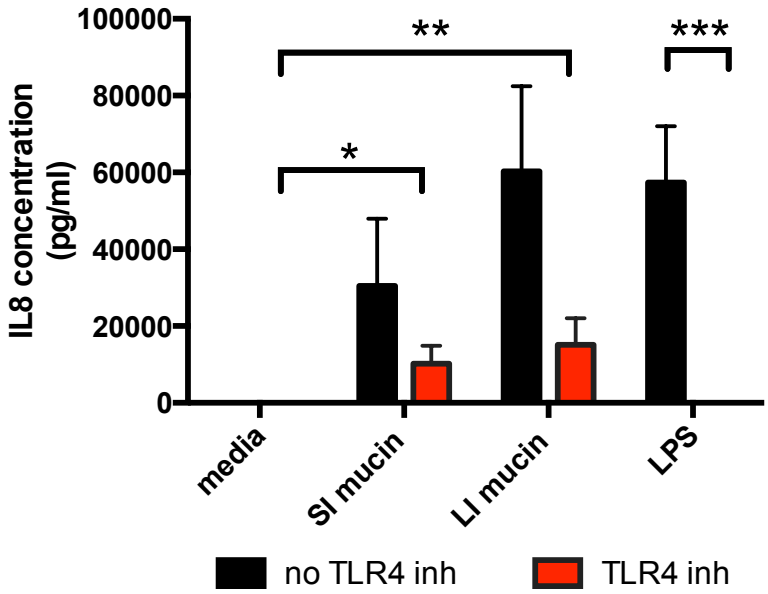

Figure 4.9 Mouse small and large intestinal mucins promote IL-8 production on moDCs in a TLR4-independent manner.

moDCs were stimulated overnight with mouse small intestinal (SI) and large intestinal (LI) mucins (50 μg/ml both), in the absence (black bars) and the presence (red bars) of the TLR4 inhibitor CLI-095. LPS (10 ng/ml) was used as a control. ELISA was performed to detect IL-8 in supernatants from untreated and mucin-treated moDCs, showing IL-8 induction in mucin-treated cells even in the presence of the TLR4 inhibitor. Results are from 8 independent experiments and statistical significance was assessed using Kruskal Wallis test followed by Dunn's multiple comparison test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$

4.2.9 DNA free- intestinal mucin samples induce IL-8 expression in a TLR4 independent manner

Additionally, it was explored whether other contaminants in intestinal mucin preparations may be inducing IL-8 expression by moDCs. Thus, bacterial DNA containing CpG motifs, which may induce intracellular TLR signalling and induce pro-inflammatory effects, could conceivably cause some contamination of samples. Isopycnic density gradient centrifugation should separate DNA from mucins during purification (Figure 4.6) but there may be some remaining DNA. Indeed, this was suggested by absorbance measurements at 260 nm (data not shown). To test samples further for potential DNA contamination, mucin-rich fractions pooled after the 2nd density gradient centrifugation were loaded in a 0.7% agarose gel and DNA visualised via staining with SafeView dye and DNA was observed in mucin-rich fractions. DNA was observed

as a single band with a high molecular weight, which may be either intestinal epithelial cell DNA or contaminant bacterial DNA (Figure 4.10).

To test whether contaminating DNA could be responsible for induction of IL-8 expression by moDCs, mucin preparations were treated with DNAse for 1 h at 37°C. DNAse activity was stopped in presence of EDTA and heating at 60°C for 5 minutes. Mucin preparations were then washed on Vivaspin filters to remove DNAse buffer, and DNA digestion verified by loading mucin preparations on 0.7% agarose gels. The DNA band observed in undigested mucins was completely removed after DNAse treatment, suggesting that DNA digestion was effective (Figure 4.10).

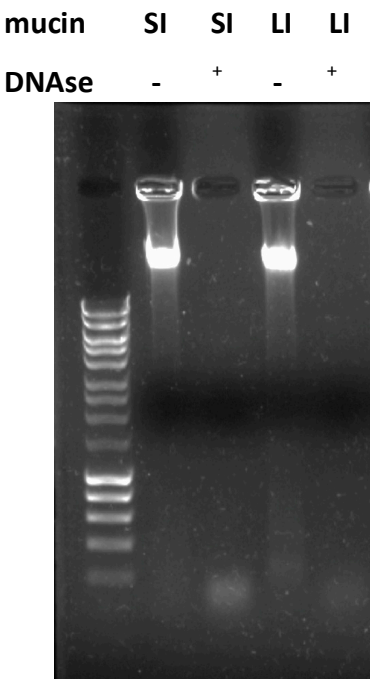

Figure 4.10 DNA can be removed from mucin preps using DNAse.

Mouse small intestinal (SI) and large intestinal (LI) mucin preps were treated with DNAse and loaded in a 0.7% agarose gel. Loading order is indicated on top of the gel (absence of DNAse = -, presence of DNAse = $^{+}$).

To ensure that mucin was not lost after DNA digestion and the further spin wash, mucin concentration was measured by SEC-MALLS. Concentrations of 194 μg/ml for small intestinal mucin and 415.8 μg/ml for large intestinal mucin were determined, showing lower molar masses compared to the undigested preps (Table 4.7). This difference may be attributed due to the removal of high molecular weight DNA that can contribute to the molar mass measurements. The concentration of mucin was decreased compared to pre-digestion; this reduction is likely due to some mucin loss in the spin filters more than a result of the DNA digestion. Small intestinal samples showed a similar endotoxin concentration compared to the undigested prep (0.3 ng/ml) whereas large intestinal preparation showed a slightly lower endotoxin concentration (9.6 ng/ml vs 13 ng/ml).

Sample	Molar mass	Mucin concentration	Endotoxin concentration
Small Intestine	1.8	194 μg/ml	0.4 ng/ml
Large intestine	2.2	415.8 μg/ml	9.6 ng/ml

Table 4.7 Characterisation of DNA-free mouse mucins.

Small and large intestine preparations treated with DNA were reduced with DTT and further analysed by SEC-MALLS. Molar mass was calculated based on the amount of light scattered and concentration is measured based on the refractive index, using Astra software. Endotoxin concentration was determined using the LAL assay.

MoDCs were then treated with DNA-free mucin preparations and IL-8 expression measured in moDCs supernatants. After being treated with DNAse, large intestinal mucin induced slightly less IL-8 expression compared to untreated mucin, although the difference was not significant. No differences were observed between DNAse-treated and untreated small intestinal mucin. Importantly, both DNA-free small and large intestinal mucin preparations significantly promoted IL-8 expression by human moDC. (Figure 4.11). Therefore, these results suggest that IL-8 induction is not a result of any bacterial or other DNA contamination in the mucin preparations.

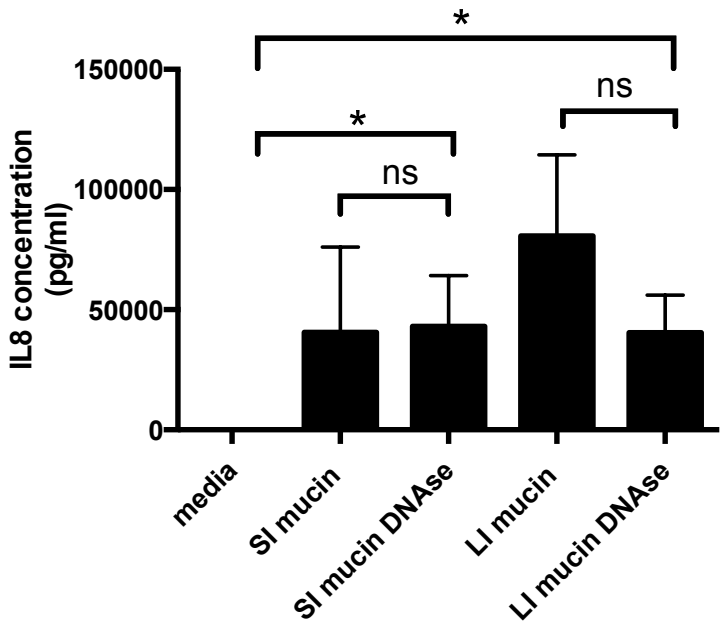

Figure 4.11 Mouse DNA-free small and large intestinal mucins promote IL-8 production by moDCs.

ELISA was performed to detect IL-8 in supernatants from untreated and mucin- treated moDCs. Results show IL-8 induction in the presence of untreated and DNAse-treated mucin (small intestinal, SI and large intestinal, LI, all 50 μg/ml). Results are from 4 independent experiments and statistical significance was assessed using Kruskal Wallis test followed by Dunn's multiple comparison test. *$p<0.05$

4.2.10 DNA-free mucin glycopeptides induce IL-8 expression in a TLR4 independent manner

Another possibility for the induction of IL-8 in moDC by mucin samples was potential the presence of other contaminating protein/s that co-purified with mucins that resulted in enhanced IL-8 expression. Furthermore, protein-rich mucin region of Muc2 could be responsible for these effects. To test this possibility, other smaller, potentially contaminating proteins and non-glycosylated protein-rich parts of Muc2, were eliminated from DNA-free mucin preparations. Thus, mucin samples were reduced, alkylated, and then treated with trypsin in order to digest the mucin and other proteins. Generation of mucin glycopeptides was confirmed by SEC-MALLS, as the molecular weight is reduced compared to the unreduced mucin (0.7-1.6MDa vs 4-6MDa) (Table 4.8).

Sample	Molar mass of the reduced original sample	Molar mass of trypsin-digested sample	Mucin concentration
Small Intestine	4.5 MDa	0.7 MDa	144.9 μg/ml
Large intestine	5.9 MDa	1.6 MDa	491.5 μg/ml

Table 4.8 Characterisation of DNA-free mouse mucin glycopeptides.
Small and large intestine preparations treated with DNAse, reduced with DTT and trypsin-digested were further analysed by SEC-MALLS. Molar mass was calculated based on the amount of light scattered and concentration is measured based on the refractive index, using Astra software. Molar masses of the original preparations (reduced with DTT) and the preparations after digestion with trypsin are shown. Endotoxin concentration was determined using the LAL assay.

MoDCs were then treated with DNA-free mucin glycopeptides overnight and IL-8 expression measured in moDCs supernatant. Both small and large intestinal mucin glycopeptides significantly induced IL-8 expression (Figure 4.12). Small intestinal mucin glycopeptides exhibited a significantly lower ability to induce IL-8 expression versus undigested small intestinal mucin samples (Figure 4.12). This may be attributed to the lower endotoxin concentration observed compared to the original mucin preparation and/or the loss of protein-rich parts of the mucin or contaminant proteins after trypsin digestion. No significant differences were observed comparing untreated large intestinal mucin and DNA-free large

intestinal mucin glycopeptides (Figure 4.12). Importantly, both small and large intestinal mucin glycopeptide samples significantly induced IL-8 compared to non-treated moDC (Figure 4.12). Together, these results indicate that IL-8 induction is not induced by contaminating DNA and that digestion of protein-rich mucin regions, and potentially contaminating proteins, does not abrogate the ability of mucins to induce IL-8.

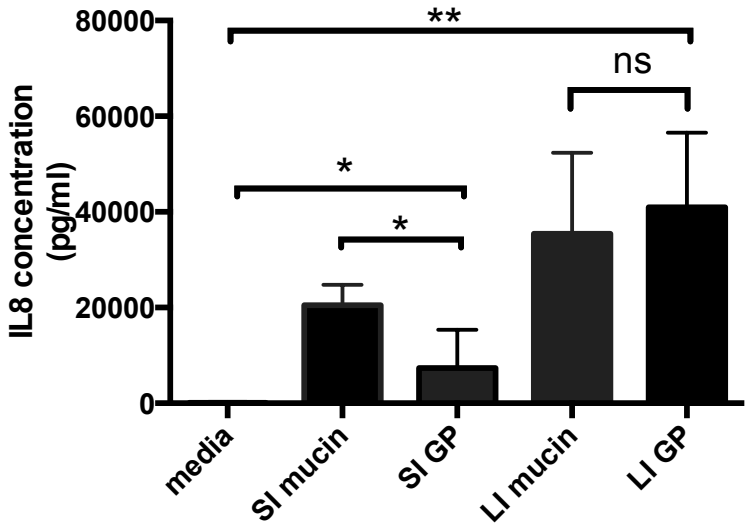

Figure 4.12 Mouse small and large intestinal mucin glycopeptides promote IL-8 production by moDCs.

ELISA was performed to detect IL-8 levels in supernatants from moDCs either untreated, treated with intact mucin (small intestinal, SI mucin and large intestinal, LI mucin) or treated with mucin glycopeptides (SI GP and LI GP). 50 μg/ml of all the different mucins were used to treat moDCs. Results are from 6 independent experiments and statistical significance was assessed using Kruskal Wallis test followed by Dunn's multiple comparison test. *$p<0.05$, and **$p<0.01$

4.2.11 Mouse intestinal mucins induce DC activation in a TLR4-independent manner

It is possible that enhanced expression of IL-8 by DCs was in part due to the ability of the mucins to activate the DCs. To determine whether treatment of DC with mucins resulted in DC activation, expression of CD86 and CD83, classical DC activation markers, was measured by flow cytometry in the presence and absence of mucin. Typically, both DC activation markers were highly induced by LPS (10 ng/ml) as a positive control. When treated with either small

intestinal or large intestinal mucin, moDCs were significantly activated, with increased populations of $CD86^+$ and $CD83^+$ DC (Figure 4.13A and B). This is consistent with the induction of the pro-inflammatory chemokine IL-8, suggesting that mucins promote pro-inflammatory features on moDCs.

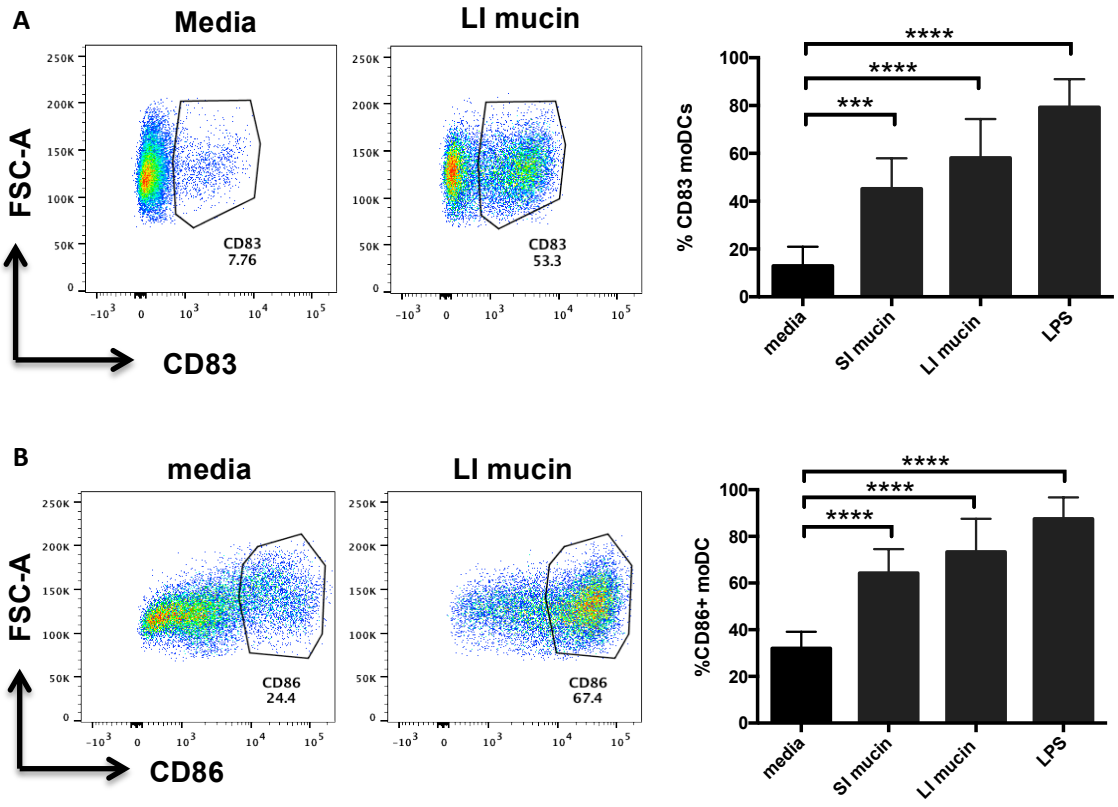

Figure 4.13 Mouse intestinal mucins induce moDC activation markers.

moDCs were treated with small intestinal (SI) and large intestinal (LI) mucins and DC activation evaluated by FACS. Representative FACS plots and percentages of $CD83^+$ (A) and $CD86^+$ (B) moDCs are shown for untreated and large intestinal mucin-treated moDCs. Results are from 8 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. ***p<0.001 and ***p<0.0001

As mucin samples were known to contain some contaminating LPS (section 4.2.7), such contamination could result in DC activation. To determine if this was the case, assays were also performed in the presence of the TLR4 inhibitor CLI-095, which effectively prevented DC activation in LPS-treated DC (Figure 4.14). Both small and large intestinal mucin preparations, significantly induced both DC activation marker, CD83 (Figure 4.14A) and CD86 (Figure 4.14B) in the presence of the TLR4 inhibitor, consistent with previously observed data with IL-8

induction. Thus, our data suggest that both small and large intestinal mucin can induce DC activation in a TLR4-independent manner.

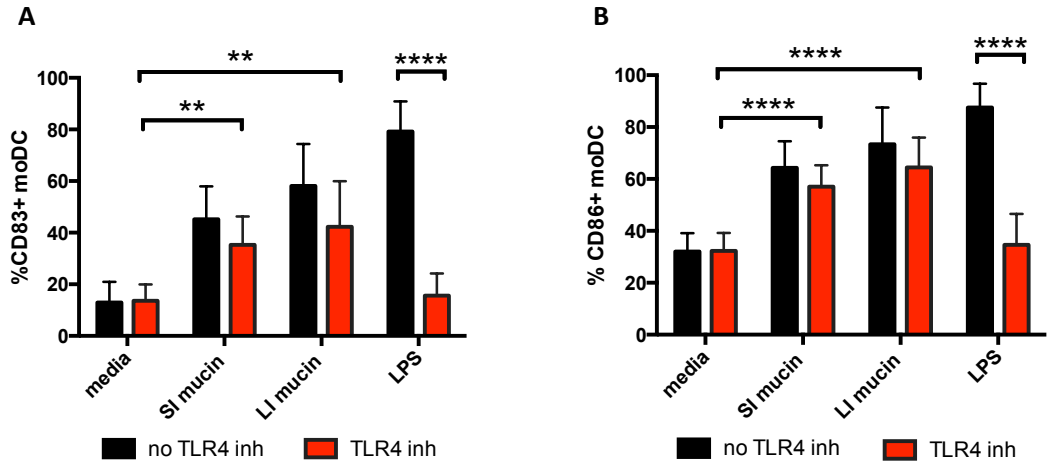

Figure 4.14 Mouse intestinal mucins induce moDC activation markers in a TLR4 independent manner.

moDCs were treated with small intestinal (SI) (50 μg/ml) and large intestinal (LI) mucins (50 μg/ml), in the absence (black bars) and the presence (red bars) of TLR4 inhibitor CLI-095 and DC activation evaluated by FACS. Percentages of $CD83^+$ (A) and $CD86^+$ (B) moDCs are shown and mucin-treated moDCs compared with untreated moDCs, in the presence of the TLR4 inhibitor. Results are from 8 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. **p<0.01 and ****p<0.0001

4.2.12 DNA-free intestinal mucins and mucin glycopeptides induce DC activation in a TLR4-independent manner

To test the potential role of DNA or other protein contamination in the mucin preparations to induce DC activation, the ability of DNAse-treated mucin and mucin glycopeptides (described above) to induce CD83 and CD86 expression by moDCs was tested. Treatment with either small or large intestinal DNA-free mucin caused a significant increase in CD83 and CD86 expression and was not significantly different to the effect induced by the untreated mucin preparation (data not shown). Similarly, DC activation was evaluated after treatment with DNA-free mucin glycopeptides. Both preparations, from small and large intestine, induced increased CD83 and CD86 expression, which was also observed in the presence of the TLR4

inhibitor (Figure 4.15A and B and data not shown). Interestingly, as seen for IL-8 production (Figure 4.12), digested small intestinal mucin samples induced significantly less CD83 expression compared to untreated small intestinal mucin, and the increase seen versus untreated moDC was non-significant (Figure 4.15A). These results suggests that although small intestinal glycopeptides seem able to induce IL-8 and DC activation, other digestible proteins or protein-rich part of the mucin may play a significant role inducing these pro-inflammatory effects. In contrast, large intestinal mucin glycopeptides still induce high levels of DC activation, suggesting that protein-rich regions of mucin and/or contaminant proteins are not responsible for these effects (Figure 4.15B).

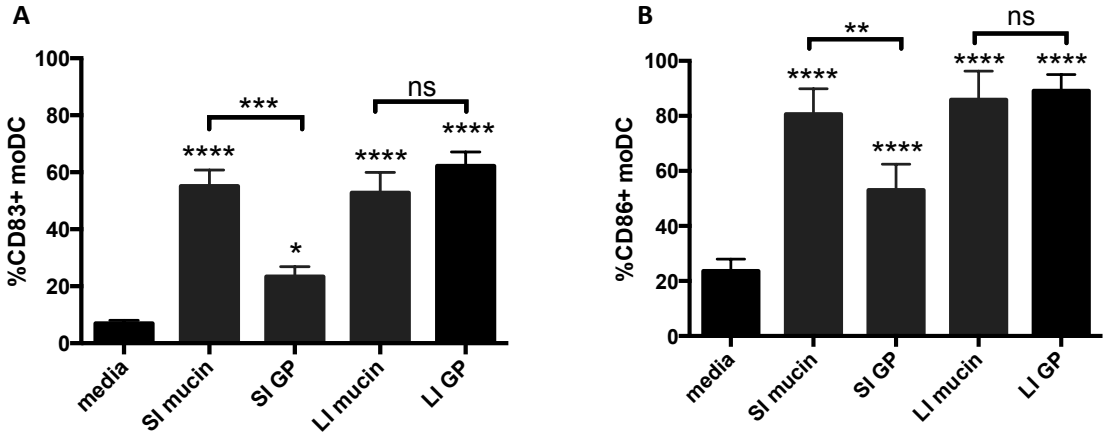

Figure 4.15 Mouse intestinal DNA-free mucin glycopeptides induce moDC activation markers.

moDCs were treated overnight with mouse intestinal mucins (50 μg/ml) and mucin glycopeptides (50ug/ml), and DC activation evaluated by FACS. A. Percentages of $CD83^+$ (A) and $CD86^+$ (B) moDCs are shown and compared between untreated, SI mucin, LI mucin and LPS-treated moDCs. Results are from 6 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. *p<0.05, ***p<0.001 and ****p<0.0001

4.2.13 Mucin-induced IL-8 is able to recruit neutrophil-like cells *in vitro*

IL-8 is a relevant chemokine during intestinal inflammation, able to recruit immune cells expressing the chemokine receptor CXCR1 such as neutrophils (Waugh and Wilson, 2008). To evaluate whether mucin treatment of DCs could induce any functional effects via IL-8, we

investigated whether DC treatment with mucins could alter the ability to attract neutrophil-like cells in a transmigration assay.

HL60 cells are a well characterized promyelocytic cell line, which can be differentiated into neutrophil-like cells after 5 days stimulation with 1.5% DMSO, an inducer of cell cycle arrest (Jacob et al., 2002, Fleck et al., 2005, Millius and Weiner, 2009). Initially, to verify appropriate differentiation of these cells, up-regulation of CD11b, which is not expressed on undifferentiated HL60 cells, was evaluated by flow cytometry (Figure 4.16). These cells were used for transmigration assays, using 3.0 μm pore transwell plates where endothelial cells EaHY926 were grown overnight forming to form a monolayer. After monolayer formation, neutrophil-like cells were added on the top of the transwell insert, whereas the lower compartment contained the supernatants of moDCs or media controls. Transmigration of neutrophil-like cells was evaluated after 24 h by counting of the cells that migrated to the media below the membrane. Serum-free media X-Vivo 15 containing recombinant IL-8 was used as a transmigration control, with increasing concentrations of IL-8 shown to correlate with increased transmigration (Figure 4.17A). For further assays, 100 ng/ml IL-8 was considered an effective concentration to induce transmigration and used as a positive control.

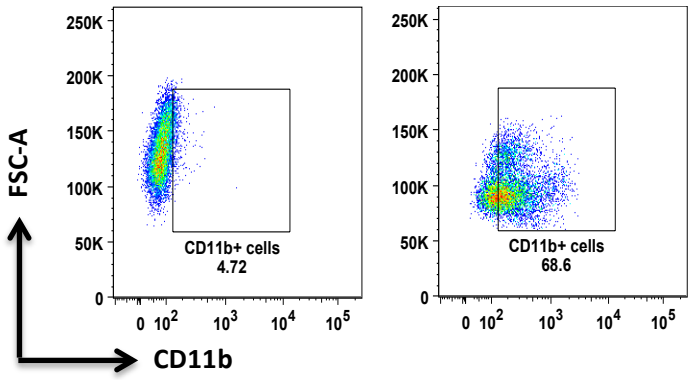

Figure 4.16 Up-regulation of CD11b in DMSO-treated HL60 cells.

HL60 cells were differentiated into neutrophil-like cells in the presence of 1.5% DMSO for 5 days. Untreated and DMSO-treated HL60 cells were stained with CD11b and up-regulation of CD11b was measured to determine differentiation. Representative flow cytometry plots showing CD11b staining are shown.

Given the similar behaviour of small and large intestinal mucins in assays, large intestinal mucin was used for subsequent functional assays. Supernatants from moDCs untreated or treated with large intestinal mucin (10 μg/ml), LPS and LPS together with large intestinal mucin were used for transmigration assays and their ability to attract neutrophil-like cells measured. Transmigration was expressed as percentages of migrated cells compared to the control. Significantly increased transmigration of these cells was observed in the presence of media containing mucin (Figure 4.17B), which is consistent with the ability of intestinal mucins to induce IL-8 expression from DCs. This result suggests that mucin-treated moDC secrete chemokines able to attract neutrophil-like cells *in vitro*.

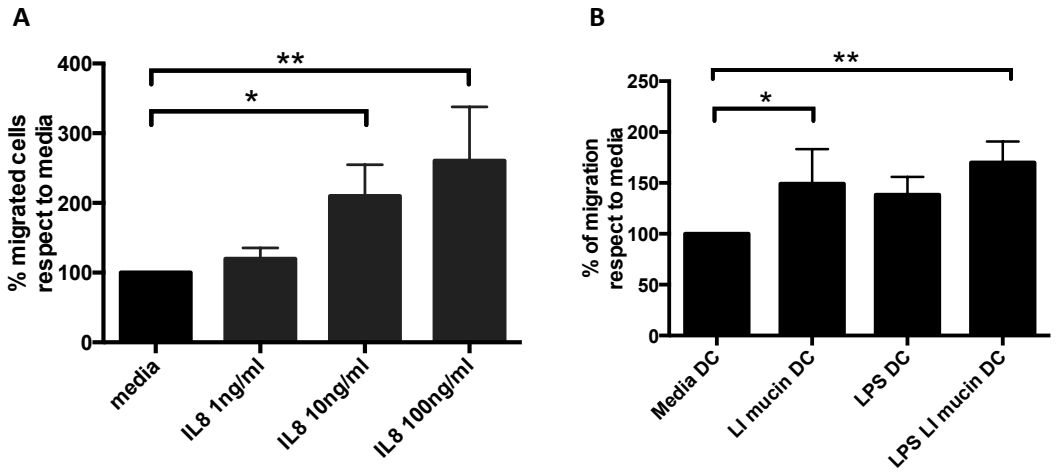

Figure 4.17 Mucin-induced secreted factors are able to recruit neutrophil-like cells in a transmigration assay.

Transmigration of differentiated neutrophil-like cells HL60 was evaluated in transmigrations assays, exposed to supernatants from monocyte-derived DC. A. Transmigration of neutrophil-like cells exposed to serum-free media X-vivo 15 containing different concentrations of recombinant IL-8. Transmigration is expressed as a percentage of migrated cells calculated respect to media alone. B. Transmigration of neutrophil-like cells exposed to supernatants from moDCs (untreated, treated with large intestinal mucin (10 μg/ml), LPS (10 ng/ml) and LPS plus large intestinal mucin. Transmigration is expressed as a percentage of migrated cells calculated respect to the transmigration observed in the presence of supernatants from untreated moDCs. Results are from 4 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. *$p<0.05$ and **$p<0.01$

To evaluate whether this increased transmigration was IL-8 dependent, assays were repeated in the presence of an anti-IL-8 monoclonal antibody. This antibody (0.4 μg/ml) effectively prevented transmigration in controls using recombinant IL-8 (100 ng/ml) (Figure 4.18A). When moDC supernatants from mucin-treated DC were added, transmigration compared to media control was fully prevented, which supports the idea that mucin-induced expression of IL-8 by DCs is able to attract neutrophil-like cells (Figure 4.18B).

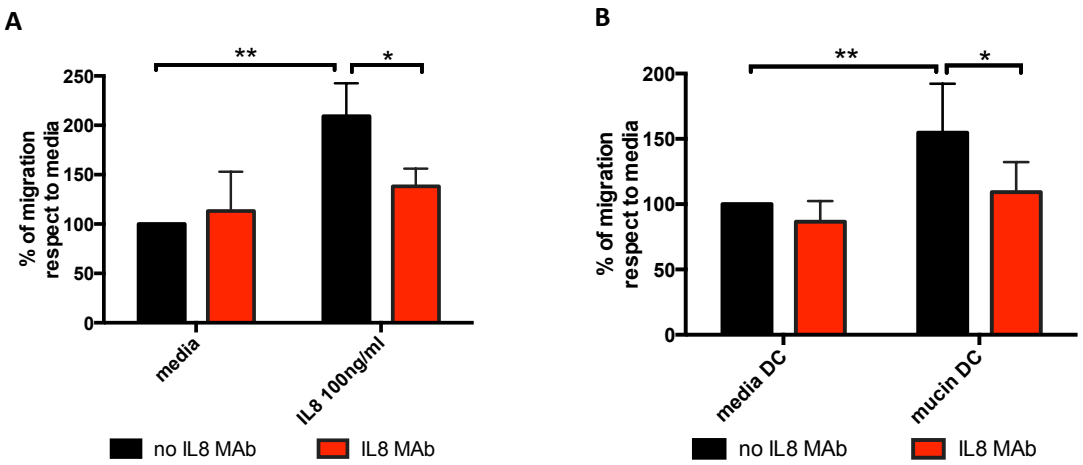

Figure 4.18 Mucin-induced neutrophil-like cell transmigration is IL-8 dependent.

Transmigration of differentiated neutrophil-like cells HL60 was evaluated in transmigrations assays, exposed to supernatants from Mo-DCs (black bars) and in the presence of anti-IL-8 blocking antibody (red bars). A. Transmigration of neutrophil-like cells exposed to serum-free media X-vivo 15 containing recombinant IL-8 (100 ng/ml) with and without the anti-IL-8 blocking antibody (0.4 ng/ml). Transmigration is expressed as a percentage of migrated cells calculated respect to media alone. B. Transmigration of neutrophil-like cells exposed to supernatants from moDCs (untreated and treated with large intestinal mucin (10 μg/ml)), in the absence and the presence of anti-IL-8 blocking antibody. Transmigration is expressed as a percentage of migrated cells calculated normalised to transmigration observed response to untreated moDCs supernatants. Results are from 4 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. *$p<0.05$ and **$p<0.01$

4.2.14 Mucin glycans may contribute to IL-8 induction and DC activation

Next, potential mechanisms by which mucins can induce DC activation and IL-8 production were analysed. It was hypothesised that the glycosylation of mucins may be important, considering that 80% of the mass of mucins are due to glycan side chains and different groups have reported potential immunomodulatory effects mediated by mucin glycans (Shan et al., 2013). Thus, to test this possibility, mucins were subjected to different treatments in order to modify or remove terminal sugars on the glycans. First, mouse large intestinal mucins were treated with sodium metaperiodate, which oxidises vicinal diols, cleaving them and producing two aldehydes. This reaction can modify terminal mucin glycans, in particular sialic acid, and potentially alter interaction with DCs. Mucin oxidation was verified by slot blotting treated samples onto nitrocellulose and detecting oxidised mucin with Schiff's reagent staining. This reagent only reacts with oxidized molecules so increased staining will correlate with increased oxidation. A dose response using increasing concentrations of sodium metaperiodate was performed and a concentration of 3 mM sodium metaperiodate was determined as adequate to fully oxidise the mucin (Figure 4.19). Mucins were treated with 3mM sodium metaperiodate for subsequent experiments.

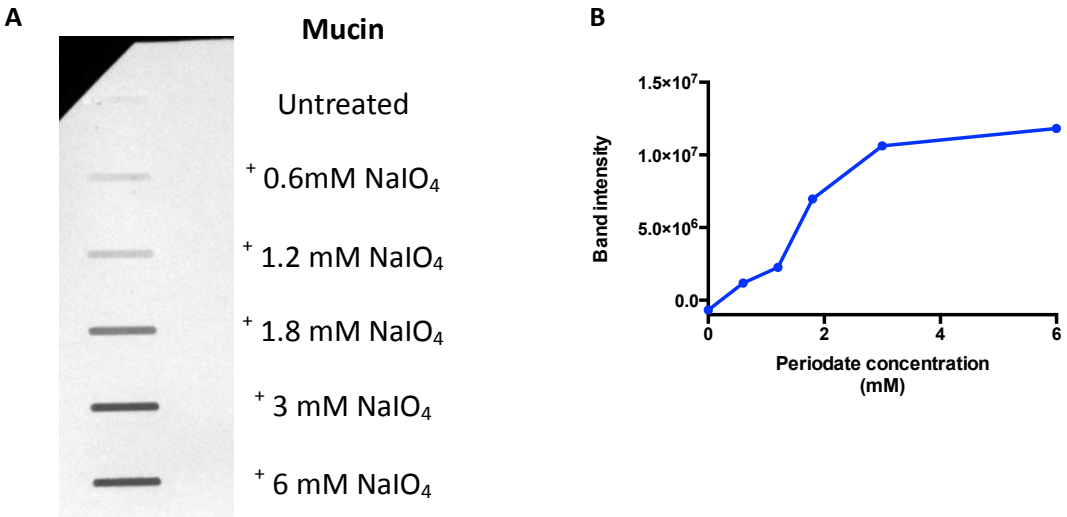

Figure 4.19 Sodium metaperiodate dose response to oxidise mouse large intestinal mucin.
To confirm mucin oxidation by sodium metaperiodate, identical concentrations of mucin were treated with increasing concentration of sodium metaperiodate ($NaIO_4$, from 0.6 mM to 6 mM), loaded onto a slot blot and detected by Schiff's reagent (A). Band intensity was calculated and plotted against sodium metaperiodate concentration (B).

MoDCs were treated with this chemically modified mucin for 24 hrs and cell viability evaluatedby FACS. When cells were treated only with 3mM sodium metaperiodate, no cell death was observed but increased CD86 expression was detected, suggesting that this chemical may activate DC. When cells were incubated with sodium metaperiodate treated mucins, alteration in the forward scatter was observed together with increased cell death, suggesting that either toxic products released during the sodium metaperiodate reaction or the oxidised mucin itself may have some lethal effects on moDCs (Figure 4.20A). Live moDCs were gated for CD86 and CD83 analysis, which suggests that oxidized mucin does not induce CD83 (Figure 4.20B) or CD86 (Figure 19C) expression compared to untreated cells. This reduction is significant compared to cells treated with native mucin in the case of CD86 but not for CD83. These results suggest that chemically modified mucins lose the ability to induce DC activation and IL-8 secretion. However, these results should be interpreted with caution, considering that changes in cell size (forward scatter) and increased cell death were observed under the treatment with oxidised mucin.

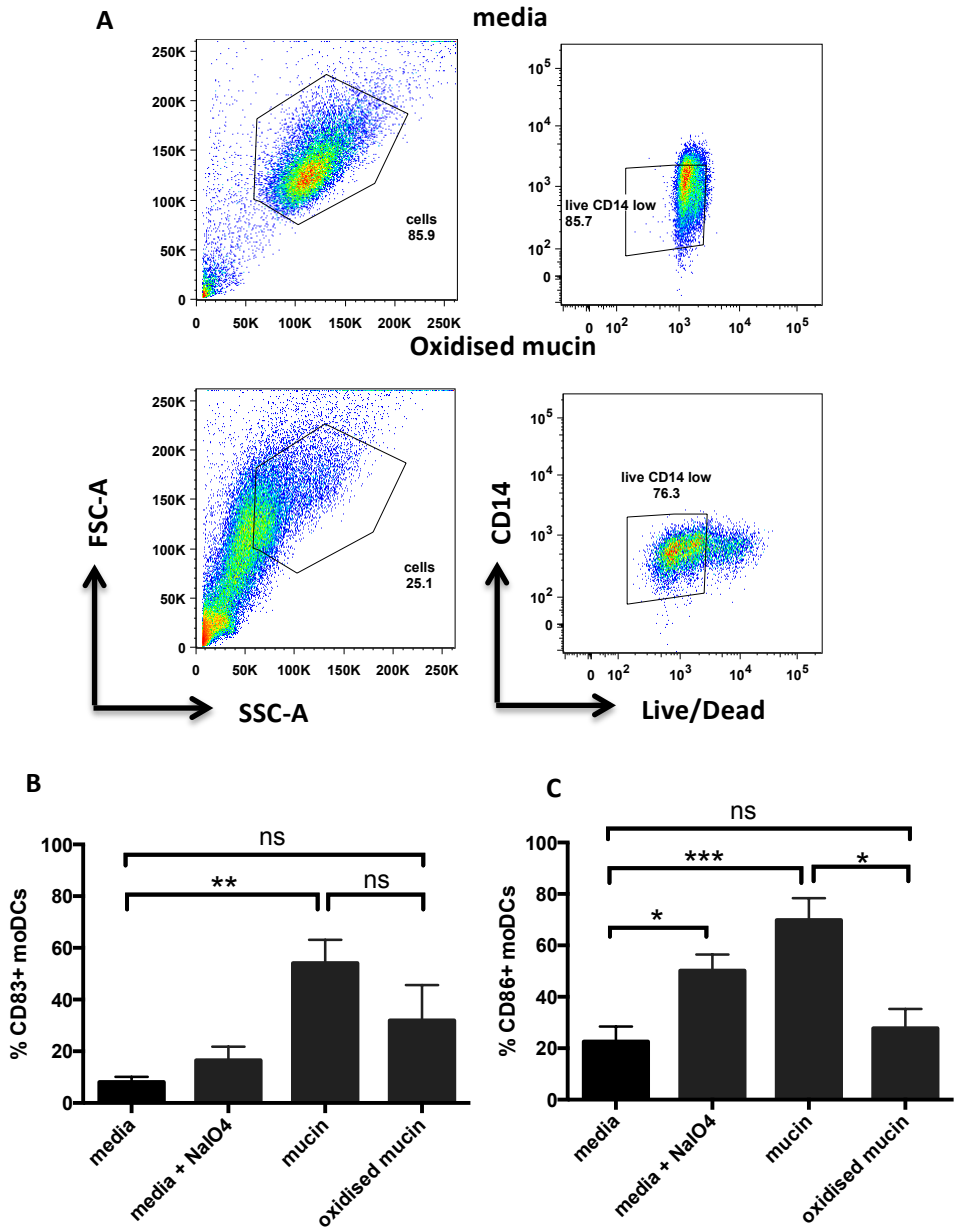

Figure 4.20 Oxidised mucin induces cell death but does not induce DC activation.

moDC viability and DC activation was evaluated in the presence of 3mM sodium metaperiodate, large intestinal mucin (50 μg/ml), oxidised mucin (50 μg/ml) and LPS (10ng/ml). (A) Representative FACS plots show FCS/SSC and live $CD14^+$ cells. Decreased viability is observed in cells treated with oxidised mucin. Percentages of $CD83^+$ (B) and $CD86^+$ (C) moDCs are shown and compared. Decreased cell activation is observed in cells treated with oxidised mucin. Results are from 4 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. *p<0.05, **p<0.01 and ***p<0.001

To evaluate whether cell death could be prevented in the assay, after the treatment with sodium metaperiodate, the sample was washed with 1X PBS in a Vivaspin filter. Some mucin may be lost in the process so mucin content and mucin oxidation were reanalysed by slot blotting. Muc2 detection suggested that mucin content is similar but oxidised mucin, detected by Schiff's reaction, seems slightly lower compared to the mucin before washing (Figure 4.21).

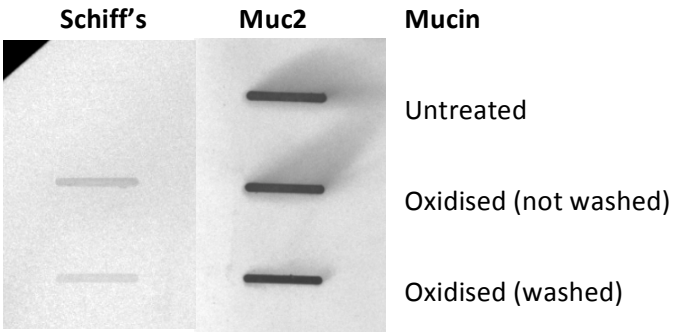

Figure 4.21 Detection of oxidised mouse large intestinal mucin after washing in a Vivaspin filter.

To confirm that oxidised mucin was not lost after washing in a spin filter, equivalent concentrations of untreated and oxidized mucin (no washed and washed) were loaded onto a slot blot. Oxidation was detected by Schiff's reagent (A) and presence of Muc2 was confirmed using an anti-Muc2 antiserum (B).

When looking at moDCs by flow cytometry, oxidised mucin washed with PBS seems to be effective in preventing cell death and decreased viability (data not shown). DC activation is partially reduced in the presence of oxidised mucin. $CD83^+$ cells are not significantly increased in the presence of oxidised mucin (Figure 4.22A) but $CD86^+$ cells are still significantly increased (Figure 4.22B). Also, when compared to cells treated with native mucin, no significant differences were found. These results suggest that oxidised mucin is still able to induce a significant increase in DC activation to some extent (significantly higher CD86 expression). In contrast, CD83 is not significantly upregulated in the presence of oxidised mucin which suggests that some of the potential to induce DC activation is lost after mucin oxidation.

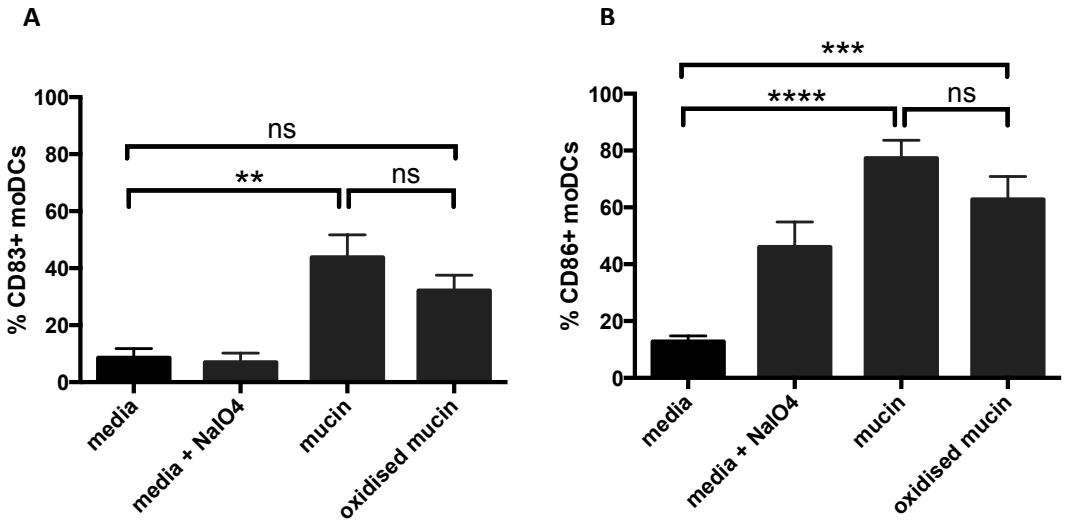

Figure 4.22 Oxidised mucin induces less DC activation compared to untreated mucin on moDCs.

DC activation was evaluated on moDCs treated with oxidised mucin. moDCs were treated with intact large intestinal mucin (50 μg/ml) or mucin oxidised with 3mM sodium metaperiodate ($NaIO_4$) (both 50 μg/ml). Percentages of $CD83^+$ (A) and $CD86^+$ (B) moDCs are shown and compared. Results are from 4 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. **p<0.01, ***p<0.001 and ****p<0.0001

IL-8 production was measured in the supernatants of the cells treated in the assays shown above. When moDCs were treated with washed and oxidised mucin, IL-8 expression is slightly enhanced by oxidised mucin, but this increase is not significant when compared to untreated moDCs (Figure 4.23A). The comparison between intact mucin and oxidised mucin is insignificant but this may be attributed to the high variability observed in the assays performed with intact mucin (n=4). These results suggest that oxidized mucin lost the ability to induce IL-8 expression on moDCs and IL-8 induction is glycosylation-dependent.

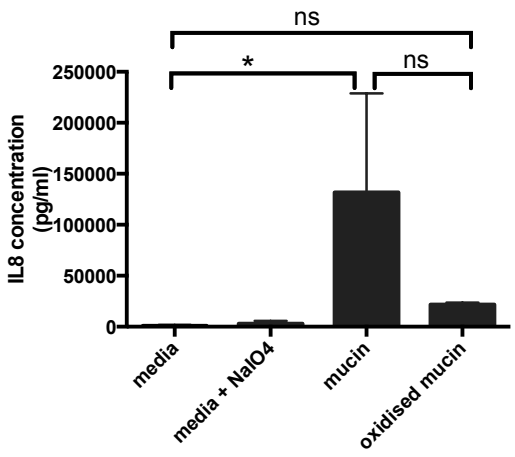

Figure 4.23 Sodium metaperiodate treated mucin is not able to induce IL-8 expression on moDCs.

MoDCs were treated with large intestinal mucin (50 μg/ml), untreated and treated with 3 mM sodium metaperiodate and IL-8 measured in moDCs supernatants by ELISA. Media containing 3 mM sodium metaperiodate was used as a control. moDCs were treated with washed and oxidised mucin. Results are from 4 independent experiments and statistical significance was assessed using the Kruskal Wallis test followed by Dunn's multiple comparison test. *$p<0.05$

4.2.15 . Studying sialic acid involvement in mucin-induced IL-8 production and DC activation

As mentioned above, a common terminal sugar present on mucins is sialic acid. Thus, to further study the contribution of glycosylation in modulation of DC function by mucin, enzymatic removal of sialic acid from the mucin was performed. To this end, mucin was treated with different concentrations of neuraminidase (Sigma) overnight at 37°C and enzymatic digestion was stopped by heating the sample at 100°C for 5 minutes. As a control, mucin was incubated overnight containing only the enzyme buffer. Effective digestion was shown by slot blotting and lectin staining.

Different lectins can detect the linkage specificity of sialic acid to the mucin glycan chains. Maackia amurensis lectin II (MAL II) preferentially binds α-2,3 attached sialic acid, whereas Sambucus Nigra lectin (SNA) binds preferentially to α-2,6 sialic acid linked to terminal galactose. Both lectins were used to test for degree of sialic acid achieved. A decrease in band intensity was observed with both lectins, with the biggest reduction in band intensity observed using the highest concentration of enzyme, indicating reduction of α-2,3 and α-2,6 linked sialic acid (Figure 4.24), thus this mucin preparation was used for further assays. No decrease in

band intensity was observed in the mucin control, suggesting that the buffer did not affect mucin glycosylation. To confirm that mucin was not degraded in the process, samples were slot blotted and detected with a Muc2 antiserum, which showed a similar signal before and after the digestion (data not shown).

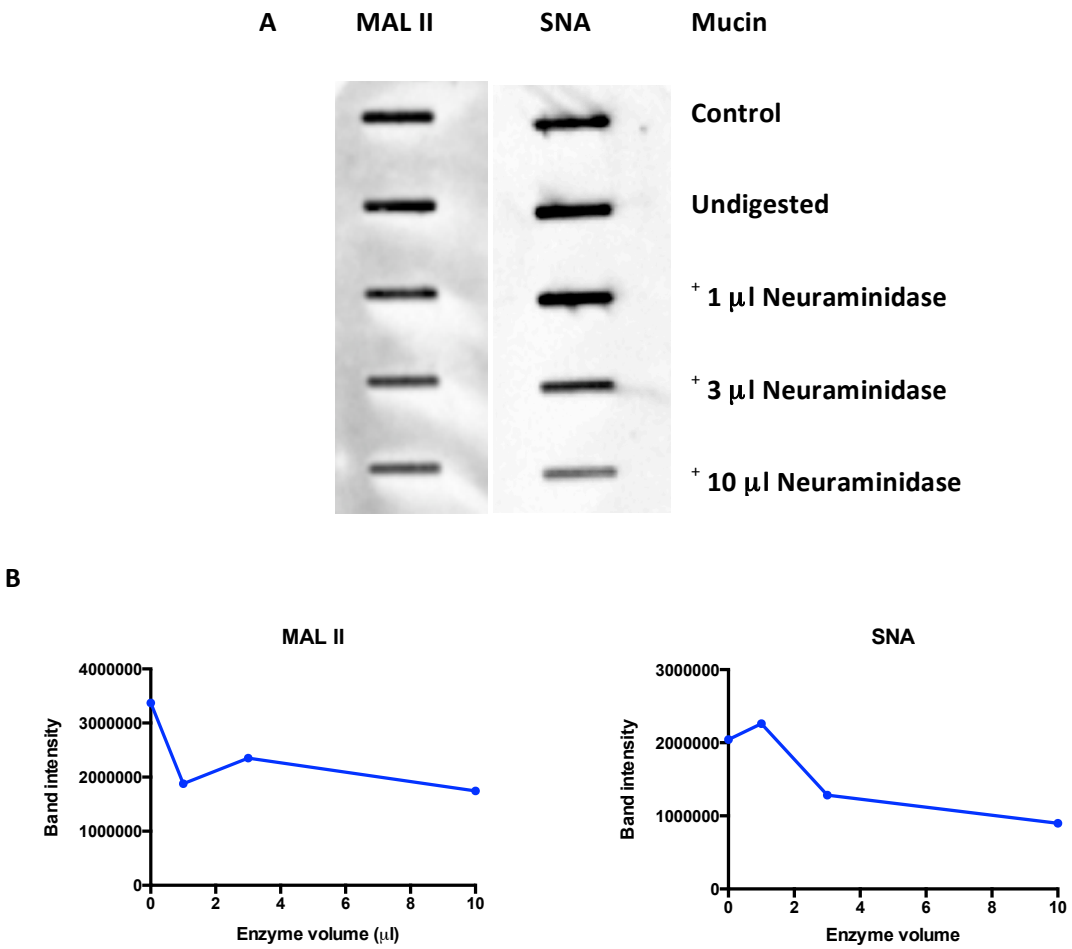

Figure 4.24 Sialic acid digestion from mucins can be verified with lectin staining.

Mucin digested overnight with different concentrations of neuraminidase (New England Biolabs) was loaded in a slot blot and stained with lectins MAL II and SNA (A). Band intensity was compared to undigested sample (B).

Additionally, to test whether this enzyme was effective to digest sialic acid, it was tested with a glycosylated protein control, fetuin, and run in an acrylamide gel and stained with Coomasie blue. Neuraminidase effectively modified the gel migration pattern of fetuin, which suggests that is effective removing sialic acid (Figure 4.26).

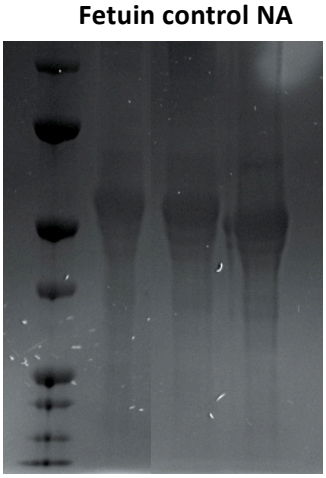

Figure 4.25 Efficacy of neuraminidase digesting fetuin.

The glycoprotein fetuin was digested for 3 hrs with neuraminidase (NA) (New England Biolabs), loaded in an acrylamide gel and stained with Coomassie blue. Fetuin control, incubated just with the respective enzyme buffer, was included. Different gel migration can be observed in the sample treated with neuraminidase.

Following sialic acid removal, moDCs were treated with neuraminidase-treated mucin, native mucin or native mucin treated in the same way as neuraminidase-treated mucin but without enzyme addition (called herein as control mucin). Supernatants were collected for IL-8 measurement by ELISA and cells were analysed by FACS. Initially, neuraminidase from Sigma was used for our assays but we found reduced IL8 production and DC activation in the control mucin, suggesting that the buffer affected the mucin ability to induce IL-8 production but not necessarily the enzyme. Thus, assays were performed using a neuraminidase from a different manufacturer (New England BioLabs, figures 4.24 and 4.25 show mucin treated with this enzyme), as both enzymes display similar effectiveness removing sialic acid from fetuin and mucin (data not shown).

Next, moDCs were treated with the NEB neuraminidase, native mucin, control mucin and neuraminidase-digested mucin overnight. Supernatants were collected for IL-8 measurement by ELISA and cells were analysed by FACS. Digested mucin still induced significantly increased IL-8 production on moDCs, and no significant decrease in IL-8 production was observed compared to moDCs treated with native mucin. No difference was observed in the presence of the mucin treated overnight in buffer only (Figure 4.26A). Digested mucin or control mucin control still induce a significant increase in the $CD83^+$ or $CD86^+$ moDC and no significant differences were found with respect to native mucin, suggesting that mucin was still able to induce DC activation (Figure 4.26B and C). These results suggest that sialic acid is not involved in the induction of IL-8 secretion and DC activation on moDCs.

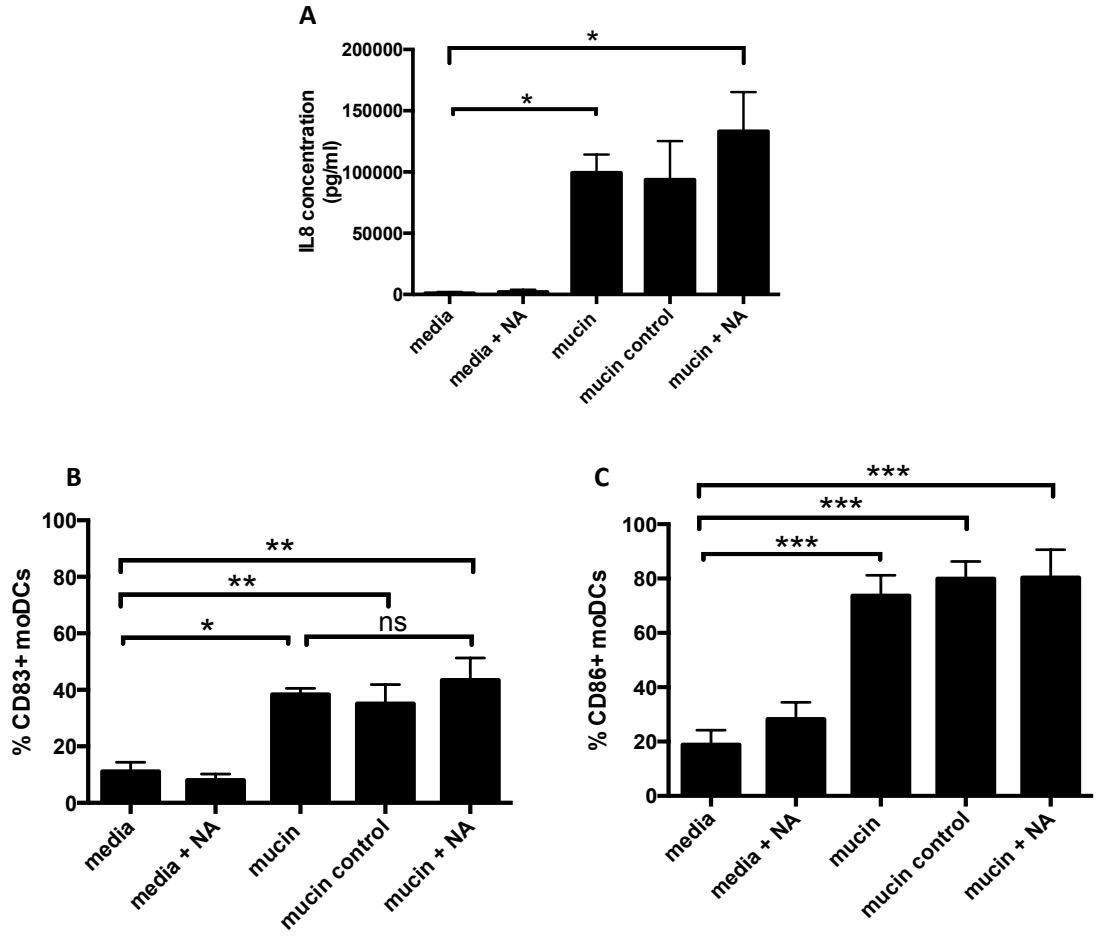

Figure 4.26 Neuraminidase-digested (NEB) mucin induces significantly higher IL-8 production and DC activation on moDCs.

IL-8 production and DC activation were evaluated on moDCs treated with mucin (50 μg/ml) digested with neuraminidase (NEB). A. IL-8 production was evaluated by ELISA on moDCs treated with intact large intestinal mucin, mucin control (treated overnight with enzyme buffer but not enzyme), neuraminidase-digested mucin (mucin $^{+}$ NA), neuraminidase (NA) and LPS. Results are from 4 independent experiments and statistical significance was assessed using the Kruskal Wallis test followed by Dunn's multiple comparison test. B and C. Percentages of $CD83^{+}$ (B) and $CD86^{+}$ (C) moDCs are shown and compared. Results are from 4 independent experiments and statistical significance was assessed using one-way ANOVA followed by Dunnet's multiple comparison test. *$p<0.05$, **$p<0.01$ and ***$p<0.001$

4.3 Discussion

4.3.1 Composition of mucin preparations

4.3.1.1 Composition of human secreted mucin preparations

Mucins were successfully purified by CsCl/GuCl density gradient centrifugation during this project but some details about the composition of different preparations should be considered. First of all, the cancer cell line LS174T used in this project does not secrete MUC2 exclusively. Thus, secretion of MUC2, MUC5AC, MUC5B and MUC6 by LS174T cells has been reported previously, consistent with tandem mass spectrometry data showed in this thesis (Bu et al., 2011, van Klinken et al., 1999, Van Seuningen et al., 2000). Compared to other colon cancer lines such as Caco-2 or LoVo, LS174T cells exhibit the highest level of MUC2 mRNA and low levels of MUC5AC mRNA, which support its use as a source of MUC2 (Bu et al., 2011).

Different secreted mucins (such as MUC2, MUC6 and MUC5AC) have similar molecular weights, due to their ability to form polymers, therefore potentially making it very difficult to separate the different mucins. This latter idea is supported by analysis of SEC-MALLS fractions from the LS174T cell-mucin preparation, where the presence of MUC2 and the gastric MUC5AC was detected in all the fractions (data not shown). Anion exchange chromatography, which allows protein separation with different electric charge, was performed in order to separate both mucins but separation was not feasible, suggesting similar net charge of both mucins (data not shown). However, relative abundance of MUC2 in the respective mucin preparations is supported by the number of unique peptides found by tandem mass spectrometry, as reported in the results section. Thus, our data together with previous reports (Bu et al. 2011) suggest that our preparation obtained from LS174T is enriched for MUC2.

Additional proteins with relatively high abundance in the samples, detected by mass spectrometry, were proteins such as keratin and agrin (data not shown). These proteins show a low molecular weight compared to glycoproteins (keratin = 54 kDa, agrin= 215 kDa) so they can potentially be separated by size exclusion chromatography if required. Agrin is a large heparan-sulphate proteoglycan located in basement membranes, which has been postulated as a tumour associated antigen based on proteomic studies on colorectal cancer cell lines, consistent with potential expression on cancer cell lines used in this project (Klein-Scory et al., 2010). On the other hand, the presence of keratin could correspond to contamination (e.g. from skin), acquired during the preparation of the samples for mass spectrometry, which is a highly sensitive technique.

4.3.1.2 Composition of murine intestinal preparations

Due to the mixed mucin composition of the human secreted mucin preparation and the altered glycosylation pattern displayed by mucins (Crocker et al., 2007), we purified mucin from mouse intestinal tissue, which may represent a closer representation of steady-state mucins. Murine small and large intestinal mucin preparations exhibited a high abundance of Muc2, which was the main mucin identified in both preparations. Low presence of other mucins was detected in both small and large intestinal preparations (low presence of Muc5ac and Muc6 in the former and presence of Muc13 in the latter), which suggest that our preparations are highly enriched in Muc2. Secreted mucins Muc5ac and Muc6 are usually found in the stomach and to a lesser extent in the large intestine (McGuckin et al., 2011) and their presence in our small intestinal preparation may be attributed to expression of these mucins near both tissues (most probably the stomach as our large intestinal preparation did not show any Muc5ac or Muc6). Muc13 is a cell surface mucin expressed along the gastrointestinal tract, which is consistent with its presence on our large intestinal preparations. Due to their low relative abundance in our preparations, it is unlikely that they interfere with our assays. Thus, our murine preparations were highly enriched in Muc2.

Moreover, the second most abundant component of our preparations was the glycoprotein IgG-Fc-binding protein (FcGBP). This glycoprotein contains mucin-like domains, rich in cysteine, and is covalently attached to intestinal mucin MUC2/Muc2 in mouse and human, although its function is unclear (Johansson et al., 2009). Our experiments do not exclude the possibility that this glycoprotein may mediate some of the effects reported in this chapter, as glycosylated domains of this protein may remain in the sample, after trypsin treatment. However, this glycoprotein is absent in our preparation from LS174T cells, suggesting that is not responsible for the changes observed in moDC.

4.3.2 Concentration of mucin preparations

An important consideration is the mucin concentration we have utilised in our DC assays. Concentration of mucin preparations purified from the cell line LS174T was relatively low, limiting the highest concentration that could be used for stimulation of DCs. For our assays, we decided that the maximum reasonable mucin volume that could be added should be around 10-20% of the total volume (8-25 μl of mucin for 125 μl), which determined the highest concentration used in every experiment. The range of mucin concentration previously reported in the literature was between 0.001 μg/ml and 1μg/ml, comparable to the

concentrations added in the present report (Ishida et al., 2008). Based on this paper, 1 μg/ml of mucin should be enough to induce biological effects. More recently, Shan et al. reported a concentration of 50 μg/ml mucin for *in vitro* assays involving different DC models. In our assays we were able to see biological effects even with 2 μg/ml but more robust and repeatable results were obtained with 50 μg/ml, which was chosen as the mucin concentration for most of the assays shown in this thesis. Due to volume and sample limitations, we only used a concentration of 10 μg/ml to treat moDCs, which supernatants were used for transmigrations assays. However, as we have shown, the amount of secreted IL-8 was able to efficiently attract neutrophil-like cells, which suggests that lower mucin concentrations may be able to induce important biological effects.

4.3.3 Potential LPS contamination of mucin preparations

Considering that the mucins preparations would be used to study modulation of DC function, a major concern was the presence of LPS in the samples, which could interfere with experiments, especially in the preparation from mouse intestine. Density gradient centrifugation and mucin purification is not performed in sterile conditions, and contamination with bacteria or other microorganisms may be a potential issue. The presence of LPS was especially a concern for the use of mucins purified from mouse intestine, which may contain co-purified bacterial components. In order to elucidate whether the pro-inflammatory effects of mucin samples on moDCs were due to LPS, we used a TLR4 inhibitor that binds intracellularly to the TLR4 receptor. This strategy was considered better than the use of polymyxin B, which binds to the lipid A region of the LPS and prevents LPS-related effects. We initially used this reagent for our assays but results were inconsistent, which may be attributed to potential interactions between polymyxin B and mucin or the inefficacy to block LPS that may be trapped into the mucin polymers. For our assays, we pre-incubated moDCs for 1h with the TLR-4-inhibitor before adding treatments with LPS or mucin, which allowed the inhibitor to enter into the cell and bind to its intracellular domain of TLR4. This time was sufficient to effectively prevent TLR-4-induced pro-inflammatory effects such as increased expression of DC activation markers (CD83 and CD86) and the chemokine IL-8. The TLR-4 inhibitor was able to effectively prevent LPS-induced effects, completely blocking effects of 10 ng/ml LPS. This concentration was at least ten times higher than the final endotoxin concentration contained in our mucin preparations, which was below 1 ng/ml (0.07 ng/ml in the SI mucin preparation and 0.9 ng/ml in the LI mucin preparation). Thus, this suggests that the TLR-4 inhibitor should have been able to prevent any endotoxin-mediated effect in our assays.

Moreover, we observed a higher endotoxin concentration on our large intestinal mucin preparations compared to our small intestinal mucin preparations, which is consistent with the increased bacterial load observed in the large intestine. Endotoxin concentration of both small intestinal and large intestinal preparations was consistent between independent preparations, with values between 0.3-3ng/ml for small intestinal preparations and 10-20ng/ml for large intestinal preparations. For the assays shown in this chapter, we used the preparations with the lowest endotoxin concentration achieved (0.3 ng/ml for SI mucin and 12 ng/ml for LI mucin), although experiments comparing these independent preparations suggest that all our preparations showed a similar potential to induce IL-8 expression and DC activation on moDCs, in a TLR-4 independent manner.

An alternative approach could be to attempt to remove LPS from mucin samples to demonstrate LPS-independent effects. Shan et al. used this strategy to deplete LPS from mucin purified from the small intestine, which they showed caused tolerogenic and anti-inflammatory effects on moDCs. To remove LPS, endotoxin removal columns can be used, although one of our concerns in the potential mucin loss in the process, as this highly polymeric molecule may stick to the column. This paper does not mention whether mucin concentration was measured after this protocol and whether the treatment with the column did not affect the sample composition (Shan et al 2013).

4.3.4 Use of monocyte-derived DC as a model of DC function

In vitro stimulation with IL-4 and GM-CSF is widely used as the standard protocol to differentiate monocytes into DC, as these cells show DC cell surface phenotype and morphology and are highly efficient antigen presenting cells (Sallusto and Lanzavecchia, 1994). This model is a useful first approach to study *in vitro* interactions as multiple conditions can be tested in the same experiments due to high cell yield, providing material for qPCR, flow cytometry analysis and analysis of secreted cytokines or chemokines, in contrast with limited DC numbers that can be obtained from the intestinal LP. It is has been proposed that monocytes migrate into inflamed tissues and can become inflammatory DCs although this is still controversial, as most of evidence in murine intestine suggest that all conventional DC subsets differentiate from pre-cDCs precursors but not monocytes, which give rise exclusively to intestinal macrophages (Varol et al., 2009; Bogunovic et al., 2009; Bain et al., 2014). Watchmaker et al. (2014) recently proposed that human CD103- SIRPα^+ DC have similar gene expression profiles to CD14$^+$ blood monocytes, which suggests that blood monocytes may give

rise to human CD103- SIRPα^+ DC. However, most evidence suggests that intestinal CD103$^+$ DCs are originated from pre-DC precursors (Watchmaker et al., 2014). Thus, although monocyte-derived DCs do not fully represent the DC subsets found in the steady state gut, which are mainly tolerogenic CD103$^+$ DC, they do serve as a useful model to determine the effects of mucins on human DCs.

4.3.5 Comparison with previous papers studying MUC2 immunodulation on DC

Two different papers from the same group have been published about the potential immunomodulatory role of MUC2 on human monocyte-derived DC (Ishida et al., 2008; Ohta et al., 2010). Both papers purified MUC2 from the cell line LS180, a colon cancer cell line similar to the LS174T cells used in this report, and incubated this mucin with monocyte-derived DC. Ishida et al. (2008) reported induction of apoptosis in the presence of MUC2 and monocyte differentiation into DC was induced by a shortened differentiation protocol, generating "FastDC" by treatment for 24 hrs with GM-CSF and IL-4, followed by stimulation with pro-inflammatory mediators (TNF-α, IL1-β, PGE2 and IFN-α) for additional 24 hrs (Dauer et al., 2003). These "FastDC" generated have shown similar characteristics to conventional monocyte-derived DC. When the effect of mucins on such cells was studied, mucins were added to the medium together with GM-CSF and IL-4 (in the first 24 hrs of differentiation) and reduced expression of the DC maturation marker, CD83, was observed together with increased apoptosis. We did not look specifically for apoptosis markers such as annexin-V but percentages of viable cells, measured by viability dye, were similar between control and mucin-treated DC. In contrast, we observe increased DC activation in the presence of mouse intestinal mucin, although as we treated cells with mucin at day 6 of monocyte differentiation, such methodological differences may explain in part the differences observed.

Moreover, Ohta et al. (2010) used a moDC differentiation protocol based on IL-4 and GM-CSF stimulation for 5-6 days, more similar to ours (Chen et al., 2004). This work reported downregulation of LPS-induced IL-12 production but no changes in IL-10 production, in the presence of MUC2. In contrast, our preliminary data from monocyte-derived DC supernantants analysed by cytometric bead array (CBA) suggests that IL-12 was not downregulated by mucins in presence of LPS (data not shown). On the other hand, this paper reported no changes in CD83 expression in the presence of MUC2, which is inconsistent with their previous work (Ishida et al., 2008), potentially due to different differentiation conditions used.

Our data is also contradictory to Shan et al. (2013), which was published during the course of this project. This work postulates that MUC2/Muc2 plays an important tolerogenic role in the gut, promoting anti-inflammatory effects on mouse and human DCs in the presence of pro-inflammatory stimuli and preventing DSS-induced colitis in Muc2-/- mice. We used a similar approach to study interactions between purified mucins and monocyte-derived DC, which were differentiated in similar conditions to Shan et al (2013). However, we were not able to replicate their results. The authors reported that both MUC2 form LS174T cells and mouse small intestinal mucin prevented inflammatory DC responses such as increased expression of DC activation markers and production of pro-inflammatory mediators, including IL-12, TNF-α, IL-6 and IL-8, in the presence of LPS, flagellin and the pro-inflammatory cytokine TNF-α. Additionally, enhanced production of the anti-inflammatory cytokines IL-10 and TGF-β and increased RALDH activity were reported in the presence of mucin and LPS in human myeloid $CD1c^+$ DCs. In our results, we have shown that both enriched human MUC2 preparations and intestinal mucins from both small and large intestine can promote pro-inflammatory features in monocyte-derived DC such as IL-8 production and DC activation. These effects were not prevented in the presence of combined LPS and mucin treatments and we did not find increased IL-10 production on moDCs in our assays (data not shown). Furthermore, our group has found that LPS can induce the expression of the integrin $\alpha v\beta 8$ on moDCs (Fenton et al, unpublished). However, we did not observe up-regulation of this integrin in the presence of mucins, which suggests that our LPS contamination is not high enough to induce $\alpha v\beta 8$ expression and/or mucin does not induce this integrin. Preliminary data suggests that our mucin preparations do not induce IL-6 (CBA data from assays with MUC2) and IL-12 (was not expressed in detectable levels), but we have not explored in detail other pro-inflammatory markers (Cerovic et al., 2013).

These discrepancies could potentially be due to the different methods to purify intestinal mucin. As discussed above, we have not specifically removed LPS from our samples; however, our mucin preparations were performed via two consecutive density gradient centrifugations, which should ensure separation from other proteins and macromolecules, and also TLR4 inhibitors did not prevent actions of the mucin. Shan et al. did perform repeated high-speed centrifugations in GuHCl, keeping both pellets and supernatants, which have been described before to separate soluble and insoluble mucins (Axelsson et al., 1998). However, Axelsson et al (1998) also included further density gradient centrifugations that were not performed by Shan et al. and thus these samples should contain more contaminants than our preparations.

We have performed preliminary mucin preparations from mouse intestine, following Shan's protocol, and mass spectrometry suggest that these samples are less pure and less enriched in Muc2 than our samples (data not shown). Additionally, no molecular characterisation is mentioned by Shan et al., and it is not clear how the authors characterised their mucin preparations. It is mentioned in their methods that they performed Western blots to confirm MUC2 presence and they ruled out the presence of other mucins. However, as we have discussed before, mass spectrometry is required to confirm the sample composition and SEC-MALLS is the most appropriated technique to quantify mucin. Moreover, Shan et al. only used reduced mucin to demonstrate Muc2 effects. We have used native polymeric mucin (not treated with DTT) for our assays but also we have reduced mucin and digested it with trypsin (for the preparation of mucin glycopeptides) and pro-inflammatory effects are still observed on moDCs. Mucin prepared following Shan et al. protocol (but not reduced) induced similar effects to our mucin preparation and thus it is unclear for us the reason of the discrepancies observed. Moreover, Shan et al. reported additional evidence supporting a tolerogenic role of mucin in gut homeostasis in the mouse model Muc2-/-, which will be discussed in the next section.

4.3.6 Genes potentially modulated by MUC2 on DC.

Preliminary PCR array results support the idea of potential modulation of gene expression by MUC2 on human monocyte-derived DC. In addition, changes in gene expression suggest both pro-inflammatory and anti-inflammatory effects on gene expression. Validation of this data was crucial to determine further experiments. Changes in gene expression observed in human monocyte-derived DC also suggested a dual effect induced by MUC2. Two genes downregulated by MUC2 treatment, *FLT3LG* and *TAPBP*, encode Flt3 ligand, an important growth factor for DCs, and tapasin (TAP binding protein), a chaperone protein involved in antigen processing and presentation, respectively. On the other hand, genes upregulated by MUC2 treatment correspond to some receptors expressed by different subsets of DC such as CD1b (associated with migratory lymph DC), CD8α (associated with antigen cross-presenting DC), and CXCR1 (which binds IL-8, also upregulated in this experiment) (Segura and Villadangos, 2009). Interestingly, both IL-8 and CXCR1 has been associated with attraction of DC into tumours and intratumoral retention, impairing DC migration and potentially weakening anti-tumour activity, although IL-8 produced by DC would be able to attract neutrophils (Alfaro et al., 2011).

Additionally, CD80 was also up- regulated, suggesting that MUC2 may induce DC activation, which was further demonstrated with mouse mucins, although up-regulation of CD80 was not as consistent as the up-regulation of CD86 or CD83. Other upregulated genes were *ITGAM*, *ICAM-1* and *ICAM-2*. *ITGAM* encodes the integrin αM subunit, also known as complement receptor 3 or CD11b, which has been used to identify lymphoid organ DC subsets, whereas *ICAM-1* and *ICAM-2* are also membrane receptors with roles in adhesion and leukocyte migration. We have not further validated some of these genes and thus it may be important to consider its validation for further assays.

4.3.7 Induction of IL8 expression by moDCs

When validating genes modulated in the PCR array, neither CXCR1 (the most upregulated gene) nor TAPBP (the most downregulated gene) showed significant differences. On the other hand, IL-8 was successfully validated and measurements of secreted IL-8 by ELISA were also significantly upregulated on MUC2-treated cells. IL-8 is a potent chemotactic factor expressed by different immune cells, including monocytes and macrophages, which can recruit cells expressing the chemokine receptors CXCR1 and CXCR2 such as neutrophils, T cells and monocytes/macrophages. IL-8 is lowly expressed during steady-state conditions, but can be highly induced by pro-inflammatory agents, such as TNF-α, IL-1, IFN-γ and LPS, and stress factors such as reactive oxygen species (Brat et al., 2005). Intriguingly, IL-8 is not only associated with inflammatory effects but also correlates with increased tumour progression (Brat et al., 2005).

TLR stimulation via the adaptor protein MyD88 can activate NF-κb and MAPK signalling pathways, which also can induce IL-8 expression. Different bacterial PAMPs can bind to TLRs including LPS to TLR4, bacterial cell wall components (e.g. peptidoglycan) to TLR2 and CpG-containing DNA to TLR9 (Takeda and Akira, 2004). Thus, the main concern for our studies was that bacterial contaminants but not mucin induced IL-8 production. As discussed earlier, we performed our experiments in the presence of a TLR4 inhibitor in order to rule out the possibility that contaminant LPS was inducing IL-8 or DC activation. Additionally, we removed DNA present in the sample, which may come either from epithelial cells or bacteria, and this did not affect IL-8 production or DC activation. We cannot exclude the possibility that other bacterial contaminants may induce the effect observed in this chapter. Some preliminary experiments were performed with a MyD88 inhibitor (NBP2-29328, Novus biologicals) but unfortunately neither our positive control (LPS) nor our samples gave consistent results, which

maybe partially attributed to the fact that the inhibitor induced cell death in our assays (data not shown). Thus, an important experiment to confirm that our effects are due to the mucin are not due to LPS is the use of a TLR2/4 inhibitor such as OxPAPC, which binds to the accessory proteins CD14, LBP and MD2. However, our human mucin preparation, that is not containing bacterial products, has the same effect inducing IL-8 expression and thus we do not think this is the case.

4.3.8 Induction of IL-8 by mucin glycans

Importantly, we have shown here that mucin glycans may be involved in the induction of IL-8 and DC activation. First, we treated our DNA-free mucin preparations with trypsin, which should degrade most of contaminant proteins and also degrade unglycosylated mucin regions (Cys domains). Our results suggest that mucin glycopeptides can induce IL-8 production and DC activation. However, small intestinal glycopeptides showed a lower ability compared to large intestinal glycopeptides to induce these effects, which may be attributed to the loss of either contaminant proteins or unglycosylated mucin regions. Both small and large intestinal glycopeptides were able to induce IL-8 and DC activation in the presence of the TLR-4 inhibitor (data not shown), which again suggests that effects seen are TLR4-independent.

We also treated mucin preparations with sodium metaperiodate, which effectively oxidises the glycans on the mucin, as shown by Schiff's staining. Initially, we tested mucin oxidised with sodium metaperiodate in our assays without using Vivaspin filters to remove remaining sodium metaperiodate and/or potential toxic products released in the oxidation reaction. However, such products appeared to induce cell death in our assays. Thus, we opted to exchange the buffer in a Vivaspin filter, removing toxic products. Oxidised and washed mucin induced some IL-8 induction but was not significant compared with the untreated moDCs. Additionally, CD83 expression was not significantly stimulated by the oxidised mucin. The remaining pro-inflammatory potential may be attributed to some contaminant LPS, remaining in the sample but assays in the presence of the TLR4 inhibitor should be performed to confirm this idea. The oxidative effect of sodium metaperiodate should only affect glycans that display vicinal diols in their chemical structure (Hong and Kim, 2000). TLR4 recognises the lipid A region of LPS, which does not contain vicinal diols and should not be affected by sodium metaperiodate. We did not observe a significant difference comparing treatments with oxidised and native mucin, although there is a trend suggesting a decreased pro-inflammatory effect in 3 out of 4 assays. Therefore, more assays are required to confirm the idea that our

oxidised mucin lost its pro-inflammatory ability and thus glycans are essential in the induction of IL-8 production.

The use of sodium metaperiodate to validate the effect of mucin glycans has also been reported by Cobo et al. (2015), who showed that treatment of MUC2 with sodium metaperiodate abrogated mucin-induced β-defensin 2 production by epithelial cells. This paper also tested whether digestion with neuraminidase and mannosidase affected β-defensin 2 production but, similar to our results, they did not observe significant differences compared to native mucin. Similarly, we did not observe a reduced ability of mucins to induce IL-8 secretion in the presence of neuraminidase-treated mucin. This finding suggests that the pro-inflammatory effects reported in this chapter are not due to sialic acid moieties present on mucins. In concordance, a free synthetic sialic acid did not induce significant induction of IL-8 production or DC (data not shown). Additionally, sialic acid did not reduce LPS stimulation of IL-8 production or DC activation, as previously shown by Ohta *et al*., 2010, which suggest that this glycan does not have immunomodulatory effects (at least on moDC). Thus, other glycans but not sialic acid may be involved in mucin-induced effects on monocyte-derived DC. An important experiment to elucidate which glycan(s) is (are) responsible for these effects, is the enzymatic removal of galactose and mannose and/or the treatment with a combination of different glycosidases.

4.3.9 A functional role of mucin-induced IL-8

Moreover, we explored whether moDC-secreted IL-8 was able to play any functional roles. As IL-8 is an important chemoattractant, we tested the ability of moDCs supernatants to recruit neutrophils, which express the chemokine receptor CXCR1. To test this possibility, we used a the well characterised cell-line HL-60, which has been described in the literature a source of different myeloid cells under different differentiation conditions. Neutrophils can be obtained under differentiation with 1.5% DMSO for 5-7 days, which induces cell cycle arrest and granulocyte differentiation (Jacob et al., 2002, Fleck et al., 2005). These cells showed upregulation of CD11b compared to untreated cells and showed chemotaxis when incubated with control media containing IL-8 (10ng/ml to 100 ng/ml). We additionally used the endothelial cell EA.hy 926 to simulate an endothelial layer in the transwell insert. Importantly, we used serum-free media (X-vivo15) for treating moDCs in order to prevent serum-dependent transmigration. Neutrophil-like HL-60 cells were washed and resuspended in serum-free media as well for the transmigration assay.

We observed in our assays that supernatants from mucin-treated moDCs were able to recruit these neutrophil-like cells and this effect was IL-8 dependent. As LPS may be partially responsible for inducing IL-8 and thus transmigration in our assays, we also used supernatants from moDC treated with mucin in the presence of the TLR4 inhibitor. These supernatants also induced significant neutrophil-like cell transmigration in our assays (data not shown), which suggests that mucin-induced IL-8 may induce neutrophil recruitment. Importantly, it will be important to repeat these assays using neutrophils from human donors in order to show that neutrophils from healthy donors are able to respond to moDC-secreted IL-8. As neutrophils are important mediators of early inflammation in the intestine, and are rapidly increased during inflammation, mucins might play a role recruiting neutrophils to the intestinal mucosa, when barrier integrity is compromised and invaded by enteric pathogens. Taken together, our results suggest that DC may recognise mucins as danger signals, which induce up-regulation of DC activation markers and secretion of IL-8, which could recruit pro-inflammatory mediators to the intestine. As intestinal DCs exhibit different and unique properties, the next chapter will explore the interactions between mouse intestinal DCs and the intestinal mucin Muc2.

5 CHAPTER 5 Regulation of mouse intestinal DC function by mouse intestinal mucin

5.1 Introduction

Intestinal mucin may play a tolerogenic role in intestinal immunity by promoting the induction of anti-inflammatory cytokines and preventing the induction of pro-inflammatory cytokines in the presence of bacterial endotoxin (LPS) (Shan *et al*., 2013). However, our data suggests that mucin Muc2 may induce pro-inflammatory features, such as induction of the chemokine IL-8 and increased expression of DC activation markers (as shown in Chapter 4), in human monocyte-derived DC and thus play important roles in the induction of host defence. Human monocyte-derived DC exhibit more pro-inflammatory features compared to tolerogenic intestinal DC, which are hyporesponsive to TLR4 stimulation. Thus, intestinal DCs could potentially show different responses to mucins.

As human intestinal DCs are difficult to get in quantities required for functional assays, we turned to the mouse system to explore how intestinal DCs interact with intestinal mucin. To determine the potential role of Muc2 in regulating intestinal DCs, we analysed intestinal DCs subsets from Muc2-/- mice and investigated whether they exhibit phenotypic differences compared to wild-type mice. Additionally, we characterised whether macrophages, which are also important antigen-presenting cells in the gut, exhibit phenotypic alterations. On the other hand, to investigate whether intestinal mucin Muc2 may induce different functional effects on intestinal DCs, such as pro-inflammatory responses and/or the induction of protective anti-microbial responses, we explored whether purified mouse intestinal mucin Muc2 induces changes in gene expression of mouse MLN DC utilising a microarray analysis. Therefore, the aims of this chapter are to determine: whether Muc2-/- mice exhibit phenotypic changes in intestinal DC subsets, that may be related with regulation of DC homeostasis by Muc2, and explore whether purified intestinal mucin induces global changes in gene expression of mouse MLN DCs, related with modulation of DC function.

5.2 Results

5.2.1 Characterisation of intestinal DC subsets on Muc2-/- mice.

To test the idea that Muc2 may regulate intestinal DC phenotype (and potentially DC function), we explored whether normal proportions of DC subsets were altered in the small intestine and colon of Muc2-/- mice. It has been reported that inflammation in Muc2-/- mice starts between 6 and 10 weeks, which depends on the environmental conditions. For our studies, mice were housed in a specific pathogen-free environment and did not shown visible signs of colitis at 6-7 weeks old (data not shown). LP cells from small intestine and colon were isolated and analysed by flow cytometry. Live $CD45^+$ cells were gated for all the different panels shown in this section (as shown in figure 5.1). Thus, further analyses were performed to characterize different immune cells including mononuclear phagocytes (DCs and macrophages) to assess changes in their normal phenotypes.

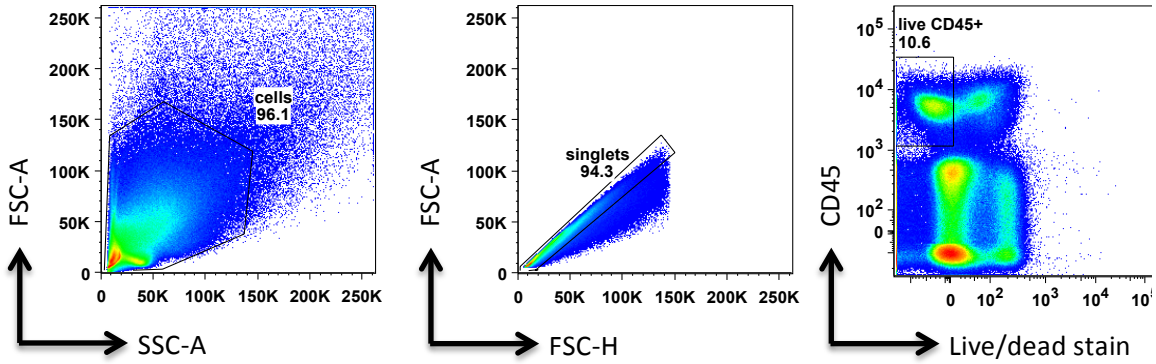

Figure 5.1 Gating strategy for intestinal cells isolated from the LP.

Cells isolated from the small intestinal or colonic LP were analysed by flow cytometry. Typically, total cells and singlets were gated, and then live (negative staining for live dead staining) and $CD45^+$ cells were selected for further analysis.

Mouse intestinal DCs present in the LP express MHC II and CD11c and can be distinguished from other mononuclear phagocytes by the lack of expression of the F4/80 and CD64 (Persson et al., 2013). Thus, we classify MHC II$^+$ CD11c$^+$ F4/80- cells as DCs and MHC II$^+$ CD11c$^+$ F4/80$^+$ cells as macrophages (Figure 5.2). We further analysed DC subsets via CD103 and CD11b expression, excluding B220$^+$ plasmacytoid DCs.

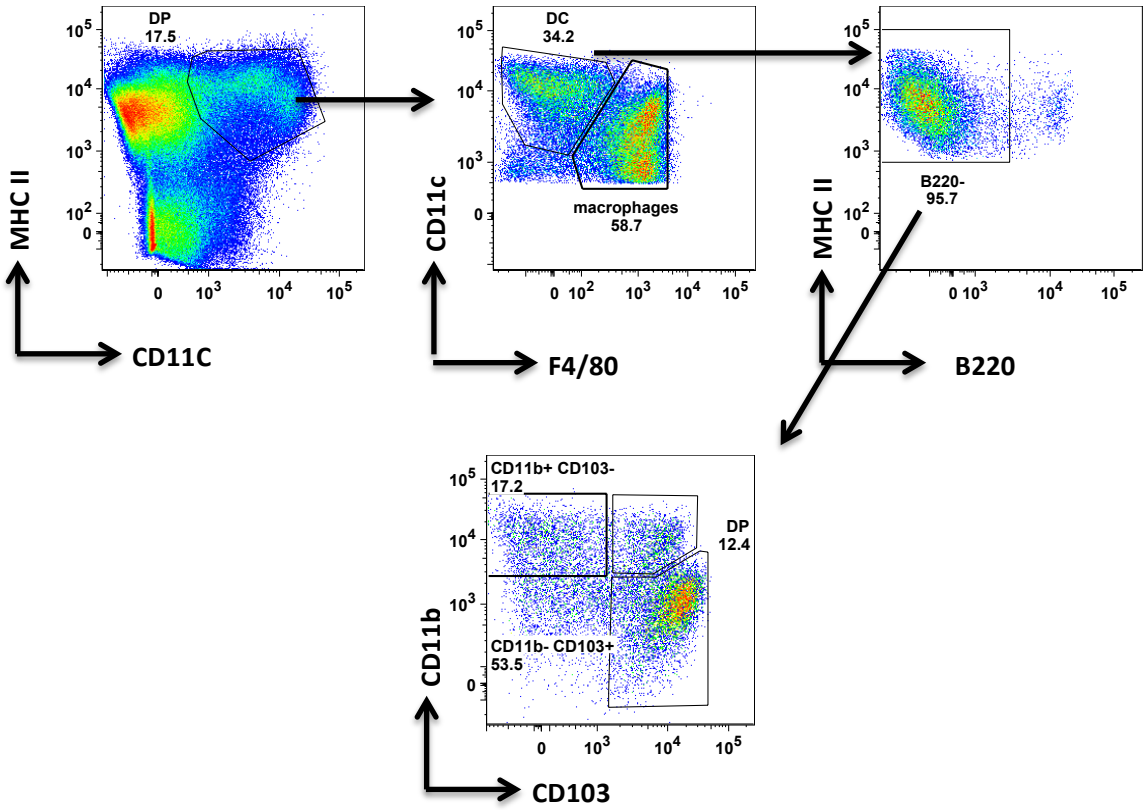

Figure 5.2 Gating strategy for LP colonic DC and macrophages.

Live CD45$^+$ cells co-expressing MHC II and CD11c were gated and then DC were discriminated from macrophages based on F4/80 expression. B220$^+$ cells (plasmacytoid DCs) were excluded and DC subsets were identified based on CD11b and CD103 expression and classified on CD103$^+$ CD11b$^-$, CD103$^+$ CD11b$^-$ and CD103$^-$ CD11b$^+$. Double negative CD103$^-$ CD11b$^-$ DCs, which constitute a minor population derived from intestinal lymphoid tissue, were not included in this analysis, as they are less likely to be in contact with the mucus barrier.

We observed that 6-7 week-old Muc2 -/- display an altered proportion of DCs (F4/80$^-$) and macrophages (F4/80$^+$), exhibiting a lower proportion of DCs compared to wild type mice in the colon (Figure 5.3A and B) but not the small intestine (data not shown). However, numbers of colonic DC and macrophages in Muc2-/- mice were not significantly different compared to control mice (Figure 3C). This decreased proportion of DCs was also observed in the colon at 10-12 week-old (data not shown). This result suggests that altered proportions of DC and macrophages are present in the colonic mucosa of Muc2-/- mice.

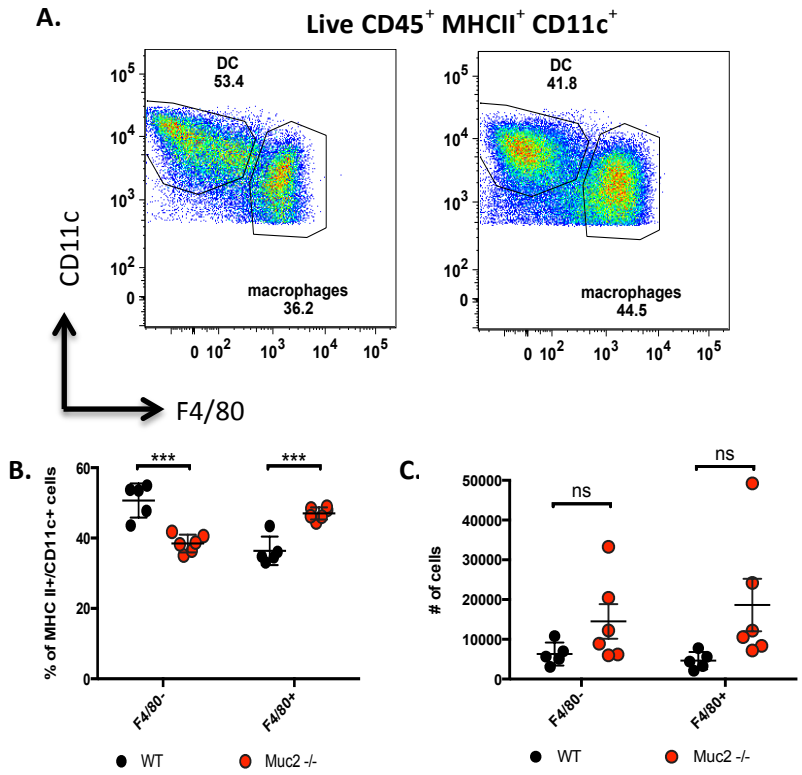

Figure 5.3 Altered proportions of macrophages and dendritic cells are observed in Muc2 -/- mice.

Colonic LP cells were isolated from WT and Muc2-/- mice at 6-7 weeks of age and analysed by flow cytometry. CD45 live cells expressing MHC II and CD11c were gated as shown in Figure 5.2. Thus, macrophages (F4/80$^+$) and DCs (F4/80$^-$) were distinguished based on the expression of F4/80. Representative plots are shown in A, percentages of F4/80$^-$ and F4/80$^+$ cells are shown in B and absolute numbers of F4/80- and F4/80+ cells are shown in C. Results are from 3 independent experiments (WT, n=5; Muc2-/-, n=6) and statistical significance was assessed using unpaired t test. ***p<0.001

Further, we analysed intestinal DC subsets on 6-7 week-old Muc2-/- mice. 6-7 weeks old Muc2 -/- mice display normal percentages and numbers of colonic DC subsets, the $CD103^+$ $CD11b^-$ subset being the most abundant subset (Figure 5.4A-C). These mice also display normal percentages of small intestinal DC subsets at 6-7 week-old (data not shown). These results suggest that DC subsets are not altered in mice lacking a normal mucus barrier.

As changes in DC subsets/function may occur during inflammation, we analysed whether older Muc2-/- mice exhibited altered proportions on intestinal DC subsets. 10-12 weeks old Muc2 -/-, with visible signs of colitis, displayed a significant decrease in the frequency of $CD103^+$ $CD11b^-$ DC and increase in the frequency of the $CD103^-$ $CD11b^+$ DC subset (Figure 5.5A and B). Numbers of $CD103^-$ $CD11b^+$ and $CD103^+$ $CD11b^+$ DC were also increased in Muc2-/- mice but numbers of $CD103^+$ $CD11b^-$ DC were not significantly different (Figure 5.5C). No difference was observed in small intestinal DC subsets of 10-12 week-old Muc2-/- mice (data not shown), suggesting that this difference is restricted to the inflamed tissue. These findings suggest that DC subsets are not altered from the early onset of inflammation and alterations in colonic DC subsets percentages occur later in the colitis progression.

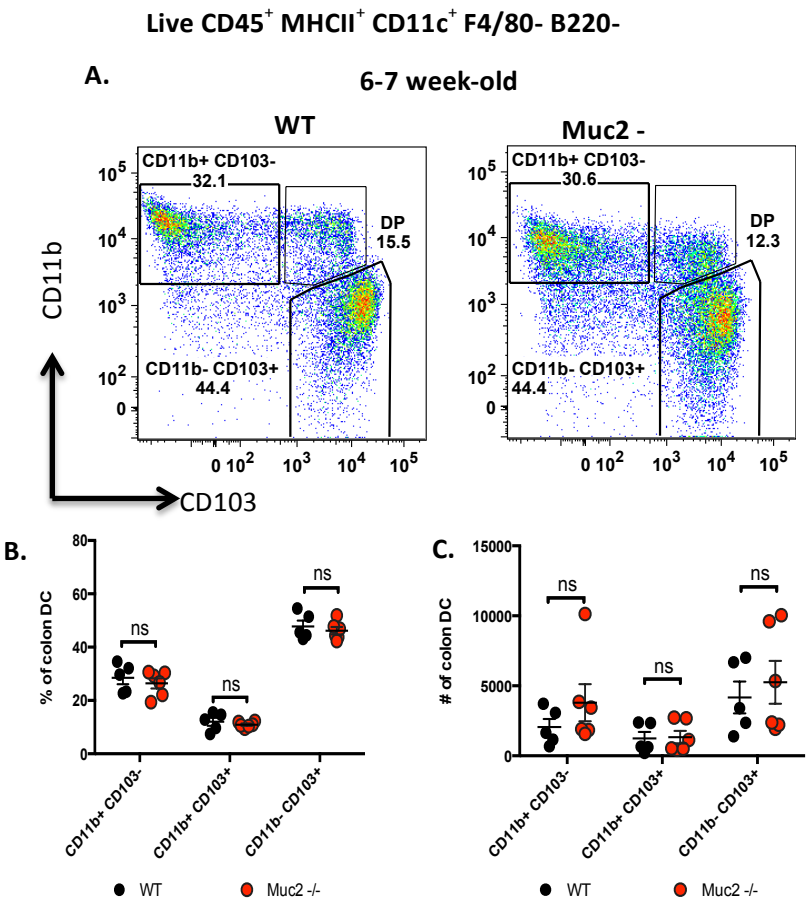

Figure 5.4 Colonic LP DC subsets are not altered in Muc2 -/- mice during early inflammation. Colonic LP DCs were isolated from Muc2-/- mice during early inflammation (6-7 week-old). Colonic LP DCs (live CD45$^+$ MHCII$^+$ CD11c$^+$ F4/80$^-$ B220$^-$) were gated on different subsets based on the expression of CD11b$^+$ and CD103 (CD103$^-$ CD11b$^+$, CD103$^+$ CD11b$^+$ and CD103$^+$ CD11b$^-$) as shown in Figure 2. Representative plots are shown in A, percentages of DC subsets are shown in B and absolute numbers of DC subsets are shown in C. Results are from 3 independent experiments (WT, n=5; Muc2-/-, n=6) and statistical analysis between WT and Muc2-/- mice was performed using unpaired t test.

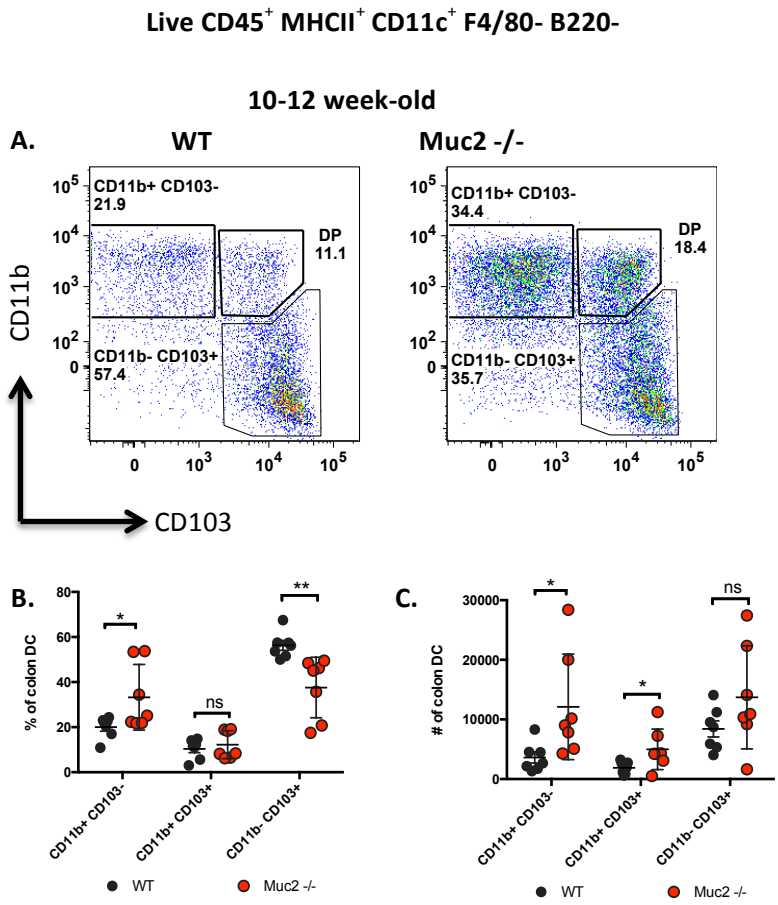

Figure 5.5 Colonic LP DC subsets are altered in Muc2 -/- mice during inflammation.

Colonic LP DCs were isolated from Muc2-/- mice during colitis (10-12 week-old). Colonic LP DCs (live $CD45^+$ $MHCII^+$ $CD11c^+$ $F4/80^-$ $B220^-$) were gated on different subsets based on the expression of $CD11b^+$ and CD103 ($CD103^-$ $CD11b^+$, $CD103^+$ $CD11b^+$ and $CD103^+$ $CD11b^-$) as shown in Figure 2. Representative plots are shown in A and percentages of DC subsets are shown in B. Results are from 3 independent experiments (WT, n=5; Muc2-/-, n=6) and statistical analysis between WT and Muc2-/- mice was performed using unpaired t test. Representative plots are shown in C and percentages of DC subsets are shown in D. Results are from 3 independent experiments (WT, n=6; Muc2-/-, n=7) and statistical significance was assessed using an unpaired t test. *p<0.05 and **p<0.01

5.2.2 Characterisation of the monocyte/macrophage axis in Muc2-/- mice

As no differences were observed in DC subsets in Muc2-/- mice, which did not show evident signs of colitis, we explored whether other innate immune cells showed any differences at this age. There is evidence that CX3CR1^{+} macrophages may extend transepithelial dendrites, sampling luminal antigens in close contact with the mucus barrier. Additionally, mature monocytes, originating in the bone marrow, circulate in the blood and migrate into the gut, replenishing the resident macrophage population. Increased extravasation and accumulation of Ly6C^{hi} monocytes in the intestinal mucosa is observed during sterile inflammation and intestinal infection (Bain et al., 2014). As we shown in Chapter 4, monocyte-derived DCs exhibit increased IL-8 production and increased expression of activation markers in the presence of intestinal mucins. Thus, it is likely that the monocyte-macrophage axis could be altered in the LP of Muc2-/- mice. For our analysis, live CD45^{+} CD11b^{+} cells were gated, excluding Ly6G^{+} and SSC high cells, and then Ly6C and MHCII expression analysed in 6-7-week-old Muc2 -/- mice (Figure 5.6).

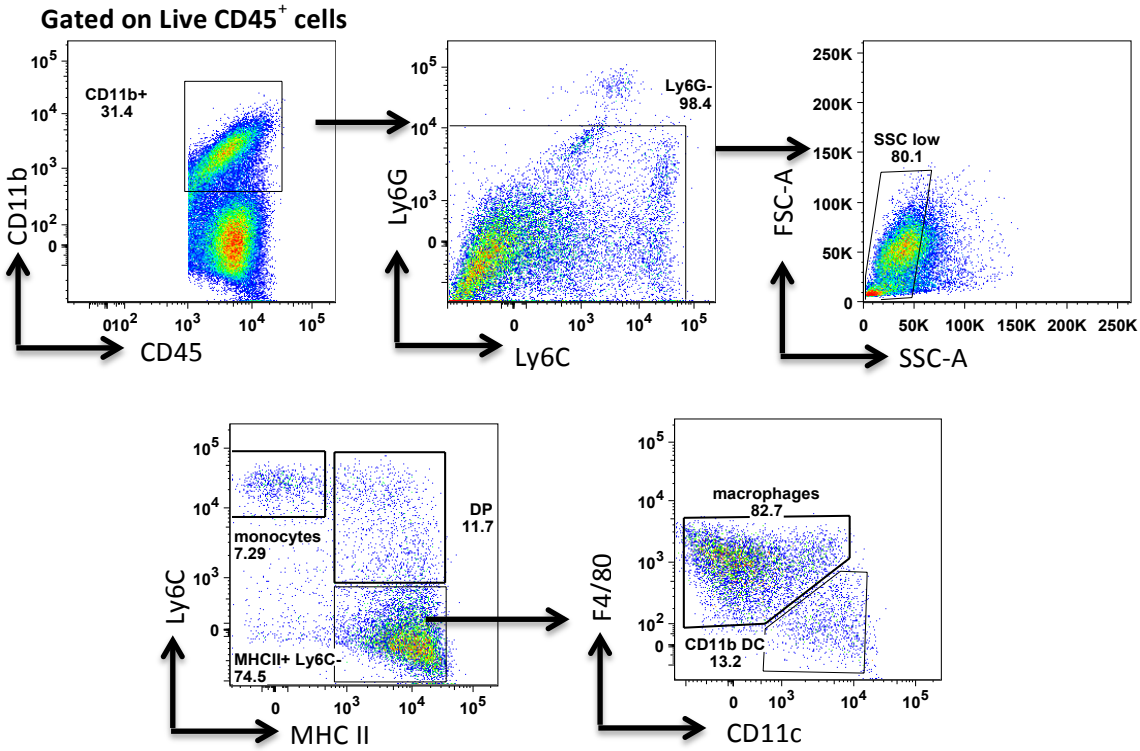

Figure 5.6 Gating strategy to identify Ly6C^{hi} monocytes and macrophages in intestinal LP.

Identification of Ly6C^{hi} monocytes and macrophages was based on expression of CD11b, excluding Ly6G and SSC^{high} cells, and then analysing Ly6C and MHCII expression. Cells were classified as monocytes (MHCII- Ly6C^{hi}), double positive intermediates (DP, MHCII^{+} Ly6C^{+}) and MHCII^{+} Ly6C^{-} cells, which were further analysed based on the expression of F4/80 (macrophages) and CD11c (CD11b^{+} dendritic cells).

6-7 week-old Muc2-/- mice showed a significantly increased proportion of Ly6C^{hi} monocytes compared to wild type mice and a significant reduction in the proportion of the MHCII^{+} Ly6C^{-} population (Figure 5.7A and B). The latter population was typically composed of macrophages (F4/80^{+}) and to a lesser extent by CD11b^{+} DC (F4/80^{-}), which did not show significant differences between Muc2-/- and WT mice (Figure 5.7A and B). At the cell number lever, all different populations anlysed were slightly increased but only double positive MHCII+ Ly6C+ cells were significantly increased (Figure 5.7C). Numbers of double pos No changes in monocyte infiltration were observed in the small intestinal LP in the Muc2-/- mice at 6-7 weeks old mice (data not shown). Older and colitic Muc2-/- mice display a similar phenotype (data

not shown). These findings suggest that there is monocyte infiltration in the colonic mucosa but not the small intestine of Muc2-/- mice but we cannot rule out that this difference is due to early inflammation.

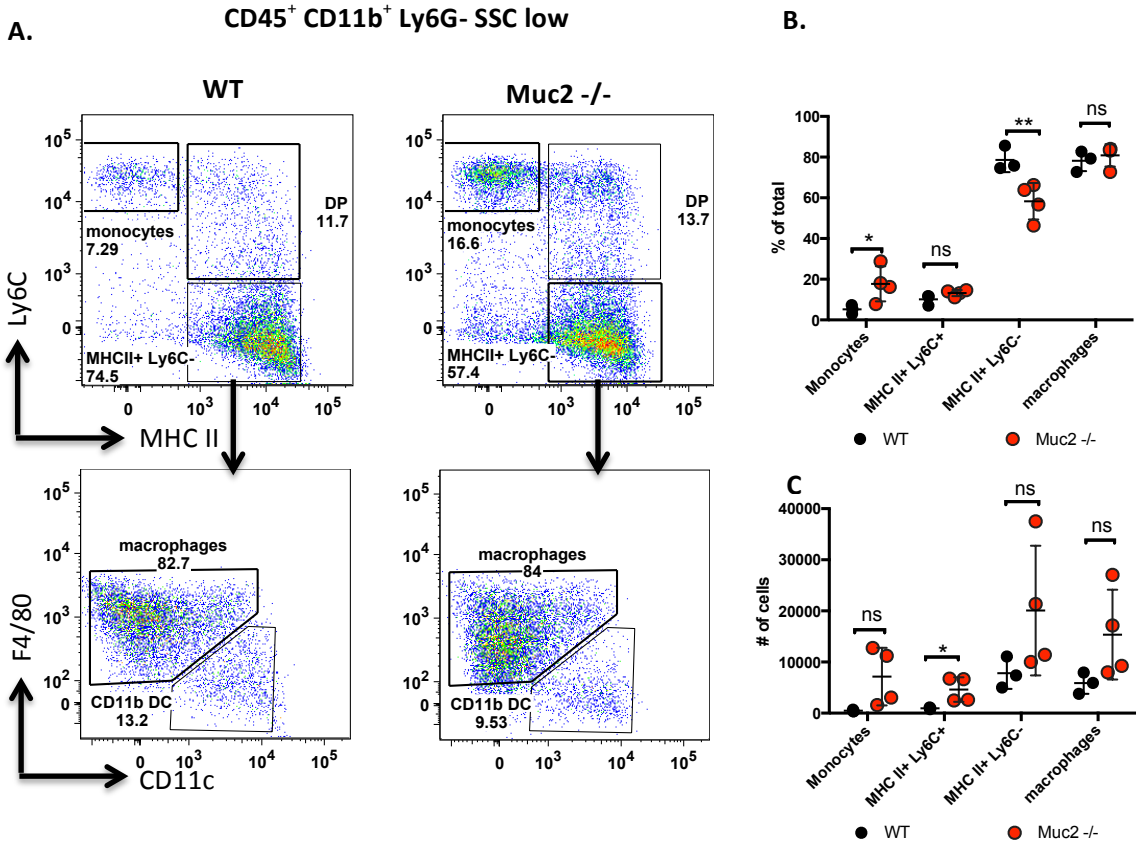

Figure 5.7 Colonic LP monocytes are increased in Muc2 -/- mice at early inflammation.

Live CD45$^+$ CD11b$^+$ Ly6G$^-$ SSC^{low} colonic LP cells were gated and analysed based on the expression of MHCII and Ly6C, as explained above in Figure 5.5. A-B. Representative plots are shown in A, percentages of MHC II/Ly6C subsets and macrophages are shown in B and absolute numbers are shown in C. Results are from 2 independent experiments (WT, n=3; Muc2-/-, n=4) and statistical significance was assessed using unpaired t test. *p<0.05 and **p<0.01

5.2.3 Comparative gene expression analysis of mouse MLN DC treated with intestinal mucin

Although our data suggest that lack of Muc2 does not alter the normal phenotype of intestinal DCs, our data suggest that intestinal mucin may modulate gene expression on human MoDCs, inducing some pro-inflammatory features such as increased production of the chemokine IL-8 and increased expression of DC activation markers. Therefore, to investigate how intestinal DCs respond to intestinal mucins and which signalling pathways could be modulated by mucins, we decided to evaluate global changes in gene expression of mouse intestinal DCs. To address this aim, DCs were purified from mouse MLN, which should contain migratory DC subsets from either small or large intestine together with resident DC populations. Intestinal macrophages lack migratory properties (Cerovic et al., 2013) and, therefore, $CD11c^+$ cells residing in the MLN should be mostly DCs. $CD11c^+$ cells were MACS-sorted and further enriched by flow cytometry sorting using anti-mouse CD11c and MHC II antibodies and a live/dead stain (7AAD). To perform the microarray we needed a concentration of 1 μg RNA and as cell yield was limited to 100,000 to 200,000 cells per mouse, mice were pooled to obtain sufficient number of cells and the required RNA. Cell purity was assessed after the sort, and > or equal to 90% of the live cells corresponded to double positive CD11c and MHC II cells (data not shown), suggesting a successful enrichment of this population, with relatively low cell death.

Sorted cells were incubated overnight in the absence or presence of mouse large intestinal mucin, using a concentration of 50 μg/ml, which has been previously shown significant effects on human moDCs (Chapter 4), and RNA isolated for microarray gene expression analysis. One of the main restrictions in order to perform the microarray was the low concentration and poor quality of the RNA extracted after overnight incubation of DCs. Typically, a total amount of 400-800ng was obtained from 1 sample containing 600,000 to 1.5 million DC and for an ideal microarray a total amount of 1 μg is required. To improve the amount of RNA, the Qiagen RNA micro kit was used, which has been designed for lower cell numbers. For the microarray, 1.5 million DC were used per treatment and after RNA extraction, 686 ng were recovered from the untreated control, whereas 1.35 μg were recovered from the mucin-treated sample.

Next, a microarray was performed using the RNA samples described above, from untreated mLN DC and mucin-treated mLN DC. This assay was performed once in order to identify potential hits to follow up and validate. Data analysis was performed by the genomic facility at the University of Manchester. The data was normalized using the Robust Multi-array Average (RMA) approach, obtaining fold-change values for the whole mouse genome. When compared to untreated cell, mucin induced mainly downregulation of genes, with 1393 genes with a fold-change more than -2, whereas 167 genes were upregulated more than 2 fold-change**.** Tables 5.1 and 5.2 show the 10 most downregulated and upregulated genes, according to Ingenuity Pathways Analysis (IPA).

Gene Title	Gene Symbol	Fold-change
platelet factor 4	Pf4	**-15.3809**
selenoprotein P, plasma, 1	Sepp1	**-7.87671**
CD200 receptor 4	Cd200r4	**-7.87002**
lipoprotein lipase	Lpl	**-7.6156**
complement component 1, q subcomponent, beta polypeptide	C1qb	**-7.61182**
FERM domain containing 4B	Frmd4b	**-7.45106**
dyskeratosis congenita 1, dyskerin homolog (human)	Dkc1	**-6.00872**
allograft inflammatory factor 1	Aif1	**-5.93825**
interferon activated gene 204	Ifi204	**-5.82117**
vascular cell adhesion molecule 1	Vcam1	**-5.29671**

Table 5.1 Most downregulated genes on mLN DC treated with mouse intestinal mucin.
Mouse gene expression was compared between untreated and mucin-treated mLN DC using a microarray. The data was normalized by the RMA approach and changes in gene expression expressed as fold change. This ranking was obtained using Ingenuity Pathway analysis, ranking from the most dowregulated gene.

Gene Title	Gene Symbol	Fold-change
Y-linked testis-specific protein 1-like /// Ssty1 family member /// spermiogenesis specific transcript on the Y 1	Ssty1 (includes other)	**13.1021**
aquaporin 4	Aqp4	**3.43358**
testis expressed gene 24	Tex24	**2.96607**
neogenin	Neo1	**2.93171**
interleukin 22 /// interleukin 10-related T cell-derived inducible factor beta	Il22 /// Iltifb	**2.84399**
Ras association (RalGDS/AF-6) domain family member 1	Rassf1	**2.83079**
myosin, light polypeptide kinase	Mylk	**2.714**
insulin-like growth factor binding protein 5	Igfbp5	**2.58227**
RIKEN cDNA E130308A19 gene	E130308A19Rik (KIAA1958?)	**2.56808**
gamma-aminobutyric acid (GABA) A receptor, subunit beta 2	Gabrb2	**2.56582**

Table 5.2 Most upregulated genes on mLN DC treated with mouse intestinal mucin.
Mouse gene expression was compared between untreated and mucin-treated mLN DC using a microarray. The data was normalized by the RMA approach and changes in gene expression expressed as fold change. This ranking was obtained using Ingenuity Pathway analysis, ranking from the most upregulated gene.

Incubation with mucin induced mostly downregulation of genes in this particular assay, including different transcription factors (STAT1/3, SOCS4, NFAT), lectins (C-type lectins, mannose and galactose receptors), interferon-related genes (interferon activated genes and interferon regulatory factor 7), integrins, chemokines (ligands and receptors) and other genes related with immune regulation (GM-CSF, TGF-β receptor 1, TRAF1 and 5, CD200R4, and annexin A1) (Appendix 1). Some genes were upregulated that have known functions in DC, particularly IRF4 and IL22. IRF4 is a crucial transcription factor required for the differentiation of intestinal $CD11b^+$ $CD103^+$ DC, the most predominant DC subset in the small intestine and known to play important functions inducing tolerance and effector responses, whereas IL22 is relevant cytokine for mucosal immunity (Persson et al., 2013). Most of the known cytokines involved in inflammation and intestinal tolerance were not altered, including pro-inflammatory cytokines such as IFNγ, TNF-α, IL17 and IL23, and anti-inflammatory cytokines such as IL10 and TGF-β. The pro-inflammatory cytokines IL12A and IL6 seem to be slightly upregulated (Appendix 1, fold changes=2.03 and 1.97, respectively) but none of the classical DC activation

markers (CD86, CD80, CD83) were upregulated. Also, one of the MHC II genes (Mb2 locus) was slightly downregulated, suggesting that mucins did not induce DC activation in this system. No difference was observed in CD11c or CD11b expression, but CD103 was downregulated (-2.23 fold change).

5.2.4 Validation of IL22 upregulation by qPCR and ELISA

IL22 is a member of IL10 family, expressed in hematopoietic cells, and plays important roles regulating barrier immunity by interacting with the IL22 receptor, expressed mainly by intestinal epithelial cells (Sonnenberg et al., 2011). Mice lacking IL-22 expression exhibit increased susceptibility to different models of induced colitis and its exogenous administration can ameliorate intestinal inflammation in a model of spontaneous colitis (Sugimoto et al., 2008, Zenewicz et al., 2008). IL-22 can also promote expression of membrane-attached mucins such as Muc1, Muc3, Muc10 and Muc13, promoting epithelial cell proliferation and tissue repair (Sonnenberg et al., 2011, Sugimoto et al., 2008), and can also promote antibacterial responses by inducing the expression of anti-microbial peptides RegIIIβ and RegIIIγ, the pro-inflammatory cytokines IL-6 and G-CSF and the chemokines CXCL1, CXCL5, CXCL9 (Aujla et al., 2008, Zheng et al., 2008). Together, these data suggest that IL-22 plays key roles promoting barrier integrity. However, it has also been reported that IL22 can promote pathogenic roles in the intestinal mucosa; for example, dysregulated IL-22 responses may promote pathogenic intestinal inflammation as in the case of Toxoplasma gondii infection, which promotes IL-23-mediated up-regulation of IL-22 on T cells and subsequent expression of the pro-inflammatory cytokine IL-18 (Munoz et al., 2009, Munoz et al., 2015). It has been reported that the main sources of IL-22 in the intestinal tissue are innate lymphoid cells (ILC) and Th22 cells, in response to IL-23 stimulation (Basu et al., 2012). However, the role of intestinal DCs as a source of IL-22 has been relatively ignored.

Based on the microarray results, modulation of IL22 expression by mucins was explored, and the fundamental importance of IL-22 in regulation of intestinal barrier. To validate results obtained by microarray, IL22 expression on $CD11c^+$/MHC II^+ mLN DC was evaluated by qPCR using RNA isolated from 3 independent experiments where mLN DCs were incubated overnight with large intestinal mucin (50 μg/ml). IL22 expression was increased in all the experiments and was significantly increased compared to untreated mLN DCs (Figure 5.8A). Additionally, IL22 protein levels were measured by ELISA on mucin-treated mLN DC supernatants and were increased compared to untreated cells in all the experiments (Figure

5.8B). However, this increase is not significant, which may be attributed to the low number of samples. It must be noted that untreated cell had almost 0 pg/ml IL22 whereas cells treated with large intestinal mucins showed from 10 to 50 pg/ml. Therefore, these results suggest that large intestinal mucins may induce IL22 on mLN DCs. Further characterization is required to understand how this induction may be relevant during steady state conditions and inflammation.

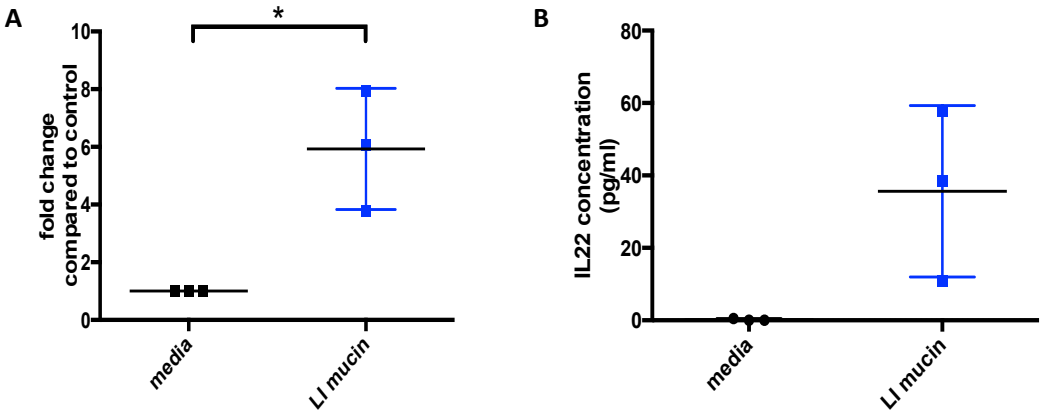

Figure 5.8 Mouse large intestinal Muc2 induces expression of the mucosal cytokine IL-22 by murine mLN DCs.

mLN DCs were treated with large intestinal mucin (LI mucin, 50 μg/ml) and IL22 expression measured by qPCR and ELISA A. qPCR was performed to detect gene expression changes on IL22 on mLN DCs treated with large intestinal mucin. Fold change was calculated in each individual experiment compared to untreated moDCs. IL22 expression at the RNA level was significantly increased in MLN DCs stimulated with large intestinal mucin (LI mucin). Results are from 3 independent experiments and statistical significance was assessed using an unpaired t test. B. ELISA was performed to detect IL22 on supernatants from untreated and large intestinal MLN DCs. IL22 expression at the protein level was not significantly increased in MLN DCs stimulated with large intestinal mucin (LI mucin). Results are from 3 independent experiments and statistical significance was assessed using an unpaired t test. *$p<0.05$

5.3 Discussion

5.3.1 DC subsets in the intestinal mucosal of Muc2-/- mice

This chapter aimed to investigate whether intestinal mucin/mucus can regulate intestinal DC function. As a first approach, we analysed whether Muc2-/- mice exhibit alteration in intestinal DC subsets, isolated from intestinal LP. We observed that neither small nor large intestinal DC subset proportions were altered in 6-7 week-old Muc2-/- mice, with predominant $CD103^+$ $CD11b^+$ DC in the small intestine and predominant $CD103^+$ $CD11b^-$ DC in the colon. Similarly, we did not observe changes in migratory DC subsets in the MLN on 6-7 week-old Muc2-/- mice (data not shown), suggesting that lack of a normal mucus barrier does not alter gut DC homeostasis and the normal DC development. In contrast, we observed an altered proportion of colonic DC in older and macroscopically inflamed mice, which exhibit a higher frequency of $CD103^-$ $CD11b^+$ DC and reduced $CD103^+$ $CD11b^-$ DC. Recently, a similar reduction in $CD103^+$ $CD11b^-$ DC has been reported in the colonic mucosa of Muc2-/- mice between 8-20 weeks old, which display mild to severe inflammation (Wenzel et al. 2014; Wenzel et al., 2015). Interestingly, the authors did not observe an increased frequency of $CD103^+$ $CD11b^+$ or $CD103^-$ $CD11b^+$ cells, but observed increased total numbers of both cell types, consistent with our results (Wenzel et al., 2014; Wenzel et al., 2015). The gating strategy used in these papers does not include appropriated macrophage markers (CD64 or F4/80) and does not exclude $B220^+$ plasmacytoid DCs, thus is a less stringent analysis compared with ours. Thus, the authors attributed the increased numbers of CD103- CD11b+ cells to an increased frequency of macrophages, monocytes and neutrophils. Here, we show that, using appropriate DC and macrophage markers, colonic CD103- CD11b+ DCs may be increased during inflammation in Muc2-/- mice. Although we observed a clear reduction in the frequency of $CD103^+$ $CD11b^-$ DC in 10-12 week-old Muc2 mice in all our experiments, the increase in $CD103^-$ $CD11b^+$ DC was most striking in one of three independent experiments, which used 12 week-old mice. Due to ethical reasons, we did not age our mice more than 10-12 weeks to avoid exacerbated colitis but it is likely that exacerbated inflammation in the colon may be responsible for the induction of these $CD103^-$ $CD11b^+$ DC. Concordantly, we observed increased numbers of CD103- CD11b+ DC and CD103+ CD11b+ DC but no significant differences in the numbers of CD103+ CD11b- DC, suggesting that Muc2-/- mice display altered proportions of intestinal DC subsets during inflammation but these changes are due to the increase in CD103- CD11b+ and CD103+ CD11b+ DCs. We did not observe any differences in the DC subsets from the small intestine on Muc2-/-, which supports the idea that the lack of Muc2 does not alter DC phenotype.

Moreover, Wenzel et al. (2015) have reported that CD103+ CD11b+ DC may be increased in the distal colon of a subgroup of colitic Muc2-/- mice displaying increased production of the pro-inflammatory cytokines, IL-6, IL-1β, TNF-α, IFN-γ and IL-17. These mice also exhibited extensive colitis. Although CD64 or F4/80 were not used for LP DC panels in this work, it is unlikely that $CD103^+$ $CD11b^+$ cells correspond to non-DC contaminant cells, as it is a phenotype exclusively associated with intestinal DCs but not macrophages. Both MLN CD103- CD11b+ and CD103+ CD11b+ DCs isolated from colitic Muc2-/- mice but not non-colitic Muc2-/- mice promoted increased production of IL-17 in co-culture assays with OT-II cells but only CD103+ CD11b+ DC promoted increased numbers of IL-17+ CD4+ T cells (Wenzel et al., 2015). Thus, this work postulates that CD103+ CD11b+ DC play role promoting extensively spread colitis in Muc2-/- mice whereas CD103- CD11b+ mononuclear phagocytes may promote localised distal colitis. This work suggests that colitis development in the Muc2-/- mouse model is quite heterogeneous, but as our data supports, altered proportion of intestinal DC subsets may contribute to the late pathogenesis of colitis.

5.3.2 Monocyte/macrophage axis in the intestinal mucosa of Muc2-/- mice

Moreover, intestinal macrophages may also be in close contact with the mucus barrier and we analysed whether these cells were altered in Muc2-/- mice. We observed an increased proportion of $MHCII^+$ $CD11c^+$ $F4/80^+$ macrophages and lower $MHCII^+$ $CD11c^+$ $F4/80^-$ DC in 6-7 week-old Muc2-/- mice compared to controls. Similarly, we analysed the monocyte-macrophage axis, which suggested that the proportion of monocytes is increased in the colonic mucosa of Muc2-/- mice, although analysis of more markers here would give more definitive results. For example, our analysis lacks the macrophage marker CX3CR1, and changes in the expression levels of this marker are associated to the development of inflammation, which reflect changes in the macrophage/monocyte composition in the gut (Bain et al., 2013). Intestinal macrophages ($MHCII^+$ $Ly6C^-$ $F4/80^+$) express heterogeneous levels of CX3CR1, including high and intermediate levels of CX3CR1, and display an anti-inflammatory phenotype, which includes high IL-10 production and increased phagocytic activity (Bain et al., 2013). During inflammation, it has been shown that CX3CR1int cells are increased in the colonic mucosa, especially the $MHCII^-$ $Ly6C^+$ monocyte gate and the $MHCII^+$ $Ly6C^+$ double positive population (Bain et al., 2013). These cells display inflammatory properties, such as production of pro-inflammatory cytokines and higher response to TLR stimulation, compared to hyporesponsive resting CX3CR1hi macrophages (Bain et al., 2013). Thus, it would be interesting to evaluate whether the population of CX3CR1int is increased and the CX3CR1hi

was decreased, as a manner to analyse development of inflammation in Muc2-/- mice. Additionally, although we excluded granulocytes/eosinophils based on the expression of Ly6C/Ly6G and excluding SSChigh cells, further experiments should include the eosinophilic marker Siglec F to more definitively remove eosinophils from our analysis.

5.3.3 Inflammatory status of Muc2-/- mice

The changes that we observed in the monocyte/macrophage axis are restricted to the colonic mucosa but, similar to our DC analysis, these changes may reflect on-going inflammation. Our analysis of the inflammatory status of 6-7 week-old Muc2-/- mice suggests that although mice do not exhibit overt macroscopic signs of colitis, they have increased neutrophil infiltration in the colonic mucosa (gated as $CD45^{+}/CD11b^{+}/Ly6C^{+}/Ly6G^{+}/MHCII^{-}$, data not shown). Thus, these mice were probably in early stages of inflammation. This finding is consistent with recent data published by Wenzel et al. (2014), which shows early neutrophil infiltration even in the absence of clinical signs of inflammation in Muc2-/- mice. Early neutrophil recruitment is consistent with data suggesting that Muc2-/- mice exhibit increased expression of chemokines, such as Ccl7, Ccl8, Ccl20, Cxcl9 and Cxcl13, in the distal colon at 2-4 weeks old (Lu et al. 2011). Similarly, Wenzel et al. (2015) reported increased expression of CXCL1, CXCL2 and CCL9 in the distal colon of colitic Muc2-/- mice.

Our analysis of Muc2-/- mice does not support the idea that mucins regulate the DC subset phenotype in Muc2-/- and thus the mucus barrier is not necessary for the correct development of DC. Our data suggest that DC subset composition in the intestine changes only after mice show signs of exacerbated colitis and these changes are restricted to the inflamed tissue. One possibility is that Muc2 is more abundant in the colon than the small intestine and thus changes are more evident in the colon than the small intestine. However, it is virtually impossible to uncouple this hypothesis from inflammation complications. The increased presence of monocytes and the increased proportion of macrophages in the colon should be interpreted as a sign of early inflammation but not necessarily as a consequence of the lack of interaction with tolerogenic signals from the mucus barrier. Data from different inflammation models and inflammatory diseases such as IBD suggest that monocyte recruitment occurs early in inflammation (Bain and Mowat, 2014), consistent with the inflammatory status of Muc2-/- mice.

5.3.4 Regulation of intestinal DC function by mucin in the Muc2-/- mouse model

The other aim of this chapter was to determine whether intestinal mucin might regulate intestinal DC functions. We have preliminary isolated $CD11c^+$ MHC II^+ MLN DC from control $Muc2^{+}/^{+}$ and Muc2-/- mice (6-week-old mice, data not shown) to analyse differences in gene expression. We preliminary analysed some genes that could be associated with expression of the pro-inflammatory cytokine IL-6 (which may be produced by $CD103^+$ $CD11b^+$ DC), as DCs from Muc2-/- mice might display augmented pro-inflammatory properties, and $\alpha v\beta 8$ integrin, which expression might be altered and affect levels of active TGF-β in the gut. These genes were not altered in MLN DCs from 6-7 week-old Muc2-/- mice but we still have not characterised other known genes relevant in intestinal immunity such as IL-10 and IL-12. Wenzel et al. reported that CD103- CD11b+ MLN DC from both non-colitic and colitic Muc2-/- mice promoted increased production of IL-6 and IFN-γ on OT-II cells, whereas CD103+ CD11b- DC promoted less production of IL-10, suggesting that DCs from Muc2-/- display altered function. Furthermore, Shan et al. (2013) reported that small intestinal LP $CD103^+$ DC (both $CD11b^+$ and $CD11b^-$) and $CD103^-$ $CD11b^+$ $CX3CR1^+$ cells (probably a mix of macrophages and DCs as CD64 or F4/80 were not included for the sorting) expressed more pro-inflammatory cytokines such as IL-12 and TNF-α and less anti-inflammatory molecules such as TGF-β1, IL-10 and ALDH1A1 compared to controls. Additionally, less small intestinal LP T_{regs} were reported. Muc2-/- mice used in this study were 6-10 weeks old and, according to this work, they did not show signs of spontaneous colitis up to 4 months. Reduction of gut bacteria with antibiotics in Muc2-/- mice did not restore normal expression of the anti-inflammatory molecules or augment T_{regs}, suggesting that these perturbations are not exclusively dependent on the contact with gut bacteria (Shan et al 2013). Furthermore, this work also postulates that oral administration of Muc2 may restore gut homeostasis in Muc2-/- mice, by increasing anti-inflammatory molecules and T_{regs}, and also may increase resistance to DSS-colitis (Shan *et al*., 2013). Thus, Shan et al. (2013) suggest that intestinal DC function is defective in Muc2-/- mice, and DC may show more pro-inflammatory properties. We do not have enough evidence to support this idea and at least from the point of view of DC subsets, our work suggest that differences observed in the Muc2-/- mice are more likely to be due to the inflammatory status of the intestinal mucosa. Therefore, further evaluation of cytokine production by the different DC subsets from non-colitic Muc2-/- mice would be important to evaluate altered DC function in our assays.

5.3.5 Potential signalling pathways regulated in MLN DCs by intestinal mucins

Here, we have performed a preliminary microarray to investigate the functional importance of interactions between intestinal Muc2 and intestinal DCs and find targets regulated by mucin. We opted for isolating $CD11c^+/MHCII^+$ DCs from the MLN to avoid potential contamination with intestinal macrophages, which also express these markers but do not migrate to the MLN (Cerovic *et al*., 2014). Additionally, we did not include $CD103^+$ in our sorting and thus our MLN DCs contained both $CD103^+$ and $CD103^-$ DC subsets. Moreover, in order to increase cell numbers and achieve the RNA concentration required for the microarray analysis, we pooled MLN from 8-10 mice in our experiments. We observed that Muc2 induced a potent down-regulatory effect of gene expression, which could be consistent with the idea that mucin glycans interact with inhibitory glycan receptors such as lectins. Interestingly, we did not observe induction of chemokines or DC activation markers, as our work with human moDCs suggested. Actually, a group of chemokines and chemokine-receptors (Xcl1, Cklf, Ccr1, Ccr5, Cxcr6 and Ccl9) seems to be downregulated, which suggest that mucin may prevent the recruitment of inflammatory mediators to the gut (Appendix 1). This is contradictory with our *in vitro* assays with human moDCs treated with mouse intestinal mucin, where we observed up-regulation of the chemokine IL-8. There are no direct murine homologues of IL-8, but it has been postulated other CXCL chemokines such as CXCL1/KC, CXCL2/MIP-2 and CXCL5-6/LIX can play similar functional roles, recruiting neutrophils (Fan et al., 2007; Hol et al., 2010; Zlotnik et al., 2006). Preliminary work on mouse splenic $CD11c^+$ DC did not show stimulation of DC activation or increased expression of the mouse IL-8 homologue, MIP-2 (data not shown). These differences may be attributed to potential redundancy of the murine IL-8 homologues and differences between the mouse and human regulation of DC function. Interestingly, Shan et al. (2013) reported similar tolerogenic effects in human moDCs and in murine DCs (from intestinal LP and bone marrow-derived DCs) but this does not seem to be the case in our current work.

On the other hand, we observed down-regulation of the anti-inflammatory Fc receptor *Fcgr2b* and *Clec7a* (Dectin-1), which have been recently proposed to form a complex together with galectin 3 that would mediate mucin-induced effects of intestinal DC, inducing β-catenin (Shan et al., 2013). We did not find changes in galectin-3 expression (*Lgals3*) but we found that other similar lectins were downregulated (*Lgals1* and *Lman1*). We did not find up-regulation of β-catenin but our data suggest that there might be downregulation of the β-catenin inhibitor, Gsk3β. Down-regulation of these genes may potentially facilitate β-catenin translocation to

the nucleus and mediation of tolerogenic effects. It is also postulated that mucin may induce CREB signalling, which in collaboration with β-catenin, may regulate IL-10 induction and IL-12 down-regulation (Shan et al 2013). However, our microarray data suggest that CREB is downregulated and we did not find IL-10 up-regulation. We found a small up-regulation of *Il12a* (fold change= 2.03) but no changes in gene expression of *Il12b*. Thus, our data supports the idea that some of these elements may be regulated by mucin but we are not able to confirm or refute the tolerogenic potential of mucins in mouse intestinal DCs described by Shan et al. (2013) with our current data.

Interestingly, Ingenuity Pathways analysis revealed that STAT signalling pathways might be regulated by intestinal mucin. Thus, our data suggest that Stat3 is downregulated and several downstream genes including the chemokine receptors *Ccr1* and *Ccr5* and the Fc receptor *Fcgr2b* are also downregulated (data not shown). Expression of *Stat1/3* is regulated by the ERK/MAPK signalling pathway and several components of this latter pathway seem to be downregulated. Additionally, phosphorylation of *Stat1* and *Stat3* is regulated by GM-CSF, and these three genes are downregulated in our microarray. We still have not validated all these potential targets but these findings support the idea that different signalling pathways related with regulation of the gut immunity may be regulated by mucin.

5.3.6 Production of IL-22 on intestinal DCs in the presence of intestinal mucin

One of the interesting targets that we found to be modulated by mucin treatment of intestinal DCs is the mucosal cytokine IL-22. Expression of this cytokine has been mainly characterised in Th22 cells and ILC3s, which are considered the major sources of this cytokine in the gut (Sonnenberg et al., 2011, Sonnenberg and Artis, 2015). Here, we found that $CD11c^+$/$MHCII^+$ MLN DCs upregulate IL-22 expression in the presence of large intestinal mucin, which was validated by qPCR and ELISA and suggests that mucin may promote important defensive roles in the gut.

There are some reports of IL-22 expression by intestinal LP $CD11c^+$ cells, which could potentially be DCs. Thus, colonic $CD11c^+$ cells appear to express IL-22 during DSS-colitis and infection with *Citrobacter rodentium*, and also in response to CpG and IL-23, suggesting that TLR ligands could promote IL-22 expression (Pickert et al., 2009, Zheng et al., 2008). ILC3s ($CCR6^+$ $CD4^{+/-}$ MHC II^+ $NKp46^-$) are enriched in the MLN, where they can potentially present antigens via MHC II to control $CD4^+$ T cell responses to commensal bacteria (Mackley et al., 2015, Hepworth et al., 2013). However, it is unlikely that our cells preparations are

contaminated with ILCs, as they do not express CD11c (Mackley et al., 2015). Interestingly, when splenic $CD11c^+$ DCs have been incubated with large intestinal mucin, we have not detected induction of IL-22 by ELISA (data not shown), which suggests that this effect may be specific for intestinal DCs. Importantly, IL-22 binding protein, a soluble negative regulator of IL-22, is highly expressed in intestinal $CD103^+$ $CD11b^+$ DCs at steady-state and is potently induced on immature human monocyte-derived DC by RA, suggesting that intestinal DCs may play a key role regulating IL-22 signalling in the gut (Martin et al., 2014).

Moreover, Cobo et al. (2014) indicates that the anti-microbial peptide β-defensin 2 is induced by mucin on intestinal epithelial cells. IL-22 is an important inducer of anti-microbial peptides in intestinal epithelial cells, and thus this evidence suggests that mucin could potentially control anti-microbial activity in the gut via IL-22. Further investigation is required to confirm which DC subset may be responsible for IL-22 production and whether LP DCs elicit a similar response to intestinal mucin. Additionally, it must be evaluated whether induced IL-22 is able to exert relevant biological functions; for example, induction of anti-microbial peptides, such as RegIIIγ (Sonnenberg et al., 2012).

5.3.7 Role of potential contaminants in the mucin preparation

As the DC number isolated from the MLN or intestine was limited, we still have not tested the potential interference of contaminants in our *in vitro* assays using mouse intestinal DCs. As we discussed in the previous chapter, LPS or bacterial components may be contaminants in our samples. However, the presence of these contaminants may better reflect physiological conditions where the mucus barrier may contain PAMPs or other accessory proteins. Intestinal DCs seem to be hyporesponsive to TLR4 stimulation and thus may not be activated by LPS bound to mucin. Also, intestinal DCs may be tolerised to different components of the intestinal barrier, including mucins. Shan et al. (2013) have shown that DCs may acquire CFSE-labelled mucin in the small intestinal LP and Peyer's patches, which suggest that DCs are constantly exposed to intestinal mucin. Moreover, important experiments in the future should analyse the effect of mucin glycopeptides and mucin with glycan modifications (enzymatic or chemical) in order to study the glycan contribution to the interaction with intestinal DCs.

6 Chapter 6 Discussion

6.1 Discussion

The intestinal barrier is a complex environment, which requires the regulation of the immune system to avoid the entrance of enteric pathogens but also avoid exacerbated immune responses against innocuous food antigens and commensal bacteria. The cross-talk between different components of the intestinal mucosa is crucial to regulate these immune responses. Therefore, regulation of gut homeostasis by DCs, IELs and the mucus barrier were a focus of this thesis in order to contribute to the current understanding of the intestinal barrier.

6.1.1 The role of DCs regulating gut IEL homeostasis

In Chapter 3, we investigated whether expression of important components of the extracellular matrix in the intestine, the integrin $\alpha v\beta 8$ and the intestinal mucin Muc2, play a role in modulating the development and/or maintenance of IELs. We have shown that expression of integrin αvβ8 on DCs, but not T cells, is important for the induction of small intestinal CD4+ CD8$\alpha\alpha$+ IELs, supporting the idea that TGF-β may induce this IEL subset. This finding supports previous data indicating that TGF-β can promote CD4+ CD8$\alpha\alpha$+ IEL differentiation, as this cytokine can modulate expression of the transcription factors ThPOK and Runx3 (Konkel et al., 2011; Reis et al, 2013; Reis et al, 2014). Additionally, our data support recent research postulating a role for intestinal DCs in regulating IEL homeostasis, as mice lacking expression of the transcription factor IRF8 (and lacking CD103+ CD11b- DC), exhibit dramatic alterations in the IEL compartment, including reduced CD4+ CD8$\alpha\alpha$+ IEL (Luda et al., 2015, submitted manuscript). Of note, 6-week-old *CD11c-cre.Itgβ8* mice do not exhibit significant differences in CD4+ CD8$\alpha\alpha$+ IEL, suggesting that other TGF-β independent mechanisms may promote expansion of this subset. This may be attributed to the systemic inflammation that these mice develop.

6.1.2 The role of the CD4+ CD8$\alpha\alpha$+ IEL subset in gut homeostasis

The role of this IEL subset *in vivo* remains unclear and quite unexplored in the literature, but some evidence supports the idea that CD4+ CD8$\alpha\alpha$+ IELs can play a regulatory role due to their "activated but resting" phenotype. These cells can produce different cytokines such as IL-10, IFN-γ and TGF-β and also display cytotoxic properties, expressing granzyme B (Cheroutre et al., 2011; Das et al., 2003). Such cells may mediate protection against infection, eliminating infected intestinal epithelial cells with increased expression of MHC II, as these IELs remain MHC II-restricted but also develop cytotoxic properties in the gut (Cheroutre et al., 2011). It

has also been reported that patients with active ulcerative colitis and coeliac disease exhibit reduced numbers of CD4+ CD8αα+ IELs in the gut (Senju et al., 1991, Carton et al., 2004). Das et al. (2003) reported that *in vitro* generated Th2-like CD4+ T cells were able to upregulate CD8αα+ in the intestinal epithelium of RAG-/- mice. These CD4+ CD8αα+ IELs were recovered from the primary recipient and transferred into immunodeficient mice, preventing T-cell-transfer mediated colitis (Das et al., 2003). Thus, the current understanding of CD4+ CD8αα+ IEL function suggests that it may be beneficial to stimulate their expansion during infection and/or inflammation. Thus, understanding the factors required for their differentiation is important for the future development of clinical therapies, aiming to expand these cells to prevent IBD and/or enhance protection from infection. Further research is required to explore the possibility that intestinal DCs may not only regulate the development and maintenance of this subset but also modulate IEL function.

6.1.3 A role of integrin αvβ8 in the regulation of gut homeostasis

Evidence provided here supports that expression of integrin αvβ8 on DCs is important for regulation of the IEL compartment, specifically in the generation of CD4+ CD8αα+ IELs. It has been shown that expression of the integrin αvβ8 on DC and effector T_{regs} is important for the activation of the latent cytokine TGF-β and thus may promote the development of T_{regs} and tolerogenic responses in the gut (Travis et al, 2007, Worthington et al., 2011a, Worthington et al., 2015). Therefore, promoting αvβ8 expression/function might be important for maintaining gut homeostasis. However, it must be considered that lack of expression of integrin αvβ8 can be protective in some diseases models such as experimental autoimmune encephalomyelitis, as TGF-β can also promote pathogenic Th17 responses (Melton et al., 2010). Moreover, preliminary work from our group suggests that patients with Crohn's disease exhibit higher expression of integrin αvβ8 (Fenton et al 2015, unpublished), which suggests that integrin αvβ8 might play a role suppressing exacerbated inflammatory responses. The Integrin αvβ8 locus does not seem to be linked to IBD according to genome-wide association studies, which suggests that integrin αvβ8 is not implicated with susceptibility to IBD (Jostins et al., 2012). Our group has recently shown that integrin αvβ8 is upregulated on effector T_{regs} and effector T_{regs} lacking integrin αvβ8 are unable to supress T cell inflammatory responses in a transfer colitis model (Worthington et al., 2015). Therefore, this evidence suggests that potentially promoting integrin αvβ8 expression in the human gut may be beneficial during inflammatory conditions although further research is required to confirm this idea.

6.1.4 Control of integrin αvβ8 expression

Understanding of modulation of αvβ8 integrin expression is important in order to design effective therapeutic treatments. Interestingly, recently it has been shown that IRF8 may control expression of integrin αvβ8 on APCs but not T cells, and IRF8-/- mice are protected from EAE, with reduced Th17 and T_{regs}, similar to *CD11c-cre.Itgβ8* mice (Yoshida et al., 2014). As mentioned above, this transcription factor regulates the differentiation of the murine intestinal DC subset CD103+ CD11b- but its role in the differentiation of human intestinal DCs is less understood. Both human CD1c+ and CD141+ DC express IRF8 but only CD1c+ DCs (proposed to be the human counterpart of CD103+ CD11b+ DC and also CD103- CD11b+ DC) are ablated in human IRF8 mutation (Collin et al., 2011, Hambleton et al., 2011, Watchmaker et al., 2014). Concordantly, our group has found that human intestinal CD1c+ (CD103+ SIRPα+ and CD103- SIRPα+ DC) exclusively express integrin αvβ8 (Fenton et al, 2015, unpublished), which suggests that there are important discrepancies between the mouse model and humans that need to be addressed.

6.1.5 Failure of the intestinal barrier during IBD

Understanding of the role of integrin αvβ8 in gut homeostasis may be important in order to supress inflammation during IBD. IBD comprises two different diseases affecting the gastrointestinal tract, ulcerative colitis and Crohn's disease. Ulcerative colitis is focalised in the colon, whereas Crohn's disease affects any part of the gastrointestinal tract (Khor et al., 2011). IBD has a strong genetic component, with 163 genetic loci associated and studies in monozygotic twins indicate that is even higher for Crohn's disease with a concordance rate of 30%-35 compared to 10-15% of ulcerative colitis (Jostins et al., 2012, Khor et al., 2011). A "multihit" model has been proposed for IBD, where several abnormalities need to converge, involving a failure of the intestinal barrier at different levels, in order to trigger IBD (Maloy and Powrie, 2011). Abnormalities associated with IBD include Paneth cell dysfunction, defects in the epithelial barrier, innate tolerance, altered PRR signalling, ER stress, oxidative stress and cytokine production (Khor et al., 2011). Environmental factors also contribute to the disease pathogenesis, such as dietary factors and an altered gut microbiota, that may affect the integrity of the intestinal barrier (Maloy and Powrie, 2011). Thus, development of effective therapies has proven to be extremely difficult due to the multi-factorial component of this disease and uncertainty of the sequential order of events during IBD pathogenesis (de Souza and Fiocchi, 2015). Current therapies include administration of the immunosuppressive agent

glucocorticosteroid and anti-TNF but neither of them represents an effective treatment for IBD. Therefore, understanding of regulation of the intestinal barrier may be of help to develop effective therapies for IBD.

6.1.6 The role of the intestinal mucus barrier in regulating gut immunity

We did not find a role of the intestinal mucin Muc2 in regulating the development and/or maintenance of IELs, which suggest that the mucus barrier is dispensable for IEL homeostasis. However, we have shown in Chapters 4 and 5 that the intestinal mucin Muc2 may play important roles in modulating the function of DC and our work provides a new point of view for the role of the intestinal mucus barrier. Different studies have postulated that Muc2 plays a tolerogenic role, preventing inflammatory responses by DC (Ishida et al., 2008; Ohta et al., 2010; Shan et al., 2013). Here, we have shown that Muc2 has the potential to induce pro-inflammatory responses, inducing IL-8 expression and DC activation in human moDCs, which may be important in the context of different inflammatory conditions such as Crohn's disease and ulcerative colitis. Thus, understanding the interactions between DCs and mucin may provide interesting molecular targets that could prevent exacerbated inflammation.

Our data suggest that DC might recognise mucins as danger signals, which might occur during disruption of barrier integrity, facilitating the contact between mucin and intestinal DCs. Aberrant glycosylation of mucins has been reported in patients with IBD and colorectal cancer, and this may contribute to disease pathogenesis (Boltin et al., 2013, Crocker et al., 2007). As we have shown, it is likely that glycosylation of mucin plays a key role in inducing pro-inflammatory responses in human moDCs and thus identification of the functionally important glycosylations might provide novel clinical targets, with blocking agents, such as specific lectins, potentially of clinical benefit. We find here that mucin-induced pro-inflammatory effects are sialic acid-independent, and further investigation is required to identify the mucin glycan responsible for the induction of IL-8 and DC activation. Such work will be important to elucidate the signalling pathways involved in these effects, which may also represent important clinical targets.

6.1.7 Differences between the human and mouse intestinal barrier

We found some discrepancies between our *in vitro* assays with human moDCs and mouse MLN DCs. Whereas in Chapter 4, we observed the induction of IL-8 and DC activation markers by human moDCs in response to intestinal mucin, a preliminary microarray shown in Chapter 5

did not show up-regulation of genes known to be associated with pro-inflammatory functions on MLN DC incubated with mucin but showed downregulation of several chemokines, integrins and transcription factors. Importantly, we validated that intestinal mucin promotes IL-22 expression, a cytokine known to play important roles in the mucosa, maintaining barrier integrity and promoting anti-microbial responses (Sonnenberg et al., 2011). These findings are consistent with recent work, suggesting that Muc2 may promote expression of the anti-microbial peptide β-defensin 2 on intestinal epithelial cells, as IL-22 can promote expression of the anti-microbial peptide RegIIIγ (Cobo et al., 2015, Sonnenberg et al., 2011). Therefore, despite the differences displayed between the mouse and human DC models, our data supports the idea that mucin may play important roles promoting gut immunity and not only tolerogenic roles. Further exploration of the regulation of murine intestinal DC function may reveal other important targets that may be analysed in the human intestinal DCs.

We have found some discrepancies using the human moDC model and mouse intestinal DCs, which may be attributed to the different properties of tolerogenic intestinal DCs compared to the more pro-inflammatory profile of the human moDC model. Moreover, we cannot rule out the possibility that differences observed between human and mouse DCs may be due to a different repertoire of glycan receptors displayed by DCs and that may affect their interactions with the intestinal mucin Muc2. Additionally, although murine and human DCs seem to share fundamental functions regulating gut homeostasis (Watchmaker et al., 2014), there might be phenotypic and functional differences in DC subsets that need to be addressed in order to translate findings in mouse models into human therapies.

Also, it has been found that human and mouse intestinal mucins display different glycosylation patterns. Human small intestinal mucin displays highly fucosylated glycans in the ileum compared to the low fucosylation observed in mouse ileum, whereas most mouse small intestinal glycans are sialylated compared to the human small intestinal mucin (Holmen Larsson et al., 2013, Robbe et al., 2004). Interestingly, core O-glycans also seem to differ between mouse and human mucin, with core 3 O-glycans (GlcNAc(β1-3)GalNAc) being the more predominant type in the human intestine and core 2 structures (Gal β1-3(GlcNAc β 1-6)GalNAc) predominant in the mouse intestine (Holmen Larsson et al., 2013, Robbe et al., 2004). However, our work suggests that both human and mouse intestinal mucins induce similar effects in human moDCs, suggesting that mucin function is conserved between mouse and human.

6.1.8 Lack of a functional intestinal mucus barrier in the Muc2-/- model

Moreover, as we observed in Muc2-/- mice, lack of the mucus barrier drives the development of spontaneous colitis, due to the increased contact between the epithelial layer and gut microbiota. We observed alterations in DC/macrophage proportions and monocyte infiltration in the colonic mucosa but not the small intestine in 6-7 week-old Muc2-/- mice. Our evidence suggest that most of dysregulated intestinal immune response could be attributed to the lack of a physical barrier more than the lack of tolerogenic signals from the mucus barrier, although other groups have shown that DC and intestinal epithelial cell function may be altered in this model (Cobo et al., 2015, Shan et al., 2013, Wenzel et al., 2015).

6.1.9 Muc2-/- mice as a model of ulcerative colitis

Additionally, some studies have postulated that the Muc2-/- model could be useful to understand intestinal inflammation, especially ulcerative colitis, as the defects in the intestinal barrier of these mice may correlate with human disease (Johansson et al., 2014, Wenzel et al., 2014). Wenzel et al. (2014) have compared inflammation observed in the Muc2-/- mouse model, observing similar changes in DC subsets and macrophage composition in patients with active ulcerative colitis. Similarly, Johansson et al. (2014) have reported a more penetrable mucus barrier in patients with active ulcerative colitis and some patients in remission, which might increase the contact of the human gut microbiota and the intestinal epithelium. However, it is unclear whether this poor quality of the mucus layer is a primary cause of IBD and whether it is a consequence of the increased inflammation observed in the gut that may alter mucin production by goblet cells due to the high turnover of the mucus layer required during inflammation and also the reduction of goblet cells observed in IBD (Johansson et al., 2014). Furthermore, MUC2 loci do not seem to be involved in susceptibility to IBD (Jostins et al., 2012), which suggests that either inflammation or other environmental agents may cause the defects observed in the mucus barrier from patients with active ulcerative colitis. Thus, although studies in the Muc2-/- model might contribute to the understanding of the pathogenesis of ulcerative colitis, results from this model may be interpreted with caution, as ulcerative colitis is a much complex and multi-factorial phenomenon.

6.1.10 A role of the microbiota regulating the intestinal mucus barrier

The colonic mucus layer forms an impenetrable barrier that excludes potential enteric pathogens. Interestingly, the interactions between the mucus barrier and the microbiota seem

to be quite complex. Germ-free mice display an abnormal and penetrable mucus layer, and colonisation studies in germ-free mice suggest that long-term bacterial colonisation is required to modulate mucin glycosylation patterns and to form an impenetrable colon mucus inner layer (Johansson et al., 2015). Additionally, bacterial composition of the gut may influence mucus penetrability, and some bacterial phyla may increase mucus penetrability such as Proteobacteria, which is increased in the colonic mucosa of patients with IBD (Jakobsson et al., 2015, Matsuoka and Kanai, 2015). Consistently, a more penetrable mucus layer has been reported in patients with ulcerative colitis, suggesting that altered properties of the mucus layer might be induced, at least in part, by the gut microbiota (Johansson et al., 2014). Modulation of gut microbiota is an attractive idea to regulate barrier integrity and prevent intestinal inflammation. Recent clinical data suggest that faecal transplants might be an effective therapy for *Clostridium difficile* infection although is unclear how this therapy works (Bowman et al., 2015). Therefore, understanding of the interactions between mucus and the microbiota may help to the improvement and optimisation of this therapy.

6.2 Thesis conclusion

The aims of this project were to investigate the modulation of immunity in the intestinal barrier. One of our major findings was that expression of integrin $\alpha v\beta 8$ expression on intestinal DCs plays an important role inducing the IEL subset CD4+ CD8$\alpha\alpha$+ in mice. Future research should be focused on the regulation of IEL function by intestinal DCs, and how this interaction may impact gut homeostasis during inflammation and/or infection. Secondly, we have found evidence supporting a role of the intestinal mucin Muc2 promoting pro-inflammatory features on human moDC, which may be important in promoting neutrophil recruitment during bacterial infection. Further elucidation of the signaling pathways involved in mucin-induced effects is required, in order to identify potential therapeutic targets for intestinal inflammation. Moreover, a microarray using murine MLN DCs treated with intestinal mucin suggest that the intestinal mucin Muc2 regulates important genes in gut immunity, including upregulation of the important mucosal cytokine IL-22, which might promote anti-microbial response. Therefore, our work provides important data about the cross-talk between different components of the intestinal barrier and how these interactions may be relevant for regulation of gut homeostasis.

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Appendix I

Table AI.1. Comparative gene expression microarray between untreated and mucin-treated mLN DC. Mouse gene expression was compared between untreated and mucin-treated mLN DC. The data was normalized by the RMA approach and changes in gene expression expressed as fold change. Different tables are shown, classifying genes with related functions or characteristics (highlighted in red) (n=1).

Integrins		
integrin beta 5	Itgb5	**-3.10433**
integrin alpha V	Itgav	**-2.45105**
integrin alpha L (CD11a)	Itgal	**-2.39207**
integrin alpha 6	Itga6	**-2.3736**
integrin alpha E, epithelial-associated (CD103)	Itgae	**-2.23614**
integrin alpha FG-GAP repeat containing 1	Itfg1	**-2.0572**

Lectins and lectin-like receptors		
C-type lectin domain family 1, member b	Clec1b	**-4.27109**
C-type lectin domain family 4, member a3	Clec4a3	**-2.81875**
C-type lectin domain family 7, member a	Clec7a	**-2.65232**
lectin, galactose binding, soluble 1	Lgals1	**-4.08288**
lectin, galactose binding, soluble 1	Lgals1	**-3.65379**
lectin, mannose-binding, 1	Lman1	**-2.10196**
lectin, mannose-binding, 1	Lman1	**-2.0673**
lectin, mannose-binding, 1	Lman1	**-2.00047**
killer cell lectin-like receptor, subfamily D, member 1	Klrd1	**-4.48095**
killer cell lectin-like receptor subfamily B member 1B	Klrb1b	**-3.51406**

Chemokine-related genes		
chemokine (C motif) ligand 1	Xcl1	**-5.18127**
chemokine-like factor	Cklf	**-3.04778**
chemokine (C-C motif) receptor 1	Ccr1	**-2.77577**
chemokine (C-C motif) receptor 5	Ccr5	**-5.08702**

chemokine (C-X-C motif) receptor 6	Cxcr6	**-2.70842**
chemokine (C-C motif) ligand 9	Ccl9	**-2.70333**

Interferon-related genes		
interferon activated gene 204	Ifi204	**-5.82117**
myeloid nuclear differentiation antigen like	Mndal	**-4.89723**
interferon activated gene 204 /// interferon activated gene 205 /// myeloid cell nuclear differentiation antigen /// myeloid nuclear differentiation antigen like	Ifi204 /// Ifi205 /// Mnda /// Mndal	**-4.45155**
interferon-induced protein with tetratricopeptide repeats 2	Ifit2	**-4.24948**
interferon-induced protein with tetratricopeptide repeats 1	Ifit1	**-4.10021**
interferon activated gene 205 /// myeloid cell nuclear differentiation antigen	Ifi205 /// Mnda	**-3.62521**
interferon (alpha and beta) receptor 2	Ifnar2	**-3.35769**
interferon, alpha-inducible protein 27 like 2A	Ifi27l2a	**-3.48386**
interferon gamma inducible protein 47	Ifi47	**-2.27974**
interferon regulatory factor 7	Irf7	**-2.06303**
interferon regulatory factor 4	Irf4	**2.05842**

Other relevant genes modulated by mucins		
annexin A1	Anxa1	**-3.40531**
complement component 1, q subcomponent, C chain	C1qc	**-3.27798**
complement component 3a receptor 1	C3ar1	**-2.06752**
colony stimulating factor 2 (granulocyte-macrophage)	Csf2	**-2.55278**
dedicator of cytokinesis 7	Dock7	**-4.06873**
dedicator of cytokinesis 9	Dock9	**-2.22312**
DNA-damage regulated autophagy modulator 2	Dram2	**-4.11637**
Fc receptor, IgG, low affinity IIb	Fcgr2b	**-3.4533**
Forkhead box P1	Foxp1	**-3.72332**
glycogen synthase kinase 3 beta	Gsk3b	**-2.00638**
histocompatibility 2, O region beta locus	H2-Ob	**-2.62167**

histocompatibility 2, class II, locus Mb2	H2-DMb2	**-2.48405**
inhibitor of kappa light polypeptide enhancer in B cells, kinase complex-associated protein	Ikbkap	**-3.63351**
interleukin 6 signal transducer	Il6st	**-2.10954**
mucosa associated lymphoid tissue lymphoma translocation gene 1	Malt1	**-3.04977**
nuclear factor of activated T cells, cytoplasmic, calcineurin dependent 3	Nfatc3	**-2.08674**
nuclear factor of activated T cells 5	Nfat5	**-2.24903**
nuclear receptor subfamily 3, group C, member 1	Nr3c1	**-3.30295**
(glucocorticoid receptor)		
platelet-derived growth factor, C polypeptide	Pdgfc	**-3.11464**
pyrin and HIN domain family, member 1	Pyhin1	**-4.63108**
signal transducer and activator of transcription 1	Stat1	**-3.16701**
Signal transducer and activator of transcription 3	Stat3	**-2.02366**
suppressor of cytokine signaling 4	Socs4	**-2.21843**
transforming growth factor, beta receptor I	Tgfbr1	**-2.6336**
transforming growth factor, beta induced	Tgfbi	**-2.01805**
transforming growth factor, beta receptor II	Tgfbr2	**-1.97838**
TNF receptor-associated factor 1	Traf1	**-2.07616**
TNF receptor-associated factor 5	Traf5	**-2.41358**

Cytokines and DC markers		
transforming growth factor, beta 1	Tgfb1	1.40426
transforming growth factor, beta 2	Tgfb2	-1.38439
transforming growth factor, beta 3	Tgfb3	1.1
interleukin 1 beta	Il1b	1.24761
interleukin 6	Il6	**1.97154**
interleukin 10	Il10	1.43382
interleukin 12a	Il12a	**2.03439**
interleukin 12b	Il12b	1.56561
interleukin 12b	Il12b	1.08152
interleukin 17B	Il17b	1.09229

interleukin 17A	Il17a	-1.25818
interleukin 23, alpha subunit p19	Il23a	1.01469
interferon gamma	Ifng	1.1
tumor necrosis factor	Tnf	1.01314
CD8 antigen, alpha chain	Cd8a	1.38123
integrin alpha X (CD11c)	Itgax	1.29937
integrin alpha M (CD11b)	Itgam	1.07878
CD80 antigen	Cd80	1.15879
CD83 antigen	Cd83	-1.0907
CD86 antigen	Cd86	1.30419