THE ROLE OF SUPPRESSOR OF CYTOKINE SIGNALLING 3 IN REGULATING TOLL LIKE RECEPTOR-MEDIATED INTESTINAL EPITHELIAL HOMEOSTASIS AND REPAIR

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ABSTRACT

THE ROLE OF SUPPRESSOR OF CYTOKINE SIGNALLING 3 IN REGULATING TOLL LIKE RECEPTOR-MEDIATED INTESTINAL EPITHELIAL HOMEOSTASIS AND REPAIR

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The surface of the alimentary tract is lined with a single layer of intestinal epithelial cells (IEC) that functions as a barrier between commensal microflora and the underlying immune system. Maintenance of IEC barrier, subsequent to injury or physiological damage is essential in maintaining homeostasis. IECs express Toll-like receptors (TLR) on their surface which are able to detect microbial ligands such as helminth proteins, Poly I:C, lipopolysaccharide and flagellin, recognised by TLR2, TLR3, TLR4 and TLR5, respectively. Recent evidence proposes TLR-induced inflammatory pathways are vital for mucosal homeostasis with dysregulated repair predisposing individuals to inflammatory bowel disease (IBD). In IBD, the expression of suppressor of cytokine signalling -3 (SOCS3), a negative feedback inhibitor of inflammatory cytokines (TNFα, IL-6) is enhanced. The aim of this study was to investigate the role of SOCS3 on TLR-induced IEC responses associated with normal homeostasis and epithelial repair. SOCS3 over-expressing IEC were developed to assess its function on epithelial repair, gene and protein expression in response to microbial stimulation. Our results support previous data implicating TLR ligands being imperative for repair of damaged epithelial surfaces, and highlight a pivotal role of SOCS3 in mediating TLR-induced epithelial repair. Our results then go onto indicate over-expression of SOCS3 in IBD may perpetuate inflammation by promoting the production of pro-inflammatory TNFα in response to commensal microflora. In the final part of this study we show IEC become tolerant to commensal flora, protecting

against incessant immune activation by commensals. In conclusion, these studies give credit to the hypothesis that SOCS3 influences microbial-induced IEC responses associated with normal homeostasis and epithelial repair. Furthermore, our data indicates SOCS3 expression must be tightly regulated permitting TLR-induced epithelial repair. The findings presented within this study offer a strong foundation for future *in vivo* studies on how SOCS3 impacts on intestinal disease.

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Most importantly, I would like to thank my dear parents Inayat and Sharifa, my siblings Seraz, Zenab and Kulsum and finally all my friends, whose patience and understanding have encouraged me throughout.

DECLERATION

I declare that this thesis was composed by myself and has not been submitted in substantially the same form for the award of a higher degree elsewhere.

Imtiyaz Thagia MSc

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1. GENERAL INTRODUCTON

1.1 The human digestive system

1.1.1 Overview of the human digestive system

The human digestive system is composed of two groups of organs; these are the gastrointestinal (GI) tract and the accessory organs. The GI tract or alimentary canal is a continuous tube that extends from the mouth to the anus through the thoracic and abdomino-pelvic cavities measuring between 5-7 metres in length. The organs of the GI tract include the mouth, oesophagus, stomach and both the small and large intestines. The accessory digestive organs include the teeth, tongue, salivary glands, liver, pancreas and gallbladder. Teeth aid in the mechanical breakdown of food, while the tongue facilitates with chewing and swallowing. The remainder of the accessory organs do not come into direct contact with food, but produce or store secretions aiding the chemical breakdown of food (Snell 2003).

The GI tract contains food from the time it is ingested until it is digested and absorbed or eliminated from the body. Muscular contractions of the wall of the GI tract physically breakdown food by churning and propelling food from the oesophagus to the anus. Additionally, the contractions also help to breakdown food by mixing it with fluids secreted into the tract. Food is also chemically broken down by enzymes secreted by the accessory digestive organs and cells lining the GI tract (Snell 2003). Organic substrates, electrolytes (inorganic ions), vitamins and water are absorbed by epithelial cells lining the lumen of the GI tract, which subsequently pass into blood or lymph and circulate to cells throughout the body. The digestive tract and glandular organs discharge waste products into the lumen of the GI tract, which after mixing with the indigestible residues, are eliminated from the body (Martini 2006).

1.1.2 Anatomy of the colon

The colon is the concluding section of the vertebrate digestive system measuring approximately 1.5 metres in length, 0.1 metres in diameter, and constituting almost one-fifth of the entire alimentary canal. The caecum operates as a valve connecting and permitting the passage of processed material between the small intestine and the colon. In humans, the colon is composed of four key segments; these are: the ascending colon, the transverse colon, the descending colon and finally, the sigmoid colon. The rectum is located at the end of the colon and faeces are stored here before they are eliminated from the body. Collectively, both the colon and the rectum are referred to as the large bowel; but they are more commonly denoted as the large intestine or the gut (Snell 2003).

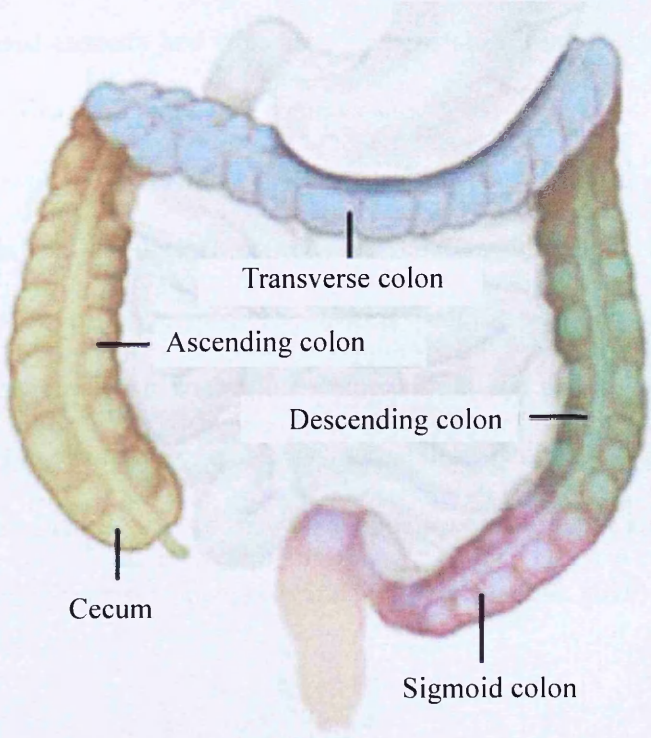


Figure 1-1 Anatomy of the human colon, composed of four segments: the ascending, transverse, descending and sigmoid colon (Encyclopædia Britannica 2003)

1.1.3 Microanatomy of the colon

The microanatomy of the colon is similar to that of other structures within the digestive tract. Visualizing the layers of the gut radially inwards these are the:

- Serosa – or adventitia, the outermost layer of the colon. It is constructed from loose connective tissue coated with mucus to thwart friction damage and abrasion from other tissues and organs.
- Muscularis externa – comprising of an inner circular layer and an outer longitudinal layer of smooth muscle. Both groups of muscle work simultaneously to create a wavelike motion (peristalsis) moving waste material along the colon.
- Submucosa – comprising collagen and elastic fibers, this layer provides increased capacity and stretching for peristalsis. Blood, lymphatic vessels and nerves also form part of the submucosa.
- Mucosa – the inner most layer comprising the glandular epithelium, lamina propria, and the muscularis mucosae. The glandular epithelium forms tubular structures, called crypts. The lamina propria supports the epithelium and comprises reticular connective tissue with elastic and collagen fibers, plasma cells, lymphocytes, granulocytes, as well as lymphatics and capillaries. The muscularis mucosae consist of a thin layer of smooth muscle between the mucosa and the submucosa (Feldman, Friedman et al. 2010).

1.1.4 Differentiation of intestinal epithelial stem cells

The luminal surface of the intestine is lined by a simple columnar epithelium, folded into a number of deep cavities or crypts of Lieberkühn, and embedded in connective tissue. In healthy adults, colorectal epithelial cells undergo continual rapid renewal where cell loss is precisely balanced with cell proliferation (Kirkland and Henderson 2001; Potten, Booth et al. 2003). The continuous supply of new cells originates from undifferentiated multipotent stem cells, anchored at the base of the crypt.

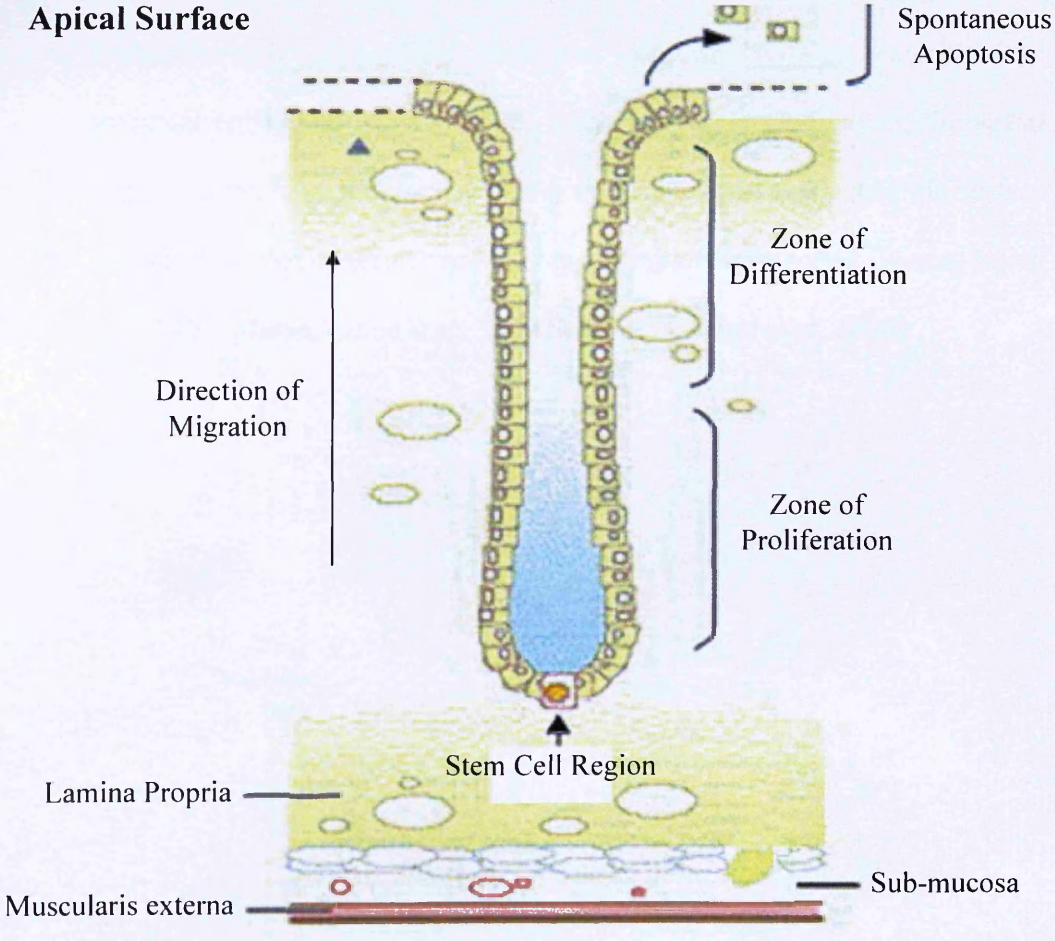


Figure 1-2 Intestinal epithelial stem cells become increasingly differentiated while migrating along the vertical axis of the crypt (Chell, Kadi et al. 2006)

These cells undergo a stringent programme of proliferation and differentiation as they migrate from the base of the crypt to the luminal surface yielding three epithelial lineages:

- Goblet cells – flat shaped cells that produce mucin protecting the epithelial surface and facilitating movement of food along the GI tract.
- Enteroendocrine cells – located in the intestinal crypt comprising ~1% of the epithelial cell population. There are at least 15 subtypes, each secreting several peptide hormones which regulate the physiological and homeostatic functions of the GI tract.
- Absorptive cells – also called colonocytes. These cells form the majority of the intestinal epithelium. A microvillus brush border develops along the apical surface as the cells differentiate along the upper crypt increasing the surface area across which molecules and ions can be rapidly transported (Potten, Booth et al. 2003; Moran, Leslie et al. 2008; Sternini, Anselmi et al. 2008).

1.1.5 Organisation of intestinal tissue

The entire surface of the colon is lined with a single layer of intestinal epithelial cells (IEC) that form intracellular tight junctions, preventing macromolecules and microbes from passing transversely across while guarding against opportunistic infections and pathogens. The immune system of the digestive tract is often referred to as the gut-associated lymphoid tissue (GALT) functioning to protect the body from invading organisms (Mayer 2000). The GALT is the largest collection of lymphoid tissues in the human body, due to the vast numbers of lymphoid and quantities of immunoglobulins produced. This is principally due to the colossal antigen load to which these cells are continually exposed to (Mayer 2000; Chehade and Mayer 2005; Forchielli and Walker 2005). GALT comprises of organised lymphoid tissues, such as mesenteric lymph nodes (MLN), Peyer's patches (PP); and more diffusely dispersed lymphocytes in the crypt region of the intestinal lamina propria (Forchielli and Walker 2005).

Figure 1-3 indicates that despite the epithelial surface, some bacteria are able to penetrate the IEC directly (1), whereas others use M (microfold) cells, which are positioned over lymphatic aggregates to contravene the barrier (2). Another newly discovered route of 'luminal sampling' uses uptake by dendritic cell (DC) projections that extend into the intestinal lumen (3) (Gewirtz and Madara 2001).

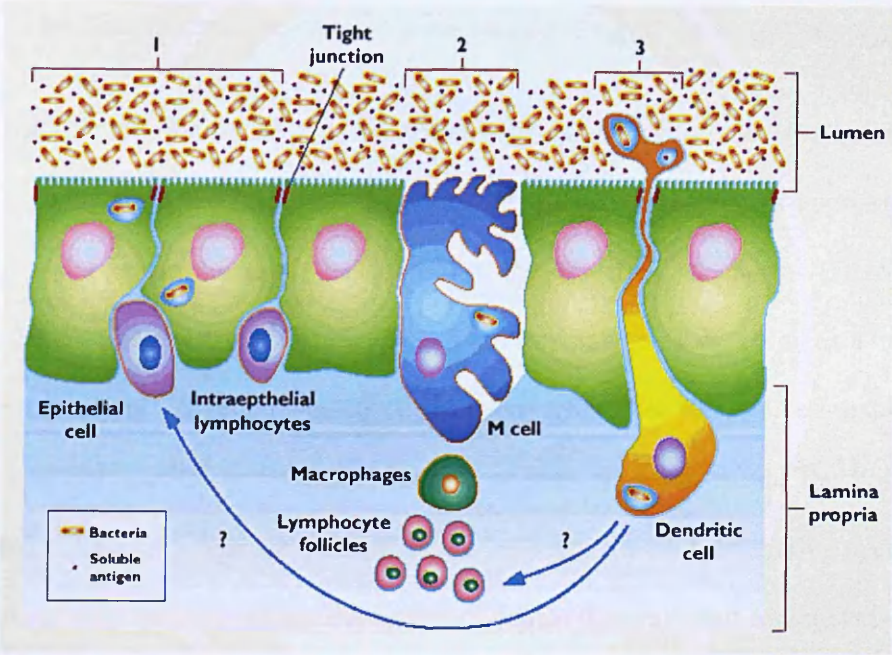


Figure 1-3 Diagram showing the mucosal layer of the colon, comprising the epithelium and the underlying lamina propria (Gewirtz and Madara 2001)

DCs express tight junction proteins such as claudin 1, occludin and zonula occludens 1, allowing them to send dendrites outside the epithelium by opening tight junctions, while maintaining epithelium integrity. These DCs are then able to acquire, process, and exhibit antigens present within the lumen (Rescigno, Rotta et al. 2001; Rescigno, Urbano et al. 2001).

1.1.6 The role of commensal flora in maintaining colonic physiology

Traditionally the function of the colon is to reclaim water and salts before they are eliminated from the body. By this phase of the digestive process approximately 90% of water is reclaimed by the colon, leaving 1.5-2L in the faeces (Harrell and Chang 2006). However, over recent decades the importance of the colon as a home to diverse microflora has been revealed. Commensal microflora has evolved making the colon a natural habitat with mutual benefits to both themselves and the host. The intestinal habitat of a healthy adult harbours between 300-500 distinctive species of microflora; with species varying between individuals (Guarner and Malagelada 2003; Fava and Danese 2011). Prevalent genera of commensals within the colon include *Bacteroides*, *Bifidobacterium*, *Clostridium* and *Eubacterium*. Whereas aerobes such as *Enterobacter*, *Enterococcus, Klebsiella, Escherichia* and *Lactobacillus* are among the subdominant genera (Pirzer, Schönhaar et al. 1991; Macpherson, Khoo et al. 1996; Guarner and Malagelada 2003). The number of microbial cells within the gut lumen is about 10 times greater than the number of eukaryotic cells in the entire human body (Bengmark 1998). The highest concentrations of genera found in the lumen are similar to those found in colonies growing under optimal conditions over the surface of laboratory plates, with concentrations peaking at 10^{11}-10^{12} flora per gram of luminal content (Guarner and Malagelada 2003; Abreu, Fukata et al. 2005). Almost 60% of faecal solid is comprised of bacteria (Stephen and Cummings 1980). Fluctuations in the composition of flora can be seen in cases of acute diarrheal illness, antibiotic treatment and to a lesser extent dietary interventions, however; an individual's flora composition usually remains stable (Brandtzaeg, Halstensen et al. 1989; Pirzer, Schönhaar et al. 1991; Guarner and Malagelada 2003).

The traditional function of the colonic microflora is the fermentation of non-digestible dietary deposits and endogenous mucus. Examples of such residues include large polysaccharides such as starch, pectin, cellulose and hemicelluloses (Cummings, Pomare et al. 1987). Oligosaccharides which have evaded the digestive process and unabsorbed alcohols and sugars are also exploited by gut flora. The outcome is the fabrication of short-chain fatty acids which can be utilised by IEC (Guarner and Malagelada 2003; Cummings, Beatty et al. 2007). Putrefaction of proteins and peptides by gut flora also generate short-chain fatty acids. Potential sources of peptides include collagen and elastin from dietary sources, sloughed epithelial cells, lysed bacteria and enzymes. A drawback to this process is the generation of a series of potentially toxic thiols, phenols, indols, ammonia, and amines all of which are generally absorbed by gut flora (Macfarlane, Cummings et al. 1986; Smith and Macfarlane 1996). The overall outcome of these processes is the reclamation of absorbable substrates and energy for the host, and a source of energy and nutrients for microflora. The symbiotic relationship provides approximately 100 calories of energy per day to the host (Smith and Macfarlane 1996; Guarner and Malagelada 2003; Munkholm 2003; Salminen, Bouley et al. 2007). Less traditional critical functions of host microflora include mucosal immune modulation and stimulation of epithelial repair and renewal.

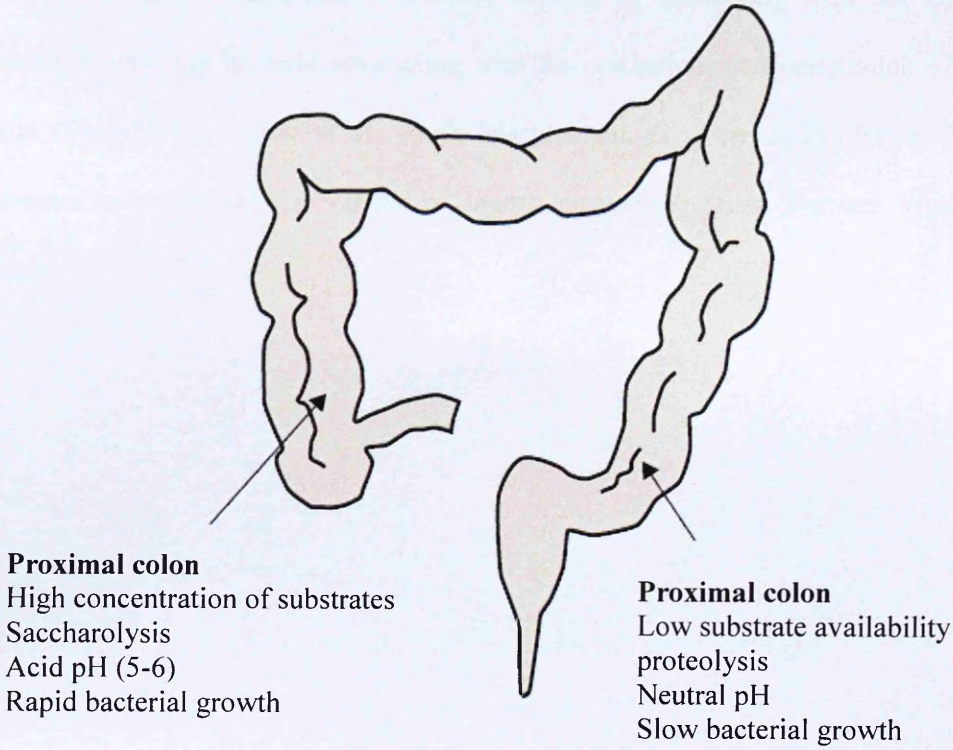


Figure 1-4 Fermentation within the colon (Guarner and Malagelada 2003)

The use of antibiotics is shown to upset the ecological balance and permit the excessive growth of the commensal *Clostridium difficile (C. difficile),* a species with potential pathogenicity linked to pseudomembranous colitis (Waaij 1989). *In vitro* studies have shown that commensal flora compete with each other for attachment sites in the brush border of IEC, preventing the attachment and subsequent invasion of pathogenic enteroinvasive bacteria (Bernet, Brassart et al. 1994). Additionally, these non-pathogenic floras compete for nutrient availability in ecological niches, limiting nutrient availability for pathogenic bacteria. Lastly, flora can also limit the growth of pathogenic bacteria by secreting antimicrobial substances termed bacteriocins (Lievin, Peiffer et al. 2000; Guarner and Malagelada 2003; Manco, Putignani et al. 2010). The immune system is not ignorant to microflora, the secretion of antibodies such as

immunoglobulin A (IgA) and T-cells are capable of interacting with our colonic microflora, limiting bacteria associating with the epithelium and penetration of host tissue (Macpherson, Gatto et al. 2000; Macpherson, Geuking et al. 2005). These processes occur through a variety of highly conserved innate immune receptors (1.2.1).

1.2 Interaction between intestinal epithelial cells and gut microbiota

1.2.1 Toll-like receptors and Toll-like receptor signalling

The intestinal immune system is highly evolved as its duel function protects the host from pathogenic organisms which could potentially cause infection, as well as coexistence with myriad commensal organisms. Studies have verified that microflora have an influential role in the preservation of IEC homeostasis and that the host is able to recognise and respond to luminal flora (Lee, Mo et al. 2006; McCole and Barrett 2007; Artis 2008). Mice bred in a germ-free environment display a decline in IEC proliferation in comparison to mice which have been colonised with conventional flora (Guarner and Malagelada 2003). Other studies demonstrate smaller specialised follicle structures, lower densities of lymphoid cells, and depleted levels of immunoglobulin within germ-free animal blood (Alam, Midtvedt et al. 1994; Abreu, Fukata et al. 2005).

Numerous papers have publicised that humans express pattern recognition receptors (PRRs) that recognise microbe-associated molecular patterns (MAMPs), especially those from pathogens, regulating the activation of both innate and adaptive immune responses (Akira, Yamamoto et al. 2003; Takeda, Kaisho et al. 2003; Takeda and Akira 2004; Beutler 2009). Cross-talk between commensal microflora and the host is mediated by Toll-like receptors (TLRs) (Abreu, Fukata et al. 2005; Abreu 2010; Brown, Wang et al. 2011). TLRs are a cohort of single membrane spanning protein receptors that together with the interleukin-1 receptor form a receptor superfamily, known as the 'interleukin-1 receptor/Toll-like receptor superfamily'; but they are generally identified as Toll-IL-1 receptors (TIR) (Kluwe, Mencin et al. 2009).

The link between TLRs and innate immunity was first identified in Drosophila. TLRs were initially recognised and classified as transmembrane receptors requisite for the establishment of dorso-ventral polarity in the developing embryo (Hashimoto, Hudson et al. 1988). Toll signalling in Drosophila displayed notable resemblance to the mammalian interleukin-1 (IL-1) signalling pathway, triggering the transcription of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), responsible for many features of inflammatory and immune responses (Belvin and Anderson 1996). Based on these parallels, it was suggested that TLR-mediated pathways could potentially be implicated in regulating immune responses. This was evidently confirmed in mutant Drosophila deficient in distinct components of the TLR signalling pathway. Mutant flies were found to be highly susceptible to fungal infection as they were unable to express drosomycin, an anti-fungal peptide (Lemaitre, Nicolas et al. 1996).

TLRs are highly conserved from Drosophila to humans, both sharing analogous structural and functional patterns. They are structurally characterised by the presence of a leucine-rich repeat (LRR) domain in their extracellular domain and a TIR domain in their intracellular domain (Rock, Hardiman et al. 1998). The LRR domain is separated from the transmembrane region by a carboxy-terminal domain. The TIR domain which spans about 200 amino acids, has contrasting degrees of similarity among TLRs and is required for intracellular signalling (Akira, Yamamoto et al. 2003; Kawai and Akira 2009). TLRs contain three box sequences which are highly conserved among family members. Box 1 is believed to be the conserved sequence among all TLRs, whereas both boxes 2 and 3 contain amino acids essential for signalling (Carpenter and O'Neill 2007).

TLRs are among the most investigated and described PRR, due to their ability to detect a variety of pathogen associated molecular patterns (PAMPs). To date, 10 TLRs have been identified in humans, with each TLR having a specific set of detectable ligands (Kawai and Akira 2007; Kumar, Kawai et al. 2009). The existence of a large number of TLRs enables the innate immune system to differentiate between PAMPs that are explicit to distinct microbial cohorts and instigate specific defence responses.

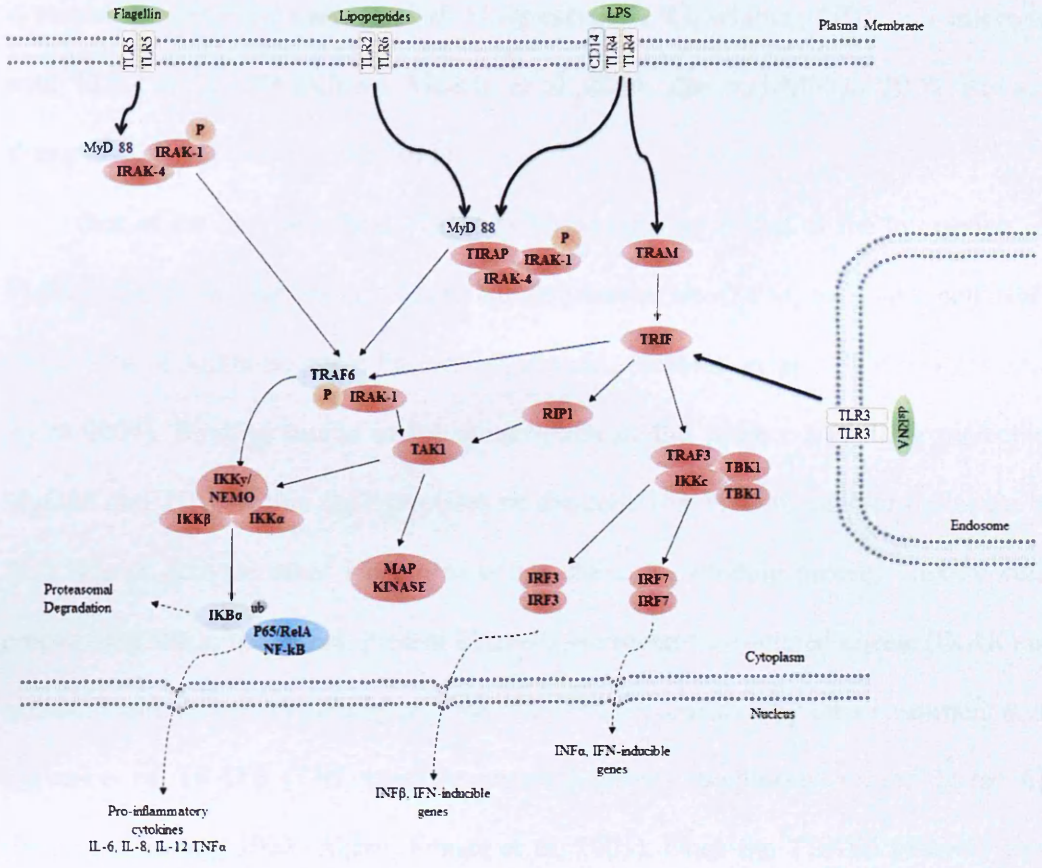


Figure 1-5 TLRs are involved in the recognition of microbial molecular patterns. Following the specific recognition of a microbial ligand by TLRs, various adaptor molecules are recruited to the TLR. This leads to the activation of signalling pathways, the transcription of inflammatory genes and the regulation of innate and adaptive immune responses. Adapted from (Zhu and Mohan 2010)

Although each TLR detects specific ligands, many of the signalling pathways that mediate intracellular responses are shared by most TLRs. For example, all TLRs signal through one or two adaptor molecules, myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor-inducing interferon-β (TRIF). MyD88

is part of the signalling cascade of all TLRs except TLR3, whereas TRIF only interacts with TLR3 and TLR4 (Kluwe, Mencin et al. 2009; Zhu and Mohan 2010; Brown, Wang et al. 2011; Lin, Li et al. 2011).

One of the best described TLR signalling pathways is that of the interaction of TLR4 with its corresponding ligand lipopolysaccharide (LPS), an outer cell wall constituent of Gram-negative bacteria (Takeuchi, Hoshino et al. 1999; Takeda and Akira 2004). Binding results in the conscription of the adaptor signalling molecule MyD88 and TRIF within the cytoplasm of the cell. The MyD88 adaptor molecule is then able to activate other molecules within the cell including protein kinases thus propagating the initial signal. Protein kinase IL-1 receptor-associated kinase (IRAK) is activated and phosphorylated by MyD88. This is then followed by the recruitment and activation of TRAF6 [TNF (tumour necrosis factor) receptor-associated factor 6] (Buer and Balling 2003; Abreu, Fukata et al. 2005). Once the TRAF6 pathway has been activated; the MAPK (mitogen-activated protein kinase) pathway is also activated due to an evolutionary conserved signalling intermediate within the TLR signalling pathway (Abreu, Fukata et al. 2005).

The upshot of this is the amplification of the initial signal and ultimately the stimulation or suppression of genes that coordinate the inflammatory response (Buer and Balling 2003). Activation of TLR4, and other TLRs lead to the transcription of a number of pro-inflammatory cytokines such as IL-6, IL-8, IL-22 and tumour necrosis factor α (TNFα) (Zhu and Mohan 2010). TLR4 has also been shown to induce cyclo-oxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) in both *vitro* and *vivo* studies; both being imperative for apoptosis and proliferation in response to intestinal mucosal injury (Fukata and Abreu 2007).

As illustrated in Figure 1-5, the mechanism by which TLR5 propagates its signal is similar to that of TLR4, but unlike TLR4, TLR5 does not require TRIF as an intracellular adaptor molecule to disseminate its signal (Kluwe, Mencin et al. 2009). Ligation of TLR5 also consequents in the activation of NF-$_k$B, driving the expression of numerous pro-inflammatory genes, such as neutrophil chemo-attractant IL-8, thus functioning to protect the cell (Vijay-Kumar, Aitken et al. 2008; Kluwe, Mencin et al. 2009).

TLR3 ligates with double stranded ribonucleic acid (dsRNA) and recruits its sole cytosolic adaptor molecule TRIF, initiating downstream signalling (Hoebe, Du et al. 2003; Leonard, Ghirlando et al. 2008). Subsequently, the transcription factors NF-$_k$B and interferon regulatory factor (IRF)-3 are activated and induce the secretion of interferon-β (IFN-β), as well as a number of other inflammatory cytokines (Leonard, Ghirlando et al. 2008).

TLR2 appears to be capable of distinguishing a significant range of microbes. This is somewhat due to its ability to form hetrodimers with TLR1 and TLR6. TLR2 and TLR6 activate downstream effector molecules similar to the previously mentioned TLRs (Kawai and Akira 2007; Zhu and Mohan 2010). TLR2$^{-/-}$ and TLR6$^{-/-}$ mice are shown to have reduced levels of IL-1β, TNFα and transforming growth factor-β (TGF-β), but elevated levels of IL-4 and IL-6 in response to *Mycobacterium tuberculosis* (*M. tuberculosis)* (Sugawara, Yamada et al. 2003).

1.2.2 TLR ligands

IEC express a cohort of PRRs, termed TLRs, which are able to communicate and respond to microbes, such as bacteria, viruses, parasites and their structural constituents (Kawai and Akira 2007; Kumar, Kawai et al. 2009). For the purposes of this study I shall be focusing on TLR2, TLR3, TLR4, TLR5 and TLR6 as these are the TLRs which are imperative for bacterial, viral and helminth detection.

1.2.2.1 TLR4 signalling

Bacteria are a large cohort of unicellular microorganisms typically a few micrometres in length. They can exist as different shapes from spheres (coccus) and rods (bacillus) to spirals. *Escherichia .coli (E. coli)* is a Gram-negative bacterium that is commonly found within the colon. Most strains of this bacterium are harmless; but some strains can cause critical food-poisoning within humans such as O157:H7 (Simon and Gorbach 1984; Borriello, Welch et al. 1988). The innocuous strains form a constitute of the normal gut flora and can aid the host by yielding vitamin K, or by occupying a niche thus averting the establishment of pathogenic bacteria within the colon (Prescott, Harley et al. 2002).

LPS is a large molecule formed from a lipid and a polysaccharide joined by a covalent bond, located on the outer membrane of Gram-negative bacteria. LPS is fundamental to the bacterium because it protects and helps stabilise the membrane structure (Prescott, Harley et al. 2002). However, LPS is also an endotoxin to humans as binding to TLR4 promotes the secretion of pro-inflammatory cytokines in many cell types (Song, *et al.*, 1999).

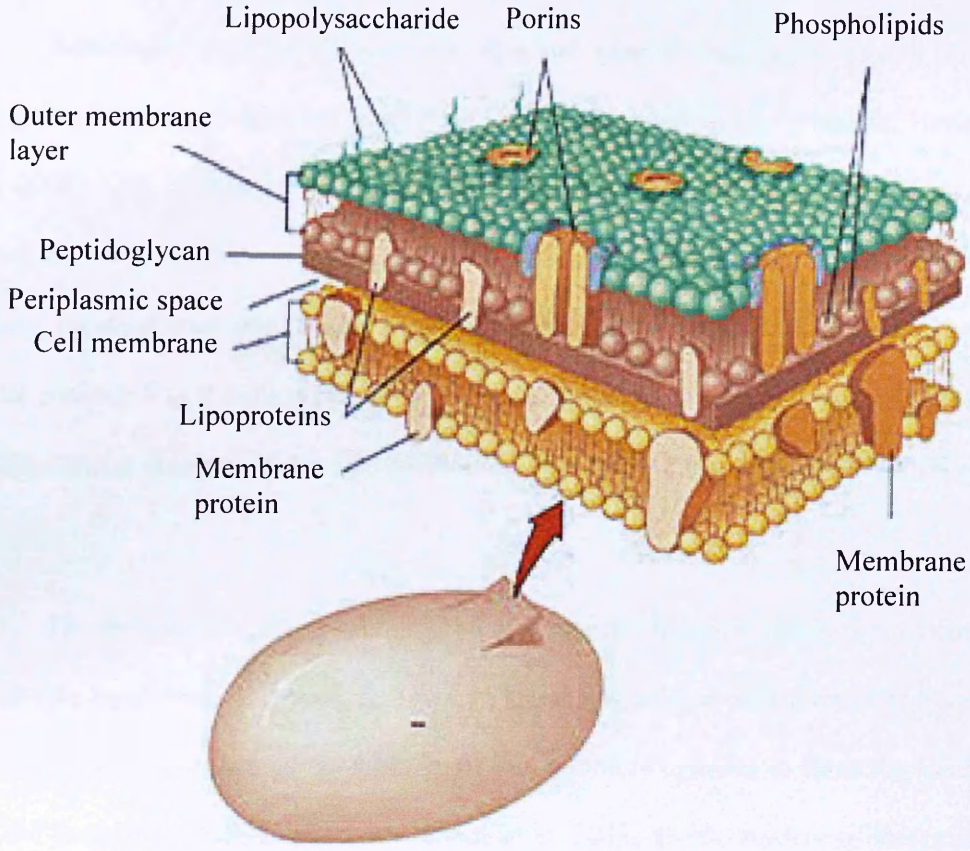


Figure 1-6 Cell membrane of a Gram-negative bacterium. The cell wall is composed of two main structures: the internal membrane (cytoplasmic membrane) and the outer membrane. A thin peptidogylcan cell wall and a periplasmic space that contains substrate-binding enzymes separate the two membranes. (Prescott, Harley et al. 2002)

As well as detecting LPS, TLR4 is also able to detect a number of other endogenous ligands such as High-mobility group protein B1 (HMGB1), fibronectin, hyaluronic acid fragments, antiphospholipid fragments and saturised fatty acids (Seki, Tsutsui et al. 2005)

1.2.2.2 TLR5 signalling

Salmonella enterica (S. enterica) is a rod shaped flagellated, aerobic Gram-negative bacterium which has more than 2000 serovars or strains (Prescott, Harley et al. 2002). One of these is *Salmonella typhimurium (S. typhimurium),* which can cause gastroenteritis within humans. This is caused by inflammation within the gastrointestinal tract and results in acute diarrhoea. Inept treatment of gastroenteritis kills between 5 to 8 million people world-wide each year, and is the principal cause of death among children under five (Simon and Gorbach 1984; Borriello, Welch et al. 1988).

The bacterial flagellum is composed of a filament that is attached to a molecular base (the basal body and hook complex). Flagellin, a protein with a mass of between 30,000 and 60,000 daltons, arranges itself into a hollow cylinder to form the filament in the bacterial flagellum (Samatey, Imada et al. 2001; Smith, Andersen-Nissen et al. 2003; Yonekura, Maki-Yonekura et al. 2003). The correct assembly of the flagella filament is fundamental for bacterial motility (Berg and Anderson 1973).

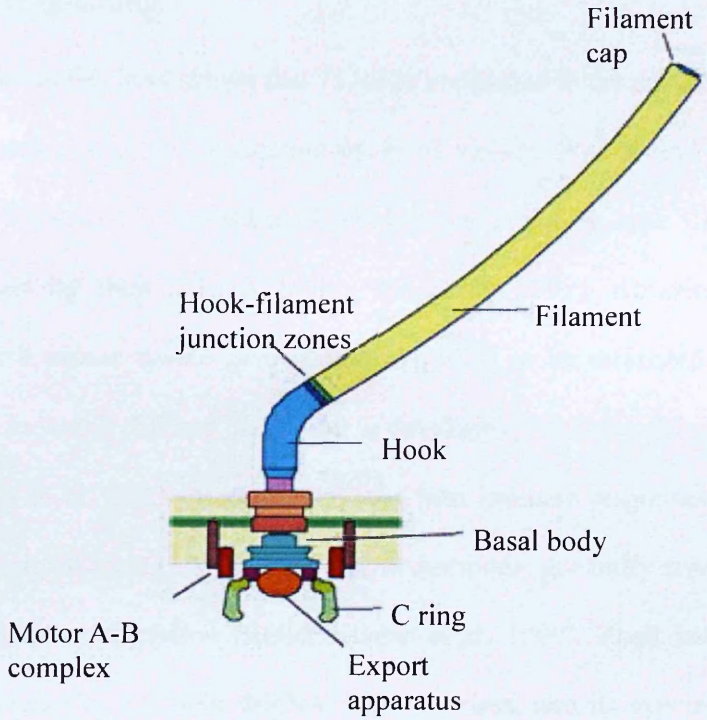


Figure 1-7 The bacterial flagellum consists of three parts, the basal body (within the cell envelope), the hook (blue) and the filament (yellow) constructed from multiple subunits of flagellin, the TLR5 ligand (Beatson, Minamino et al. 2006)

1.2.2.3 TLR3 signalling

Numerous studies have shown that TLR3 is implicated in the recognition of viral dsRNA, produced during the replication cycle of viruses (Kawai and Akira 2007; Beutler 2009). Rotavirus is a member of the *Reoviridae* family, type III viruses that are characterised by their dsRNA (Arias, Isa et al. 2002). Rotavirus is highly pathological as it causes severe gastroenteritis, leading to an estimated 600, 000 to 870,000 deaths in young children every year in developing countries (Morris and Estes 2001; Arias, Isa et al. 2002). Rotavirus evades host immune responses resulting in altering the functional activity of IEC, leading to diarrhoea, generally considered to be linked to enterocyte obliteration (Rollo, Kumar et al. 1999). Zhou and colleagues (2007) have shown that genomic dsRNA from rotavirus, and its synthetic analogue polyinosinic:polycytidylic acid (Poly I:C), provokes severe mucosal damage of the small intestine (Zhou, Wei et al. 2007). Ligation of TLR3 expressing IEC with dsRNA are also shown to upregulate the secretion of IL-15, causing an increase in intestinal epithelial lymphocytes (IELs) and augmenting the cytotoxicity of IELs (Zhou, Wei et al. 2007). TLR3-deficient mice are shown to have a limited response to synthetic Poly I:C (Alexopoulou, Holt et al. 2001). These results imply that as well as dsRNA, its synthetic analogue poly I: C is able to cause a breakdown of mucosal homeostasis when ligating with its complementary receptor TLR3.

1.2.3.4 TLR2/6 signalling

Gastrointestinal nematode parasites cause some of the most prevalent human diseases world-wide. Infections tend to be chronic, with high rates of reinfection after drug treatment. *Trichuris trichiura (T. trichiura)*, the human whipworm currently infects more than 1 billion people globally (De Silva, Chan et al. 1997). Most of our current understanding of immune-regulatory mechanisms underlying resistance or susceptibility to *T. trichiura* infection has come from the laboratory *Trichuris muris (T. muris)*, a parasite mouse model as both share similar cross-reactive antigens (Else and deSchoolmeester 2003). The parasite generally lives partially buried within the epithelium of the large intestine, forming syncytial tunnels through which they move and feed. *T. muris* excretory/secretory proteins (ES) form a heterogeneous solution of worm proteins containing substances that are shown to express structures bearing mannose and *N*-acetylglucosamine residues; products recognised by colonic epithelial TLR (TLR2, TLR4 and TLR6) (deSchoolmeester, Martinez-Pomares et al. 2009). TLR2 is involved in the recognition of a diverse array of microbial molecules, representative of a broad group of species such as Gram-positive and Gram-negative bacteria, as well as yeast and mycoplasma. TLR2 is able to recognise a number of components such as lipoteichoic acid, lipoprotein and peptidoglycan, all cell-wall components of gram-positive bacteria as well as lipoarabinomannan from mycobacteria and zymosan from yeast (Takeuchi, Hoshino et al. 1999; Ozinsky, Underhill et al. 2000; Morr, Takeuchi et al. 2002). Unlike other TLR, TLR2 is able to form hetrodimers with TLR1 and TLR6 expanding the ligand spectrum enabling the innate immune system to identify diverse structures present in various pathogens (Farhat, Riekenberg et al. 2008).

Numerous studies have demonstrated exposure to helminths prevent 2.4,6-trinitrobenzene sulfonic acid (TNBS)-type colitis (Khan *et al.*, 2002; Elliot *et al.*, 2003; Moreels *et al.*, 2004); suggesting helminth products promote mucosal healing in animal models of colitis. Other studies have shown after exposure to *T. muris,* some mice strains expel the parasite and develop resistance (e.g. BALB/c and BALB/k). Whereas, other strains of mice fail to expel the parasite permitting chronic infection. Numerous studies have also indicated resistance or susceptibility to *T. muris* is dependent upon T-helper 2 (Th2) and Th1 immune responses, respectively (Else, Hültner et al. 1992; Else, Finkelman et al. 1994; Helmby, Takeda et al. 2001).

Mice infected with *T. muris* also display a profound increase in the rate of epithelial cell turnover, termed 'epithelial escalator' during parasite expulsion (Cliffe, Humphreys et al. 2005). One study showed an almost doubling of IEC proliferation, whereas only an insignificant elevation in IEC proliferation in mice which did not expel the parasite. This was most notable in IL-$13^{-/-}$ mice, which did not expel the parasite displaying a reduced rate of IEC turnover, similar levels to those observed in naturally susceptible wild-type mice (Bancroft, Artis et al. 2000). The expression of IFN-γ produced in mice which do not naturally expel the parasites and initiate a Th1 response acts not only to counter-regulate the potential protective Th2 response, but through the induction of C-X-C motif chemokine 10 (CXCL10), slows the turnover of IEC. *In vitro* studies have shown blocking CXCL10 increases IEC turnover rate, causing worm expulsion without altering the current Th1 cytokine response generally observed in susceptible mice (Cliffe, Humphreys et al. 2005).

Studies have also shown IL-4, IL-9 and IL-13 all play an important role in parasite expulsion (Bancroft, McKenzie et al. 1998; Bancroft, Artis et al. 2000), whereas IFN-γ is associated with chronic infection (Lee, Wakelin et al. 1983; Else,

Finkelman et al. 1994). *In vivo* studies have shown that obstruction of TNFα in normal resistant mice significantly delays worm expulsion for the duration of treatment. Furthermore, knockdown of the TNFα receptor in mice does not allow parasite expulsion, initiating a predominant Th1 response, indicating lack of TNF signalling considerably modifies the phenotype of the response (Artis, Humphreys et al. 1999).

1.2.3 Establishment of gut microbiota

All organs within the human body undertake a remarkable transition at birth, from an isolated intra-uterine environment to the drastically distinctive environment of the outside world. This is also observed in the GI tract of a foetus where up until birth, the GI tract is sterile. However, upon delivery and swiftly thereafter, there is a rapid transition to primary colonisation of the neonatal GI tract, posing a critical challenge to the immune system of the newborn. Studies have shown that the gut can be colonised by as much as 10^8 to 10^{10} bacteria per gram of faeces within the first few days of birth (Bettelheim, Breadon et al. 1974; Bezirtzoglou 1997; Fanaro, Chierici et al. 2003). The GI tract is initially colonised by facultative aerobes. But after consuming all the oxygen, these bacteria create an anaerobic environment making it a favourable environment for strict anaerobic species, generally belonging to the *Clostridium, Bacteroides* and *Bifidobacterium* generas. As these anaerobes multiply, the facultative bacteria are no longer able to tolerate the environmental changes and consequently, decline in number (Adlerberth and Wold 2009; O'Toole and Claesson 2010). Studies have shown children born by vaginal delivery have higher levels of *Bifidobacteria* and lower levels of *C. difficile* compared to infants born by caesarean section (Grölund, Lehtonen et al. 1999; Penders, Thijs et al. 2006). Studies have also shown hospitalisation and prematurity are associated with increased counts of *C. difficile* (Penders, Thijs et al. 2006). Other factors which influence the composition of intestinal microbiota in neonates are modes of infant feeding and hygiene measures (Orrhage and Nord 1999). The microbiota of breast-fed children are dominated by *Bifidobacteria*, perhaps due to the presence of *Bifidobacteria* growth factors in breast milk (Coppa, Bruni et al. 2004; Morelli 2008). Infants also have significantly lower levels of *C. difficile, E. coli, B. fragilis* group species, and *Lactobacilli* than formula-

fed infants (Penders, Thijs et al. 2006). However, after introduction of solid foods and weaning, the microflora of breast-fed infants become comparable to that of formula fed infants (Penders, Thijs et al. 2006; Manco, Putignani et al. 2010).

Given the limited exposure to antigens *in utero,* the infant to a significant extent must depend upon the innate immune system for protection, as the innate immune system can direct the adaptive immune response (Janeway Jr and Medzhitov 2002; Firth, Shewen et al. 2005; Levy 2007). The GI tract of the foetus and newborn also express TLRs on their IEC surface to detect the presence of microbial components (Imler and Hoffmann 2001; Akira, Yamamoto et al. 2003; Doyle and O'Neill 2006). Human small IEC are known to express basolateral TLR2 and TLR4 at 18 to 21 weeks after gestation (Fusunyan, Nanthakumar et al. 2001). Other studies have shown human and mouse tissue cultured foetal IEC express TLR4 and MyD88, with elevated NF-κB activation and production of CXCL8 in comparison to adult IEC (Nanthakumar, Fusunyan et al. 2000; Lotz, Gütle et al. 2006). The robust inflammatory response to LPS could cause a significant risk after birth, when the newborn is rapidly being colonised by microflora (Fanaro, Chierici et al. 2003; Penders, Thijs et al. 2006).

Exposure of perinatal IEC to LPS has been shown to result in the loss of IEC responsiveness to LPS. This was linked to down regulation in the expression of IRAK1, a crucial intermediate of TLR4 signalling (Lotz, Gütle et al. 2006). Mice which were delivered vaginally, in comparison to caesarean mice, were more exposed to the mother's microflora and quickly became unresponsive to LPS by down regulating the signal transduction protein IRAK1. However, mice which were delivered via caesarean and therefore had no previous exposure to gut flora, were shown not to downregulate IRAK1 and remained responsive to LPS (Chassin, Kocur

et al. 2010). The authors of this study showed that the regulation of IRAK1 is mediated by microRNA mIR-146a, which is itself upregulated in response to LPS. This suggests upregulation of mIR-146a in neonates is fundamental for protecting the gut from mucosal damage after induction of large concentrations of LPS when the gut becomes colonised. (Chassin, Kocur et al. 2010).

The developmental expression of TLR4 in neonates is of great importance as this may lead to necrotizing enterocolitis (NEC), an intestinal disease that ensues in preterm newborns (Jilling, Simon et al. 2006; Lin and Stoll 2006). Failure of preterm neonates to correctly downregulate responses to LPS appears to significantly contribute to neonates susceptibility to NEC. Neonatal intestinal immunity can be radically altered by breastfeeding. It has been suggested that breast-milk helps mediate intestinal immunomodulation permitting sub-clinical infections that progressively inspires immunological memory while limiting inflammation (Newburg 2005; Newburg and Walker 2007). Human breast-milk also contains factors which influence TLR signalling, such as soluble TLR2, acting as a decoy receptor, competitively inhibiting signalling through membrane bound TLR2 (LeBouder, Rey-Nores et al. 2003).

The innate immune system in newborns is highly immature, which results in increased susceptibility to numerous infections. However, the immune system of the gut does allow the colonisation of microflora building up tolerance and establishment of mucosal homeostasis. Mechanisms by which IEC develop tolerance will be investigated in chapter 6.

1.2.4 Oral tolerance

Miscellaneous interactions between the epithelium, GALT and microflora are implicated in sculpting the memory mechanism of systematic immunity. Following antigen feeding, systematic administration of the same antigen results in a reduced inflammatory response compared with unfed, control mice. This occurrence has been termed 'oral tolerance' (Higgins and Weiner 1988; Chen, Kuchroo et al. 1994). Microflora have also been implicated in oral tolerance, as this effect persists for several months in mice with conventional flora, whereas in germ-free mice systematic unresponsiveness persists for only a few days after ingesting the same antigen (Moreau and Gaboriau-Routhiau 1996). Germ-free mice orally administered with ovalbumin (OVA) as a tolerogen, before a systematic dose with OVA, show an abrogated Th1 mediated immune response such as reduction of IgG2a and IFN-γ, while the Th2-mediated immune response is maintained generating IgE, IgG1 and IL-4 against OVA. This aberration can be remedied after colonisation with conventional flora, such as *Bifidobacterium*, one of the predominant intestinal floras. This restores the susceptibility of these Th2 responses to oral tolerance induction, but the process is only effective when such reconstitutions are performed in neonates, not in mice of an older age (Sudo, Sawamura et al. 1997; Guarner and Malagelada 2003). The results from this study indicate intestinal microflora play a fundamental role in generating a Th2 immune response, and are altogether quite susceptible to the induction of oral tolerance, perhaps influencing development of the immune system at the neonatal stage.

Endotoxin shock is often a fatal consequence of bacterial infection, that may occur when LPS enters the blood stream (bacteremia), causing a systemic

inflammatory reaction. However, some studies have shown that moderate concentrations of LPS do not induce TLR4-mediated responses in several human IEC lines such as HT-29, Caco-2 and NCM460 (Rakoff-Nahoum, Paglino et al. 2004; Rhee, Keates et al. 2004; Rhee, Kim et al. 2006), indicating IEC are able to tolerate higher exposure levels of LPS than many other cell types. Potential mechanisms of this hyporesponsiveness include studies suggesting mouse intestinal crypt epithelial mIC_{c12} cells harbour TLR4 at the golgi apparatus rather than at the cell surface. This consequently means LPS has to be internalised before the signal can be propagated, therefore receptors are shielded from direct interaction with the immense levels of LPS within the lumen (Hornef, Normark et al. 2003; van Aubel, Keestra et al. 2007).

1.3 Suppressor of Cytokine Signalling – 3

1.3.1 Structure and function of Suppressor of cytokine signalling - 3

Cytokines are known to regulate many physiological responses and conserve homeostasis; they do this by influencing proliferation, differentiation, and functional activity of cells of the immune system, as well as those of most other tissues and organs. The kinetics and extent of cytokine signalling via the Janus Kinase (JAK)/ signal transducer and activator of transcription (STAT) pathway must be tightly controlled. The suppressor of cytokine signalling (SOCS3) family comprising eight members, SOCS1 to SOCS7 and cytokine inducible SH2 (CIS); are a collection of intracellular proteins, whose expression is induced in response to a wide range of growth factors, cytokines and hormones (Alexander and Hilton 2004; Piessevaux, Lavens et al. 2008). SOCS proteins were initially identified as inhibitors of the JAK-STAT signalling pathway, operating in a negative feedback loop (Endo, Masuhara et al. 1997; Krebs and Hilton 2001). This hypothesis was supported when $SOCS1^{-/-}$ mice succumbed to a multifaceted neonatal inflammatory disease, illustrated by the hyperactivation of T-cells and hyper-responsiveness to IFN-γ (Naka, Matsumoto et al. 1998; Alexander, Starr et al. 1999; Marine, Topham et al. 1999). $SOCS2^{-/-}$ mice display enhanced growth, characteristic of a heightened response to insulin-like growth factor-1 (IGF-1) and/or growth hormones (Horvat and Medrano 2001). $SOCS3^{-/-}$ knockdown results in mid-gestation embryonic lethality due to placental insufficiency; mice are also found to display disproportionate erythropoiesis (Marine, McKay et al. 1999; Roberts, Robb et al. 2001). Furthermore, embryonic lethality can be rescued by substituting the SOCS3-deficient placenta with $SOCS3^{+/+}$ placenta, signifying an essential role of SOCS3 in placental development (Takahashi, Carpino et al. 2003).

The SOCS3 protein is composed of three domains, a variable N-terminal region, a central Src homology 2 (SH2) domain and a 40 amino acid carboxyl–terminal module, termed the SOCS box. The N-terminal domain contains an extended SH2 subdomain (ESS) that aids the SOCS3 protein with substrate binding (Yasukawa, Sasaki et al. 2000; Alexander 2002; Kile, Schulman et al. 2002; Babon, McManus et al. 2006).

The kinase inhibitory region (KIR) of the SOCS3 protein is located in the N-terminal, adjacent to the SH2 domain. The KIR domain contains a 12 amino acid region, which is proposed to act as a pseudo-substrate, binding to the catalytic cleft, thus blocking subsequent JAK enzymatic activity (Croker, Kiu et al. 2008). Although SOCS3 interacts with both JAK and receptor, a two-step model of interaction has been proposed whereby the SOCS3-SH2 domain is first recruited to the cytoplasmic domain of the receptor, this is followed by a subsequent bi-model binding to JAK through the KIR and SH2 domain resulting in a higher affinity interaction, thus impeding the enzymatic activity of JAK (Krebs and Hilton 2001; Croker, Kiu et al. 2008). Point mutations in KIR are show to abrogate the actions of SOCS3 without affecting SH2 domain binding (Sasaki, Yasukawa et al. 1999; Yasukawa, Sasaki et al. 2000).

Studies have identified two small regions at the N-termini and C-terminus of the SOCS3-SH2 domain. These are termed N- and C-extended SH2 subdomain (N-ESS and C-ESS, respectively); both are required for phosphotyrosine binding (Sasaki, Yasukawa et al. 1999; Yasukawa, Sasaki et al. 2000; Croker, Kiu et al. 2008). The N-ESS forms a 15-residue alpha helix, which directs the phosphotyrosine-binding loop influencing its orientation. Conservation of these important residues indicates N-ESS

is highly likely to be a mutual structural feature among SOCS-SH2 domains (Babon, Yao et al. 2005; Babon, McManus et al. 2006; Croker, Kiu et al. 2008).

All SOCS proteins contain a 40 amino acid SOCS box motif, which in most cases, is located in the C-terminus of the protein. The SOCS box consists of three α-helices packed together (Croker, Kiu et al. 2008; Piessevaux, Lavens et al. 2008).

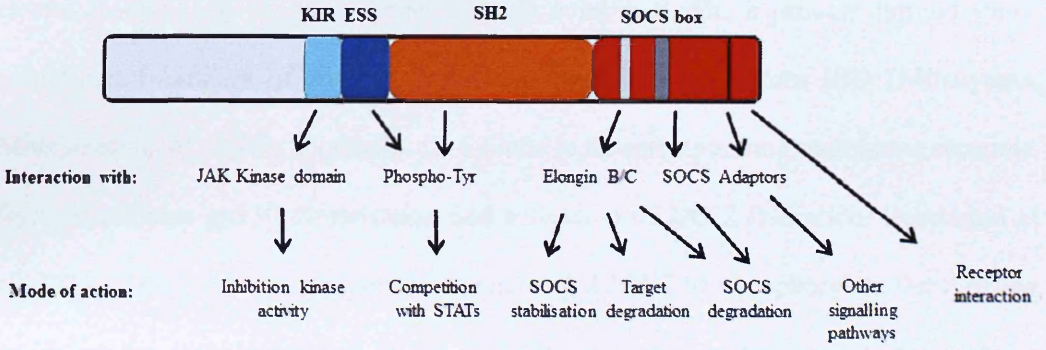


Figure 1-8 Diagram depicting the various domains of the SOCS3 protein, sites of interaction and mode of action. Adapted from (Piessevaux, Lavens et al. 2008)

Perturbations in SOCS3 are shown in several intestinal pathologies including inflammatory bowel disease (IBD) and colorectal cancer (CRC). IBD is a term used to describe a cohort of diseases in which there is chronic recurrent colonic inflammation within the bowel, Ulcerative colitis (UC) is a phenotype of this, and is associated with an increased risk of developing CRC (Eaden and Mayberry 2000; Eaden, Abrams et al. 2001). Destruction of the epithelial layer can be caused by chronic inflammation. Cytokines released in response to inflammation stimulate the proliferation of IEC to compensate for the loss of IEC. This chronically stimulated state of the IEC layer may ultimately lead to the development of UC-CRC (Sartor

2006; Schottelius and Dinter 2006; Li, de Haar et al. 2010). There is considerable evidence to suggest cytokines, predominantly IL-6 directly stimulate the proliferation of IEC (Schneider, Hoeflich et al. 2000). Elevated levels of IL-6 have been detected in both serum samples and colonic biopsy specimens of patients with UC, with levels of IL-6 correlating with disease severity (Chung and Chang 2003; Carey, Jurickova et al. 2007). A mutation in the IL-6 receptor (IL-6R) is associated with an increased risk of colon cancer in humans (Landi, Gemignani et al. 2006). In addition to the membrane bound IL-6R, IL-6 can also signal through soluble IL-6R, a process termed trans-signalling. Inhibitors of soluble IL-6R are known to ameliorate IBD (Mitsuyama, Matsumoto et al. 2006). Typically, IL-6 binds to its corresponding membrane receptor, ligation initiates gp130 dimerisation and activation of JAK2 (Heinrich, Behrmann et al. 2003). This subsequently permits the activated JAK2 to phosphorylate the tyrosine reside on the cytoplasmic tail of the receptor, presenting a docking site for proteins with an SH2 domain, for instance, the STAT3 protein (Mitsuyama, Matsumoto et al. 2006). STAT3 is able to bind to the receptor where it is activated through phosphorylation through JAK, leading to homo-hetro-dimerisation of STAT3 (pSTAT3). Mice with mutated STAT3 binding sites are shown to have impaired healing after acute injury with dextran sulphate sodium (DSS) (Tebbutt, Giraud et al. 2002). Mice with mutated binding sites on gp130 are found to develop spontaneous gastric tumours associated with increased STAT3 (Judd, Alderman et al. 2004). The pSTAT3 hetero-dimers translocate to the nucleus, where they interact with specific DNA sequences and induce the transcription of target genes (Krebs and Hilton 2001; Yoshimura, Naka et al. 2007; Li, de Haar et al. 2010).

SOCS3 is transcribed by the IL-6/pSTAT3 pathway and serves as a major negative feedback inhibitor of the JAK/STAT signalling cascade (Suzuki, Hanada et

al. 2001; Li, de Haar et al. 2010). STAT3 activates the transcription of *Socs3*mRNA by binding to the promoter region of the *Socs3* gene (Aman and Leonard 1997; Starr, Willson et al. 1997). SOCS3 then translocates out of the nucleus where it inhibits signalling by binding to STAT3 no longer allowing it to bind to the receptor (Schmitz, Weissenbach et al. 2000). SOCS3 also inhibits signalling by binding to phosphotyrosine sites on the cytoplasmic domain of the receptor and blocking STAT3 recruitment to the receptor. The mechanism of SOCS3 action was established by investigating the primary SOCS3-binding sites within the gp130 signalling pathway (Nicholson, Willson et al. 1999). This was achieved by establishing the affinity to which SOCS3 binds to several phosphopeptides derived from the JAKs, STATs and the gp130 subunit of the IL-6R. It was discovered that SOCS3 binds with highest affinity to a phosphopeptide in an area surrounding Y757 within the gp130, while exhibiting weaker binding affinity to STAT3. Furthermore, when Y757 is mutated to phenylalanine the inhibition of SOCS3 on gp130 is greatly reduced (Nicholson, Willson et al. 1999; Schmitz, Weissenbach et al. 2000).

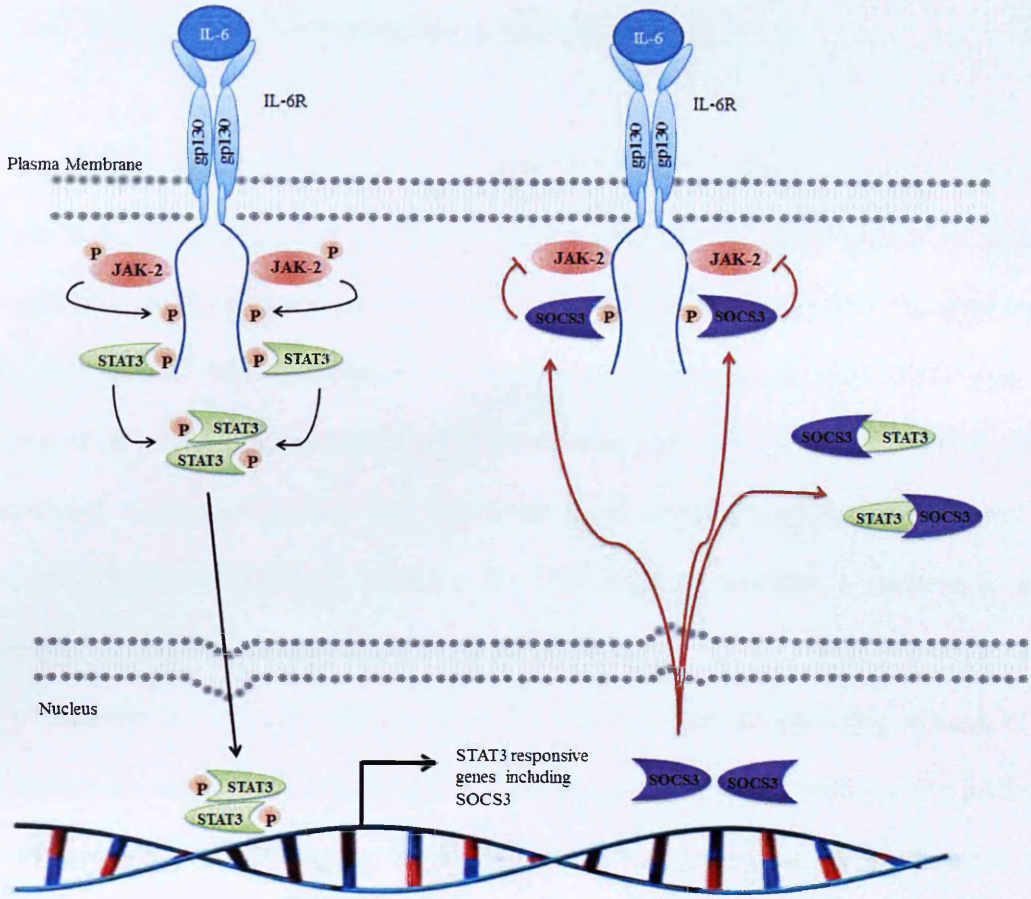


Figure 1-9 The molecular mechanism by which SOCS3 negatively regulates IL-6 signalling. Adapted from (Yoshimura, Naka et al. 2007)

1.3.2 Role of SOCS3 in mediating TLR signalling

SOCS3 appears to influence secondary effects of LPS by modulating cytokine signalling, but may also play a role in direct regulation of TLR signalling. The majority of studies determining the role of SOCS3 in TLR signalling have focussed on immune cells (Yoshimura, Ohishi et al. 2004; Qin, Roberts et al. 2007; Yoshimura, Naka et al. 2007). Macrophages stimulated with LPS trigger the production of numerous cytokines including IL-6 and IL-10; all of which upregulate the expression of SOCS3. These cytokines influence the LPS response in either a positive or a negative manner (Dimitriou, Clemenza et al. 2008).

Studies have shown SOCS3 is a key regulator of the opposing actions of cytokines in macrophages. IL-6R and IL-10R signalling pathways both involve JAK-STAT3 signalling (Yoshimura, Ohishi et al. 2004). IL-6 is a pro-inflammatory cytokine and plays a functional role in many inflammatory diseases. Whereas IL-10 is an immunoregulatory cytokine, which has anti-inflammatory properties (Dimitriou, Clemenza et al. 2008). Although both cytokines exhibit opposite effects, both require the transcription factor STAT3 for functional activity (Takeda, Clausen et al. 1999).

SOCS3 binds to phosphorylated Y759 on the IL-6R subunit gp130, thus uncoupling IL-6 mediated STAT3 activation (Nicholson, De Souza et al. 2000; Yasukawa, Ohishi et al. 2003). In contrast, SOCS3 does not bind to IL-10R. Yasukawa and colleagues (2003) showed that macrophages lacking the *SOCS3 gene* or possessing a mutation in the gp130 binding site cause prolonged STAT3 activation and an anti-inflammatory response similar to that observed in response to IL-10 (Yasukawa, Ohishi et al. 2003). Therefore, SOCS3 is vital in defining the dichotomous effects of LPS-induced IL-6 and IL-10 responses in macrophages. However, the exact

mechanism by which SOCS3 function results in differential STAT3 activity remains unclear.

Taken together, these studies indicate SOCS3 may not directly target TLR signalling intermediates in immune cells, but may influence secondary effects of TLR signalling by modulating cytokine signalling in macrophages. In the subsequent chapters I will investigate the role of SOCS3 in regulating TLR-mediated intestinal epithelial homeostasis and repair.

1.3.3 Role of SOCS3 in proteosomal degradation

Zhang and colleagues (2008) have suggested that SOCS3 may also promote the degredation of specific signalling proteins. They had suggested that the SOCS box, located at the C-terminal of SOCS3, acts as an adaptor molecule that recruits activated signalling molecules to the proteosome (Zhang, Farley et al. 1999). SOCS3 associates with a complex containing elongins B and C (elongin BC) via the SOCS box (Kamura, Sato et al. 1998; Zhang, Farley et al. 1999). This subsequently allows cullin-2 to associate with elongin BC to form the E3 ligase scaffold. As SOCS3 contains an SH2 domain that interacts with tyrosine-phosphorylated signalling molecules, SOCS3 then acts as an adaptor that facilitates ubiquitination and subsequent degredation of the associated signalling molecule (Krebs and Hilton 2001; Babon, Sabo et al. 2008; Linossi and Nicholson 2012).

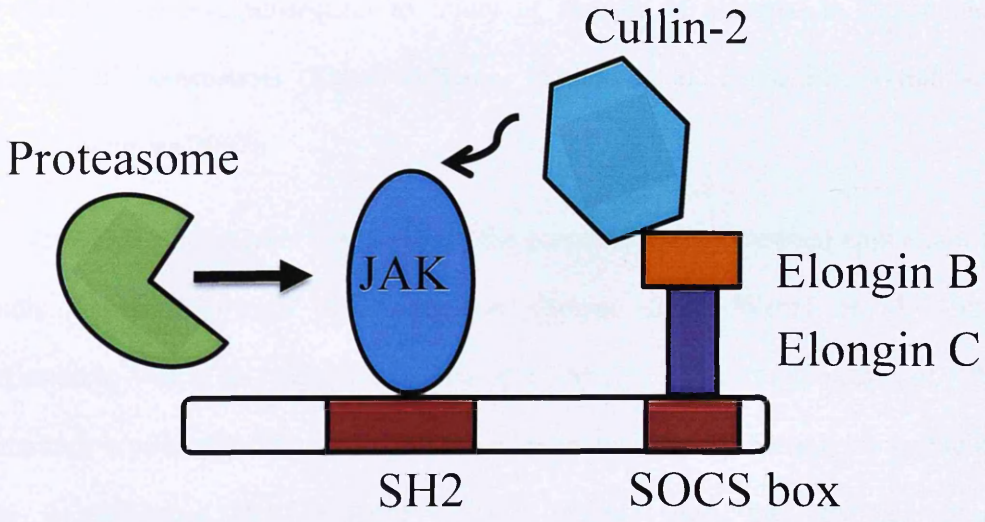


Figure 1-10 Proposed mechanism by which SOCS3 targets signalling molecules for proteosomal degradation by the proteasome (Krebs and Hilton 2001)

1.4 Tissue homeostasis and repair following damage

1.4.1 Intestinal epithelial homeostasis and repair following injury

The mucosal surface epithelial cells of the alimentary tract are highly proliferative with complete turnover every 4-5 days (Tsujii and DuBois 1995). The proliferative compartment is localised to the crypt region and is separated from a gradient of increasingly differentiated epithelial cells along the functional villus compartment (Dignass 2001). The integrity of the mucosal surface of the colon is essential in forming a barrier between the diverse immunogenic and noxious compounds present within the gastrointestinal lumen and the underlying mucosal immune system (Sturm and Dignass 2002). Damage or impairment of the intestinal surface barrier is observed in the course of a number of diseases, and these may lead to an increased infiltration and immunological intolerance of luminal contents, leading to inflammation and disequilibrium of homeostasis of the host. Thus, rapid resealing of the mucosal surface, subsequent to injury or damage is essential in maintaining conventional homeostasis (Rakoff-Nahoum, Paglino et al. 2004; Ben-Neriah and Schmidt-Supprian 2007).

Numerous studies have demonstrated the potential of the intestinal epithelium to rapidly re-establish itself after extensive damage (Feil, Wenzl et al. 1987; McCormack, Viar et al. 1992; Nusrat, Delp et al. 1992). The continuity of the epithelium is re-established by at least three distinctive phases. Firstly, the epithelial cells neighbouring the damaged surface migrate into the denuded area, dedifferentiate form pseudopodia-like structures, re-organise their cytoskeleton and then re-differentiate after closure of the damaged wound. This process is termed restitution and can arise within minutes to hours after injury in both *in vitro* and *in*

vivo. Restitution is followed by the second phase proliferation; this is when IEC divide to replenish the reduced cell numbers. Thirdly, the undifferentiated epithelial cells mature and differentiate to maintain the functional activity of the epithelium (Dignass 2001; Dignass, Baumgart et al. 2004; Sturm and Dignass 2008).

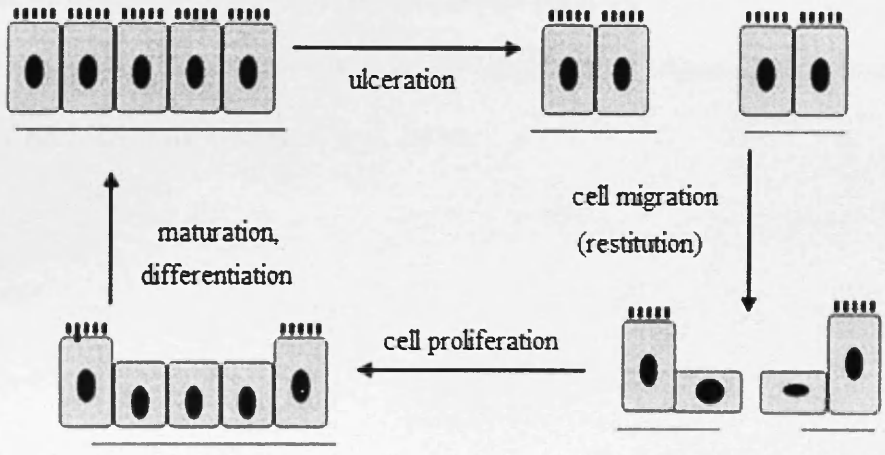


Figure 1-11 Simplified model of repair of superficial epithelial cell injury within the intestine (Dignass 2001)

The illustration provides a simplified model of repair to better understand the physiology and pathophysiology of intestinal epithelial wound repair. However, the three distinctive phases do overlap and moreover, additional repair mechanisms that involve inflammatory processes may also be requisite as a result of deeper lesions or penetrating injuries (Dignass 2001).

The repair of the intestinal surface barrier is regulated by a broad spectrum of structurally distinct regulatory peptides, non-peptide factors, extracellular matrix factors and direct cell-cell interactions. Cytokines and growth factors have both been

shown to play a role in regulating differential epithelial cell functions to conserve habitual homeostasis and integrity of the intestinal epithelium. Epidermal growth factor (EGF), TGF-α, and TGF-β to name a few have been shown to enhance epithelial cell restitution (Podolsky 1997; Wilson and Gibson 1997). Other compounds such as short-chain fatty acids, phospholipids and adenine nucleotides, have also been shown to modulate intestinal epithelial repair mechanisms. Some of these factors are released by injured or dying IEC, whereas others reach the intestinal mucosa via the intestinal lumen or bloodstream (Wilson and Gibson 1997; Dignass 2001; Sturm and Dignass 2002; Dignass, Baumgart et al. 2004).

1.4.2 TLR signalling and intestinal epithelial regeneration

In addition to their role in mammalian host defence from adverse microbes, TLRs are also involved in several features of mammalian homeostasis such as development, the recognition of cellular tissue injury, tissue repair and regeneration (Zhang and Schluesener 2006; Rakoff-Nahoum and Medzhitov 2008). Other studies have demonstrated that TLR signalling is essential for maintaining tissue integrity and repair of damaged tissue in models of chemical, radiation and colonic injury (Fukata, Michelsen et al. 2005; Rakoff-Nahoum and Medzhitov 2008). Studies in wound healing models have shown that the absence of TLR4 had notable consequences on both the inflammatory and repair phases of lesions (Mollen, Anand et al. 2006). This can be supported by both Rakoff-Nahoum (2004) and Abreu (2005) who have both indicated that mice deficient in TLR4 or MyD88 are at an increased risk of epithelial injury following the application of DSS (Rakoff-Nahoum, Paglino et al. 2004; Abreu, Fukata et al. 2005). Similarly, gut-sterilised mice exhibit a promotion in intestinal injury and DSS-induced death as MyD88-defecient mice inferring PAMPs from intestinal microbiota stimulate cytoprotective pathways to prevent epithelial injury (Rakoff-Nahoum, Paglino et al. 2004; Kluwe, Mencin et al. 2009). Studies have also shown germ-free mice have a reduced rate of crypt cell production, with crypts also containing fewer cells than mice colonized with conventional flora; indicating commensal flora effect IEC proliferation and subsequent repair (Alam, Midtvedt et al. 1994).

Numerous studies have shown the importance of TLR5 signalling to be paramount for maintaining tight junction assembly and cytoprotection within IEC layers (Jones, Edwards et al. 2004; Otte, Cario et al. 2004; Vijay-Kumar, Sanders et al.

2007). Vijay-Kumar and colleagues (2008) had shown that mice systematically injected with purified flagellin do not display the clinical features of inflammation. Furthermore flagellin is shown to shield mice against pathological, chemical and ionizing radiation (Vijay-Kumar, Aitken et al. 2008). More recent studies have also supported the role of flagellin pre-treatment in protecting the intestinal mucosa from injury resulting from irradiation-induced apoptosis (Jones, Sloane et al. 2011).

Numerous studies have demonstrated that exposure to helminths prevents TNBS-type colitis (Khan *et al.,* 2002; Elliot *et al.,* 2003; Moreels *et al.,* 2004); suggesting helminth products promote mucosal healing in animal models of colitis. Colitis is supressed by inhibiting inflammatory cytokines, such as TNFα and IL-12 or stimulating the secretion of immunoregulatory cytokines such as TGF-β and IL-10 (Elliott, Summers et al. 2007; Ruyssers, De Winter et al. 2008).

Looking at the molecular mechanism coupling TLR signalling to epithelial homeostasis, it has been shown that stimulation of epithelial TLR induce the activation of a group of receptors with tyrosine kinase activity. Notably, all four members of the epidermal growth factor receptor (EGFR) family, including ErbB-1, ErbB-2, ErbB-3 and ErbB-4 display elevated phosphorylation levels (Shaykhiev, Behr et al. 2008). EGFR is known to play a predominant role in mediating wound healing induced by TLR ligation in IEC, since specific obstruction of the receptor tyrosine kinase (RTK) associated TLR completely abolishes the effect of ligation on epithelial migration and repair (Rakoff-Nahoum, Paglino et al. 2004).

In addition to recognising PAMPs, TLR are also able to recognise damage-associated molecular patterns (DAMPs), a group of endogenous molecules released from injured or inflamed tissue; implying TLR may also appear to regulate

inflammatory responses during the wound healing process. Such DAMPs that are able to ligate with TLR include hyaluronan, HMGB1, S100 protein 60 and heat shock proteins (Miyake 2007; Kluwe, Mencin et al. 2009). However, there is still on-going debate with respect to some endogenous ligands, as many of these have either been purified in bacterial systems or have a high affinity to bacterial products, such as LPS, which proposes that the bacterial products rather than the suggested ligands themselves mediate their TLR-activating effect (Tsan 2007; Kluwe, Mencin et al. 2009).

Previous studies have demonstrated that flagellin treatment is also a potent activator of host defence/cytoprotective gene expression in IEC (Zeng, Carlson et al. 2003; Zeng, Wu et al. 2006). Research conducted by Vijay-Kumar (2008) and colleagues have demonstrated that clinical features, such as loss of body weight, shortening of the colon and robust inflammation in mice induced by the ingestion of DSS were all greatly abrogated by a single administration of flagellin upon initial exposure to DSS indicating flagellin can protect the gut from chemical injury (Vijay-Kumar, Aitken et al. 2008).

1.4.3 TLR signalling in epithelial regeneration of other tissues

The involvement of TLRs in the regeneration of other tissues has also been studied recently. Shaykhiev and colleagues (2008) had found that *Staphylococcus aureus (S. aureus)* signals via TLR2 and TLR5, both signalling pathways triggering a cohort of non-immune lung epithelial cell responses including cell migration, proliferation, wound repair, and survival of epithelial cells (Shaykhiev, Behr et al. 2008). Jiang and colleagues (2005) had shown that TLR signalling is required for liver regeneration after partial hepatectomy; with other studies exhibiting the significance of TLR signalling displaying protection from bleomycin- and hyperoxic-induced liver injury (Jiang, Liang et al. 2005; Seki, Tsutsui et al. 2005). In the central nervous system, TLR have also been shown to coordinate the protective responses after axonal and crush injuries of the brain and spinal cord (Babcock, Wirenfeldt et al. 2006; Kigerl, Lai et al. 2007; Kim, Kim et al. 2007).

1.5 Role of microbes in intestinal diseases

1.5.1 Inflammatory bowel disease

The ability of the host to initiate an inflammatory response following injury or exposure to foreign antigens is imperative for host homeostasis and survival. However, a continually heightened immune response such as that perceived in chronic inflammatory conditions severely weakens host organ activity, eventually leading to disease progression (Medzhitov 2008). IBD refers to chronic inflammation that affects the GI tract (Barbosa and Rescigno 2010; Maloy and Powrie 2011). There are two principal clinical forms of IBD, Crohn's disease (CD) characterised by transmural, patchy inflammation generally affecting the ileum and the colon, but can occur anywhere within the GI tract; and ulcerative colitis (UC) which is more superficial with pathology restricted to the colonic mucosa (Podolsky 1991; Hanauer 2006; Strober, Fuss et al. 2007). Manifestations of both diseases include abdominal pain, diarrhoea rectal bleeding, and malnutrition. Studies have shown that the incidence of CD is 8/100, 000 and UC 11/100, 000 within the UK population (Garcia Rodriguez, Gonzalez-Perez et al. 2005).

The exact aetiology of IBD remains undecided, but three distinct categories are known to contribution to disease pathology; these have been identified as: the host immune system, genetic factors and environmental factors such as the gut microbiota (Baumgart and Carding 2007; Hill and Artis 2009). Swidsinski and colleagues (2007) have shown that the colonic mucus layer of patients with IBD is greatly reduced, and unevenly distributed in patients with colitis, leaving significant areas of the epithelium exposed to luminal contents (Swidsinski, Loening Baucke et al. 2007). This may be attributed to a decline in the population of goblet cells as observed in the colonic epithelium of patients with UC (Kolios, Valatas et al. 2002).

Patients with IBD have also been reported to exhibit less diversity in microflora, possibly associated with a relatively lessened proportion of protective bacteria. The molecular-phylogenetic classification of colonic microflora permitted the identification of phyla selectively depleted in biopsy specimens of IBD patients, namely the *Firmicutes* group (Manichanh, Rigottier-Gois et al. 2006; Sokol, Pigneur et al. 2008). A number of other studies have shown *Yersinia enterocolitica (Y. enterocolitica)* and other species of *Yersinia* are associated with CD (Lamps, Madhusudhan et al. 2003; Saebo, Vik et al. 2005; Zippi, Colaiacomo et al. 2006). Overall, at least 18 different bacterial species or genera have been implicated in CD (Man, Kaakoush et al. 2011).

TLR play a major role in the detection of microbiota, additionally stimulation of these receptors is requisite in maintaining intestinal epithelial homeostasis, often interrupted in IBD (Fukata and Abreu 2007; Danese and Mantovani 2010). Studies have shown mutations in TLR4 are associated with CD and UC (Fukata, Michelsen et al. 2005). Other studies have shown that TLR4 is upregulated in IEC of active UC and CD patients (Cario and Podolsky 2000). TLR4 signalling induces COX-2, PGE_2 and reactive oxygen species (ROS) fundamental for the development of CRC and colitis associated cancer (CAC) (Gupta and DuBois 2001; Fukata 2006; Wang and DuBois 2009). Several studies have also found that polymorphisms in TLR4 (D299G) are associated with increased risk of both UC and CD (Franchimont, Vermeire et al. 2004; Török, Glas et al. 2004; Gazouli, Mantzaris et al. 2005; Ouburg, Mallant-Hent et al. 2005).

Numerous studies have demonstrated flagellin is endotoxic to humans as binding to TLR5 instigates the transcription of pro-inflammatory genes, which may play a role in triggering acute flares of inflammation in IBD (Kluwe, Mencin et al. 2009). The

implication of flagellin in IBD, has been supported by polymorphisms in TLR5 negatively correlating with CD and commensal-derived flagellin being identified as a dominant antigen to this disorder (Erridge, Duncan et al. 2010).

A number of studies have shown that the expression profile of other TLRs, such as TLR2, TLR3 and TLR8, are dissimilar between IEC of patients with CD and non-IBD controls (Cario and Podolsky 2000; Szebeni, Veres et al. 2008; Steenholdt, Andresen et al. 2009). Taken together, emerging evidence suggests that defective TLR sensing of microbial components may lead to susceptibility to CD and UC.

IBD is sometimes described as a dysregulated immune response to commensal microflora in genetically susceptible individuals. Numerous studies have shown that there is a strong relationship between inflammation and progression to colon cancer, as observed in patients with IBD (Itzkowitz and Yio 2004; Yang and Pei 2006). The severity of inflammation correlates with the risk of cancer in IBD patients (Itzkowitz 2002; Rutter, Saunders et al. 2004). Mouse models of IBD have shown commensal flora are required for the initiation of colitis and the promotion of dysplasia or cancer (Sellon, Tonkonogy et al. 1998; Itzkowitz and Yio 2004). Conversely, germ-free rats are less prone to dysplasia and cancer following exposure to carcinogens (Reddy, Narisawa et al. 1976; Laqueur, Matsumoto et al. 1981).

1.5.2 Role of microbes in colorectal cancer

Colorectal cancer is one of the most common causes of cancer morbidity and mortality within the western world; with 36, 500 new cases being diagnosed each year within the UK. Almost three quarters of these cases arise in individuals over the age of 75 (UK 2013).

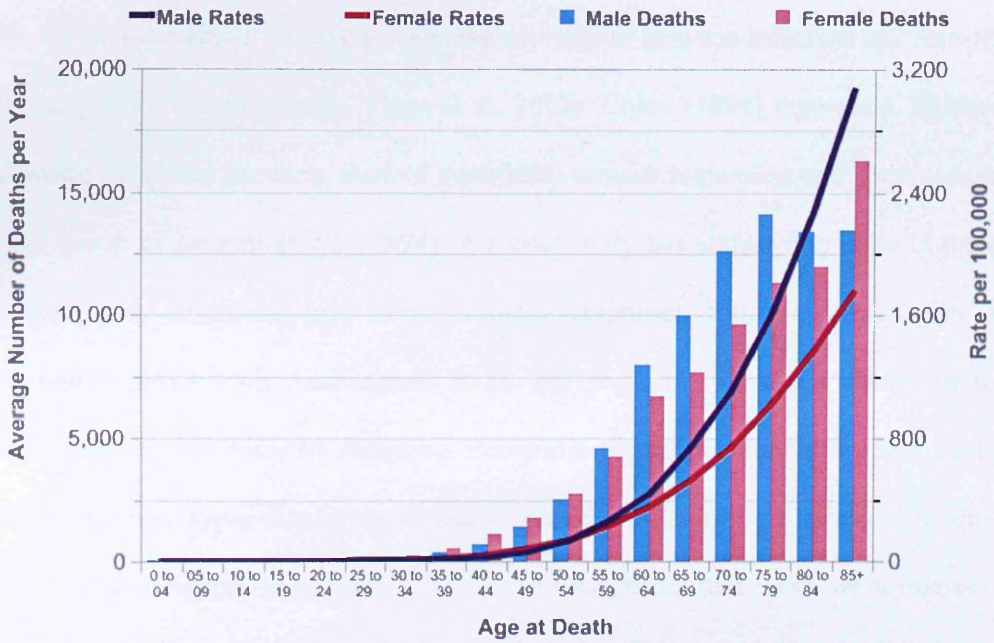


Figure 1-12 Average number of deaths per year and age-specific mortality rates of colorectal cancer, by sex, UK, 2008-2010 (UK 2013)

Over the last few decades there has been a steady increase in the incidence of colon cancer within the western world (Slattery 2000; Giovannucci 2002). This may be attributed to changes in diet and the types of bacteria that reside within our gut (Munkholm 2003). Although there is no recognised bacterial pathogen for human colorectal cancer; some bacteria have been shown to cause infection which may herald

cancer. *Streptococcus bovis (S. bovis)* is an element of the human enteric flora and patients with colon cancer have an increased faecal carriage of *S. bovis* in contrast to healthy patients or those with non-malignant enteric disease. It has been suggested that *S. bovis* carriage maybe associated with the promotion of intestinal carcinogenesis (Klein, Warman et al. 1987; Ellmerich, Schöller et al. 2000; Biarc, Nguyen et al. 2004).

The role of microbes in mediating an anti-tumour effect was first recognised in the 18th when Deidier observed a positive correlation between infection and remission of malignant cancers (Garay, Viens et al. 2007). Coley (1894) then went further by showing microbial products showed significant tumour regression and even cure in a large group of patients (Coley 1894). A recent study has shown that both TLR4 and MyD88 play a crucial role in anti-tumour responses following irradiation and chemotherapy (Apetoh, Ghiringhelli et al. 2007). TLR4$^{-/-}$ mice are shown to have significantly larger tumours following oxaliplatin therapy or irradiation than control-mice, with both types displaying similar levels of growth in the absence of treatment (Apetoh, Ghiringhelli et al. 2007). It has been suggested that there are a number of mechanisms through which TLR agonists mediate their anti-tumour activity. TLR agonists are shown to directly kill both tumour cells and ancillary cells located in the tumour microenvironment (Andaloussi, Sonabend et al. 2006; Salaun, Coste et al. 2006; Rakoff-Nahoum and Medzhitov 2008). TLR activation can also lead to tumour regression by increasing vascular permeability, recruitment of leukocytes, activation of natural killer- (NK-) and cytotoxic T-cells, and increasing the sensitivity of tumour cells to assisted killing through TNFα, TRAIL and perforin/granzyme B (Smyth, Dunn et al. 2006; Akazawa, Ebihara et al. 2007; Garay, Viens et al. 2007).

Studies have shown that TLR ligands enhance the growth of adoptively transferred tumours (Harmey, Bucana et al. 2002; Luo, Maeda et al. 2004; Sfondrini, Rossini et al. 2006; Huang, Zhao et al. 2007). A spontaneously metastasising mammary adenocarcinoma cell line was first injected into a mouse model and LPS was then systematically injected into the mouse. The results showed LPS administration increased both migration and invasion to secondary locations (Harmey, Bucana et al. 2002). A similar model, but using a colonic adenocarcinoma cell line, showed intraperitoneal injection of LPS displayed an increase in proliferation and a decrease in apoptosis of metastatic tumours (Luo, Maeda et al. 2004). Other studies have shown TLR4 expression is strongly upregulated in the epithelial compartment of tumours of patients with UC and colon cancer, as well as in murine models of inflammation-associated bowel cancer (Fukata, Chen et al. 2007). Furthermore, $TLR4^{-/-}$ mice display overwhelmingly reduced dysplasia, number and size of tumours (Fukata, Chen et al. 2007). Similar findings have also been reported in a study which explored the role of Myd88 in colon cancers. MyD88 deficiency was found to lessen carcinogenesis in $Apc^{Min/+}$ mice, a non-inflammatory model of colon cancer as well as in azoxymethane (AOM) models. The study also showed MyD88-deficiency depicted a reduction in size and number of polyps. Additionally, although polyps showed parallel levels of proliferation between MyD88 and wild-type mice, higher levels of apoptosis was observed in polyps of the MyD88- deficient mice in comparison to control mice (Rakoff-Nahoum and Medzhitov 2007).

Rhee and colleagues (2008) investigated whether TLR5 signalling inspires innate immune responses in regulating anti-tumour activity in mouse xenograft models of human colon cancer. They showed blocking TLR5 dependent signalling significantly promoted tumour growth and impeded tumour necrosis whereas ligation

of TLR5 with its ligand flagellin drastically regressed tumour progression. Indicating engagement of TLR5 with flagellin elicits inhibitory activity against human colon adenocarcinoma cells (Rhee, Im et al. 2008).

1.5.3 Role of microbes in necrotising enterocolitis

Necrotising enterocolitis (NEC) is among the most common and distressing diseases in neonates. NEC was first described over a century ago, but the disease has still remained an enigma. Pathogenesis of NEC is still unconfirmed; treatment is problematic and regularly proves ineffective; with no successful prevention approach agreed upon. An epidemiological study conducted by Rees and colleagues (2010) had shown that NEC prevalence decreases significantly with increasing birth rate and increasing gestation (Rees, Eaton et al. 2010). There are a multitude of NEC-like conditions with a diverse presentation of symptoms. However, the most characteristic signs and symptoms of 'classical' NEC in preterm infants include abdominal distension, feeding intolerance and bloody stools 8 to 10 days after birth (Neu and Walker 2011).

The pathophysiology of NEC remains poorly understood. However, epidemiological studies strongly indicate a multifactorial cause (Claud and Walker 2001). Immature intestinal motility and digestion could possibly predispose preterm infants to NEC. Animal and human foetal studies have suggested that the development of GI motility commences in the second trimester, but matures in the third trimester (Sase, Lee et al. 2005; Sase, Miwa et al. 2005). Studies have shown premature infants have immature motility patterns when compared with full-term infants, although enteral feeding can mature these responses (Berseth 1989; Ittmann, Amarnath et al. 1992; Al Tawil and Berseth 1996; Lin and Stoll 2006). Studies in animal models of NEC and human foetal-cell cultures have proposed that foetuses and preterm infants have a disproportionate inflammatory response to microbial flora (Otte and Podolsky 2004).

Another hypothesis suggested to initiate NEC is the inappropriate initial microbial colonisation of the GI tract in preterm infants, predominantly since NEC occurs about 8 to 10 days after birth, at a time when anaerobic bacteria have colonized the intestinal lumen (Claud and Walker 2001). Additionally, experimental NEC does not transpire in germ-free animals (Morowitz, Poroyko et al. 2010). The evaluation of faecal microflora from unaffected preterm infants and infants suffering from NEC has shown that the ailment is associated with both unusual intestinal microbial species and a global reduction in the diversity of microbiota (Vollaard and Clasener 1994; Wang, Hoenig et al. 2009; Mshvildadze, Neu et al. 2010). The reduction in microbial diversity and alteration in microbial species may limit colonisation resistance, making the infant more susceptible to colonisation by hospital-acquired pathogens that can cause intestinal inflammation. Additionally, commensal flora as well as pathogens have been shown to elicit a disproportionate inflammatory response as compared with mature enterocytes (Claud, Lu et al. 2004). The disparity in responses appears to be arbitrated to a developmental immaturity in the expression of I_kB (a molecule that impedes NF-$_k$B activated cytokines) (Claud, Lu et al. 2004; Neu and Walker 2011).

Numerous strategies have been proposed for the prevention of NEC. These approaches include using enteral antibiotics; withholding enteral feeding and feeding infant milk expressed from mothers (Neu and Walker 2011). A recent study reported probiotics decrease the incidence of NEC, but not mortality from NEC. Another newly proposed preventative strategy for NEC is to supplement feeding with prebiotics, or nutrients that have the potential to enhance the growth of beneficial intestinal microflora (Gibson, Probert et al. 2004). The efficacy of prebiotics as a preventative measure for NEC is still unclear because prebiotics enhance the proliferation of endogenous flora, such as *Bifidobacteria.* However; they demand appropriate

preliminary colonisation of the gut, that which is lacking in NEC infants (Moro, Minoli et al. 2002).

1.6 Probiotics and prebiotics

1.6.1 Probiotics

The term probiotic refers to 'a live microbial food ingredient that is beneficial to health' (Salminen, Bouley et al. 1998). Examples of probiotics include selected lactic acid bacteria (LAB) such as *Lactobacillus*, *Streptococcus* and *Bifidobacterium* which can be ingested in fermented milk products or as a supplement (Rafter 2003; Rijkers, Bengmark et al. 2010). The health-promoting benefits credited to probiotics are widespread, and include serum cholesterol reduction, the improvement of constipation and alleviation of symptoms due to lactose intolerance (Rafter 2003).

In addition to these favourable effects, several experimental observations have also implied a potential protective effect of LAB against the progression of colon tumours (Sanders 1994; Brady, Gallaher et al. 2000; Wollowski, Rechkemmer et al. 2001). In animals, LAB ingestion was shown to block carcinogen-induced pre-neoplastic lesions and tumours (Goldin, Gualtieri et al. 1996; Challa, Rao et al. 1997; Rowland, Rumney et al. 1998). Additionally, direct evidence for anti-tumour properties of LAB have been attained using pre-implanted tumour cells in animal models. Studies indicate feeding cultures containing LAB or fermented milk inhibits the growth of tumour cells injected into mice (Kato, Kobayashi et al. 1981). In addition to a probable role in the prevention of cancer, probiotics have also been suggested to boost the immune system and impede the growth of existing tumours (Geier, Butler et al. 2006). Probiotics containing LAB are shown to increase the survival rate of mice injected with tumour cells, correlating with an increase in the number of NK cells, T cells, MHC class II+ cells and CD4-CD8+ T cells (Lee, Kim et al. 2004).

Epidemiological studies looking at the association between fermented milk products and colorectal cancer have found that the consumption of large quantities of dairy products containing *Bifidobacterium* and *Lactobacillus* may correlate with lower incidence of colon cancer (Shahani and Ayebo 1980). In a two population-based case study of colon cancer, an inverse relationship was observed for yogurt when the study was adjusted for possible co-founding variables (Peters, Pike et al. 1992). In another case-control study, an inverse relationship was observed again between the consumption of yogurt and risk of colon adenomas in both males and females (Boutron, Faivre et al. 1996).

There have been a number of clinical trials that have demonstrated that probiotics improve health in patients with IBD for both CD and UC patients (Venturi, Gionchetti et al. 1999; Guslandi, Mezzi et al. 2000; Kruis, Frič et al. 2004; Tursi, Brandimarte et al. 2004; Furrie, Macfarlane et al. 2005; Karimi, Peña et al. 2005). Guslandi and colleagues (2005) had shown that patients given *Saccharomyces boulardii (S. boulardii)* as a probiotic displayed relapse in only 6% of CD patients *vs.* 38% of patients given conventional treatment only (Guslandi, Mezzi et al. 2000). Another study showed probiotic treatment given to UC patients was just as effective as conventional treatments (mesalazine and balsalazide) in maintaining remission (Kruis, Frič et al. 2004; Tursi, Brandimarte et al. 2004). Other studies have also noted the anti-inflammatory properties of probiotics; such as improvement of clinical appearances of chronic inflammation in patients, decreases in TNFα, improvement of histology in TNBS models of rat colitis, reduction in the number of activated T-lymphocytes in the lamina propria of CD mucosa and reduced production of pro-inflammatory cytokines to name a few (Sheil, McCarthy et al. 2004; Furrie, Macfarlane et al. 2005; Peran, Sierra et al. 2007). Probiotics have also been shown to decrease luminal pH in patients

with CD thus antagonising pathogenic bacteria; as well as promoting the production of peptides that inhibit a broad range of pathogens such as *Staphylococcus, Enterococcus, Listeria, Bacillus* and *Salmonella* (Venturi, Gionchetti et al. 1999; Flynn, van Sinderen et al. 2002).

1.6.2 Prebiotics

A prebiotic was first defined as 'a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health (Gibson and Roberfroid 1995). Since its introduction, there have been a number of oligosaccharides and polysaccharides that have claimed to have prebiotic activity, without due consideration to the criteria required. Not all carbohydrates are prebiotics, and clear criteria need to be recognised for the classification of foods as prebiotics. These criteria are:

(1) Resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption;
(2) Is fermented by intestinal microflora;
(3) Selectively stimulates the growth and/or activity of intestinal bacteria associated with health and well-being.

Common prebiotics include inulin, oligosaccharides, lactulose and resistant starch. Dietary fibre has also been shown to convey prebiotic responses (Lim, Ferguson et al. 2005). Numerous studies have suggested colonic microflora are a risk factor for colon cancer. However, modifying the metabolic activity or composition of colonic microflora through the use of prebiotics could be a preventative measure in reducing the prevalence of colorectal cancer. Mechanisms by which prebiotics may bestow beneficial effects include detoxification of exogenous carcinogens, decreasing luminal pH, improving solubility of and uptake of ions, namely magnesium, calcium and iron; and modulation of immune responses (Lim, Ferguson et al. 2005).

Numerous studies have looked at the protective effects of prebiotics, namely fructooligosaccharides and inulin on colon cancer. Femia and colleagues (2002) had reported that the expression of genes involved in colon carcinogenesis - Glutathione *S*-transferase (GST) and GST placental enzyme pi type, were lowered when rats were fed with prebiotics individually, and in combination with *Bifidobacterium lactis (B. lactis)* Bb12 and *Lactobacillus rhamnosus (L. rhamnosus)* (Femia, Luceri et al. 2002). Additionally, inducible nitric oxide synthase (iNOS), found to play a significant role in colon tumour growth was also reduced in tumours of mice fed with prebiotics (Ahn and Ohshima 2001). COX-2, an enzyme generally upregulated in cancer, is also found to be increased in the tumours of control rats but not in those fed with prebiotics (DuBois, Radhika et al. 1996; Ahn and Ohshima 2001).

Numerous studies have observed the effect of prebiotics on experimental colitis (Ewaschuk and Dieleman 2006; Geier, Butler et al. 2007). Inulin and lactulose have both been shown to attenuate inflammation in IL-$10^{-/-}$ mice and DSS-induced colitis respectively (Madsen, Doyle et al. 1999; Videla, Vilaseca et al. 2001; Ewaschuk and Dieleman 2006). Another study, again using lactulose showed a dose-dependent positive effect of lactulose on DSS-induced colitis in rodents, including improvement in areas of colonic ulceration, reduced diarrhoea, increased body weight, and a reduction in myeloperoxidase activity and microscopic colitis (Rumi, Tsubouchi et al. 2004). In another experiment of TNBS-induced colitis, rats which were fed for two weeks with lactulose prior to the induction of colitis had reduced myeloperoxidase activity, colonic TNFα and leukotriene B production (Camuesco, Peran et al. 2006). Some studies have explored the use of an insoluble mixture of hemicellulose-rich dietary fibre and glutamine-rich protein termed germinated barley foodstuff (GBF) on epithelial repair. Fukudo and colleagues (2002) had discovered that feeding GBF to

rodents with DSS-induced colitis significantly reduced colonic inflammation scores and increased butyrate concentrations in cecal contents of rodents (Fukuda, Kanauchi et al. 2002). Another study in rodents with DSS-induced colitis also observed similar results, and further concluded that the dietary fibre component rather than the protein component of GBF is responsible for the beneficial effects of GBF (Kanauchi, Mitsuyama et al. 2003).

There have been a number of prebiotics investigated in clinical trials, primarily in UC patients (Ewaschuk and Dieleman 2006; Geier, Butler et al. 2006; Geier, Butler et al. 2007). GBF has been shown to lower the severity of UC both clinically and endoscopically (Bamba, Kanauchi et al. 2002). GBF has also been found to prolong the period of remission in UC and stimulate the growth of *Bifidobacterium* and *Eubacterium* (Kanauchi, Suga et al. 2002; Kanauchi, Mitsuyama et al. 2003; Hanai, Kanauchi et al. 2004).

1.7 Role of SOCS3 in intestinal disease

Chronic inflammation, that which is observed in IBD has been associated with an increased risk of developing CRC (Eaden and Mayberry 2000; Eaden, Abrams et al. 2001; Li, de Haar et al. 2012). Numerous studies have begun to unravel the core mechanisms that may drive the development of IBD-CRC (Breynaert, Vermeire et al. 2008; Brackmann, Aamodt et al. 2010; Saleh and Trinchieri 2010). The activation of the immune system in IBD triggers the production of mediators that promote the destruction of the epithelium further enhancing inflammation. Other inflammatory mediators secreted by immune cells or IEC themselves augment wound-repair by encouraging IEC proliferation. It is a combination of these events which leads to the development of IBD-CRC (Eaden 2004; Sartor 2006).

Studies have uncovered the molecular mechanism of intracellular signalling pathways of inflammatory cytokines such as TNFα and IL-6, implying signalling transpires through the JAK-STAT signalling pathway (Krebs and Hilton 2001; Yoshimura, Naka et al. 2007; Li, de Haar et al. 2010). SOCS3, a natural endogenous regulator of inflammatory mediated signalling has been shown to limit transcription factor activation/translocation in response to inflammatory stimuli (Sasaki, Yasukawa et al. 1999; Suzuki, Hanada et al. 2001; Jarnicki, Putoczki et al. 2010; Li, de Haar et al. 2012). Therefore; SOCS3 provides a self-regulating mechanism to control inflammation within IEC.

There is compelling evidence to support the role of SOCS3 in IBD, as increased expression of SOCS3 is detected in mouse models of inflammation (Suzuki, Hanada et al. 2001). Furthermore, inflamed biopsy specimens of UC patients express greater

levels of SOCS3 mRNA than non-inflamed biopsies (Miyanaka, Ueno et al. 2007; Li, de Haar et al. 2010).

Studies investigating the progression from UC-CRC have shown that the numbers of SOCS3-positive IEC are significantly reduced during UC-CRC in comparison to patients with UC. With reduced expression of SOCS3 also detected at dysplastic and cancerous regions of the colon (Li, de Haar et al. 2010). The decline in SOCS3 expression is due to epigenetic regulation via enhanced DNA methylation (Li, de Haar et al. 2010; Li, Deuring et al. 2012).

STAT3 has already been demonstrated to have a stimulatory effect on cell survival and proliferation (Grivennikov and Karin 2010). Therefore, restricting SOCS3 may be associated with the development of cancer. Numerous studies have supported the role of SOCS3 as a tumour suppressor, for example targeted deletion of SOCS3 in IEC promotes tumour incidence and growth in the colon; with methylation silencing of SOCS3 shared in multiple tumour types (Ogata, Chinen et al. 2006; Rigby, Simmons et al. 2007). *In vitro* studies have shown that SOCS3 is a potent suppressor of proliferation in both transformed and non-transformed IEC lines (Rigby, Simmons et al. 2007).

Taken together, SOCS3 is a critical regulator of cytokine signalling and the subsequent immune response. Since, SOCS3 regulates the activity of a number of cytokines linked to IBD and cancer, SOCS3 also plays a fundamental role in the promotion of these diseases.

1.8 Hypothesis

"SOCS3 impacts on TLR-induced intestinal epithelial responses associated with normal homeostasis and epithelial repair".

1.9 Aims

To investigate the above hypothesis (1.8) we aimed to determine:

1. Whether SOCS3 influences microbial-induced epithelial wound repair.
2. Whether SOCS3 influences IEC cytokine production in response to microbial challenge.
3. Whether SOCS3 mediates IEC tolerance to TLR ligation

1.10 Specific Aims

1. To address whether SOCS3 influences microbial-induced epithelial wound repair; we aimed to:

A) Determine the effect of TLR ligands on epithelial wound repair in a model system

B) Determine the effect of SOCS3 on epithelial wound repair.

2. To investigate whether SOCS3 influences IEC cytokine production in response to microbial challenge; we aimed to:

A) Identify cytokines upregulated in response to microbial stimulation

B) Determine the effect of SOCS3 on microbial-induced cytokine production and signalling.

3. To investigate whether SOCS3 mediates IEC tolerance to TLR ligation; we aimed to:

A) Monitor TLR and SOCS3 expression levels in IEC following TLR ligation.

B) Assess SOCS3 and cytokine expression in a model of conventional and disrupted IEC monolayers

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell lines

For the purposes of this study, colon adenocarcinoma cell lines were purchased from the European Collection of Cell Cultures (ECACC). The cell lines used as part of this study were:

- The human colorectal cancer SW480 epithelial cell line derived from a grade 3-4 colon adenocarcinoma. SW480 IEC were chosen as they are epithelial like in morphology and have extensively been used as an *in vitro* model to investigate cell signalling in colorectal studies.
- The human colorectal cancer Caco-2 epithelial cell line derived from a grade 2 colon adenocarcinoma. *In vitro,* Caco-2 IEC undergo a process of spontaneous differentiation leading to the formation of an IEC monolayer. These monolayers express several functional and morphological features of mature enterocytes. The characteristic features of Caco-2 IEC forming monolayers will be used as an *in vitro* model to investigate epithelial wound repair.

2.1.2 TLR ligands

Lipopolysaccharide (LPS) from *E. coli* (Sigma Aldrich)

Flagellin (FLA) from *S. typhimurium* (InvivoGen)

Poly I:C HMW (InvivoGen)

T.muris excretory/secretary proteins (ES) (gift from Kathryn Else, University of Manchester)

2.1.3 Antibodies

Antibody	Manufacturer	Product code	Molecular weight (kDa)	Source	Working dilution
B-actin	Cell Signalling Technologies	4967	45	Rabbit	1:1000
SOCS3	Cell Signalling Technologies	2923	26	Rabbit	1:1000
pSTAT3	Cell Signalling Technologies	9145	79, 86	Rabbit	1:1000
STAT3	Cell Signalling Technologies	9139	79, 86	Mouse	1:1000
NF-κB	Cell Signalling Technologies	3037	65-80	Rabbit	1:1000
Alexa Fluor® 488 Donkey Anti-Rabbit IgG	Life Technologies	A21206			1:200
rabbit IgG-HRP	Santa Cruz	sc-2030			1:5000
mouse IgG-HRP	Santa Cruz	sc-2031			1:5000

Table 2-1 List of manufacturers, product codes, molecular weight, source and working dilution of primary and secondary antibodies

2.1.4 Primers

Primers were designed and checked for specificity using the National Centre for Biotechnology Information (NCBI). The primer sequences used to amplify genes were:

RPLPO (142bp) Forward: GCAATGTTGCCAGTGTCTG and Reverse: GCCTTGACCTTTTCAGCAA

SOCS3 (119bp) Forward: TCGATTCGGGACCAGCCCCC and Reverse: TGCTGTGGGTGACCATGGCG

TNFα (82bp) Forward: AGGTTCTCTTCCTCTCACATAC and Reverse: ATCATGCTTTCAGTGCTCATG

TNFR2 (221bp) Forward: GAGTGGTGAACTGTGTCATGA and Reverse: GAGCTCGGCGCTGTGATC;

TLR4 (134bp) Forward: GCCCTGCGTGGAGGTGGTTC and Reverse: AGCTGCCTAAATGCCTCAGGGGA

TGFβ (131bp) Forward: GTCACCGGAGTTGTGCGGCA and Reverse: CGGCCGGTAGTGAACCCGTT

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Culturing cell lines

The human colorectal cancer SW480 epithelial cell line was maintained in Leibovitz growth medium (Gibco) supplemented with 10% foetal bovine serum (FBS) (Life Technologies), 50 units/ml of penicillin and streptomycin (Sigma Aldrich) and 0.11mg/ml of sodium pyruvate. Cells were allowed to differentiate under an atmosphere of 100% air at 37°C in a humidified incubator (incu safe).

The human colorectal cancer Caco-2 epithelial cell line was maintained in Minimal Essential Medium-MEM (Gibco) supplemented with 10% FBS, 50 units/ml of penicillin and streptomycin, and 1% MEM Non-Essential Amino Acids (Gibco). Cells were allowed to differentiate under an atmosphere of 95% (v/v) air-5% CO_2 at 37°C in a humidified incubator (incu safe).

2.2.1.2 Trypsinisation of cells

To maintain cell growth, cells were routinely passaged once cells had reached 70-80% confluence to prevent cells from becoming over-confluent and terminally differentiated. In order to split cells, either for passaging or seeding for subsequent experiments, medium was removed from flask and cells were washed three times with Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} or Mg^{2+} (Gibco). Trypsin-EDTA (1X) with Phenol Red (Gibco) was then added to the flask (0.5mls Trypsin per T25 flask). Cells were incubated with trypsin for 6 minutes at 37°C, to allow cells to detach from flask. Once cells were no longer adherent, 5mls of growth

medium was added to inactivate trypsin. Cells were then pipetted gently to create a single cell suspension and allow for an accurate cell count using a haemocytometer. Cells were then seeded into a new T25 flask containing 6mls of new growth medium at the required density, depending upon when cells would next be passaged. For the purposes of our experiments, cells were only used between passage 20 and 50, to reduce divergence from parent lines.

2.2.1.3 Frozen cell stocks

To reduce divergence from parent cell lines, cells were frozen down for future use after 5-6 passages. To create a frozen stock, the cell suspension was transferred to a 15ml centrifuge tube and centrifuged at 1000 RPM for 5 minutes to form a pellet. The medium was removed and the pellet resuspended in 1ml of appropriate medium containing 40% FBS and 10% Dimethyl sulfoxide (DMSO) (Sigma Aldrich). The cell suspension was immediately transferred to a 1.8ml polypropylene cryo-vial and stored in a NALAGENE Freezing container filled with isopropanol overnight at -80°C to allow gradual freezing before being transferred to liquid nitrogen (-180°C) for long term storage.

2.2.2 Plasmid preparation for transfection

2.2.2.1 Transforming *E.coli* with the GS50726 pIERESneo-Human_SOCS3 plasmid

The GS50726 pIERESneo-Human_SOCS3 plasmid was transformed into *E. coli* using the One shot ®OmniMAX™-T1Chemically competent *E. coli* kit (Life Technologies). To do this, one vial of the OneShot®OmniMAX™-T1 chemically competent *E. coli* was thawed slowly on ice. Five microlitres of the GS50726 pIERESneo-Human_SOCS3 plasmid was then added to the vial and mixed gently. The vial was incubated on ice for 30 minutes. Cells were heat-shocked at 42°C for 30 seconds without shaking. Cells were again placed on ice for 2 minutes. Two hundred and fifty microlitres of pre-warmed S.O.C Medium was then added to the transformation.

2.2.2.2 Clone selection using LB plates

The transformation was streaked on an LB plate containing 50µg/ml of ampicillin (Sigma Aldrich). Care was taken not to streak too much transformed bacteria on plate as it may not be absorbed into the medium. Plate was then incubated upside down at 37° for 16-24h. As the SOCS3 plasmid also contains an ampicillin resistance cassette, *E. coli* which had acquired the SOCS3-plasmid will selectively grow on the LB plate forming a single cell clone.

2.2.2.3 Amplification of clone

Using a sterile loop, a single clone was transferred into a 25ml universal tube containing 5ml of LB Broth (Life Technologies) with ampicillin at a final concentration of 50µg/ml. Universal tube was incubated at 37°C overnight in a shaking incubator.

2.2.2.4 Isolation of plasmid DNA

The illustra plasmidPrep Mini Spin Kit (GE Healthcare) was be used to extract and purify the plasmid DNA, with DNA eluted in 15µl of elution buffer.

2.2.2.5 Linearisation of plasmid DNA

The NanoDrop (Thermo Scientific) was used to determine the concentration and purity of extracted DNA. Following on from this, a reaction mixture containing the Ahd I restriction enzyme (NEB) was used to linearise the plasmid, incubating at 37°C overnight. To do this, 5µl of NEB Buffer 4, 0.5µl of BSA, 3µl of enzyme and plasmid were added together. dH_2O was then added to bring the total volume to 50µl, mixture was incubated at 37°C for 16h. The mixture was then incubated at 65°C for 20 minutes the following day to stop the reaction. Some of the plasmid was retained before linearisation so DNA could be electrophoresed on an agarose gel to confirm plasmid had been linearised.

2.2.2.6 Ethanol precipitation

Ethanol precipitation was carried out to purify DNA from the aqueous solution by adding ethanol as an anti-solvent. Five microlitres of 3M sodium acetate and 110µl of neat ethanol was added to the tube, and mixed gently. The tube was then incubated at -20°C for 90 minutes. After 90 minutes, the tube was centrifuged in the cold room (4°C) at full speed for 20 minutes. Three hundred microlitres of 80% ethanol was then added to the tube, again in the cold room. The tube was then centrifuged at full speed for 5 minutes in the cold room. The supernatant was removed from tube in the culture hood, and the DNA pellet was resuspended in 30µl of autoclaved dH_2O and stored on ice until further use.

2.2.2.7 Confirming plasmid has been linearised

Concentration and purity of plasmid-prep was again established using the NanoDrop. DNA was reconstituted to 1µg/µl, and aliquoted in 20µl volumes in labelled eppendorf tubes and stored at -20°C until further use. Both uncut and linearised plasmids were electrophoresed on an agarose gel with a 100bp ladder to confirm plasmid had been linearised.

2.2.3 Transfecting IECs with plasmid DNA

Lipofectamine™ 2000 (Life Technologies) transfection reagent was used to transfect IEC with plasmid DNA. On the day before transfection, cells were plated in the appropriate medium without antibiotics such that they would be 80-90% confluent at the time of transfection. For each transfection sample, DNA was diluted in the appropriate amount of medium without serum. Lipofectamine was mixed gently before use and diluted in the appropriate amount of medium without serum. Both solutions were mixed gently and incubated for 5 minutes at room temperature (RT). After 5 minutes, the diluted DNA and Lipofectamine were combined, mixed gently and incubated for 20 minutes at RT to allow complex formation to occur. The DNA-Lipofectamine complexes were then added to each well/flask containing cells and medium without serum and incubated at 37°C for 5h. After 5h, medium was removed from wells/flask to avoid reagent cytotoxicity and replaced with fresh complete serum containing medium.

2.2.4 Quantitative PCR

2.2.4.1 RNA extraction

Total RNA from each sample was extracted using the TRI Reagent® RNA Isolation Reagent (Sigma Aldrich) method; briefly, 1ml of TRI regent was used to lyse adherent cells. After addition of reagent, the pipette tip was used to scrape the base of the well, and pipetted up and down a few times to form a homogenous lysate. The lysate was then incubated for 10 minutes at RT to permit the complete dissociation of nucleoprotein complexes. 0.2mls of chloroform was added for each 1ml of TRI reagent used for the initial homogenisation. The lysates were left at RT for 10 minutes. Samples were then centrifuged at 12,000g for 15 minutes at 4°C. The resultant aqueous phase was carefully removed and transferred to a new tube. The RNA was then precipitated from the aqueous phase by mixing with 0.5mls of isopropyl alcohol (Sigma Aldrich) per 1ml of TRI reagent used in initial homogenisation. Samples were mixed and incubated at RT for 10 minutes to allow RNA to precipitate. Samples were then centrifuged at 12, 000g for 10 minutes at 4°C, to pellet the RNA. The supernatant was removed and the RNA pellet was washed by adding 1ml of 75% ethanol (Fisher Scientific). Samples were vortexed and centrifuged at 7, 500g for 5 minutes at 4°C. Supernatant was again removed and samples were washed a second time as before in 75% ethanol. The RNA pellet was allowed to dry for 10 minutes at RT before resuspending in 20µl of RNase free-water (Life Technologies).

2.2.4.2 Examining concentration and purity of extracted RNA

The RNA concentration and purity (260/280) were determined by measuring the absorbance of the sample using the NanoDrop 2000. To confirm the quality of RNA, 0.5mg of each sample was run on a 1% non-denaturing agarose gel in TBE (Tris Borate Ethylenediaminetetracetic acid EDTA) containing a 1x solution of GelRed™ Nucleic Acid Gel Stain and bands visualized under UV light. For good quality RNA, two bands should be present, representing the ribosomal 18S and 28S RNA. Samples with degraded RNA were signified with an unspecific smear on the gel instead of two distinct bands, which were then excluded from subsequent steps.

2.2.4.3 cDNA synthesis

Two micrograms of RNA was then reverse transcribed into cDNA using the Enhanced Avian RT First Strand Synthesis Kit (Sigma Aldrich). One microlitre of oligo dT (Fisher Scientific) was added to 2µg of RNA to be reverse transcribed. The total volume was made up to 10µl using nuclease-free water. The mixture was incubated at 70°C for 10 minutes before being placed on ice for 5 minutes. Two microlitres of 10x M-MLV Reverse Transcriptase Buffer, 1µl of dNTP (10mM) (Sigma Aldrich), 1µl of M-MLV Reverse Transcriptase enzyme and 6µl of nuclease-free water were then added bringing the total volume to 20µl. The samples were briefly centrifuged before being incubated at 42°C for 1h and 80°C for 10 minutes to denature the M-MLV reverse transcriptase enzyme. The samples were then stored at -20°C for future use.

2.2.4.4 Relative Quantitative Real Time PCR

2.2.4.4.1 Basic principle of SYBR Green I based QPCR

Relative Quantitate Real time PCR was performed using the SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma Aldrich). The diagram below represents a schematic representation of the steps involved in the real-time SYBR-Green reaction.

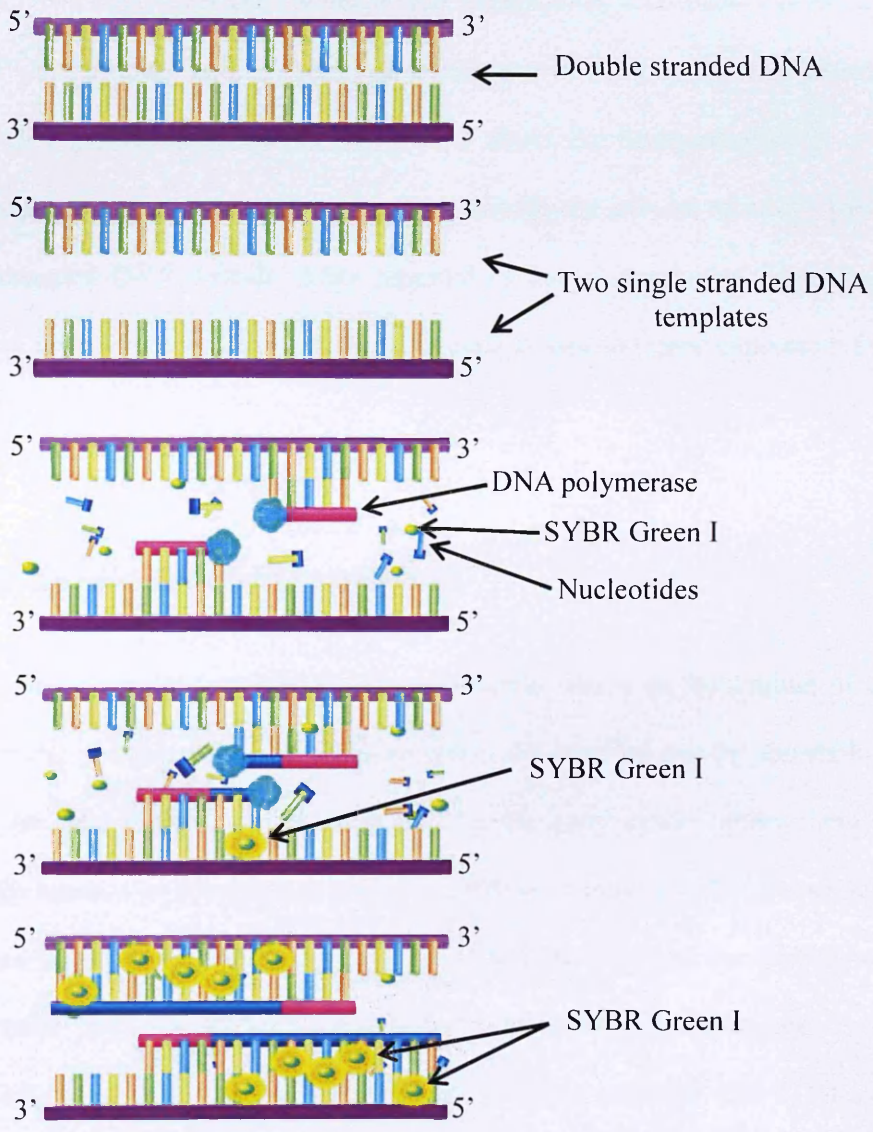


Figure 2-1 Schematic representation of the steps involved in the real-time SYBR-Green reaction (Sigma Aldrich)

Double stranded DNA is denatured by heating to 94°C. During this period, the double-stranded DNA helix separates into two single-stranded DNA templates. The temperature then falls to 60°C during which the DNA polymerase enzyme anneals to the DNA strands and extends the sequence specific primers with the incorporation of nucleotides that are complementary to the template yielding a double-stranded DNA complex. The SYBR Green I is then able to bind to the newly synthesised double-stranded DNA complexes and fluoresce. The fluorescence accumulates as the cycling of the PCR continues and is measured at the end of each cycle. The intensity of fluorescence generated by the SYBR Green I above the background level or cycle threshold (C_t value) is measured and used to quantify the amount of newly generated double-stranded DNA strands. After repeated cycles of denaturing, annealing and extension, approximately 40 times, the C_t is used to measure gene expression (Sigma Aldrich).

2.2.4.4.2 SYBR Green I data parameters

To detect gene expression between test samples based on the number of cycles passed before possible detection of fluorescence, the baseline and the threshold must first be set. The baseline is the noise level in the early cycles where there is no detectable increase in fluorescence due to amplification products. The threshold is an adjustable value above the background but significantly below the plateau of the amplification plot. The threshold is usually placed within the linear region of the amplification, and is usually determined by the computer software. The C_t for a given amplification curve is defined as the cycle number at which the fluorescence generated within a particular reaction surpasses the defined threshold and signifies a point at

which there is a significant increase in detectable product. The fewer number of cycles required to reach the detectable level of fluorescence, indicates a greater initial copy number of the target nucleic acid.

2.2.4.4.3 Endogenous control reference gene

This is a gene whose expression level does not differ between samples, such as housekeeping genes. The use of a reference gene or endogenous control is important in order to standardise the quantity and monitor the quality of cDNA added to each reaction. Initially, three housekeeping genes RPLPO, 18S and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) were tested for this study. Validation experiments indicated RPLPO was the best housekeeping gene to determine the relative levels of gene expression. Hence, RPLPO was used as an internal reference gene for this study.

2.2.4.4.4 Calculating relative gene expression

The C_t number for both the gene of interest and the housekeeping gene were verified for each sample. This would permit the expression of the gene of interest to be normalised to the endogenous housekeeping gene. The comparative method, sometimes called the $2^{-\Delta\Delta Ct}$ method, was used for calculating the relative gene expression. This involved comparing the ΔCt of the samples with a non-treated sample. This involved calculating the $\Delta\Delta Ct$, by subtracting $\Delta Ct_{non\text{-}treated}$ from the ΔCt_{sample}. Changes in C_t values for the gene of interest were normalised relative to RPLPO. In all experiments, gene expression was expressed as fold-change relative to no treatment control.

2.2.4.4.5 Primer design

Primers for genes were designed from sequences in the Genebank database. The forward and reverse sequences of the primers for each gene are shown in section 2.1.4. All primers sets were designed such that the PCR product size was a maximum of 250 base pairs and primers only spanned exon-exon junctions. However, it was later discovered that TNFR2 primers might be amplifying residual genomic DNA as they were buried in one exon. Therefore, TNFR2 primers were validated for subsequent experiments. All primer sets were tested by BLAST searching to ensure primers did not bind to other sequences and that primer dimers were not formed. Primer sequences were then sent to Sigma Aldrich for production. An attempt was made to design TNFα primers, but this proved unsuccessful. Primers were therefore purchased from Primerdesign. RPLPO was selected as a housekeeping gene because in preliminary experiments, mRNA expression of RPLPO did not change following treatment.

2.2.4.4.6 Primer concentration optimisation for qPCR

Before primers could be used to detect gene expression for qPCR, they first had to be optimised to determine the concentration of forward and reverse primers that produced the smallest C_t number with the highest fluorescent signal. For the purpose of these experiments, the real time PCR reactions were initially performed with 1μl of cDNA template and different combinations of forward and reverse primer concentrations as depicted in table 2-2.

500nM Forward 500nM Reverse	500nM Forward 1000nM Reverse	500nM Forward 1500nM Reverse
1000nM Forward 500nM Reverse	1000nM Forward 1000nM Reverse	1000nM Forward 1500nM Reverse
1500nM Forward 500nM Reverse	1500nM Forward 1000nM Reverse	1500nM Forward 1500nM Reverse

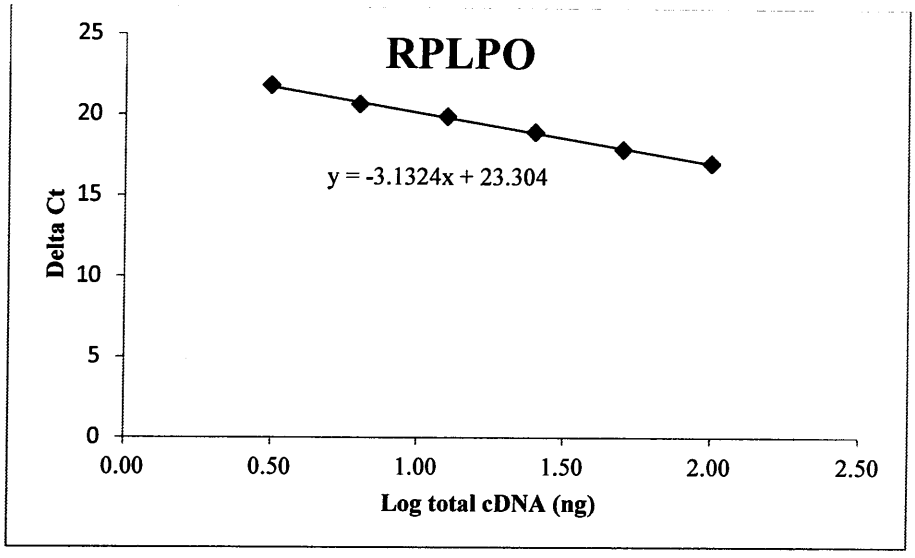
Table 2-2 Combination of forward and reverse primer concentrations for optimisation experiments for qPCR

Primer optimisation was performed for all primers with experiments indicating the optimum combination of primer concentrations for the quantitate amplification of RPLPO, SOCS3, TNFR2, TLR4 and TGFβ to be 1000nM forward and 1000nM reverse for each gene.

2.2.4.4.7 Primer validation

For the ΔΔCt calculation to be valid, the amplification efficiencies of the target and the endogenous reference genes must be roughly the same. This can be established by observing how ΔCt_{sample} varies with template dilution. The ΔCt is plotted against the logarithm (log) of the template amount. If the gradient of the cDNA dilution versus ΔCt is near to 0 (<0.1) then this indicates the amplification of the housekeeping and the target genes are comparable.

To validate primers, cDNA was diluted 1 in 2 as a serial dilution. Six dilutions were performed ranging from 100ng to 3.125ng. The same cDNA was used for all validation experiments for all genes of interest.



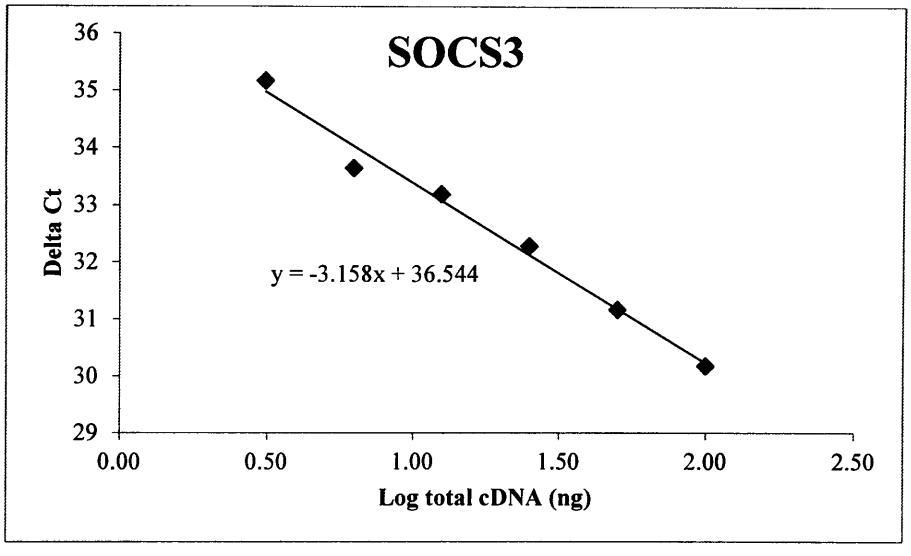


Figure 2-2 Example of a validation experiment for the SOCS3 primer in relation to the RPLPO housekeeping gene

Figure 2-2 shows the variation of ΔCt value for SOCS3 in relation to RPLPO housekeeping gene when template input is diluted. The x-axis depicts the $\log_{10}$ of the cDNA added, whereas the y-axis depicts the ΔCt value. For the $2^{-\Delta\Delta Ct}$ method to be valid, the amplification efficiencies of SOCS3 and RPLPO must be equal in the range

of template input used. This is confirmed when the slope value is less than 0.1. The slope value for RPLPO was -3.1324 and SOCS3 was -3.158. The ΔCt values for SOCS3 in relation to RPLPO are therefore acceptable when the range of cDNA is between 100 and 3.125ng.

Validation experiments were also performed for TNFR2, TLR4 and TGFβ primers in relation to RPLPO as the internal reference gene; the ΔCt values were acceptable when the range of cDNA was between 100 and 3.125ng.

2.2.4.4.8 SYBR Green I cDNA amplification reaction

Relative Quantitate real-time PCR was performed in a 15μl reaction volume in a 96-well plate in the C1000 Real Time Thermocycler (Bio-Rad). The mixture was prepared according to the following recipe:

7.5μl	2x SYBR Green I Mastermix
1.5μl (10μM) Forward primer	Final concentration of 1000nM
1.5μl (10μM) Reverse primer	Final concentration of 1000nM
1μl of cDNA	
H_2O (Nuclease free water) to 15μl	

The concentrations of primers were used according to the optimised experiments (section 2.2.4.4.6). Cycling conditions were 94°C for 2 minutes, 40 x 94°C for 15 seconds and 60°C for 1 minute. No Template Control (NTC) were always included in each real-time PCR run to ensure amplification was not due to reagent contamination.

2.2.5 SDS-PAGE and Western blotting

2.2.5.1 Preparation of lysates and electrophoresis

The Abcam method of SDS-PAGE and western blotting was used to determine protein expression levels. Following treatment, the 6-well cell culture dish was placed on ice and washed once with ice-cold Phosphate Buffered Saline (PBS) (Gibco). It was important to remove as much PBS after washing, as this would dilute the lysis buffer. Wells were then lysed with 50µl of ice-cold lysis buffer (50mM Tris-HCL pH7.5, 150mM NaCl, 1% Nonidet P40, 10% Glycerol and 5mM EDTA) containing phosphatase inhibitor (Sigma Aldrich) and protease inhibitor (Sigma Aldrich). Adherent cells were scrapped off the base of the plate using a pipette; the cell suspension was then gently transferred to a pre-cooled tube and centrifuged at 12,000 RPM for 20 minutes at 4°C. Tubes were removed from centrifuge and placed on ice, supernatant was aspirated to a fresh tube and placed on ice, discarding pellet.

2.2.5.2 Determining protein concentration

Before performing immunoblotting, it was necessary to determine the concentration of protein present in the samples to be tested. Bradford reagent (Sigma Aldrich) was used to determine protein concentration of samples; this involved a coloumetric assay for measuring total protein concentration. Briefly, 6 protein standards of between 100µg and 0 were generated in duplicate using BSA in a final volume of 100µl. Five hundred microlitres of Bradford reagent was then added to standards and samples, and incubated at RT for 5 minutes. After 5 minutes, absorbance of standards and samples were measured at 595nm. A standard curve was

then generated by plotting the 595 nm values (y-axis) versus their concentration in μg/ml (x-axis). The standard curve was then used to determine the unknown sample concentration. Protein aliquots of 25μg were generated and boiled at 95°C for 10 minutes with 4x Sample buffer (Life Technologies) and 2-Mercaptoethanol (Sigma Aldrich).

2.2.5.3 Western blotting technique

Proteins were run alongside the Precision Plus Protein™ All Blue Standard (Bio-Rad) to monitor electrophoretic separation and molecular weight sizing of separated proteins on a 10% SDS-PAGE gel at 40mA for 1h. Proteins were subsequently electro-transferred onto a nitrocellulose membrane (Macherey-Nagel) using the semi-dry Trans-Blot® Turbo™ Transfer system (Bio-Rad) using 10x Fast Transfer Buffer (Life Technologies) for 30 minutes. The membrane was treated with 3% bovine serum albumin (BSA) (PAA) in TBST (Tris Buffered Saline-Tween) for 1h at RT, then incubated with primary antibody (SOCS3, 1:1000, Cell Signalling Technology, Rabbit, 26kDa), (β-actin, 1:1000, Cell Signalling Technology, Rabbit, 45kDa), (NF-$_k$B, 1:1000, Cell Signalling Technology, Rabbit, 65-80kDa), (pSTAT3, 1:1000, Cell Signalling Technology, Rabbit, 79, 86kDa) or (STAT3, 1:1000, Cell Signalling Technology, Mouse, 79, 86kDa) overnight at 4°C. Membrane was then washed three times for 10 minutes in TBST and incubated in anti-rabbit IgG-HRP (Santa Cruz, 1:5000) or anti-mouse IgG-HRP (Santa Cruz, 1:5000) conjugated secondary antibody for 1h at RT. The membrane was again washed three times in TBST and once in TBS, 10 minutes for each wash. The Pierce® ECL Western Blotting Substrate kit (Thermo Scientific) was then used to detect Horseradish peroxidase

(HRP), this was later detected and quantified using the ChemiDoc XRS imaging system (Bio-Rad). Western blots were carried out in a minimum of three independent experiments.

To blot for loading control (β-actin) or normalise pSTAT3 expression to total STAT3 expression, primary antibody was removed from probed membranes using mild stripping buffer (1.5% glycine, 1% SDS and 1% Tween-20) for 10 minutes two times at RT, before being washed two times for 10 minutes each in PBS and two times for 5 minutes each in TBST. Consequently, membranes were ready to be blocked in 3% BSA in TBST then probed with the next primary and secondary antibody as described above.

Protein expression levels were again quantified through densitometry using the ChemiDoc XRS imaging system.

2.2.6 Proliferation assay

The CyQUANT® Cell proliferation Assay kit (Molecular Probes, Invitrogen) which includes the CyQUANT® GR dye and cell lysis buffer were used to determine the proliferation capabilities of cells after treatment with microbial ligands. The CyQUANT method works by incorporating the fluorescent dye into total DNA, which correlates directly to the number of cells in culture.

Stock cell densities were determined by visual counting using a haemocytometer and Trypan blue (Sigma Aldrich). Cells were then resuspended in medium and plated in 24-well culture plates at a concentration of 25,000 cells per well in 500µl volumes; and incubated overnight at 37°C, thus allowing cells to adhere to the well surface. Following microbial stimulation, no treatment cultures were also included allowing comparison; plates were harvested after 48h by removing growth medium and freezing plates at -80°C.

To establish the number of cells; a standard calibration curve of known cell number was constructed. A cell suspension 2 x 10^6 cells was centrifuged (Harrier 18/80) for 5 minutes at 1000 RPM. The suspension was carefully removed and the pellet frozen for 1h at -80°C. The dye in cell lysis buffer was made up immediately prior to reading plates by diluting the dye stock solution 20-fold in cell lysis buffer; followed by diluting this solution 20-fold in distilled water. The pellet was thawed at RT and 1.6mls of the dye/lysis buffer was added to the pellet, and the lysate resuspended by brief vortexing, to generate a suspension of 1.25 x 10^6 cells/ml. A cell dilution series ranging from 500,000 to 10,000 cells was formed in duplicate in a 24-well cell culture plate with the dye/lysis buffer cell suspension mixture in final volumes of 400µl per well. A 400µl control well with no cells (dye/lysis buffer only) was also prepared and the plate was incubated in darkness for 4 minutes at RT. The

plate was then read by measuring fluorescence using a plate-reader with excitation/emission maxima: 480/520nm for dye bound to nucleic acids (WALLAC VICTOR^2 1420 Multicaler). A standard calibration curve was generated by plotting measured fluorescence values versus cell number from the standard curve as described previously. Four hundred microlitres of dye/lysis buffer was added to each well of the sample plate, and incubated in darkness for 4 minutes at RT, absorbance readings for sample plate was then obtained (WALLAC VICTOR^2 1420 Multicaler).

To evaluate cellular proliferation in response to treatment; values had been expressed as percentage change in cell number relative to no treatment controls for treatment.

2.2.7 Wound healing assay

An *in vitro* wound healing assay was performed using the modified method as described by Han and colleagues (2000). Four reference lines were drawn horizontally across the outer bottom of a 24-well cell culture plate. Caco-2 cells (2 x 10^5 cells/well) were then seeded at 80-90% confluence and allowed to form monolayers for 7 days at 37°C. After 7 days, linear wounds were made with a sterile 10µl plastic pipette tip perpendicular to lines drawn on the bottom of the plate. To remove floating cells, cells were washed three times in PBS. Cells were then treated as required; with no treatment controls also included to allow comparison. Medium and treatment within wells was replaced with freshly prepared medium and respective treatment after 24, 48 and 72h with the no treatment wells also replaced with complete medium to remove floating cells, replenish nutrients and restore treatment levels.

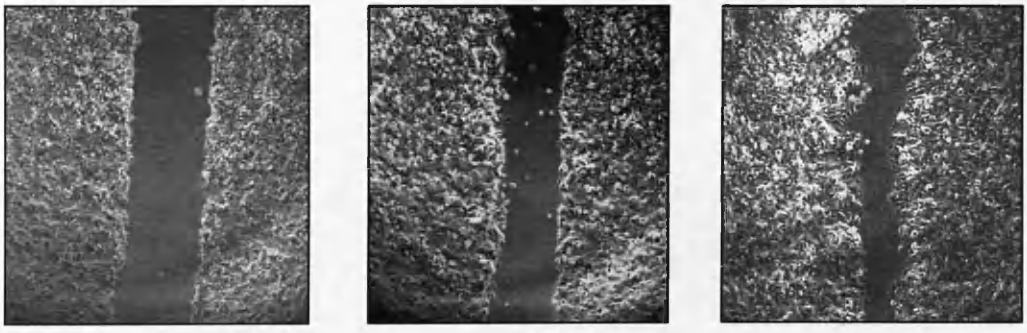


Figure 2-3 Phase-contrast images of Caco-2 monolayer's with images taken at (a) 0 Hours (b) 24 Hours (c) 48 Hours after wounding (x10 magnification)

Images of wounds were obtained using the confocal microscope (Leica DMIRE2 inverted microscope) at 10x magnification using standard protocols at the predetermined location at various times after wounding. To attain an accurate

determination of wound repair in response to ligand treatment, the area of the acellular region was measured using ImageJ and data calculated as % wound healed Vs. 0h.

To investigate the effect of SOCS3 overexpression on wound repair, IEC were transfected with the pBIG2i plasmid either containing the entire coding sequence for SOCS3 (S3) or empty vector (EV) control. To establish levels of SOCS3 after monolayers have formed (i.e. 7 days), Caco-2 IEC were seeded in a 24-well culture plate at 2 x 10^5 cells/well in 1ml of medium without antibiotics such that they were 80-90% confluent minimising post-transfection proliferation and ready to be transfected the next day. Caco-2 IEC were then transfected with 1μg of EV or S3 plasmid as described in section 2.2.2. After 7 days, total RNA was extracted, cDNA generated and SOCS3 mRNA expression levels monitored in triplicate between EV and S3 cultures as described previously (section 2.2.4)

2.2.8 ELISA Assay

Supernatant collected from IEC which had previously been treated with TLR ligands was used for the quantitative measurement of hTNFα in a sandwich ELISA format using the Human TNFα ELISA Development Kit purchased from PeproTech (900-K25).

To prepare plate, capture antibody was diluted with PBS to a concentration of 1μg/ml, 100μl of this solution was used to coat each well of an ELISA plate, and sealed overnight at RT. The following day, liquid was aspirated from wells and washed 4 times in wash buffer (0.05% Tween-20 in PBS) with plate inverted and blotted on paper to remove residual buffer. The TNFα standard was then prepared from 1.5ng/ml to zero in diluent (0.005% Tween-20, 0.1% BSA in PBS). One hundred microlitres of standard or sample was immediately added to each well in duplicate and incubated at RT for two hours. After 2h, plate was washed as before then incubated with 100μl of detection antibody in diluent to a concentration of 0.5μg/ml for 2h at RT. Wells were again aspirated and washed as previously described. Avidin Peroxidase was diluted 1:2000 in diluent, 100μl was added to each well and incubated for 45 minutes at RT. Wells were again washed as before, 100μl of ABTS liquid substrate solution (Sigma Aldrich) was then added to each well and incubated at RT for colour development before reading ELISA plate at 405nm with wavelength correction set at 650nm. Experiment was repeated three times with data pooled together and a student *t test* used to determine statistical effect in contrast to no treatment with $p \leq 0.05$.

2.2.9 Immunocytochemistry

Coverslips were sterilized with ethanol, and carefully placed in each well of a 12-well plate. IEC were then seeded onto coverlsips at 1 x 10^5 IEC/well and allowed to adhere. Following TLR treatment, medium was removed and IEC were washed 3 times for 5 minutes each in 1ml of PBS. IEC were then incubated in 1ml of ethanol:methanol for 10 minutes at -20°C. IEC were again washed as before and blocked in 1ml of 2% BSA in PBS-Tween-20 for 30 minutes at RT. IEC were washed in PBS, and incubated with 500µl of primary antibody ((Anti-Human sTNF Receptor Type II(Peprotech 500-P168)) at 0.5µg/ml in blocking solution at 4°C overnight. IEC were washed in PBS, then incubated with Alexa Fluor® 488 Donkey Anti-Rabbit IgG secondary antibody (Life Technologies, 1:200) for 1h at RT in the dark. Secondary antibody solution was then decanted and IEC washed as described previously. To counterstain IEC, wells were incubated with 500µl of propidium iodide (Life Technologies, P3566, 1:3000 in PBS) for 1 minute, then washed once with PBS. Coverslips were then mounted onto slides and sealed with nail varnish to prevent drying and movement under microscope. Slides were stored in the dark at 4°C ready to be viewed using the Zeiss LSM 510 Meta Confocal Microscope.

3. GENERATION OF IECs OVER-EXPRESSING SOCS3

3.1 Summary

To determine the role of SOCS3 in IEC replenishment and repair, we initially wanted to generate an *in vitro* model system. This enabled the investigation of the role of SOCS3 in mediating cellular responses and epithelial repair following microbial induced TLR signalling. We used models of transient and stable IEC transfection.

We were successfully able to overexpress SOCS3 in both SW480 (8.2) and Caco-2 (5.9) IEC. Transient transfection has its disadvantages in that cells only overexpress the inserted gene for a limited period. Cells are therefore required to be transfected for each experiment which could result in experimental variation in terms of levels of gene expression.

Stable transfection involves DNA to be integrated into the host genome therefore cells continue to express traits caused by the genetic material, even after many generations of cell division. SW480 IEC were successfully transfected, resulting in a 12-fold increase in mRNA and a 25% increase in SOCS3 protein expression in comparison to $SOCS3^{norm}$ IEC. However we were unable to stably transfect Caco-2 IECs. To investigate the effect of SOCS3 on TLR-mediated wound repair, IEC were transfected and monolayers allowed to form for 7 days. SOCS3 mRNA expression levels were then tested before wounding. qPCR data showed Caco-2 IEC were successfully transfected with SOCS3 mRNA expression 5.3 times greater in SOCS3 transfected cultures than empty vector control cultures.

In the following chapters, both transient and stable IEC (SW480 and Caco-2) will be used in conjunction with control (EV or $SOCS3^{norm}$) IEC to investigate the role of SOCS3 in mediating cellular responses following microbial-induced TLR signalling.

3.2 Introduction

Studies have begun to investigate the mechanisms that push the development of IBD (Breynaert, Vermeire et al. 2008; Saleh and Trinchieri 2010; Li, de Haar et al. 2011; Li, de Haar et al. 2012). The activation of the immune system in IBD initiates the production of mediators that promote the destruction of the epithelium further augmenting inflammation. Studies have uncovered the molecular mechanism of intracellular signalling pathways of inflammatory cytokines such as TNFα and IL-6. Binding of these inflammatory cytokines to their corresponding receptors activates JAK2; subsequently permitting the phosphorylation of the transcription factor STAT3 (Mitsuyama, Matsumoto et al. 2006; Croker, Kiu et al. 2008). The pSTAT3 then translocates to the nucleus, where it interact with specific DNA sequences and induces the transcription of target genes (Krebs and Hilton 2001; Yoshimura, Naka et al. 2007; Li, de Haar et al. 2010).

SOCS3 is a natural endogenous regulator of inflammatory cytokine-mediated signalling, and has been shown to block TNFα-induced NF-$_k$B translocation and IL-6 induced STAT3 phosphorylation (Rigby, Simmons et al. 2007). SOCS3 inhibits signalling through the JAK/STAT signalling pathway by inhibiting both JAK kinase and STAT phosphorylation (Sasaki, Yasukawa et al. 1999; Li, de Haar et al. 2012).

Suzuki and colleagues (2001) have shown that SOCS3 expression is upregulated in both animal and human intestinal inflammation (Suzuki, Hanada et al. 2001). There is also compelling evidence to support the role of SOCS3 as a tumour suppressor in the intestine, with studies showing targeted removal of SOCS3 encouraging tumour incidence and growth in the bowel; and methylation silencing of SOCS3 shared in multiple tumours (Ogata, Chinen et al. 2006; Rigby, Simmons et al. 2007). In human

lung cancer cells, restoring SOCS3 expression decreased STAT3 activation and reduced tumour growth (He, You et al. 2003).

Numerous studies have shown that commensal bacteria are recognised by IEC which express TLRs (Abreu, Fukata et al. 2005; Kawai and Akira 2007; Beutler 2009). Studies have shown that TLR signalling plays a fundamental role in the maintenance of intestinal epithelial homeostasis, with commensal flora and TLR signalling protecting from intestinal epithelial injury. Conversely, germ-free mice also have a lower incidence of tumour burden within the colon (Rakoff-Nahoum, Paglino et al. 2004).

As evidence also supports TLRs as imperative mediators of mucosal homeostasis, repair of damaged tissue and protection against the development of colon cancer; this project seeks to investigate the dual and perhaps integrated role of SOCS3 and TLRs in mediating intestinal epithelial homeostasis and repair following damage.

The aim of the first section was to develop IEC which constitutively overexpress SOCS3; enabling me to investigate the role of SOCS3 in mediating cellular responses and epithelial repair following microbial induced TLR signalling.

3.3 Aim: To develop SOCS3 overexpressing IECs

3.4 Methods

3.4.1 Generating transient SOCS3 overexpressing IEC

3.4.1.1 SOCS3 vector containing pBIG2i plasmid

The plasmid (pBIG2i) either containing the entire coding sequence for SOCS3 (S3) or a control empty vector (EV) was a gift from Dr. Richard Furlenetto. The S3 plasmid had previously been used to overexpress SOCS3 in IEC lines (Rigby, Simmons et al. 2007).

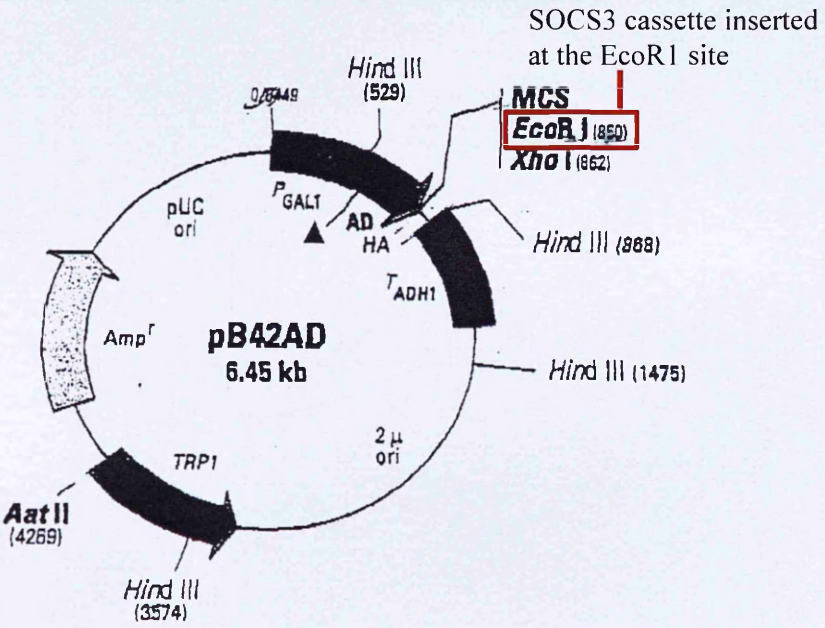


Figure 3-1 Diagram displaying the pBIG2i plasmid

The diagram above displays the pBIG2i plasmid, either containing the entire coding sequence of hSOCS3 cloned into the EcoRI site (S3) or a control empty vector (EV).

3.4.1.2 Transient transfection using the SOCS3 vector containing pBIG2i plasmid

SW480 and Caco-2 IEC were seeded in 24-well culture plates at 2 x 10^5 cells/well in 1ml of medium without antibiotics such that they were 80-90% confluent minimising post-transfection proliferation and ready to be transfected the following day. IECs were then transfected with 1µg of plasmid (pBIG2i) either containing the entire coding sequence for SOCS3 (S3) or a control empty vector (EV) using the method described in section 2.2.3. To validate overexpression of SOCS3, mRNA expression was compared between S3 and EV plasmid transfected IEC.

3.4.2 Generating stable SOCS3-overexpressing IEC

3.4.2.1 GS50726 pIERESneo-Human_SOCS3 plasmid

The GS50726 pIERESneo-Human_SOCS3 plasmid, which contains the SOCS3 gene, was cloned into the Eco RV digested pIERESneo plasmid. The plasmid was purchased from Epoch Life Sciences. The pIERESneo contains the internal ribosome entry site (IRES) of the encephalomyocarditis (ECMV) allowing the translation of two open reading frames from one mRNA. The expression cassette of pIERESneo contains the human cytomegalovirus (CMV) major intermediate early promoter followed by a multiple cloning site (MCS), a small intron known to enhance the stability of mRNA, the neomycinphosphotransferase (NPT II) gene, and the polyadenylation signal. Upon arrival, the plasmid was resuspended in 20µl of autoclaved dH20 then stored on ice.

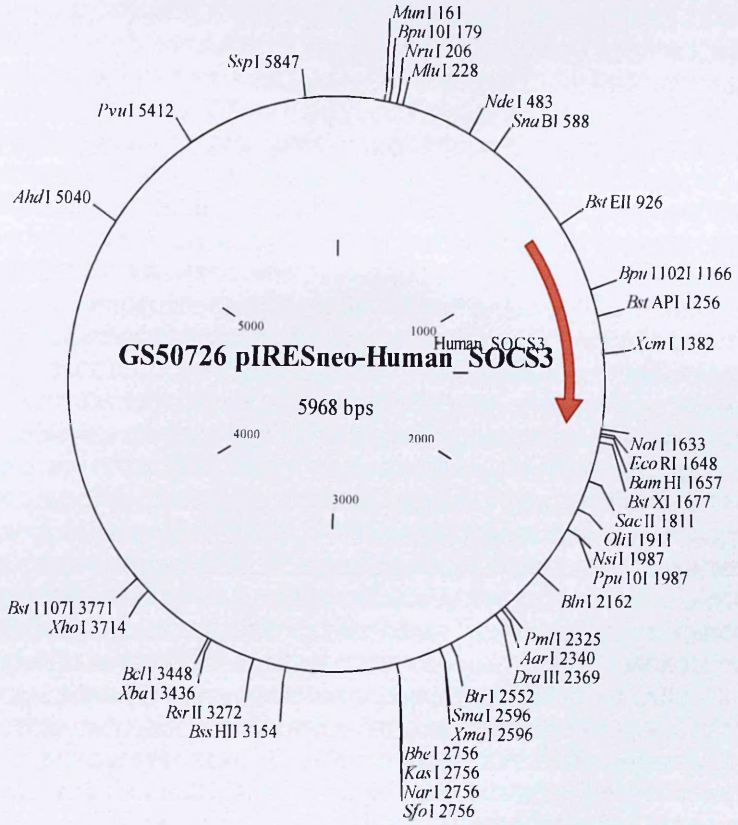


Figure 3-2 GS50726 pIERESneo-Human_SOCS3 plasmid

Base sequence of the GS50726 pIERESneo-Human_SOCS3 plasmid which contains 5968 base pairs

GACGGATCGGGAGATCTCCCGATCCCCTATGGTCGACTCTCAGTACAATCTGCTCTGATGCCGCATAGT
TAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAATTTAAGCTA
CAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTC
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GGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCT
GACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGA
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ATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACA
TGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGC
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CCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAA
CTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTTGGT
ACCGAGCTCGGATCGATATCGCCACCATGGTCACCCACAGCAAGTTTCCCGCCGCCGGGATGAGCCGCC
CCCTGGACACCAGCCTGCGCCTCAAGACCTTCAGCTCCAAGAGCGAGTACCAGCTGGTGGTGAACGCAG

TGCGCAAGCTGCAGGAGAGCGGCTTCTACTGGAGCGCAGTGACCGGCGGCGAGGCGAACCTGCTGCTCA
GTGCCGAGCCCGCCGGCACCTTTCTGATCCGCGACAGCTCGGACCAGCGCCACTTCTTCACGCTCAGCG
TCAAGACCCAGTCTGGGACCAAGAACCTGCGCATCCAGTGTGAGGGGGGCAGCTTCTCTCTGCAGAGCG
ATCCCCGGAGCACGCAGCCCGTGCCCCGCTTCGACTGCGTGCTCAAGCTGGTGCACCACTACATGCCGC
CCCCTGGAGCCCCCTCCTTCCCCTCGCCACCTACTGAACCCTCCTCCGAGGTGCCCGAGCAGCCGTCTG
CCCAGCCACTCCCTGGGAGTCCCCCCAGAAGAGCCTATTACATCTACTCCGGGGGCGAGAAGATCCCCC
TGGTGTTGAGCCGGCCCCTCTCCTCCAACGTGGCCACTCTTCAGCATCTCTGTCGGAAGACCGTCAACG
GCCACCTGGACTCCTATGAGAAAGTCACCCAGCTGCCGGGGCCCATTCGGGAGTTCCTGGACCAGTACG
ATGCCCCGCTTGATTACAAGGATGACGACGATAAGTAAGATATCTGCGGCCGCGTCGACGGAATTCAGT
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GAGTACTCCCTCTCAAAAGCGGGCATGACTTCTGCGCTAAGATTGTCAGTTTCCAAAAACGAGGAGGAT
TTGATATTCACCTGGCCCGCGGTGATGCCTTTGAGGGTGGCCGCGTCCATCTGGTCAGAAAAGACAATC
TTTTTGTTGTCAAGCTTGAGGTGTGGCAGGCTTGAGATCTGGCCATACACTTGAGTGACAATGACATCC
ACTTTGCCTTTCTCTCCACAGGTGTCCACTCCCAGGTCCAACTGCAGGTCGAGCATGCATCTAGGGCGG
CCAATTCCGCCCCTCTCCCTCCCCCCCCCCTAACGTTACTGGCCGAAGCCGCTTGGAATAAGGCCGGTG
TGCGTTTGTCTATATGTGATTTTCCACCATATTGCCGTCTTTTGGCAATGTGAGGGCCCGGAAACCTGG
CCCTGTCTTCTTGACGAGCATTCCTAGGGGTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAA
TGTCGTGAAGGAAGCAGTTCCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGACCCTTTGCAG
GCAGCGGAACCCCCCACCTGGCGACAGGTGCCTCTGCGGCCAAAAGCCACGTGTATAAGATACACCTGC
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TGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTA
TCCATCATGGCTGATGCAATGCGGCGGCTGCATCGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGAT
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CGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGCCGCT
CCCGATTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACGAGTTCTTCTGAGGGGATCAATTCTCTAGA
GCTCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCT
TCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGT
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CCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCG
CTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATC
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TGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCA
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GCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGG

TTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTC
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TGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAG
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ACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCC
CATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGT
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TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCC
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TTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACC
CAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGC
CGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTG
AAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT
AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTC

Figure 3-3 Genetic sequence of the GS50726 pIERESneo-Human_SOCS3 plasmid, with the SOCS3 coding sequence highlighted.

3.4.2.2 Generation of stable SOCS3^{hi} IEC

IECs (1 x 10^6) were seeded in a T25 flask in growth medium (without antibiotics). Twenty four hours later cells were transfected with 4μg of GS50726 pIERESneo-Human_SOCS3 plasmid (2.2.3). After 5h, medium was replaced with serum-containing media and antibiotics to avoid cytotoxicity to cells. After two days, IEC were trypsinized, and sub-cultured with selection media containing 0.3, 0.4 or 0.5 mg/ml of G418 (Neomycin) antibiotic. A control experiment where non-transfected IEC (SOCS3^{norm}) are incubated with selection media containing 0.3, 0.4 or 0.5 mg/ml of G418 antibiotic was also included.

The highest concentration of G418 antibiotic which kills SOCS3^{norm} IEC while allowing the SOCS3^{hi} IEC to survive indicates the SOCS3^{hi} IEC have acquired the plasmid and are therefore antibiotic resistant to the G418.

3.4.2.3 Confirmation of SOCS3 overexpression

To validate overexpression of SOCS3, protein and mRNA levels were measured in SOCS3^{norm} and SOCS3^{hi} IEC using qPCR and Western blot respectively. Once overexpression of SOCS3 was confirmed; IECs were continually incubated with the highest concentration of G418 antibiotic at which SOCS3-transfected IEC (SOCS3^{hi}) were able to survive while killing the SOCS3^{norm} IEC.

3.5 Results

3.5.1 SW480 and Caco-2 IEC were both shown to transiently overexpress SOCS3 mRNA

The expression of SOCS3 mRNA was normalised relative to RPLPO (housekeeping gene) mRNA, and results displayed as relative increase in SOCS3 plasmid transfected SW480 IEC vs. EV plasmid transfected SW480 IEC. A student *t test* was used to determine if there was a significant difference in contrast to EV transfection.

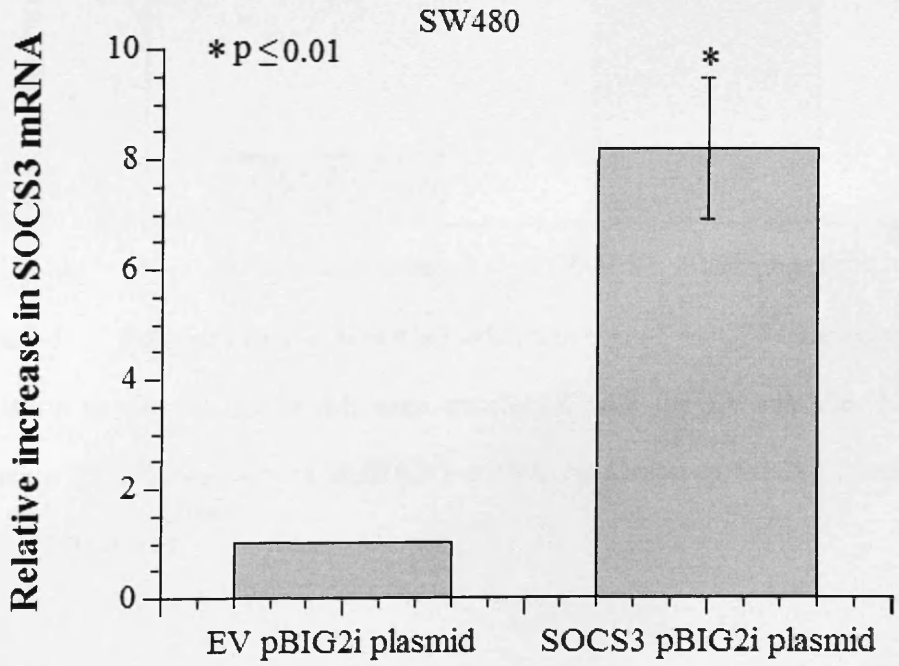

Figure 3-4 Relative increase in SOCS3 mRNA in SW480 SOCS3 transfected IEC in relation to SW480 IEC which were transfected with the EV plasmid. Results indicate an 8.2 relative-increase in SOCS3 mRNA expression in SOCS3 transfected IEC ($p \leq 0.01$) (n=3).

Data from figure 3-4 suggest that the transfection process was successful as SW480 IEC transfected with the SOCS3 plasmid significantly showed an 8.2 (±1.3) relative-increase in SOCS3 mRNA expression in comparison to SW480 IEC which were transfected with the EV plasmid ($p < 0.01$).

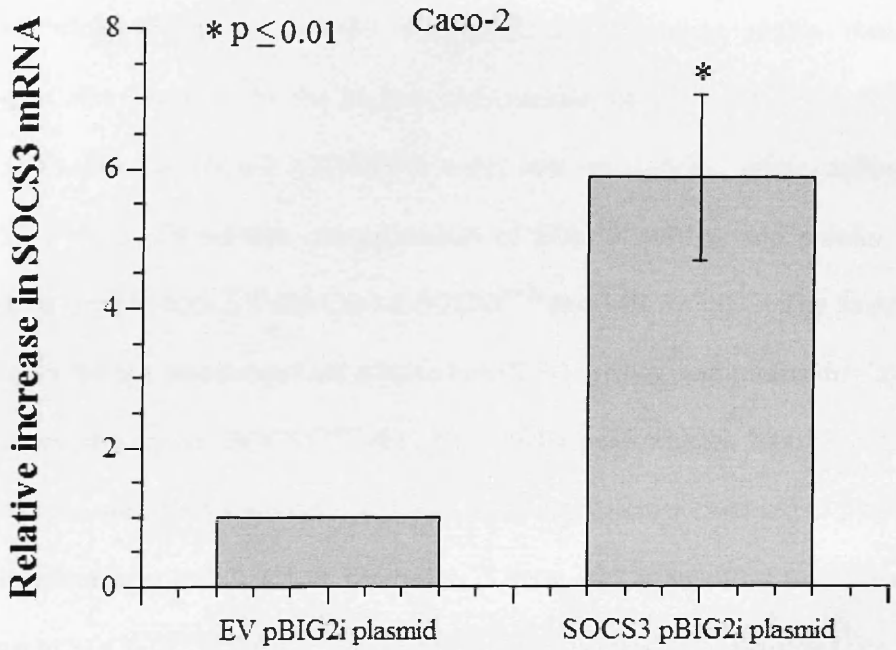

Figure 3-5 Relative increase in SOCS3 mRNA in Caco-2 SOCS3 transfected IEC in relation to Caco-2 IEC which were transfected with the EV plasmid. Results indicate a 5.9 relative-increase in SOCS3 mRNA expression in SOCS3 transfected IEC ($p \leq 0.01$) (n=3).

Data from figure 3-5 suggests that the transfection process was successful as Caco-2 IEC transfected with the SOCS3 plasmid showed a 5.9 (±1.2) relative-increase in SOCS3 mRNA expression in comparison to Caco-2 IEC which were transfected with the EV plasmid ($p < 0.01$).

3.5.2 SW480 IEC were shown to stably overexpress SOCS3 at both mRNA and protein levels

Selection media containing 0.4mg/ml was found to be the highest concentration of G418 antibiotic at which SOCS3-transfected SW480 ($SOCS3^{hi}$) were able to survive while killing the SW480 $SOCS3^{norm}$ IEC. Selection media containing 0.5mg/ml was found to be the highest concentration of G418 antibiotic at which SOCS3-transfected Caco-2 ($SOCS3^{hi}$) were able to survive while killing the $SOCS3^{norm}$ IEC. To validate overexpression of SOCS3, mRNA and protein levels were measured in both SW480/Caco-2 $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC. The expression of SOCS3 mRNA was normalised relative to RPLPO mRNA, and results displayed as relative expression vs. $SOCS3^{norm}$ IEC. SDS-PAGE and western blotting was also used to measure SOCS3 protein expression with expression normalised to β-actin. A student *t test* was then used to determine if there was a significant difference in contrast to $SOCS3^{norm}$IEC.

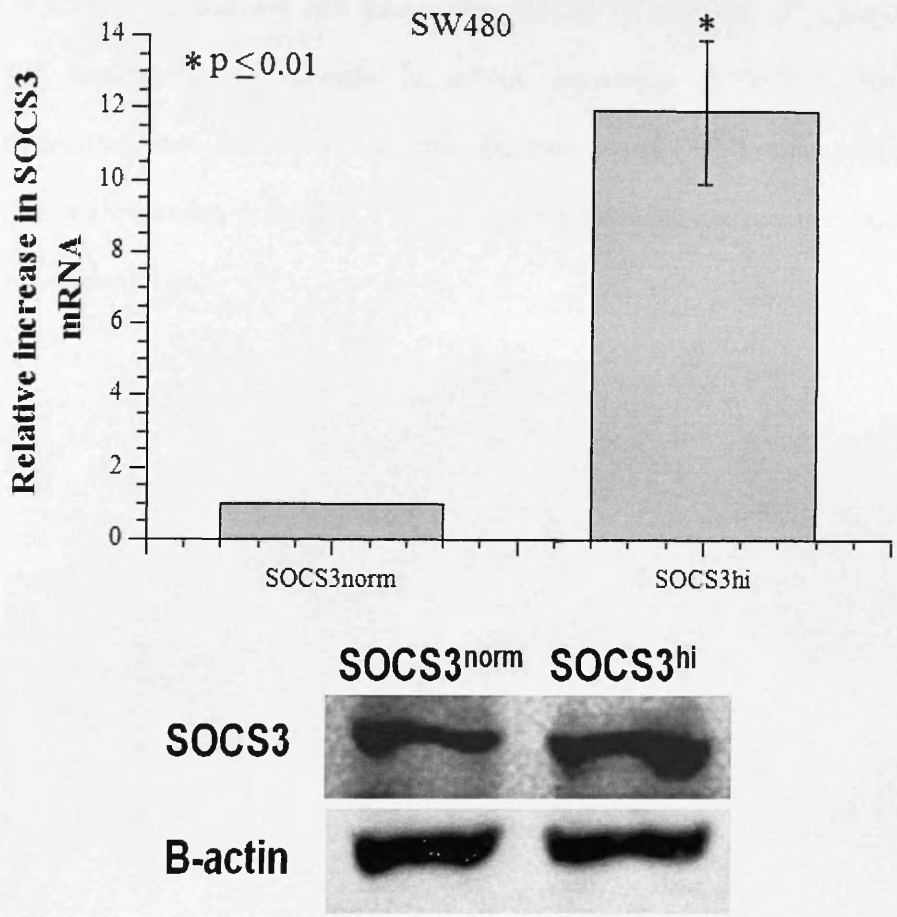

Figure 3-6 qPCR and western blots illustrating SOCS3 expression in SW480 SOCS3norm and SOCS3hi IEC, with β-actin used as a loading control for immunoblotting. SOCS3 is shown to be overexpressed at both mRNA and protein levels in SOCS3hi SW480 IEC ($p \leq 0.01$) (n=3)

SW480 $SOCS3^{hi}$ IEC depicted a 12 (±2) fold increase in SOCS3 mRNA in comparison to $SOCS3^{norm}$ IEC. Immunoblotting also showed a 25% (±9%) increase in SOCS3 protein expression when compared to $SOCS3^{norm}$ SW480 IEC (Figure 3-6). Once overexpression of SOCS3 was confirmed; SW480 $SOCS3^{hi}$ IEC were maintained with 0.4mg/ml of G418 antibiotic.

SOCS3 expression was also measured in $SOCS3^{norm}$ and $SOCS3^{hi}$ Caco-2 IEC. RT-PCR data showed a decrease in mRNA expression (0.53±0.19) however; immunoblotting data showed a negligible increase in SOCS3 protein expression (2±15%) in comparison to $SOCS3^{norm}$ IEC indicating transfection was not successful (data not shown).

3.5.3 Caco-2 IEC were shown to overexpress SOCS3 mRNA after forming monolayers

Caco-2 IEC were transfected and allowed to form monolayers for seven days. SOCS3 mRNA expression levels were then monitored before wounding to ensure Caco-2 IEC were still overexpressing SOCS3 (section 4.5.7). The expression of SOCS3 mRNA was normalised relative to RPLPO (housekeeping gene) mRNA, and results displayed as relative increase in SOCS3 plasmid transfected Caco-2 IEC vs. EV plasmid transfected Caco-2 IEC. A student *t test* was then used to determine if there was a significant difference in contrast to EV transfection.

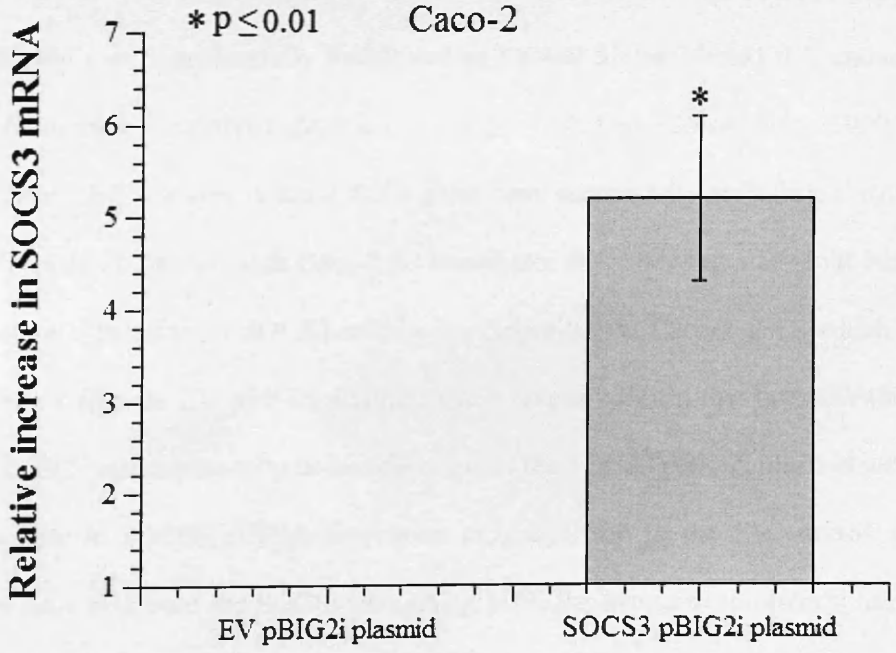

Figure 3-7 Relative increase in SOCS3 mRNA in Caco-2 SOCS3 transfected IEC in relation to Caco-2 IEC which were transfected with the EV plasmid then allowed to form monolayers for 7 days. Results indicate a 5.2 relative-increase in SOCS3 mRNA expression in SOCS3 transfected IEC ($p \leq 0.01$) (n=3).

3.6 Discussion

SOCS3 is a natural endogenous regulator of inflammatory cytokine mediated signalling within IEC and SOCS3 elicits its effects by inhibiting signalling through the JAK-STAT3 pathway (Suzuki, Hanada et al. 2001; Rigby, Simmons et al. 2007). There is overwhelming evidence to support the role of SOCS3 as a tumour suppressor within IECs, as targeted deletion of SOCS3 promotes tumour incidence and growth within the colon (Ogata, Chinen et al. 2006; Rigby, Simmons et al. 2007).

To examine the upregulation of SOCS3; actively dividing SW480 and Caco-2 IECs were transiently transfected with either SOCS3 pBIG2i plasmid (S3) or a control empty vector (EV) pBIG2i plasmid to ensure plasmid uptake. Relative SOCS3 mRNA levels were then compared to IECs which were transfected with EV plasmids. SW480 IECs were successfully transfected as SW480 S3 transfected IEC showed an 8.2 fold increase in relative expression of SOCS3 mRNA in comparison to SW480 EV transfected IEC cultures. Caco-2 IECs were also successfully transfected with the SOCS3 pBIG2i plasmid with Caco-2 S3 transfected IEC showing a 5.9 fold increase in relative expression of SOCS3 mRNA in comparison to Caco-2 IECs which were transfected with the EV pBIG2i plasmid. These results confirm that both SW480 and Caco-2 IECs can successfully be transfected with the SOCS3 pBIG2i plasmid yielding an increase in SOCS3 mRNA expression in comparison to the EV control. Other groups have also used the SOCS3 containing pBIG2i plasmid to transiently transfect Caco-2 and IEC6 IEC and constitutively overexpress SOCS3. (Rigby, Simmons et al. 2007).

During transient transfection, foreign DNA is delivered into the nucleus but is not integrated into the host genome, thus the new gene will not be replicated following

cell division. The downside to transient transfection is that cells will only express the transiently transfected gene for a finite period, after which the foreign gene is lost through cell division. Being able to transiently upregulate SOCS3 will be good for short term experiments e.g. proliferation and signalling. However, it is not good for long term experiments e.g. wound healing (Wurm 2004; Recillas-Targa 2006; Kim and Eberwine 2010). Therefore, cells would have to be repeatedly transfected which could result in experimental variation in terms of levels of gene expression. Stable transfection is another technique whereby foreign DNA is delivered to the nucleus by passage through the nucleus and nuclear membranes. The foreign DNA is then integrated into the host genome with cells continuing to express traits caused by the genetic material, even after many generations of cell division (Recillas-Targa 2006; Kim and Eberwine 2010). To overcome variation between transient experiments, efforts were made to generate IECs which were stably overexpressing SOCS3.

Actively dividing SW480 and Caco-2 IECs were stably transfected with GS50726 pIERESneo-Human_SOCS3 plasmids ($SOCS3^{hi}$). To validate overexpression, relative SOCS3 mRNA and protein expression levels were compared to IECs which had not been transfected with the SOCS3 overexpressing plasmid ($SOCS3^{norm}$). Results indicated SW480 IECs were successfully transfected as $SW480^{hi}$ IECs significantly expressed a 12 fold-increase in relative expression of SOCS3 mRNA in comparison to $SW480^{norm}$ IECs cultures. Protein expression of SOCS3 was also verified, and $SW480^{hi}$ IEC expressed a 25% fold-increase in SOCS3 protein expression in comparison to $SW480^{norm}$ IEC cultures. Efforts were also made to stably transfect Caco-2 IECs. However, this proved unsuccessful as $SOCS3^{hi}$ IECs did not show a significant overexpression of SOCS3 in comparison to $SOCS3^{norm}$ IECs. Caco-

2 $SOCS3^{hi}$ IEC showed a 0.53 fold-decrease in SOCS3 mRNA and a 2% increase in SOCS3 protein expression in comparison to $SOCS3^{norm}$ IECs.

To evaluate SOCS3s role in TLR-induced wound repair, Caco-2 IEC would have to be transiently transfected, monolayers allowed to form then SOCS3 levels monitored to ensure IEC were still overexpressing SOCS3 before wounding. Results indicated that Caco-2 IEC transfected with the SOCS3 pBIG2i plasmid were still overexpressing SOCS3 (5.2) in comparison to Caco-2 IEC which were transfected with the EV pBIG2i plasmid.

Results indicate IEC can be transfected to upregulate the expression of SOCS3. In the following chapters, both transient and stable IECs (SW480 and Caco-2) which overexpress SOCS3 will be used to investigate the role of SOCS3 in mediating cellular responses following microbial-induced TLR signalling.

4. SUPPRESSOR OF CYTOKINE SIGNALLING 3 (SOCS3) INFLUENCES MICROBIAL-INDUCED INTESTINAL EPITHELIAL RESTITUTION AND WOUND REPAIR

4.1 Summary

TLR signalling is imperative for repair of damaged tissue with microbial signalling inducing cell migration, wound repair and proliferation of IEC. SOCS3, a mediator of cytokine signalling is upregulated in both animal and human models of inflammation. Conversely, lack of epithelial SOCS3 promotes proliferation and tumourgenesis in the colon.

In these studies we have used model human SW480 and Caco-2 cell lines over-expressing SOCS3 (described in Chapter 3) to develop an *in vitro* model and assess SOCS3 and TLR ligands on epithelial wound repair. Additionally, the role of SOCS3 on TLR-induced IEC proliferation was also assessed.

Proliferation data indicates that SOCS3 limits LPS and *T. muris* excretory/secretory protein (ES) induced proliferation. Wound healing data implies ES promotes both proliferative and restitutive wound repair, whereas LPS and flagellin only promote restitutive wound repair in our model. This could be due to ES ligating with multiple TLR (TLR2, TLR4 and TLR6) whereas LPS (TLR4) and flagellin (TLR5) binding exclusively to a single receptor. It was also established wound repair was due to direct TLR ligation not secondary effects of induced cytokines. Data from this study also indicated SOCS3 was shown to limit *T. muris* induced wound repair.

In conclusion our results support previous data implicating the role of TLR ligands being imperative for the repair of damaged epithelial surfaces, and highlights a pivotal role of SOCS3 in mediating TLR-induced epithelial repair.

4.2 Introduction

The integrity of the intestinal epithelium is pivotal as this establishes a barrier that separates the luminal contents with its immunogenic, noxious compounds and commensal flora from the underlying immune system (Sturm and Dignass 2002). Injury or impairment of the intestinal surface barrier is perceived in the course of a number of diseases, which may consequently result in an elevated penetration of luminal contents, leading to inflammation and disequilibrium of the homeostasis of the host. Therefore, rapid repair of the epithelium following injury or damage is imperative to conserve normal homeostasis (Rakoff-Nahoum, Paglino et al. 2004; Ben-Neriah and Schmidt-Supprian 2007). Chronic relapse and remittance of inflammation causes damage to the intestinal epithelium, a hallmark of IBD (Hanauer 2006).

The continuity of the surface epithelium following injury is re-established by three distinctive phases (Wilson and Gibson 1997; Dignass 2001). Firstly, restitution where IEC migrate into the damaged area, this can arise within minutes to hours after injury. Restitution is followed by proliferation where IEC divide to replenish reduced cell numbers. Thirdly, differentiation where IEC mature and differentiation to maintain the functional activity of the epithelium (Dignass 2001; Dignass, Baumgart et al. 2004).

IECs are able to communicate with microbes in the colon via a cohort of pattern recognition receptors termed Toll-like receptors (TLR). TLRs are involved in several features of mammalian homeostasis such as recognition of cellular tissue injury, proliferation, tissue repair and regeneration, all fundamental for healing (Schottelius and Dinter 2006; Zhang and Schluesener 2006; Rakoff-Nahoum and Medzhitov 2008).

TLR signalling has also been shown to be imperative for repair of damaged tissue following chemical, radiation and colonic injury (Fukata, Michelsen et al. 2005; Rakoff-Nahoum and Medzhitov 2008).

Wound healing models have shown that the absence of TLR4 had notable consequences on both the inflammatory and repair phases of lesions (Mollen, Anand et al. 2006). This can be supported by other researchers which have demonstrated that mice deficient in TLR4 expression are at an increased risk of injury following DSS induced mucosal injury (Rakoff-Nahoum, Paglino et al. 2004; Abreu, Fukata et al. 2005). Microbial signalling via TLR5 within human epithelial cells induce a series of non-immune epithelial responses including cell migration, wound repair and proliferation (Shaykhiev, Behr et al. 2008). Numerous studies have also demonstrated that exposure to helminths prevents TNBS-type colitis (Elliott, Summers et al. 2007; Ruyssers, De Winter et al. 2008; Motomura, Wang et al. 2009); suggesting helminth products promote mucosal healing in animal models of colitis. These studies indicate microbes play a pivitol role in miantaining mucosal homeosis and restoration of epithelial integrity following damage.

Following tissue injury, TLRs are also able to activate numerous intracellular signalling molecules with one of these being NF-$_k$B (Barnes and Karin 1997). NF-$_k$B is important because several inflammatory cytokine genes implicated in the pathogenesis of IBD including TNF-α, IL-6 and IL-12 are all known to contain an NF-$_k$B binding site and have been shown to be transcriptionally regulated by NF-$_k$B (Barnes and Karin 1997; Schottelius and Dinter 2006). The bacterial components LPS, an outer cell membrane constituent of Gram-negative bacteria, derived from *E. coli* and flagellin a major constituent of the flagellum of the Gram-negative bacterium *S. typhimurium* were both chosen as both exist as part of the normal flora within the human colon.

Helminth products from *T. muris* were also chosen as exposure to helminth products are shown to prevent TNBS-type colitis (Khan, Blennerhasset et al. 2002; Elliott, Li et al. 2003).

SOCS3 a natural endogenous regulator of inflammatory cytokine-mediated signalling is upregulated in both animal and human intestinal inflammation (Suzuki, Hanada et al. 2001). Additionally, elevated levels of SOCS3 mRNA expression have been detected in inflamed biopsies of patients with UC compared to non-inflamed biopsies (Miyanaka, Ueno et al. 2007). Lack of epithelial SOCS3 promotes proliferation and tumourgenesis in the colon (Rigby, Simmons et al. 2007) and conversely, SOCS3 overexpression can hamper skin epithelial wound healing, enhancing inflammation (Ivory, Wallace et al. 2008; Linke, Goren et al. 2009). *In vivo* studies have shown SOCS3 overexpression limits proliferation of IEC and colon cancer cell lines (Rigby, Simmons et al. 2007).

Conclusively, these studies suggest that although SOCS3 may have a significant anti-tumourgenic role, inhibiting inflammation-associated CRC (Rigby, Simmons et al. 2007) SOCS3 may also inhibit repair and restitution of the epithelial barrier associated with UC. This section aims to develop an *in vitro* model to investigate the function of SOCS3 overexpression on TLR-mediated epithelial wound healing.

4.3 Aim: Investigate SOCS3 overexpression on TLR-mediated wound repair

4.4 Methods

4.4.1 Assessment of SOCS3 on TLR-induced IEC proliferation

SW480 IEC were seeded overnight then transfected. Cells were then treated with 1μg/ml LPS, 1μg/ml flagellin or 10μg/ml *T. muris excretory/secretary* products (ES) (gift from Kathryn Else, University of Manchester) for 48h.Concentrations were selected because these concentrations were identified as having optimal effects on wound healing. Cell number was determined using method described in section 2.2.6. To evaluate cellular proliferation in response to treatment; values had been expressed as percentage change in cell number relative to no treatment controls for treatment.

4.4.2 Assessment of TLR ligands and SOCS3 on epithelial wound repair

Caco-2 IEC were seeded at 80% confluence to minimise post-transfection proliferation and minimise loss of SOCS3 overexpression. IEC were transfected, and monolayers allowed to form for 7 days. Monolayers were scored then treated with 10μg/ml LPS, 1μg/ml flagellin or either 10 or 5μg/ml of ES. Confocal imaging was carried out at 0, 6, 24, 48 and 72h (x 10 magnifications) post wounding. Ligand containing media was replaced prior to measurements taken at 24h, 48h and 72h to remove floating cells, replenish nutrients and restore treatment concentrations. The area of the acellular region was measured using ImageJ and data calculated as % wound healed Vs. 0h.

4.4.3 Assessing wound repair as a consequence of direct TLR ligation or secondary effects of induced cytokines

Caco-2 cells were treated with LPS (10μg/ml) or flagellin (1μg/ml). After 24, 48 and 72h, supernatant was collected and centrifuged to remove floating cells. Monolayers were scored, washed and treated with either freshly prepared medium containing TLR ligands or supernatants collected from cells treated with ligands for 24h. Photomicrographs of wounds were taken at 0, 6, 24, 48 and 72h (at x10 magnification) post wounding. Media, ligands and supernatant were replaced prior to measurements taken at 24, 48 and 72h to remove floating cells, replenish nutrients and restore treatment concentrations. The area of the acellular region was measured using ImageJ and data calculated as % wound healed Vs. 0h.

4.4.4 Assessment of restitutional vs. proliferative wound repair

Immediately following wounding, Caco-2 monolayers were treated with varying concentrations of hydroxyurea (10, 5 and 2μM) to limit restitution or mitomycin C (10, 5 and 2μg/ml) to limit proliferative wound repair. Photomicrographs of wounds were taken at 0, 6, 24, 48 and 72h (at x10 magnification) post wounding. Inhibitor-containing media was replaced prior to measurements taken at 24, 48 and 72h to remove floating cells, replenish nutrients and restore inhibitor concentrations. The area of the acellular region was measured using ImageJ and data calculated as % wound healed vs. 0h.

4.4.5 Assessing proliferative inhibitor on TLR-mediated epithelial repair

Immediately following wounding, Caco-2 monolayers were treated with LPS (10μg/ml) or flagellin (1μg/ml). A second set of wells were also treated in a similar manner but with the addition of mitomycin C (10μg/ml). Photomicrographs of wounds were taken at 0, 6, 24, 48 and 72h (at x10 magnification) post wounding. TLR ligand/inhibitor-containing media was replaced prior to measurements taken at 24, 48 and 72h to remove floating cells, replenish nutrients and restore TLR ligand/inhibitor concentrations. The area of the acellular region was measured using ImageJ and data calculated as % wound healed vs. 0h.

Experiment was repeated again, but photomicrographs of wounds were taken at 0, 4, 8, 12, 16, 20 and 24h (at x10 magnification) post wounding. The area of the acellular region was measured using ImageJ and data calculated as % wound healed vs. 0h.

4.5 Results

4.5.1 SOCS3 overexpression limits LPS and ES induced IEC proliferation

To evaluate cellular proliferation in response to ligand treatment; values have been expressed as percentage change in cell number relative to no treatment controls for treatment. The bar graph shows the percentage change in cell proliferation when human SW480 IECs were transfected with EV or SOCS3 over-expressing plasmids then incubated with ES (10μg/ml), LPS (1μg/ml) or flagellin (1μg/ml) for 48h. An unpaired student *t test* was used to determine if there was significant effect in contrast to no treatment controls with $p \leq 0.05$.

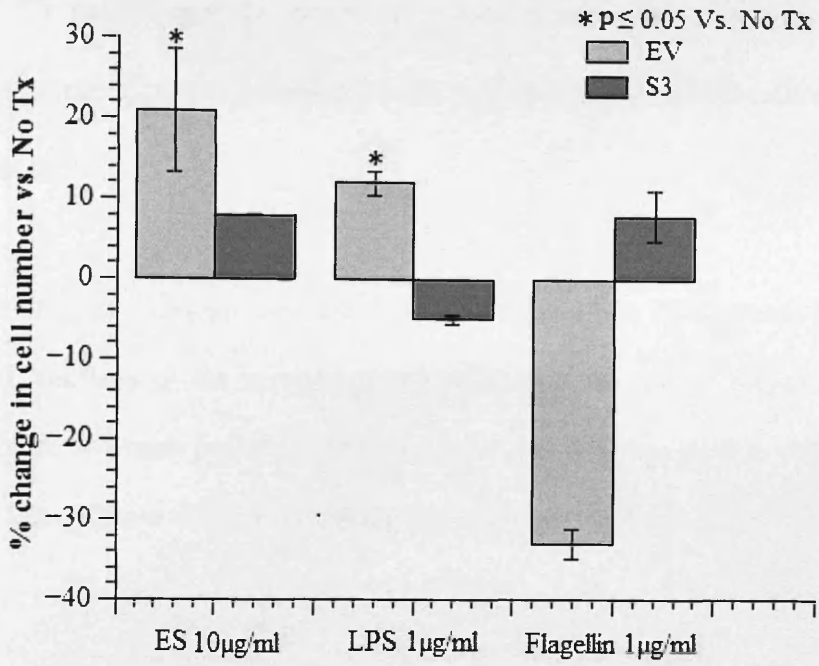

Figure 4-1 Effect of ES, LPS and flagellin on proliferation of SW480 IECs transfected with plasmid either containing the entire coding sequence for SOCS3 (S3) or a control empty vector (EV) after 48h. * = indicates a significant change Vs. No Tx ($p \leq 0.05$) (n=4)

From the bar chart we can conclude that both ES (8%) and LPS (21%) were shown to significantly promote IEC proliferation in comparison to no treatment, however this was no longer observed after treatment in SOCS3 overexpressing IEC as $p > 0.05$. Flagellin was shown to have no significant effect on cell proliferation, even in SOCS3 overexpressing IEC.

4.5.2 LPS and Flagellin promote initial (restitutive) wound healing whereas *T. muris* promotes both restitutive and proliferative wound healing.

To attain an accurate determination of wound repair in response to ligand treatment, the area of the acellular region was measured using ImageJ and data calculated as % wound healed vs. 0h, a student *t test* was then used to determine if there was a significant effect in contrast to no treatment controls.

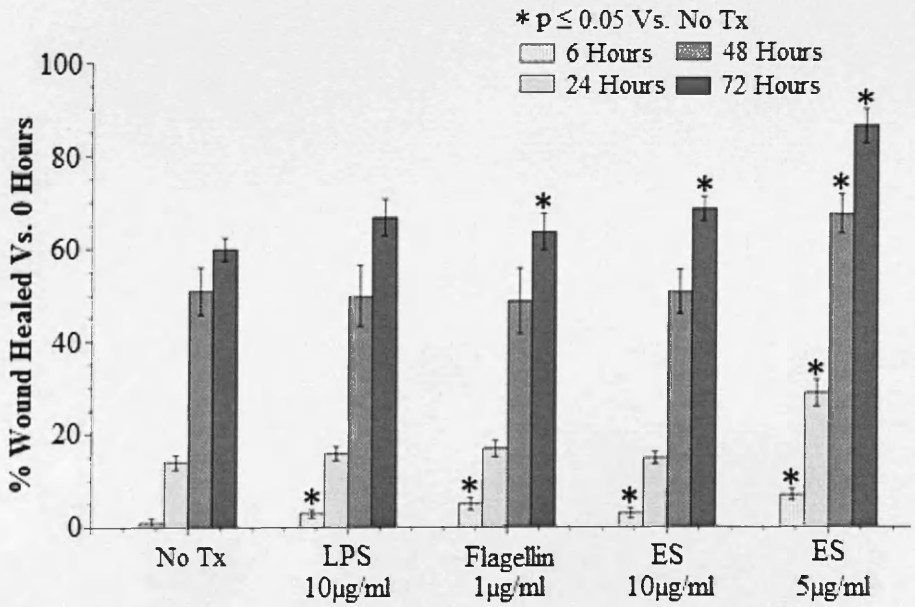

Figure 4-2 Effect of LPS, flagellin and ES treatment on wound healing of Caco-2 monolayers. * = indicates a significant change Vs. No Tx ($p \leq 0.05$) (n=16)

Figure 4-2 depicts treatment with LPS (10µg/ml) had a 2% significant increase in wound repair after 6h as $p < 0.05$. A significant increase in wound repair was also observed when Caco-2 monolayers were scarred then treated with flagellin (1µg/ml)

after 6 and 72h, with repair 4 and 3% greater respectively than no treatment cultures. A 2 and 9% significant increase in wound repair was also observed after treatment with ES (10μg/ml) after 6 and 72h as $p \leq 0.05$. Treatment with ES (5μg/ml) was shown to promote wound repair by 6, 15, 17 and 27% respectively for all time points as $p \leq 0.05$.

4.5.3 Increased wound repair is due to direct TLR ligation, not secondary effects of induced cytokines.

In figure 4-2 data indicated that TLR ligation promotes epithelial wound repair. To confirm wound repair is due to direct TLR signalling and not a secondary effect of TLR ligation, scored monolayers were treated with either freshly prepared medium containing TLR ligands or supernatants collected from cells treated with ligands. Again, the acellular region was measured using ImageJ and data calculated as % wound healed Vs. 0h, a student *t test* was then used to determine if there was a significant effect in contrast to direct treatment.

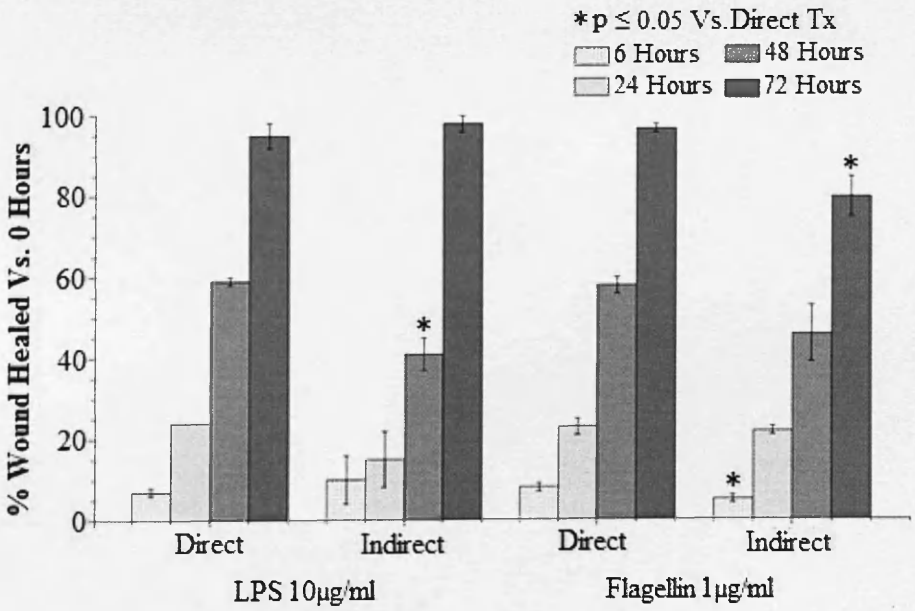

Figure 4-3 Effect of LPS and flagellin indirect and direct treatment on wound healing of Caco-2 monolayers * = indicates a significant change Vs. Direct Tx ($p \leq 0.05$) (n=4)

The bar graph in figure 4-3 indicates that supernatant collected from IEC which had previously been treated with LPS (10μg/ml) as having an 18% significant decrease in wound repair at 48h in comparison to IEC which were directly treated with medium containing LPS. A 3 and 17% significant decrease in wound repair was also observed 6 and 72h after treatment with supernatant which had previously been treated with flagellin (1μg/ml) in comparison to direct flagellin stimulation.

4.5.4 Mitomycin C (10μg/ml) is shown to inhibit proliferative wound repair

Data from figure 4-2 indicates TLR signalling helps mediate wound healing. To determine whether TLR signalling encourages restitutive or proliferative wound repair Caco-2 monolayers were wounded then treated with varying concentrations of hydroxyurea (10, 5, and 2μM) to inhibit restitution or mitomycin C (10, 5 and 2μg/ml) to limit proliferative wound repair. Hydroxyurea works by inactivating ribonucleotide reductase, blocking the synthesis of deoxynucleotides, inhibiting DNA synthesis and inducing cell death in S-phase. Mitomycin C is an inter-strand cross-linking, alkylating agent that targets guanine nucleoside in the 5′CpG-3 sequence. To attain an accurate determination of wound repair in response to inhibitor treatment, the area of the acellular region was measured using ImageJ and data calculated as % wound healed Vs. 0h, a student *t test* was then used to determine if there was a significant effect in contrast to no treatment controls.

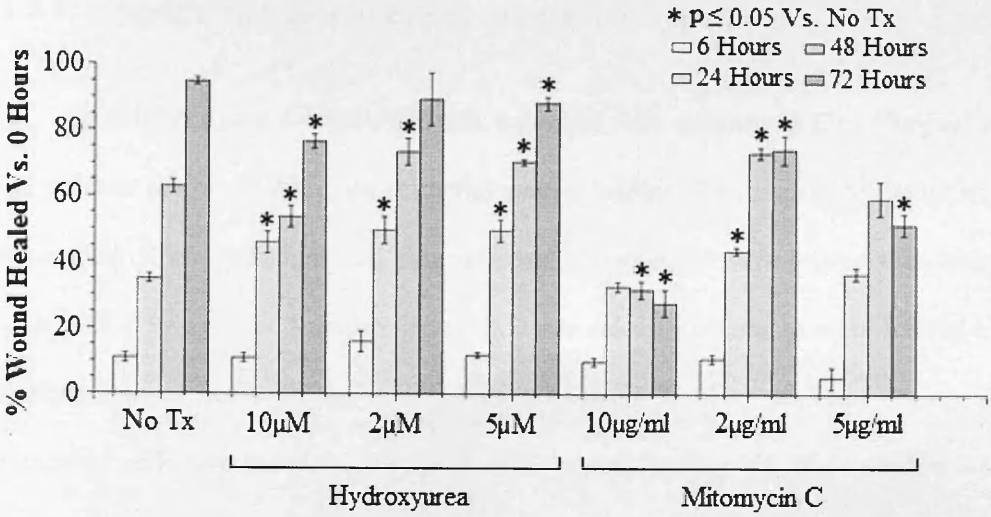

Figure 4-4 Effect of hydroxyurea (10, 5 and 2μM) and mitomycin C (10, 5 and 2μg/ml) treatment on wound healing of Caco-2 monolayers. * = indicates a significant change Vs. No Tx ($p \leq 0.05$) (n=4)

To identify the inhibitor which had the greatest inhibitory effect and its optimum effective inhibitory concentration on IEC wound repair, values have been expressed as % wound healed Vs. 0h. The experiment was repeated 4 times (n=4) with all results pooled together. The bar graph in figure 4-4 indicates mitomycin C at 10μg/ml had the greatest inhibitory effect on proliferative wound repair at 24, 48 and 72h post wounding with repair 2, 31 and 67% respectively significantly less than wounds which had not been treated with mitomycin C.

4.5.5 Flagellin may promote restitutive wound repair

Data from figure 4-4 indicated that treatment with mitomycin C at 10µg/ml had the greatest inhibitory effect on epithelial wound healing. To investigate the effect of mitomycin C on TLR-mediated wound repair, monolayers were scored then treated with LPS (10µg/ml) or flagellin (1µg/ml), a second set of cultures were treated in a similar manner but with the addition of mitomycin C. The acellular region was measured as before and data calculated as % wound healing Vs. 0h, a student *t test* was then used to determine if there was a significant effect in contrast to cultures which were only treated with mitomycin C.

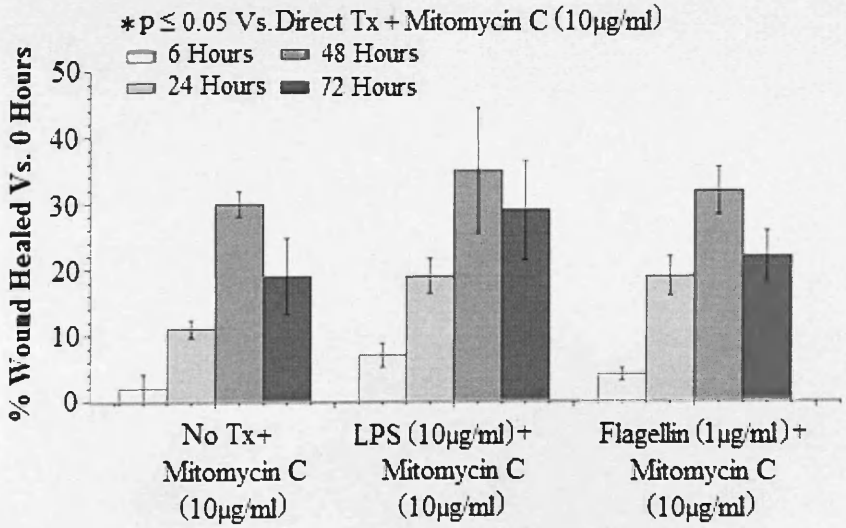

Figure 4-5 Effect of mitomycin C, LPS and flagellin treatment on wound healing of Caco-2 monolayers. * = indicates a significant change Vs. No Tx + mitomycin C Tx ($p \leq 0.05$) (n=3)

To evaluate TLR-mediated wound healing in response to mitomycin C (10μg/ml) treatment, values were expressed as % wound healed Vs. 0h. The experiment was repeated 3 times (n=3) with all results pooled together. From figure 4-5 we can conclude that treatment with mitomycin C (10μg/ml) and TLR ligands (LPS and flagellin) had no significant effect in wound repair in comparison to cultures which were only treated with mitomycin C. However, data hints treatment with flagellin (1μg/ml) may promote restitutive wound repair at 24h ($p= 0.06$) as repair was 8% greater in flagellin-treated cultures than cultures which had only been treated with mitomycin C.

4.5.6 Flagellin is shown to promote proliferative wound repair

Data from figure 4-5 indicated treatment with mitomycin C (10µg/ml) significantly inhibited flagellin (1µg/ml) induced wound repair at 6h with repair 6% significantly lower in mitomycin C (10µg/ml) treated cultures. To further investigate the effect of mitomycin C (10µg/ml) on TLR-mediated proliferative repair (0-24 h) the acellular region was measured every four hours and data calculated as % wound healed Vs. 0h, a student *t test* was then used to determine if there was a significant effect in contrast to cultures which were only treated with mitomycin C.

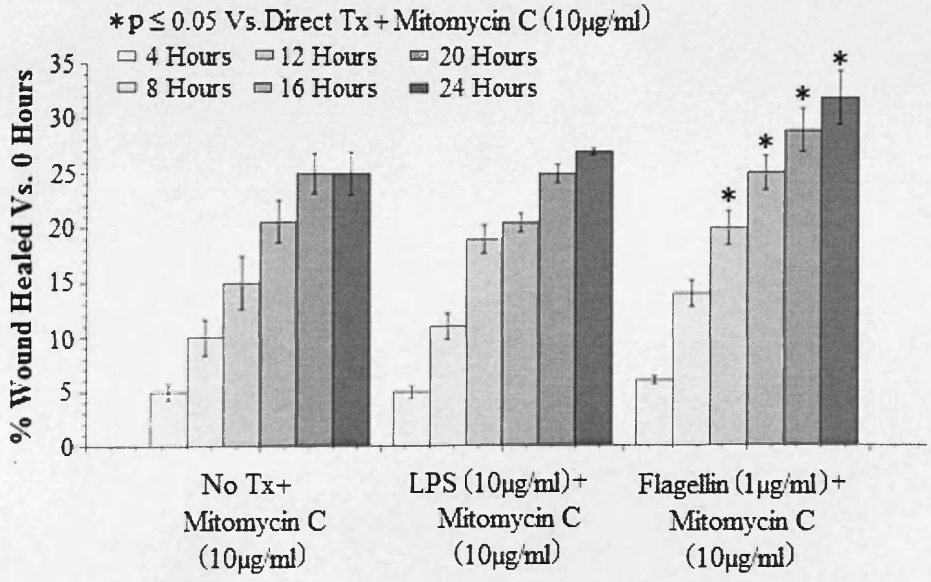

Figure 4-6 Effect of mitomycin C, LPS and flagellin treatment on wound healing of Caco-2 monolayers. * = indicates a significant change Vs. No Tx + mitomycin C Tx ($p \leq 0.05$) (n=3)

From figure 4-6 we can conclude that treatment with flagellin (1µg/ml) and mitomycin C (10µg/ml) was shown to significantly promote wound repair by 5, 4, 4 and 7% at 12, 16, 20 and 24h respectively post wounding in comparison to cultures

which had only been treated with mitomycin C (10μg/ml) as $p<0.05$. Treatment with LPS (1μg/ml) and mitomycin C (10μg/ml) had no significant effect on wound repair in comparison to cultures which had only been treated with mitomycin C (10μg/ml).

4.5.7 SOCS3 limits *T. muris*-induced wound repair.

To evaluate SOCS3s role in TLR-induced wound repair, Caco-2 IEC were transfected with SOCS3 over-expressing plasmids or empty vector (EV) controls. Again, the acellular region was measured using ImageJ and data calculated as % wound healed Vs. 0h, a student *t test* was then used to determine if there was a significant effect in contrast to EV plasmid transfected IECs.

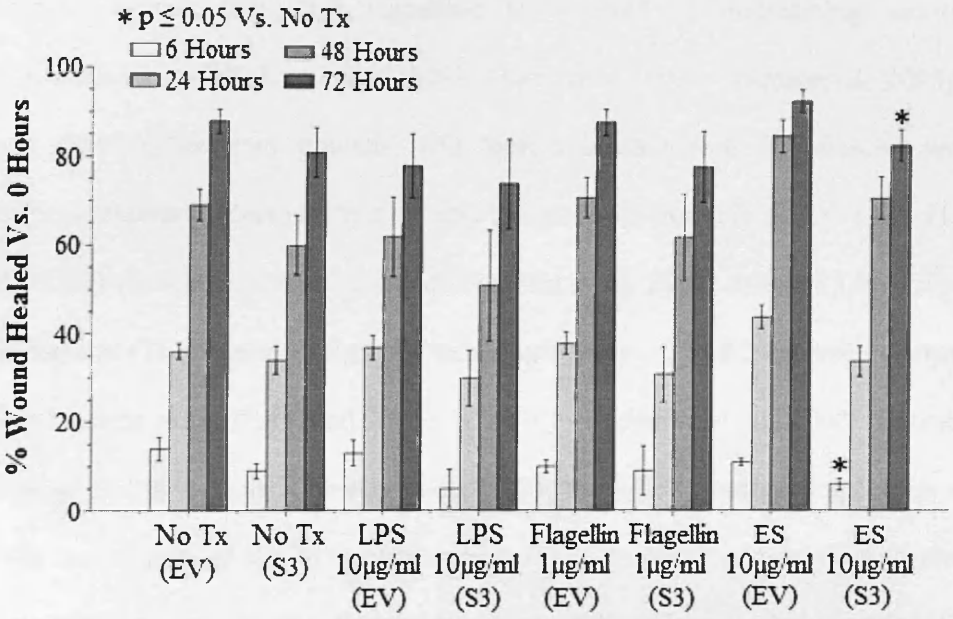

Figure 4-7 Effect of LPS, flagellin and ES treatment on wound healing of Caco-2 monolayers after transfecting Caco-2 IEC with empty (EV) or SOCS3 vector (S3) plasmids. * = indicates a significant change vs. Empty vector ($P \leq 0.05$) (n=9)

Overexpression of SOCS3 had no significant effect on flagellin-induced wound repair at all times ($p>0.05$). Likewise, over-expression of SOCS3 had no significant effect on LPS-induced wound repair. However, SOCS3 limited ES- induced wound repair at 6 and 72h by 8% and 16% respectively, as $p<0.05$.

4.6 Discussion

Previous studies have demonstrated germ free mice are more susceptible to DSS-induced intestinal injury inferring intestinal microbiota stimulate cytoprotective pathways to promote epithelial repair (Rakoff-Nahoum, Paglino et al. 2004; Kluwe, Mencin et al. 2009). Conversely, germ-free mice also have a lower incidence of tumour burden within the colon (Rakoff-Nahoum, Paglino et al. 2004). Mounting evidence suggests that TLR signalling is involved in maintaining intestinal homeostasis (Rakoff-Nahoum *et al.,* 2008, Zhang *et al.,* 2006, Fukata *et al.,* 2005). *T. muris* excretory/secretory protiens (ES) form a heterogeneous solution of worm proteins containing substances that are able to ligate with multiple TLR (TLR2, TLR4 and TLR6) (deSchoolmeester, Martinez-Pomares et al. 2009) whereas LPS (TLR4) and flagellin (TLR5) bind exclusively to a single receptor. TLR2 has been shown to heterodimerize with TLR1 and TLR6 (Ozinsky, Underhill et al. 2000; Takeuchi, Kawai et al. 2001; Morr, Takeuchi et al. 2002) to expand the ligand spectrum and permit the immune system to identify numerous structures of bactererial lipopeptides in various pathogens (Farhat, Riekenberg et al. 2008). Here, we demonstrate TLR ligation promotes both proliferative and restitutive wound repair. LPS and flagellin are shown to promote restitutive healing whereas ES is shown to promote both restitutive and proliferative types of wound healing. This could be due to ES ligating with multiple TLR whereas LPS and flagellin binding exclusively to a single receptor.

According to the *hygiene hypothesis,* lack of exposure to intestinal parasites such as helminths, negatively effects immune development, which may predispose individuals to immunological diseases later in life. This is a proposed rationale for the high incidence of IBD in industrialized countries compared with developing countries

(Talley *et al.,* 2011). Several studies exploring the therapeutic potential of helminth infection on IBD have been conducted; with positive findings indicating the potential use of helminths as a therapeutic tool (Ruyssers *et al.,* 2008, Weinstock *et al.,* 2005, Weinstock *et al.,* 2009). Treatment with living helminths, however, may have its disadvantages such as continual infection or invasion of parasite to other host tissues, where they may cause pathology. Therefore, treatment with immunologically active helminth proteins might overcome complications of therapies associated with living parasites. Both *in vivo* infection to helminths and *in vitro* exposure to ES stimulate colonic epithelial cells to produce IFN-*y,* TNFα and CCL2 mRNA and protein (deSchoolmeester *et al.,* 2006; Artis *et al.,* 2008; Cruickshank *et al.,* 2009).

These cytokines and chemokines then have the potential to recruit and activate immune cells including dendritic cells (DC) from the colonic lamina propria to the epithelial layer directing the appropriate immune response. Mice resistant to *T.muris* infection have significantly increased levels of epithelial-derived chemokines compared with susceptible mice (Cruickshank *et al.,* 2012), allowing for a more rapid recruitment and localisation of colonic DC to the epithelium following infection in comparison with susceptible mice. These results provide further evidence that IEC are the initial responders and are critical in determining the ensuing immune response, as well as maintaining barrier function. Our studies show the direct effect of TLR ligation on IEC; indicating increased wound repair is due to direct TLR ligation, and not secondary effects of TLR-induced cytokines, supporting previous studies that IEC directly respond to ES. However, we cannot guarantee that there will not be any TLR ligands in the supernatant collected from IEC which were previously treated with TLR ligands, but TLR-induced cytokines should be present.

In vitro studies have shown that SOCS3 is a potent supressor of proliferation in both transformed and non-transformed IEC lines (Rigby *et al.*, 2007). Furthermore, other studies have shown that SOCS3 overexpression can hamper skin epithelial wound repair (Ivory *et al.*, 2008, Linke *et al.*, 2010, Linke *et al.*, 2010B). Our results expand upon previous studies where SOCS3 limited spontaneous proliferation of IEC (Rigby, 2007) by demonstrating SOCS3 can also limit microbial-induced proliferation and wound repair and support previous studies showing SOCS3 limits cytokine-induced and spontaneous IEC proliferation (Rigby, 2007).

Normal gut homeostasis is likely to be due to maintenance of TLR signalling and regulation of these signalling pathways may be dependent on oscillatory behaviour of regulatory molecules such as SOCS3 (Yoshiura *et al.*, 2007) as without this regulatory behaviour, TLR signals are likely to induce pathological states. The regulation of SOCS3 levels may subsequently influence IEC turnover or repair of epithelium following injury. For example, targeted deletion of SOCS3 in IEC promotes tumour incidence and growth in the colon (Rigby, 2007); with methylation-silencing of SOCS3 shared in multiple tumour types (Ogata *et al.*, 2006). Unconditional cell proliferation could be due to SOCS3 no longer providing a negative feedback mechanism in response to perpetual TLR ligation within the intestine. SOCS3 levels are comparatively low in the colon of germ-free mice (Rigby, unpublished data), presumably as TLR signalling which would normally initiate the expression of SOCS3, no longer present. On the other hand; the overexpression of SOCS3 observed in IBD (Suzuki et al, 2001) is likely to be limiting microbial-induced wound repair. This is supported by the fact that germ-free mice show severe mortality and morbidity when given DSS because of defective epithelial repair responses,

indicating controlled microbial stimulation is essential for intestinal homeostasis (Rakoff Nahoum *et al* 2004).

In conclusion, our results support previous data implicating the role of TLR ligands being imperative for the repair of damaged epithelial surfaces, and highlight a pivotal role of SOCS3 in mediating TLR-induced wound repair.

5. SUPPRESSOR OF CYTOKINE SIGNALLING 3 (SOCS3) ENHANCES TOLL-LIKE RECEPTOR 5 (TLR5) INDUCED TNFα PRODUCTION IN INTESTINAL EPITHELIAL CELLS

5.1 Summary

The homeostatic influence of microflora on IEC turnover occurs in a highly regulated environment whereby signals from the microflora are regulated and processed by IEC. Breakdown in this regulated network of interactions is implicated in the onset of intestinal diseases such as IBD and CRC.

Ligation of TLR causes the activation of NF-$_k$B, consequently influencing the expression of a number of inflammatory cytokine such as TNFα, IL-6 and IL-8. SOCS3, an endogenous modulator of epithelial turnover acts as a regulator of inflammatory cytokine-mediated signalling by restricting TNFα-induced NF-$_k$B translocation and IL-6 induced STAT3 phosphorylation. TNFα signals through its transmembrane receptor TNFR2, with TNFR2 expression upregulated in IBD and AOM/DSS models of colitis associated cancer.

These studies aimed to assess the effects of IEC-derived SOCS3 upon cytokine responses following TLR stimulation Our results indicate activation of TLR5 signalling pathways significantly increased the expression of TNFα and not TGFβ mRNA in IEC compared with TLR3 and TLR4, in a dose dependent manner; and that SOCS3 further enhances the production of TLR5-induced TNFα mRNA. Increases in TNFα protein in the supernatant were not detectable possibly due to secreted TNFα either binding to its receptor, or protein destruction through SOCS3 driven proteasomal degradation. Our results also show TLR5 ligation significantly promotes TNFR2 mRNA expression within IEC but SOCS3 limits TLR5-induced TNFR2 mRNA expression. These results may indicate increased expression of SOCS3 in IBD may perpetuate 'inflammation' by promoting the production of TNFα in response to bacteria through down-regulating expression of TNFR2 on IEC.

5.2 Introduction

Intestinal epithelial cells (IEC) lining the human colon are in perpetual communication with commensal microflora. This host-commensal relationship is known to contribute to preserving the steady-state equilibrium necessary for gut physiology and fortification against damage and invasion (Barbosa *et al.,* 2010, Shaykhiev *et al.,* 2008). The homeostatic influence of microbiota with IEC occurs in an actively regulated environment, dictated both by signals from the microbiota, and by the fine-tuned regulatory activity of IEC and IEC-conditioned components of the immune system. Failure of this regulated network of interactions is implicated in the onset of IBD (Barbosa *et al.,* 2010). Damage to the intestinal barrier is a hallmark of IBD, a cohort of diseases in which there is chronic relapse and remittance of inflammation within the bowel, characteristic of both UC and CD (Hanauer, 2006).

IEC are able to converse with microbes in the colon via a cohort of pattern recognition receptors termed Toll-like receptors (TLR). The detection of microbial ligands such as Poly I: C, LPS and flagellin are recognised by TLR3, TLR4 and TLR5 respectively (Abreu *et al.,* 2005). The core mechanism through which TLR5 signals is similar to that of other TLR. Ligation of TLR5 by flagellin leads to the activation of MAP kinase and NF-$_k$B, influencing the expression of a number of pro-inflammatory cytokines such as TNFα, IL-6 and IL-8 (Vijay-Kumar *et al.,* 2008). As well as maintaining mammalian host defence from hostile microbes, TLR are also responsible for several aspects of mammalian homeostasis for example development, recognition of tissue injury, tissue repair and renewal (Rakoff-Nahoum *et al.,* 2008, Zhang *et al.,* 2006). Studies have demonstrated the importance of TLR5 signalling to be imperative in conserving intact epithelial barrier function, with TLR5 knock-out mice exhibiting

deficient repair following injury and development of spontaneous colitis (Li *et al.*, 2010, Vijay-Kumar *et al.*, 2007). Colitis is supressed by inhibiting inflammatory cytokines (TNFα and IL-12) or stimulating the production of immunoregulatory cytokines (TGFβ and IL-10) (Elliott, Summers et al. 2007; Ruyssers, De Winter et al. 2008).

Germ-free mice and mice deficient in MyD88, an adaptor molecule crucial for TLR signalling given DSS to induce intestinal injury display increased colonic bleeding and epithelial injury compared to control animals (Rakoff-Nahoum *et al.*, 2004). TLR2 and TLR4 knockout mice also have a reduced capacity to repair colonic mucosa, compared with wild-type controls upon DSS administration, though not to the same extent as $MyD88^{-/-}$ mice (Rakoff-Nahoum *et al.*, 2004). $MyD88^{-/-}$ mice administered with AOM a model used to simulate intestinal colonic tumourgenesis are shown to have a lower incidence of tumour formation in comparison to wild-type control mice. Signifying MyD88-dependent signalling pathways are necessary in contributing to carcinogen-induced colonic tumourgenesis (Rakoff-Nahoum *et al.*, 2007).

Suppressor of cytokine signalling-3 (SOCS3), an endogenous modulator of epithelial turnover is enhanced in IBD (Suzuki, 2001; Karin *et al.*, 2005, Rakoff-Nahoum *et al.*, 2006). SOCS3 acts as a regulator of inflammatory cytokine-mediated signalling by restricting TNFα-induced $NF\text{-}_kB$ translocation and IL-6-induced STAT3 phosphorylation (Rigby *et al.*, 2007). There is much evidence to support the role of SOCS3 as a tumour suppressor in the intestine, for example targeted deletion of SOCS3 in IEC promotes tumour incidence and growth in the colon; with methylated silencing of SOCS3 shared in multiple tumour types (Rigby *et al.*, 2007, Ogata *et al.*,

2006). *In vitro* studies have depicted SOCS3 as a potent suppressor of proliferation in both transformed and non-transformed IEC lines (Rigby *et al.*, 2007).

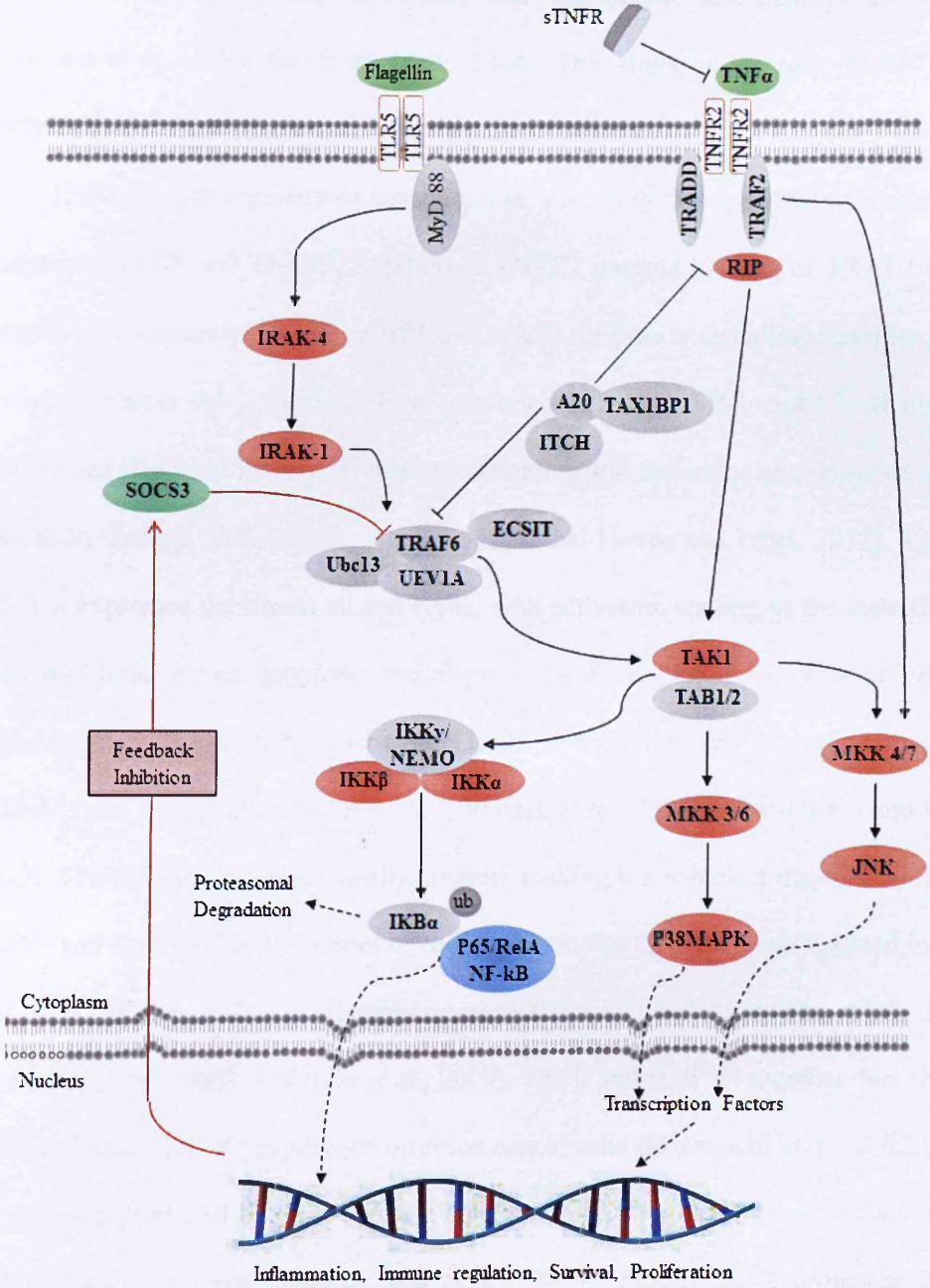

Figure 5-1 TLR5 and TNFR2 receptor signalling pathways, causing the expression of SOCS3 and its negative feedback mechanism on both signalling pathways (Luo, Maeda et al. 2004; Croker, Kiu et al. 2008; Onizawa, Nagaishi et al. 2009).

It has been well documented that levels of TNFα are elevated in serum and intestinal mucosa of patients with IBD, with neutralisation of TNFα associated with improved health, particularly in patients with CD (Stillie and Stadnyk 2009, Van Dullemen *et al.,* 1995, Sandborn *et al.,* 2004). This supports the role of anti-TNFα therapies in their effectiveness in treating patients with IBD.

TNFα is a key regulator of inflammation, signalling through two transmembrane receptors TNFR1 and TNFR2. Ligation of TNFR2 permits binding of TRAF2 to the receptor, subsequently TRAF3, cIAP1 and cIAP2 all form a signalling complex. This complex induces the activation of the transcription factors AP-1 and NF-$_k$B through MAPK and IKK respectively. TNFR2 trimerization and activation also leads to NF-$_k$B activation through NIK (Hauer, *et al.,* 2005; Cabal-Hierro and Lazo, 2012). TNFR1 (p55) is expressed on almost all cell types, with activation leading to the induction of both proliferative and apoptotic processes (Chan *et al.,* 2000; Grell *et al.,* 2012). Alternatively, TNFR2 (p75) activation leads to transcriptional activation of genes linked to cell proliferation and survival (Wallach *et al.,* 2005; Cabal-Hierro and Lazo, 2012). TNFR2 can be proteolytically cleaved, making it a soluble antagonist of TNFα (Stillie and Stadnyk 2009). Studies have also shown that TNFR2 is upregulated in IBD and AOM/DSS models of inflammation-associated cancer (Hernandez *et al.,* 2000; Mizoguchi *et al.,* 2002; Onizawa *et al.,* 2009). TNFα and IL-6 act together, but neither alone to induce TNFR2 expression on colon cancer cells (Mizoguchi *et al.,* 2002).

TGFβ produced by most immune cells is a potent regulator of proliferation and differentiation of most immune cells (Chen 2006). Studies have proposed TGFβ functions as a negative regulator of TLR4-induced macrophage activation; with mice lacking TGFβ associated with LPS hyperresponsiveness, subsequently leading to overexpression of pro-inflammatory cytokines (Comalada, Cardó et al. 2003;

McCartney-Francis, Jin et al. 2004). Activation of TLR4 signalling and its downstream components without negative regulation of TGFβ may therefore promote abnormal immune responses and contribute to unregulated inflammation.

5.3 Aim: To investigate whether SOCS3 influences IEC cytokine production in response to microbial challenge

5.4 Methods

5.4.1 Assessment of TLR ligands and SOCS3 on cytokine and receptor expression

SW480 $SOC3^{norm}$ and $SOCS3^{hi}$ IEC were seeded in a 24-well plate at 2 x 10^5 cells/well. IEC were allowed to proliferate for 48h before serum-starving overnight. IEC were then treated with varying concentrations of LPS (0.1, 1 and 10μg/ml), flagellin (1, 0.1 and 0.01μg/ml) and poly I:C (0.1μg/ml), for 2h. TNFα, TGFβ and TNFR2 mRNA expression levels were then measured using QPCR.

Following treatment, supernatant was collected and TNFα levels measured using an ELISA assay.

5.4.2 Assessment of TLR ligands and SOCS3 on transcription factor expression

SW480 $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC were seeded in a 12-well plate at 4 x 10^5 cells/well. IEC were allowed to proliferate for 48h before serum-starving overnight. IEC were then treated with varying concentrations of LPS (10, 1 and 0.1μg/ml) and flagellin (1, 0.1 and 0.01μg/ml) for 2h. STAT3 phosphorylation and NF-$_k$B expression were then measured using SDS-PAGE and western blotting. Values will be expressed as fold-change in transcription factor protein expression vs. no treatment ± standard error of the mean (SEM).

5.4.3 Assessment of TNFR2 protein expression.

SW480 IEC were seeded in a 12-well plate at 1 x 10^5 cells/well and allowed to adhere overnight to coverslips. IEC were then treated with flagellin (0.1μg/ml) for 1, 2, 4, 6, and 12h with no treatment controls also included to allow comparison. Immunocytochemistry was then used to observe distribution of TNFR2, with IEC TNFR2 expression visualised using the Zeiss LSM 510 Meta Confocal Microscope.

5.5 Results

5.5.1 TLR5 ligation is shown to promote TNFα mRNA expression

Inflammatory TNFα and regulatory TGFβ cytokine mRNA expression were assessed in response to TLR ligand treatment. Expression was normalised to RPLPO mRNA, and results displayed as fold-change in cytokine relative to no treatment. A student *t test* was used to determine statistical difference in contrast to no treatment with $p \leq 0.05$.

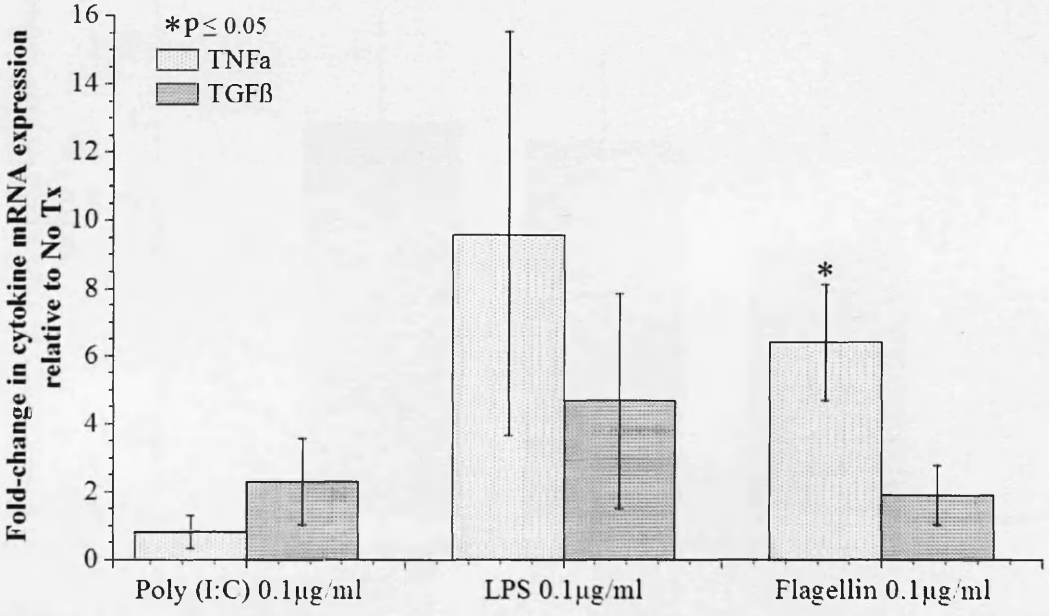

Figure 5-2 Fold-change in cytokine mRNA expression after TLR ligation (*=p ≤0.05 vs. No Tx) (n=3)

Data from figure 5-2 indicate treatment with flagellin, but not poly I:C or LPS caused a significant increase in TNFα mRNA expression (6.4). No significant increases in TGFβ mRNA expression were observed after ligation of TLR3, TLR4 or TLR5.

5.5.2 TLR5 ligation had no effect on TGFβ mRNA expression

TGFβ mRNA expression was observed when IEC were treated with varying concentrations of flagellin (0.01μg/ml 0.1μg/ml and 1μg/ml) for 2h. Figure 5-3 shows fold-change in TGFβ mRNA expression relative to no treatment. An unpaired student *t test* was used to determine if there was significant effect in contrast to no treatment controls with $p \leq 0.05$.

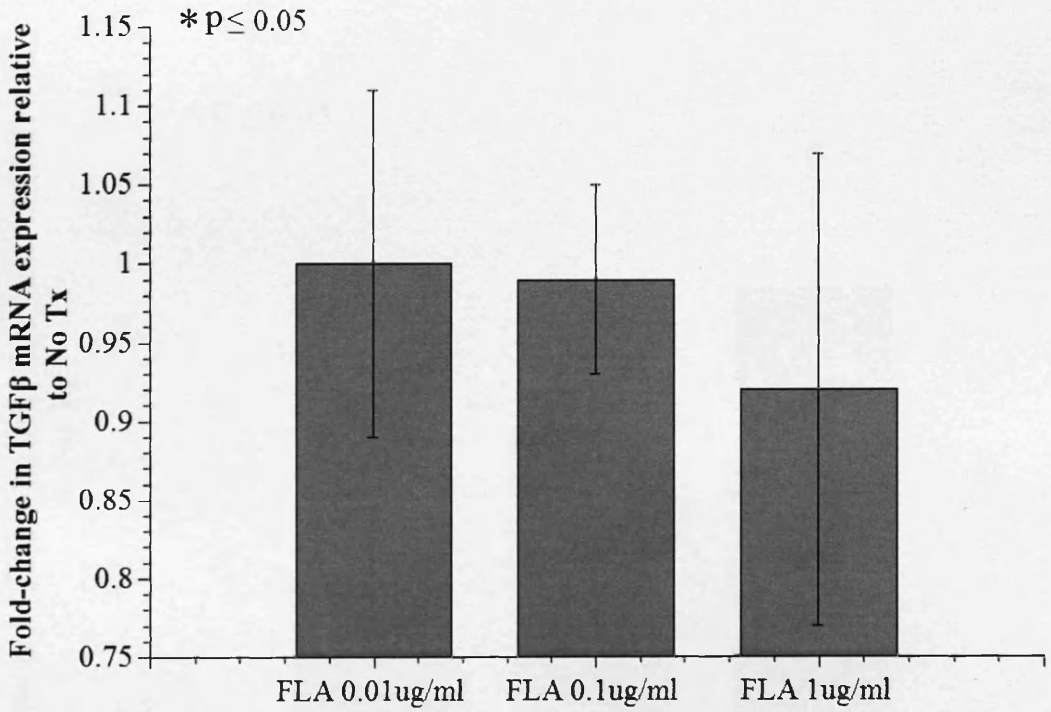

Figure 5-3 Fold-change in TGFβ mRNA expression after TLR5 ligation. (* = $p \leq 0.05$ vs. No Tx) (n=3)

From figure 5-3 we can conclude that all concentrations of flagellin (0.01, 0.1 and 1μg/ml) had no effect on TGFβ mRNA expression as $p > 0.05$.

5.5.3 TLR5 induces TNFα mRNA expression in a dose dependent manner

TNFα mRNA expression was observed when IEC were treated with varying concentrations of flagellin (0.01μg/ml 0.1μg/ml and 1μg/ml) for 2h. Figure 5-4 shows fold-change in TNFα mRNA expression relative to no treatment. An unpaired student *t test* was used to determine if there was significant effect in contrast to no treatment controls with $p \leq 0.05$.

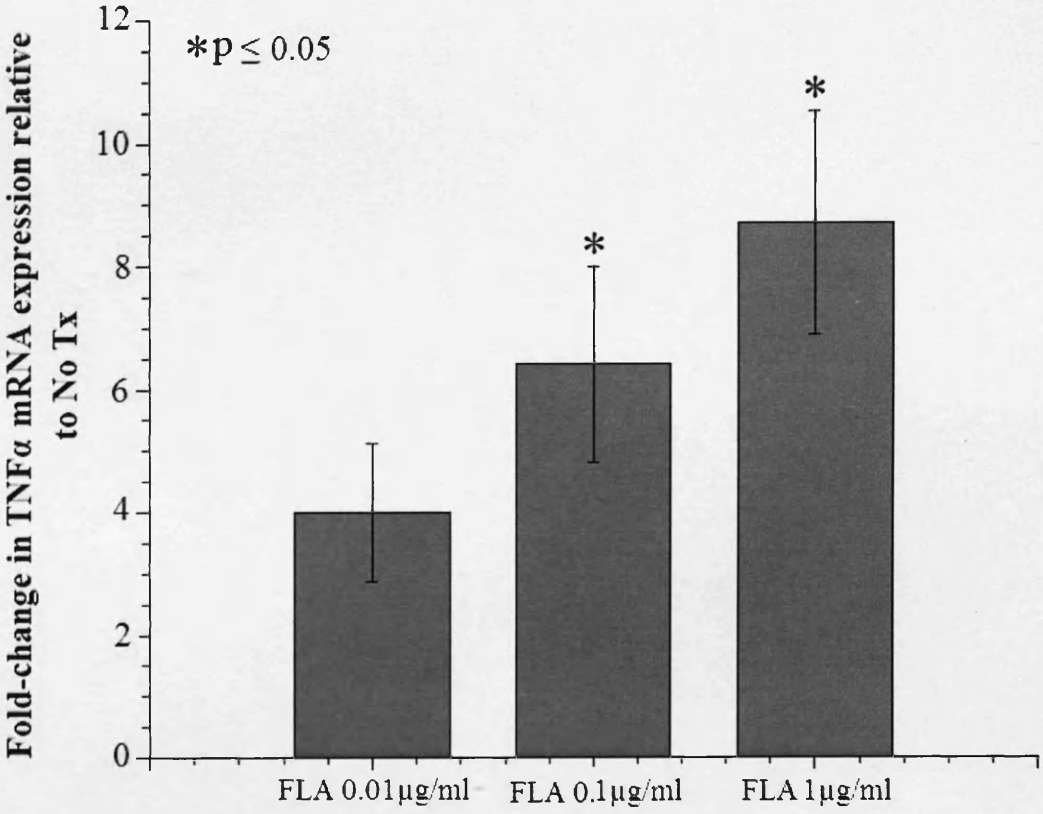

Figure 5-4 Fold-change in TNFα mRNA expression after TLR5 ligation. (∗= $p \leq 0.05$ vs. No Tx) (n=3)

From figure 5-4 we can conclude that 0.1μg/ml of flagellin significantly promotes TNFα mRNA expression in IEC (6.4 ± 1.6, $p \leq 0.03$) when compared to no

treatment control. Additionally, treatment with 1μg/ml of flagellin was also shown to significantly promote TNFα mRNA expression in IEC (8.7 ± 1.8, $p \leq 0.01$) when compared to no treatment control. Treatment with the lowest concentration of flagellin (0.01μg/ml) hinted of an increase in TNFα mRNA expression in IEC (4.0 ± 1.1, $p = 0.06$).

5.5.4 SOCS3 enhances TLR5 induced TNFα mRNA expression

To establish how SOCS3 affects IEC TLR signalling, TNFα mRNA expression was measured after $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC were treated with varying concentrations flagellin for 2h. Figure 5-5 shows fold-change in TNFα mRNA expression relative to no treatment. An unpaired student *t test* was used to determine statistical effect in contrast to $SOCS3^{norm}$ IEC with $p \leq 0.05$.

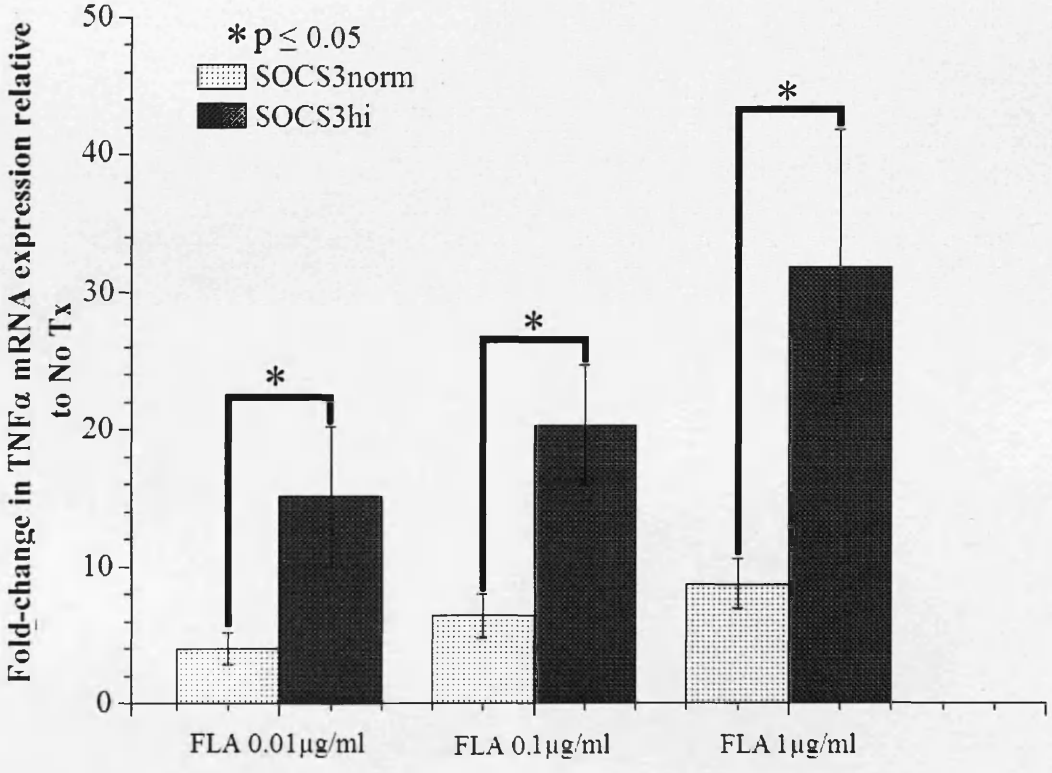

Figure 5-5 Effect of varying concentrations of flagellin treatment (0.01, 0.1 and 1µg/ml) on the expression of TNFα mRNA in $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC (*= $p \leq 0.05$ vs. $SOCS3^{norm}$ IEC (n=5).

From figure 5-5 we can conclude that SOCS3 overexpression enhanced the mRNA expression of TNFα in a dose dependent manner after TLR5 ligation. Treatment with the lowest concentration of flagellin (0.01µg/ml) showed a 3.8 fold-

increase in TNFα mRNA expression in $SOCS3^{hi}$ IEC in comparison to $SOCS3^{norm}$ IEC. Fold-increases of 3.2 and 3.7 were also observed in $SOCS3^{hi}$ IEC in comparison to $SOCS3^{norm}$ IEC after treatment with 0.1 and 1μg/ml of flagellin respectively.

5.5.5 SOCS3 driven increases in mRNA do not appear to translate to increase in secreted TNFα

Data from figure 5-5 indicates SOCS3 overexpression enhances flagellin-induced mRNA expression. To determine if increases in TNFα mRNA translated into secreted protein, soluble TNFα was detected in the supernatant. Figure 5-6 shows TNFα secretion after $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC were treated with varying concentrations of flagellin. An unpaired student *t test* was used to determine statistical effect in contrast to $SOCS3^{norm}$ IEC with $p \leq 0.05$.

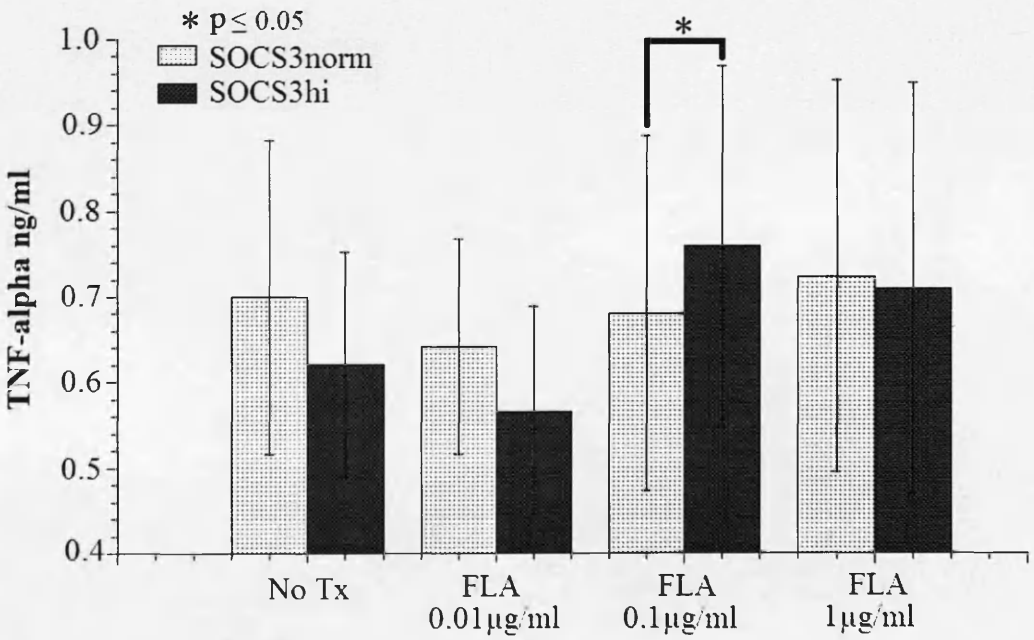

Figure 5-6 Effect of flagellin treatment on TNFα secretion from $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC (*= $p \leq 0.05$ vs. $SOCS3^{norm}$ IEC) (n=6).

Modest increases in sTNFα were detected by ELISA, but increases in secreted protein were nowhere near as marked as increases in mRNA, leading us to speculate that increases in TNFα mRNA were not reflected in the secreted form maybe due to

either rapid binding to membrane receptor or post-translational regulation, possibly regulated by SOCS3.

5.5.6 SOCS3 overexpression had no significant effect on LPS or FLA induced-pSTAT3 expression

To observe the effect of TLR ligation and SOCS3 status on the phosphorylation of STAT3, $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC were treated with varying concentrations of LPS and flagellin for 2h. Figure 5-7 shows fold-change in TLR-induced pSTAT3 expression in $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC in comparison to no treatment. An unpaired student *t test* was used to determine statistical effect in contrast to $SOCS3^{norm}$ IEC with $p \leq 0.05$.

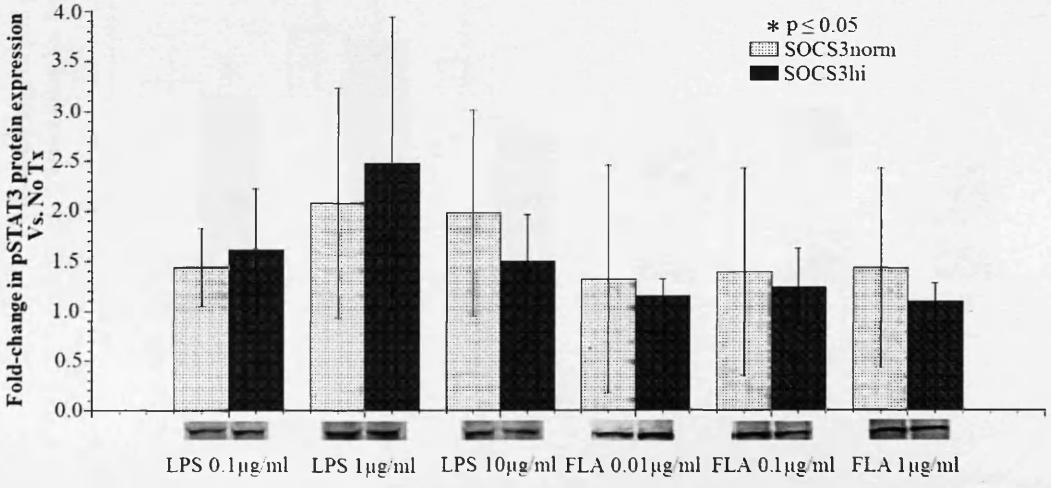

Figure 5-7 Effect of LPS and flagellin, treatment on STAT3 phosphorylation in $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC ($* = p \leq 0.05$ vs. $SOCS3^{norm}$ No Tx) (n=3).

From figure 5-7 we can conclude that SOCS3 overexpression had no significant effect on LPS or flagellin induced pSTAT3 expression as $p > 0.05$.

5.5.7 Flagellin does not induce NF-$_k$B p65 expression

To observe the effect of TLR ligation and SOCS3 status on NF-$_k$B p65 expression, SOCS3^{norm} and SOCS3^{hi} IEC were treated with varying concentrations of LPS and flagellin for 2h. Figure 5-8 shows fold-change in TLR-induced NF-$_k$B p65 expression in SOCS3^{norm} and SOCS3^{hi} IEC in comparison to SOCS3^{norm} no treatment. An unpaired student *t test* was used to determine statistical effect in contrast to SOCS3^{norm} no treatment IEC with $p \leq 0.05$.

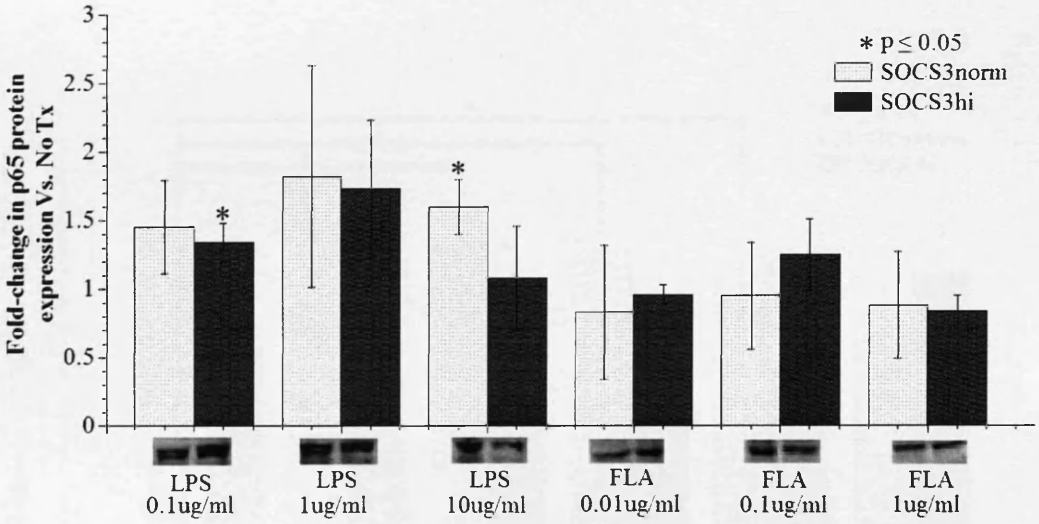

Figure 5-8 Effect of LPS and flagellin, treatment on NF-$_k$B p65 in SOCS3^{norm} and SOCS3^{hi} IEC. (* = $p \leq 0.05$ vs. SOCS3^{norm} No Tx) (n=3).

Figure 5-8 shows that LPS (10µg/ml) caused an increased in NF-$_k$B p65 expression (1.6 ± 0.2, $p \leq 0.04$) in SOCS3^{norm} IEC; with SOCS3-overexpression again causing an increase in NF-$_k$B p65 expression (1.34 ± 0.14, $p \leq 0.05$) after LPS stimulation (0.1µg/ml). All concentrations of flagellin (0.01, 0.1 and 1µg/ml) were shown to have no significant effect on NF-$_k$B p65 expression.

5.5.8 SOCS3 blocked TLR3, and TLR5-induced TNFR2

SOCS3 overexpression was shown to enhance TLR5-induced TNFα mRNA expression in IEC; however this was not translated to secreted protein possibly due to TNFα binding to its receptor TNFR2. To establish how SOCS3 affects TNFR2 transcription in response to varying concentrations of flagellin, Poly I: C and LPS, $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC were treated for 2h. An unpaired student *t test* was used to determine statistical effect in contrast to no treatment $SOCS3^{norm}$ IEC with $p \leq 0.05$.

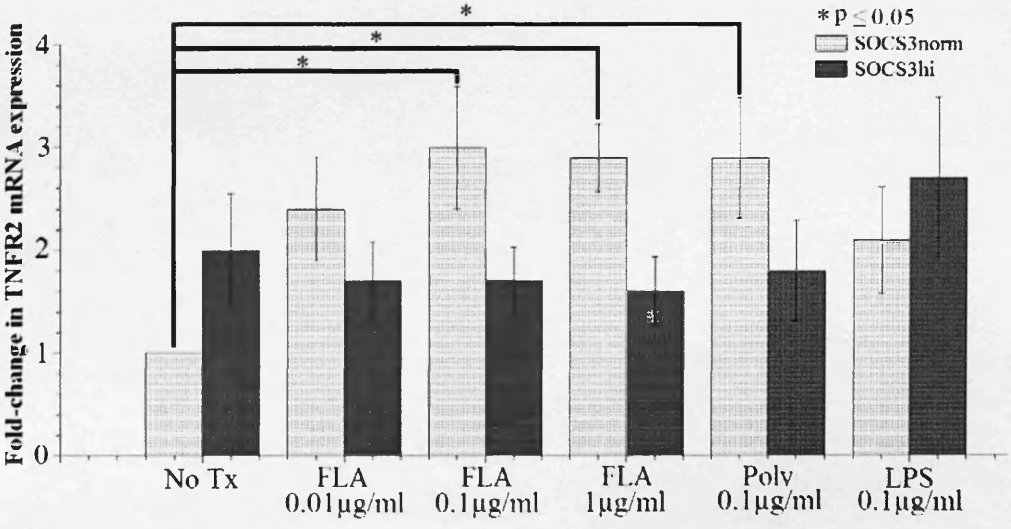

Figure 5-9 Effect of flagellin, Poly I:C and LPS treatment on the expression of TNFR2 mRNA in $SOCS3^{norm}$ and $SOCS3^{hi}$ IEC (* = $p \leq 0.05$ vs. $SOCS3^{norm}$ No Tx) (n=6).

Results from figure 5-9 indicate that in $SOCS3^{norm}$ IEC treatment with most concentrations of flagellin (0.1 and 1µg/ml) and Poly I:C (0.1µg/ml) were shown to significantly enhance TNFR2 mRNA expression by 3.0, 2.9 and 2.9 respectively, as p

<0.05. However, overexpression in TNFR2 mRNA expression in response to TLR3 and TLR5 ligation were no longer observed in SOCS3^{hi} IEC.

5.5.9 Flagellin was shown to down- then up-regulate TNFR2 protein expression

Data from figure 5-9 indicated TLR5 ligation increased TNFR2 mRNA expression. To determine whether TLR5 ligation also induced the expression of TNFR2; immunocytochemistry was used to observe TNFR2 expression in $SOCS3^{norm}$ IEC after treatment with flagellin for 1, 2, 4, 6, and 12h.

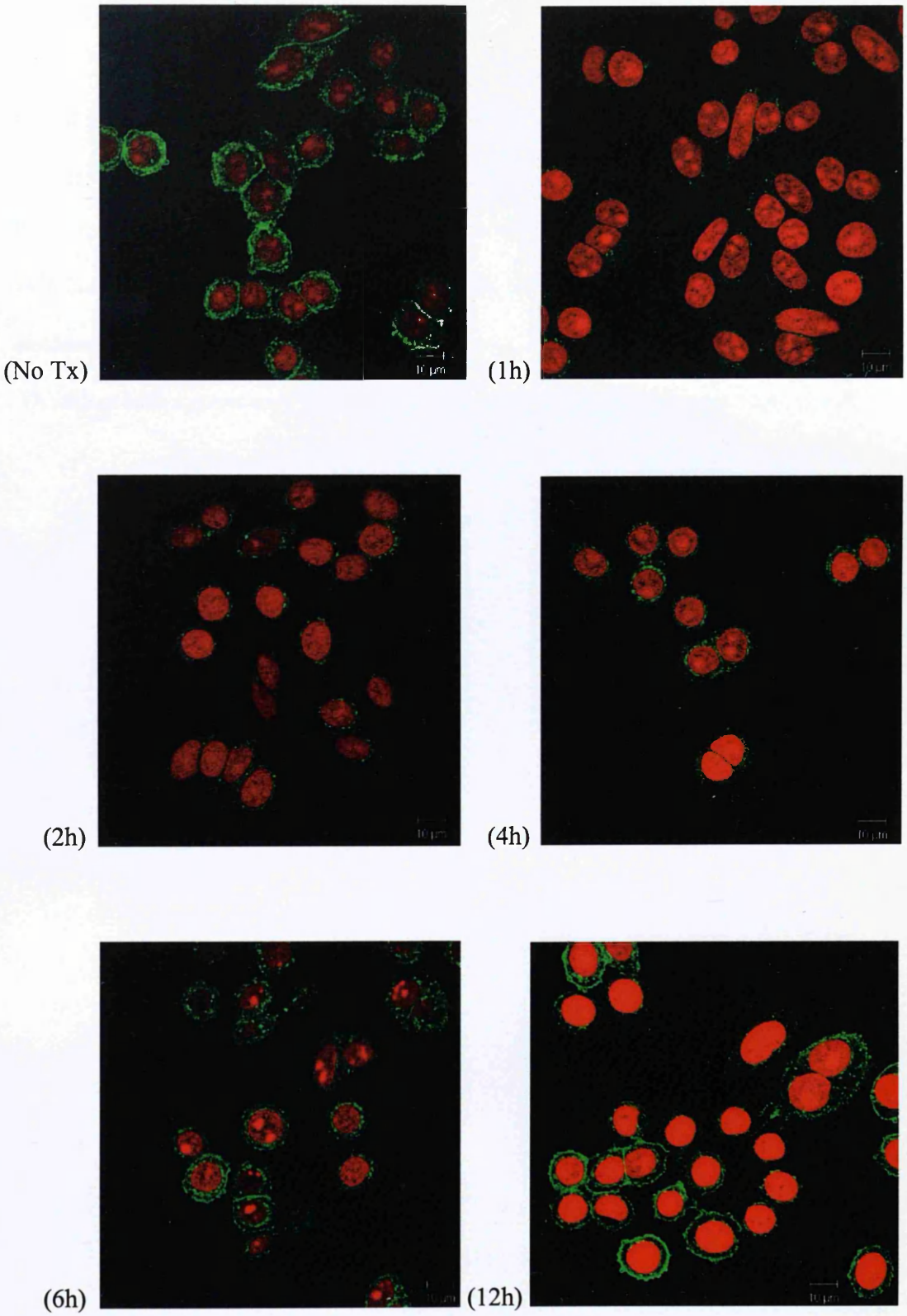

Figure 5-10 Distribution of TNFR2 in SOCS3norm IEC after exposure to flagellin (0.1μg/ml) for 1, 2, 4, 6, and 12h using immunocytochemistry. Green represents TNFR2, Red represents propidium iodide (n=3).

From figure 5-10 we can conclude that baseline levels of TNFR2 show distribution of receptor at both cytoplasmic and membrane locations. Treatment of IEC with flagellin (0.1μg/ml) depicted a down-regulation in TNFR2 distribution at both cytoplasmic and membrane locations after 1 and 2hrs. Cytoplasmic and membrane TNFR2 protein expression steadily began to increase again back to no treatment levels after stimulating with flagellin for 4, 6 and 12h; with greatest levels of TNFR2 at both cytoplasmic and membrane locations observed 12h post-stimulation.

5.6 Discussion

TLR signalling pathways have been shown to be important activators of NF-$_k$B, influencing the expression of a number of 'pro-inflammatory' genes such as TNFα, IL-6 and IL-8 (Vijay-Kumar *et al.*, 2008) essential for immunity and tissue repair. However, if persistently activated, excessive levels of these cytokines can lead to certain pathologies. TGFβ is an immunoregulatory cytokine regulating proliferation, differentiation and function of both immune and non-immune cells (Hartsough and Mulder 1997; Letterio and Roberts 1998; Yue, Sun et al. 2004). Studies have shown TGFβ is a potent inhibitor of DC activation. TGFβ-conditioned myeloid DCs appear to be tolorogenic as they do not respond to microbial stimuli and protect mice against lethal LPS-induced inflammation (Neurath, Fuss et al. 1996). Our studies indicate TLR4 or TLR5 ligation has not effect on TGFβ mRNA expression. Here, we also demonstrate that TLR ligation with flagellin and not Poly I:C or LPS causes an increase in TNFα mRNA expression. Moreover, SOCS3 enhances TLR5 induced TNFα in a dose dependent manner.

Increases in TNFα mRNA upon flagellin stimulation did not appear to translate to increases in secreted TNFα. This led us to hypothesise that this was perhaps due to TNFα binding to its receptor. Numerous studies have shown that TNFα signals through two transmembrane receptors TNFR1 and TNFR2. *In vitro* studies using colonic IEC have demonstrated that stimulation with TNFα causes an upregulation of both TNFR2 mRNA and sTNFR2 protein (Hamilton, Simmons et al. 2011). Other studies demonstrate TNFR2 can be proteollytically cleaved making it a soluble antagonist of TNFα (Stillie and Stadnyk 2009). This suggests a possible rationale that although sTNFα maybe secreted upon flagellin stimulation; TNFα may not be

detectable by the ELISA assay as sTNFR2 could act as a decoy receptor. Alternatively it could also be due to a post-translational regulatory mechanism.

SOCS3, an endogenous negative feedback inhibitor of inflammatory cytokine-mediated signalling is shown to limits transcription factor activation/translocation in response to inflammatory stimuli within IEC. Examples of inhibitory effects include TNFα-induced NF-$_k$B translocation and IL-6 induced STAT3 phosphorylation (Rigby, Simmons et al. 2007; Li, de Haar et al. 2010). There is overwhelming evidence to support the role of SOCS3 as a regulator of intestinal epithelial homeostasis, including IEC overexpression in IBD (Suzuki, Hanada et al. 2001), targeted deletion of IEC SOCS3 promoting tumour incidence and growth (Rigby, Simmons et al. 2007); and epigenetic silencing of SOCS3 observed in intestinal tumours (Li, de Haar et al. 2012; Li, Deuring et al. 2012).

Toll-like receptors have been shown to influence IEC turnover through promoting phosphorylation of STAT3 and activating NF-$_k$B regulating the transcription of several hundred genes with *k*B binding sites (Suzuki, Hanada et al. 2001; Ghosh and Karin 2002; Abreu, Thomas et al. 2003). Our results indicate SOCS3 overexpression had no effect on TLR4 or TLR5 induced-STAT3 phosphorylation and that TLR4, but not TLR5 signalling promoted NF-$_k$B activation, although SOCS3 had little effect on TLR4 induced NF-$_k$B in our system.

TNFR2 activation leads to transcriptional factors triggering genes linked to cell proliferation and survival (Rodriguez-Vita and Lawrence 2010; Hamilton, Simmons et al. 2011). Studies have shown that TNFR2 is upregulated in IBD and AOM/DSS models of inflammation associated cancer (Crespo, Cayón et al. 2001; Mizoguchi, Mizoguchi et al. 2002; Onizawa, Nagaishi et al. 2009). Hamilton and colleagues (2011) have shown that overexpression of SOCS3 decreases cytokine induced TNFR2

expression, limits proliferation of colon cancer cells and decreases anchorage-independent growth of colon cancer cells, even cells overexpressing TNFR2 (Hamilton, Simmons et al. 2011). Our results indicate that both TLR3 and TLR5 ligation show increases in TNFR2 mRNA expression, with SOCS3 limiting this upregulation. The limiting effect of SOCS3 on TNFR2 expression could account for the increase in TNFα, or equally the increase in TNFα could account for limitation of TNFR2 upregulation. IHC data indicates TLR5-induced TNFα causes a rapid downregulation of TNFR2, and expression is restored to habitual levels by 6h post treatment, indicating overexpression of TNFα may negatively inhibit its receptor, limiting unwarranted signalling.

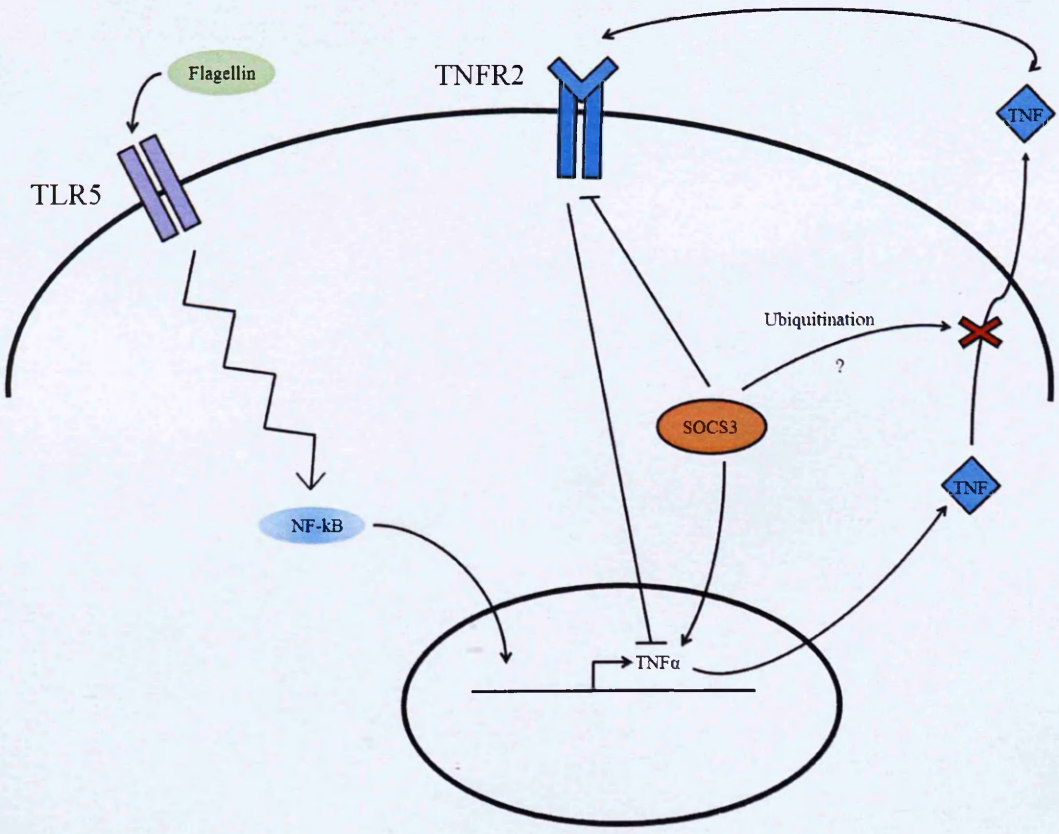

Figure 5-11 Possible mode of SOCS3 action in TLR5-induced TNFR2 signalling

The dual roles of SOCS3 in cancer and inflammatory diseases have been recognised. Our findings indicate SOCS3 may perpetuate microbial-induced TNFα which may drive mucosal inflammation, thus promoting pathological symptoms of UC. Furthermore, as silencing of SOCS3 promotes cancer, SOCS3 limitation of microbial-induced TNFR2 may be one mechanism by which SOCS3 limits tumour formation, but promotes pro-mitotic inflammation.

6. IEC SOCS3 MAY MEDIATE HYPO-RESPONSIVENESS TO COMMENSAL MICROFLORA

6.1 Summary

Innate immune interactions of IEC with microbial constituents drive the expression of pro-inflammatory genes, and secretion of cytokines which subsequently activate the adaptive immune response. The intestinal epithelium regulated inflammatory signalling relative to immune cells generates 'tolerance' to commensal flora. Breakdown of tolerance to commensal flora is associated with the pathogenesis of IBD and NEC.

These studies aimed to assess whether SOCS3 mediates IEC tolerance to TLR ligation and assess in what way differentiation status of IEC affects cytokine, and SOCS3 expression. Our results indicate pre-treatment with LPS is shown to attenuate LPS-induced SOCS3 and TLR4 expression, suggesting IEC become tolerant or hyporesponsive to successive stimulations of LPS. An *in vitro* model was developed to assess TLR4-induced SOCS3 and cytokine expression in conventional (differentiated) and damaged (proliferating) IEC monolayers. Our results show damaged monolayers express greater levels of LPS-induced SOCS3 and TNFα than conventional monolayers, parallel to observations in IBD. However; TGFβ is shown to be upregulated in both damaged and conventional monolayers following TLR ligation, supporting its role as an TLR-induced immunoregulatory cytokine. These results suggest IEC become tolerant to commensal flora, protecting against incessant immune activation by commensals. Furthermore, expression of microbial-induced inflammatory cytokines and their regulator, SOCS3 are influenced by epithelial integrity.

6.2 Introduction

The intestinal epithelium serves as a physical barrier that separates luminal microflora and food antigens from the internal milieu. As well as constituting a barrier to luminal contents, IEC are active contributors to the intestinal innate immune response, responding to signals from both the luminal (apical) and lamina propria (basolateral) environments (Madara 1997; Hecht 1999). Interaction of IEC with pathogenic bacteria provoke the expression of pro-inflammatory genes, and secretion of cytokines and chemokines, recruitment of inflammatory cells, and subsequent activation of the adaptive immune system (Jung, Eckmann et al. 1995; Madara 1997; Kim, Eckmann et al. 1998; Elewaut, DiDonato et al. 1999). The innate immune responses of IEC have evolved restricting infection by luminal pathogens. IEC are able to detect molecules associated with pathogens via a cohort of pattern recognition receptors termed Toll-like receptors (TLR). The detection of microbial ligands such as lipopolysaccharide (LPS) is recognised by TLR4 (Abreu, Fukata et al. 2005; Kawai and Akira 2007; Beutler 2009). Stimulation of TLR influences the expression of a number of pro-inflammatory cytokine such as TNFα, IL-6 and IL-8 (Kawai and Akira 2007; Beutler 2009).

Although none of the TLR microbial ligands are unique to pathogens, with many also common to non-pathogenic organisms, this proves an enigma to the intestinal epithelium which is constitutively exposed to vast numbers of microbes and their products (Rakoff-Nahoum, Paglino et al. 2004). Despite this, under physiological conditions, the intestinal epithelium does not appear to activate the pro-inflammatory-signalling cascade in response to commensal flora. Therefore, mechanisms must exist to regulate the intestinal immune system between mucosal defence from adverse

microbes and 'tolerance' to commensal flora. Idiopathic IBD is described as chronic intestinal inflammation in the absence of identified bacterial pathogens.

Studies have shown that several microbial components trigger innate immune responses and can incite endotoxin shock-like symptoms in experimental animals. However, these microbial components also generate tolerance following successive stimulation, conceivably safeguarding the host from dysregulated immune responses (Biberstine, Darr et al. 1996; Zeisberger and Roth 1998).

The transition from a sterile inter-uterine environment to a world that is rich in microbial antigens elevates the risk of microbial infection due to the immaturity of the immune system in newborns. The newborn faces a multifaceted array of immunological demands, including fortification against infection and avoidance of harmful inflammatory immune responses. One of the tolerance mechanisms proposed is that shortly after birth, TLR4 is down regulated in the neonate intestine, presumably resulting in the "hyporesponsive" nature of IEC (Levy 2007). Cario and colleagues (2000) had previously demonstrated that TLR4 is sparsely expressed on human IEC. However, TLR4 expression is up-regulated in both CD and UC. TLR5 remains unaffected, indicating IBD is associated with dysregulated TLR4 expression (Cario and Podolsky 2000). Other researchers have shown that TLR4 is primarily expressed on the surface of IEC, but can become internalised making it less responsiveness to LPS (Abreu, Vora et al. 2001; Abreu, Fukata et al. 2005).

Necrotising enterocolitis (NEC) is one of the most common and distressing gastrointestinal emergencies in neonates. The exact pathogenesis of NEC is still unconfirmed, however one hypothesis suggests that NEC occurrence may be due to inappropriate initial microbial colonisation or the naivety of the under-developed

immune system of the gastrointestinal tract in newborns, as NEC arises 8-10 days after birth (Claud and Walker 2001). Intestinal samples of NEC neonates are shown to have increased pro-inflammatory cytokines, suggesting these mediators play a part in NEC development. Another study has depicted rat models of NEC overexpressing TLR2, as well as NF-$_k$B in IEC correlating with severity of mucosal damage (Martin and Walker 2006; Schultz, Bonnard et al. 2007). Additionally, IEC expression of TLR and cytokines precedes histological injury in experimental NEC (Liu, Zhu et al. 2009). NEC is also associated with increased expression of TLR4 in the intestinal mucosa (Leaphart, Cavallo et al. 2007). These findings demonstrate the critical role of TLR expression in the development of NEC.

Endotoxin shock is often a fatal consequence of bacterial infection, that may occur when LPS enters the blood stream (bacteremia), causing systemic inflammatory reaction. Although, some studies have indicated modest concentrations of LPS do not induce TLR4-mediated responses in several cell lines, suggesting IEC are more tolerant to LPS than other cell lines.

Transforming growth factor-β (TGFβ), is produced by most immune cells, and is a potent regulator of proliferation and differentiation of immune cells (Chen 2006). TGFβ is shown to negatively regulate TLR4-induced macrophage activation. Furthermore, mice lacking TGFβ display elevated levels of TLR4 mRNA expression and are LPS hyperresponsive, subsequently expressing increased levels of pro-inflammatory cytokines (Comalada, Cardó et al. 2003; McCartney-Francis, Jin et al. 2004).

Hamilton and colleagues (2009) had shown that the helminth *Fasciola hepatica (F. hepatica)*, the causative agent of fasciolosis is evolving as a significant disease in

humans (Mas-Coma, Bargues et al. 1999). The *F. hepatica* tegumental antigen (FhTeg) renders dendritic cells hyporesponsive to TLR activation. Furthermore, FhTeg is also shown to suppress LPS induced NF-$_k$B activation in dendritic cells (Hamilton, Dowling et al. 2009). Other studies have gone on to demonstrate that FhTeg's inhibitory effects on TLR4 signalling are not only limited to the suppression of NF-$_k$B activation, but also MAPK, an important intermediate in TLR4 signalling (Vukman, Adams et al. 2013). FhTeg is also shown to induce the expression of SOCS3 in mast cells, impairing ability to drive Th1 immune responses, a characteristic feature of *Fasciola* infection (Vukman, Adams et al. 2013). The upregulation of SOCS3 following pre-treatment with FhTeg could provide a rationale for the hyperresponsiveness of LPS-induced cytokine secretion.

Conclusively, these studies suggest that neonatal IEC become hyporesponsive to microbial-induced TLR signalling following birth. Since aberrations of TLR and inflammatory cytokine expression are associated with NEC development; this chapter aims to investigate the potential role of SOCS3 as a mediator of hyporesponsiveness to TLR ligation. Furthermore, SOCS3 and cytokine expression levels will also be assessed in a model of conventional and disrupted IEC monolayers.

6.3 Aim: To investigate whether SOCS3 mediates IEC tolerance to TLR ligation

6.4 Methods

6.4.1 Assessment of TLR ligand pre-treatment on SOCS3 and TLR receptor expression

These experiments utilised the Caco-2 IEC which were seeded in 12-well plates at 2 x 10^5 cells/well and allowed to adhere overnight. IEC were either pre-treated with LPS 10μg/ml for 6h, or left in complete medium, then subjected to a subsequent 10μg/ml LPS treatment for varying time points (1, 2, 3, 4, and 6h), or not. SOCS3 and TLR4 mRNA levels were then measured using qPCR. Experiment was repeated 3 times (n=3) with all results pooled together.

6.4.2 Assessment of IEC differentiation status on SOCS3, TGFβ and TNFα expression

Proliferating Caco-2 cells which had been seeded overnight, and Caco-2 cells which had been allowed to differentiate for 7 days were treated with 1μg/ml LPS for 2h. SOCS3, TGFβ and TNFα mRNA levels were then measured using qPCR. Experiment was repeated 3 times (n=3) with all results pooled together.

6.5 Results

6.5.1 SOCS3 expression upregulated following treatment with LPS

To evaluate the effect of treatment with LPS on the expression of SOCS3 in proliferating IEC, SOCS3 mRNA was assessed. A unpaired student *t test* was used to determine if there was significant effect in contrast to No Tx with $p \leq 0.05$.

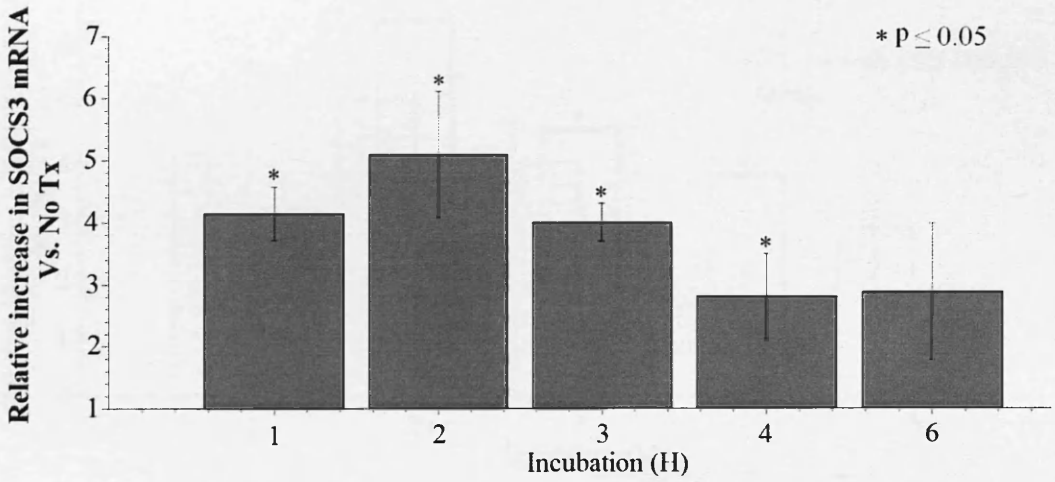

Figure 6-1 Effect of LPS treatment (1, 2, 3, 4 and 6h) on SOCS3 mRNA expression vs. No Treatment. A unpaired student *t test* was used to determine significant effect in contrast to no treatment cultures. * = indicates a significant change Vs. No Tx IEC cultures ($p \leq 0.05$) (n=3)

In figure 6-1 we can conclude that SOCS3 mRNA expression showed fold-increases of 4.2, 5.1, 4.0 and 2.8 following stimulation with LPS for 1, 2, 3, 4 and 6h respectively, in comparison to no treatment cultures.

6.5.2 LPS pre-treatment attenuated IEC SOCS3 expression following LPS stimulation.

To evaluate the effect of pre-treatment with LPS on the expression of SOCS3 in proliferating IEC, SOCS3 mRNA was assessed. A student *t test* was used to determine if there was significant effect in contrast to Naïve IEC with $p \leq 0.05$.

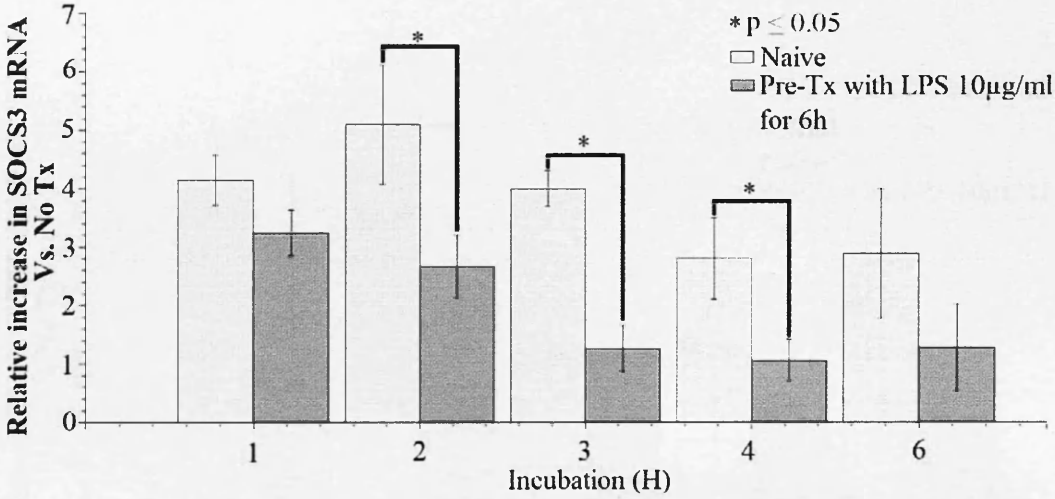

Figure 6-2 Effect of LPS pre-treatment (6h) and subsequent LPS treatment (1, 2, 3, 4 and 6h) on SOCS3 mRNA expression vs. No Treatment. A student t test was used to determine significant effect in contrast to pre-treated cultures with $p \leq 0.05$. * = indicates a significant change Vs. pre-treated cultures ($p \leq 0.05$) (n=3)

Figure 6-2 shows that pre-treatment with LPS for 6h was shown to attenuate SOCS3 mRNA transcription by 48, 68 and 62% at 2, 3 and 4h respectively, in comparison to naive cells.

6.5.3 TLR4 IEC expression attenuated response following pre- treatment with LPS

To evaluate the expression of TLR on proliferating Caco-2 IEC which had either been pre-treated with 10μg/ml LPS or not, then treated again with LPS for varying periods, values have been expressed as relative increase in TLR4 mRNA vs. No Treatment. A student *t test* was used to determine if there was significant effect in contrast to Naïve IEC with $p \leq 0.05$.

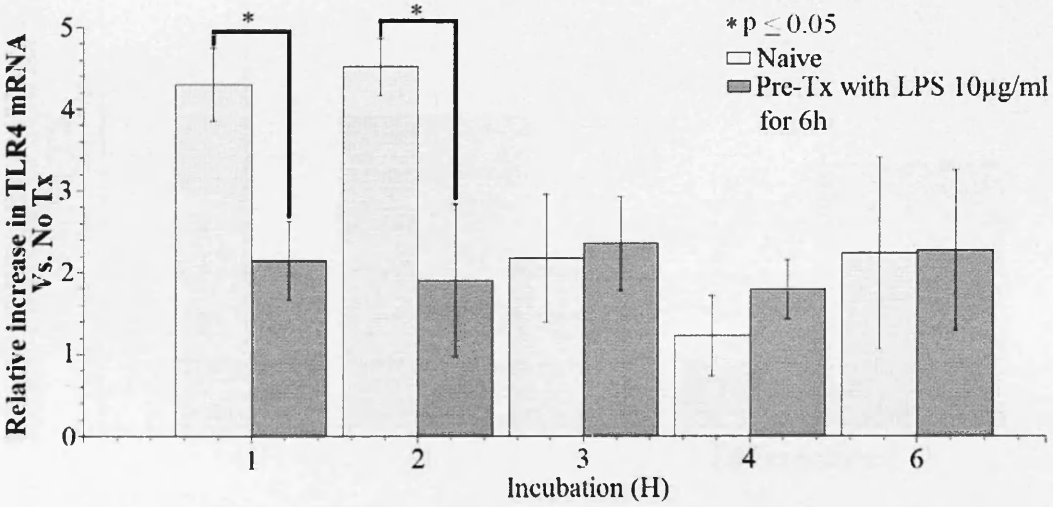

Figure 6-3 Effect of LPS pre-treatment (6h) and subsequent LPS stimulation (1, 2, 3, 4 and 6h) on TLR4 mRNA expression. * = indicates a significant change Vs. pre-treated cultures ($p \leq 0.05$) (n=3)

From figure 6-3 we can conclude that pre-treatment with LPS for 6h was shown to inhibit TLR4 mRNA expression by 50 and 58% at 1 and 2h respectively, in comparison to naive cultures which were also treated with LPS for 1 or 2h with $p < 0.05$.

6.5.4 Proliferating IEC express greater levels of SOCS3 than differentiated IEC following TLR4 ligation

To evaluate SOCS3 expression in differentiated and proliferating Caco-2 cells in response to 1µg/ml LPS treatment, values have been expressed as relative increases in SOCS3 mRNA vs. proliferating No Treatment IEC. A student *t test* was used to determine if there was significant effect in contrast to No Tx with $p \leq 0.05$.

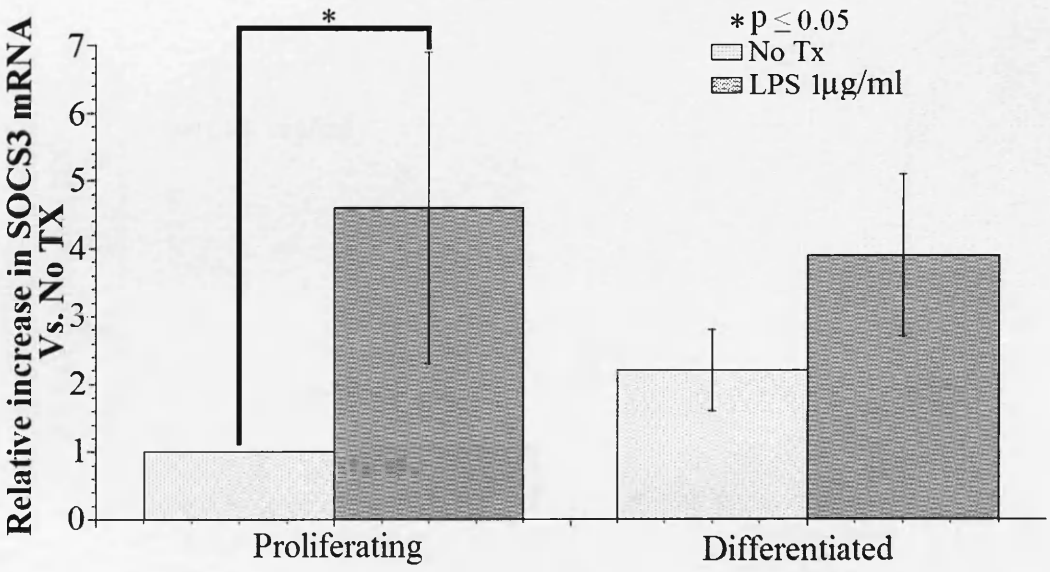

Figure 6-4 Effect of Caco-2 IEC differentiation status and LPS treatment (2h) on SOCS3 mRNA expression. * = indicates a significant change Vs. No Tx IEC ($p \leq 0.05$) (n=3)

From figure 6-4 we can conclude that treatment with LPS for 2h depicted a 4.6 fold-increase in SOCS3 mRNA expression in proliferating IEC in comparison to no treatment IEC cultures. Treatment with LPS for 2h was shown to have no significant effect on SOCS3mRNA expression in differentiated IEC.

6.5.5 Proliferating IEC express greater levels of TNFα than differentiated IEC following TLR4 ligation

To evaluate TNFα expression in differentiated and proliferating Caco-2 cells in response to 1μg/ml LPS treatment, values have been expressed as relative increases in TNFα mRNA vs. proliferating No Treatment IEC. A student *t test* was used to determine if there was significant effect in contrast to No Tx with $p \leq 0.05$.

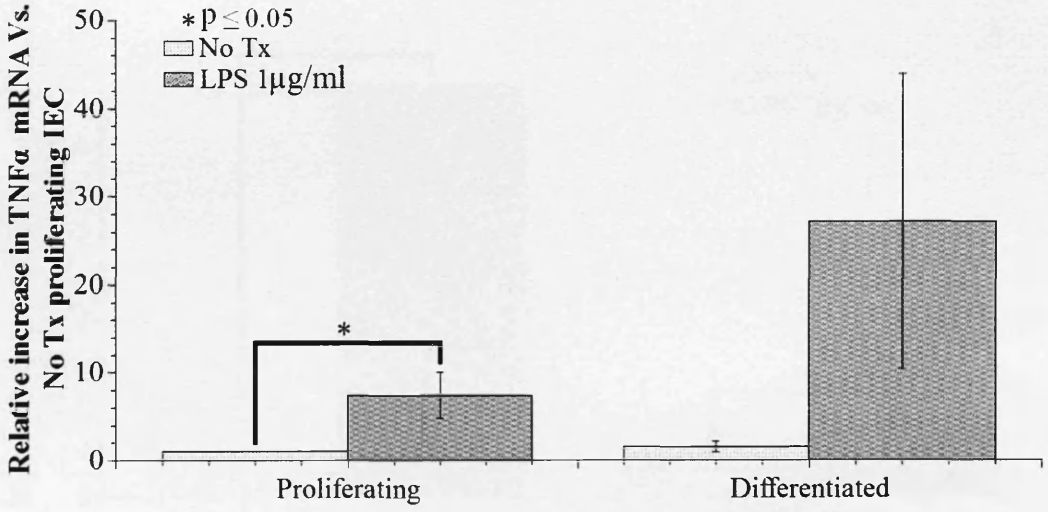

Figure 6-5 Effect of Caco-2 IEC differentiation status and LPS treatment (2h) on TNFα mRNA expression. * = indicates a significant change Vs. No Tx ($p \leq 0.05$) (n=3)

From figure 6-5 we can conclude that proliferating IEC showed a 7.4 fold-increase in TNFα mRNA expression in comparison to no treatment IEC cultures. Treatment with LPS for 2h was shown to have no significant effect of TNFα mRNA expression in differentiated IEC.

6.5.6 TGFβ is upregulated in both proliferating and differentiated IEC following TLR4 ligation

To evaluate TGFβ expression in differentiated and proliferating Caco-2 cells in response to 1μg/ml LPS treatment, values have been expressed as relative increases in TGFβ mRNA vs. differentiated No Treatment IEC. A student *t test* was used to determine if there was significant effect in contrast to No Tx with $p \leq 0.05$.

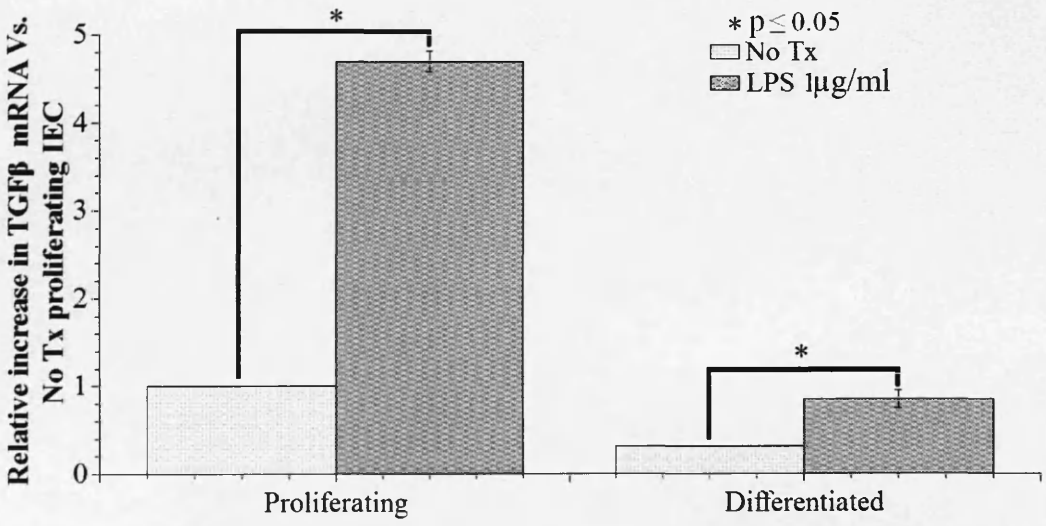

Figure 6-6 Effect of Caco-2 IEC differentiation status and LPS treatment (2h) on TGFβ mRNA expression. * = indicates a significant change Vs. No Tx ($p \leq 0.05$) (n=3)

From figure 6-6 we can conclude that treatment with LPS for 2h depicted a 4.5 fold-increase in TGFβ mRNA expression in proliferating IEC in comparison to no treatment IEC cultures. Treatment with LPS for 2h was also shown to depict a 2.7

fold-increase of TGFβ mRNA expression in differentiated IEC in comparison to no treatment IEC cultures.

6.6 Discussion

Previous studies have demonstrated that the gut can be colonized by as much as 10^8 to 10^{10} microflora per gram of faeces within the first few days of birth (Bettelheim, Breadon et al. 1974; Bezirtzoglou 1997; Fanaro, Chierici et al. 2003). Recognition of these microbial components such as LPS by TLR4 results in the expression of a number of pro-inflammatory genes such as TNFα, IL-6 and IL-8 (Vijay-Kumar, Aitken et al. 2008). However, if persistently activated, excessive levels of these cytokines may initiate certain pathologies such as IBD and NEC. Failure of pre-term neonates to correctly down-regulate responses to LPS appears to significantly contribute to neonates susceptibility to NEC (Jilling, Simon et al. 2006; Lin and Stoll 2006). Nevertheless, in most cases microflora do not typically elicit an inflammatory response from IEC.

The mechanisms by which IEC are protected against incessant immune activation by commensals and LPS is of great importance. Studies by Levy (2007) have shown that following birth, neonates down-regulate the expression of IEC TLR4, resulting in the "hyporesponsiveness" nature of IEC (Levy 2007). Our results also indirectly support these findings by indicating that IEC down-regulate the expression of TLR4 mRNA following successive treatments of LPS, with IEC developing LPS tolerance.

SOCS3, inhibits TNFα-induced NF-$_k$B translocation and IL-6 induced STAT phosphorylation (Rigby, Simmons et al. 2007; Li, de Haar et al. 2010). There is much evidence to support the role of SOCS3 as a governor of IEC homeostasis. SOCS3 is upregulated in IBD (Suzuki, Hanada et al. 2001) and targeted removal of SOCS3 is shown to encourage intestinal tumour incidence and development (Rigby, Simmons et

al. 2007). Baetz and colleagues (2004) have shown that LPS induces the expression of SOCS3 in macrophages (Baetz, Frey et al. 2004). Our results mirror these findings by demonstrating stimulation with LPS promotes the expression of SOCS3 mRNA in IEC. Furthermore, our results also indicate pre-treatment with LPS attenuates LPS-induced SOCS3 mRNA expression. SOCS3 mRNA expression following LPS stimulation suggests it is oscillatory in behaviour. Other studies have also depicted similar findings with SOCS3 mRNA expression oscillating with peaks at 50 and 70 minutes after serum stimulation. Moreover, western blot analysis also showed SOCS3 protein expression displaying oscillations with peaks at 2 and 4h after serum stimulation, indicating a delay of about 1h between mRNA and protein synthesis (Yoshiura, Ohtsuka et al. 2007). It is worth noting that the reduction of SOCS3 mRNA expression following pre-treatment could entirely be due to down-regulation of signalling only at the receptor level and not down-regulation of both TLR4 and SOCS3.

Epithelial homeostasis, that which is the physiological equilibrium of IEC proliferation, differentiation and apoptosis, is fundamental for the growth and maintenance of complex tissues. However, there are a number of instances when this delicate equilibrium is pathologically disrupted, leading to distorted epithelial architecture, a loss of epithelial barrier function, and in severe cases- tumourgenesis (Rakoff-Nahoum, Paglino et al. 2004; Artis 2008; Maloy and Powrie 2011; Koch and Nusrat 2012). A distinguishing feature of IBD is that the rate of IEC proliferation and apoptosis is enhanced, correlating with disease severity (Serafini, Kirk et al. 1981; Sipos, Béla Molnár et al. 2005). Pathologically heightened IEC apoptosis in IBD is thought to potentiate the epithelial barrier defect, permitting the translocation of luminal antigens exuberating the inflammatory immune response (Koch and Nusrat

2012). Differentiation of IEC is also affected during chronic colitis to such a degree, that mucin depletion has been proposed as a possible diagnostic marker for UC (McCormick, Horton et al. 1990). The JAK-STAT3 signalling pathway is regulated by SOCS3 and has been identified as a key regulator of inflammatory cytokines (Rigby, Simmons et al. 2007; Li, de Haar et al. 2010). To analyse LPS-induced SOCS3 and inflammatory cytokine expression in conventional and damaged monolayers, an *in vitro* model was developed where IEC were either allowed to differentiate and form monolayers for 7 days (conventional) or seeded the day before where IEC were proliferating, signifying loss of barrier integrity (damaged) as observed in IBD.

Our results (Fig 6.4) indicate that LPS stimulation promotes a 4.6 fold-increase in expression of SOCS3 in proliferating IEC, indicating proliferating IEC are responsive to LPS. LPS did not induce SOCS3 expression in differentiated cells, possibly due to polarisation of Caco-2 cells in monolayers, and limited apical exposure to LPS. Further evidence to suggest that differentiated Caco-2 IEC are less responsive to TLR stimulation can be seen in Figures 6.5 and 6.6.

TNFα, is upregulated in inflammatory disorders, and has developed as a key therapeutic target for IBD (Ghosh 2005; Sfikakis 2010). Numerous studies within macrophages have shown TNFα signalling transpires through NF-$_k$B and MAPK. Studies have also shown TNFα increases IEC apoptosis and antigen sampling. Conversely, it also limits IEC proliferation and barrier function (Söderholm, Streutker et al. 2004; Wang, Graham et al. 2005; Kolinska, Lisa et al. 2008; Nava, Koch et al. 2010). Our results (Fig 6.5) indicate TLR ligation promotes a 7.4 fold-increase in TNFα expression in proliferating IEC; however TLR4 ligation had no impact on TNFα in differentiated IEC. This emulates observations in IBD where loss of barrier integrity causes an upregulation of TNFα.

TGFβ, an immunoregulatory cytokine has beneficial effects on epithelial homeostasis by rescuing epithelial barrier function in the presence of IFN-*y* (Planchon, Martins et al. 1994). Other studies have suggested rather than moderating the junctions of epithelial cells directly, TGFβ protects the epithelium by preventing the expression of inflammatory cytokines following microbial challenge (Jarry, Bossard et al. 2008). Our results (Fig 6.6) indicate TLR4 ligation promotes a 4.5 fold-increase in TGFβ in proliferating IEC; with a fold-increase of 2.7 in TGFβ also observed in differentiated IEC following TLR4 ligation. Data supports the role of TGFβ as an immunoregulatory cytokine limiting expression of other inflammatory cytokines following microbial challenge. Furthermore, a greater increase in TGFβ expression is detected in proliferating IEC than differentiated IEC possibly due to polarisation of Caco-2 cells in monolayers, limiting apical exposure to LPS

Conclusively, our results indicate IEC become hyporesponsive following successive stimulations with LPS, making IEC tolerant to LPS and protected against incessant immune activation by commensals and LPS. Furthermore, cytokines and their endogenous feedback inhibitor, SOCS3 vary in expression levels conditional to differentiation status and juxtaposition of IEC during TLR4 ligation.

7. GENERAL DISCUSSION AND FUTURE WORK

IEC are able to communicate with microflora in the colon via a cohort of pattern recognition receptors, termed TLR. Numerous studies have demonstrated that TLR through interaction with commensals and their products play a pivotal role in inducing tissue regeneration following injury (Rakoff-Nahoum, Paglino et al. 2004; Rakoff-Nahoum and Medzhitov 2007). Conversely, TLR knockdown or germ-free mice display overwhelmingly reduced dysplasia, number and size of tumours (Fukata, Chen et al. 2007). Taken together, these studies indicate controlled microbial stimulation via TLR are essential for intestinal homeostasis. SOCS3 is also key in regulating intestinal homeostasis indicated by its irregular expression in mouse models of inflammation (Suzuki, Hanada et al. 2001; Li, de Haar et al. 2011). Furthermore, studies have also depicted SOCS3 as a tumour suppressor, including in the colon (Rigby, Simmons et al. 2007).

Several studies have investigated the effect of TLR ligation and SOCS3 expression of immune cells such as dendritic cells and macrophages (Krutzik, Tan et al. 2005; Liu, Zhang et al. 2008; Posselt, Schwarz et al. 2011; Shibata, Motoi et al. 2011). TLR ligation is shown to promote both cytokine and chemokine production in dendritic cells, with SOCS3 influencing cytokine production (Alexander and Hilton 2004; Strengell, Lehtonen et al. 2006; Barr, Brown et al. 2007; Yoshimura, Naka et al. 2007; Liu, Zhang et al. 2008), but our study is novel in that no other study to date has assessed the innate immune roles of TLR and SOCS3 in IEC. IEC form the first point of contact between commensal microflora and the underlying immune system, thus our study focussed on microbial-induced TLR signalling in IEC as this is likely to present a pivotal interaction with regard to the ensuing immune response.

As numerous studies substantiate TLR signalling as imperative mediators of mucosal homeostasis, repair of damaged tissue and fortification against the occurrence

of bowel cancer; this project aimed to investigate the duel and perhaps integrated roles of SOCS3 and TLR in mediating epithelial repair following damage. In chapter 3, IEC models which constitutively overexpressed SOCS3 were developed enabling the role of SOCS3 in mediating cellular responses and epithelial repair following microbial induced TLR signalling to be explored.

To address these issues, stable SOCS3 transfections which involved DNA integrating into the host genome by recombination were developed, making IEC continually overexpress SOCS3. Both transient and stably transfected IEC (SW480 and Caco-2) were then used to investigate the role of SOCS3 in mediating cellular responses following microbial-induced TLR signalling.

In vitro wound healing models show that following injury, IEC migrate into the damaged area (restitution), this is then followed by proliferation and differentiation to preserve the functional activity of the epithelium (Dignass 2001; Dignass, Baumgart et al. 2004). TLR signalling is essential for maintaining tissue integrity and repair of damaged tissue in models of colonic injury (Fukata, Michelsen et al. 2005; Rakoff-Nahoum and Medzhitov 2008). Furthermore, TLR knockdown and germ-free animals depict deficient repair of damaged tissue (Rakoff-Nahoum, Paglino et al. 2004; Abreu, Fukata et al. 2005; Kluwe, Mencin et al. 2009). Chapter 4 involved developing an *in vitro* model of wound repair, permitting investigations on the role of SOCS3 on TLR-mediated wound healing. Our data supports other studies indicating microbial-induced TLR signalling is fundamental for epithelial repair following injury. Furthermore, novel findings in this study provide a direct link between TLR and inadequate repair mechanisms by indicating SOCS3 plays a pivotal role in inhibiting microbial-induced proliferation and epithelial repair. These findings provide a rationale for the

pathogenesis of IBD, indicating aberrations in SOCS3 expression limit microbial-induced wound repair.

Helminth products were shown to promote both proliferative and restitutive repair, whereas LPS and flagellin only enhanced restitutive repair. A plausible explanation for this observation could be helminth-derived ES proteins contain substances that are able to ligate with multiple TLR (TLR2, TLR4 and TLR6) (DeSchoolmeester, Martinez-Pomares et al. 2009). Further studies would use anti-TLR2, TLR4 and TLR6 antibodies to block distinct TLR signalling pathways permitting further investigation of SOCS3 on ES-induced epithelial repair. Immune-mediated diseases such as IBD are increasingly prevalent as populations adopt highly clean lifestyles (Elliott, Summers et al. 2007). Helminths interact with both host innate and adaptive immune responses activating immune-regulatory pathways dampening later pathways that drive aberrant inflammation (Weinstock and Elliott 2009). Helminth exposure can avert TNBS-induced colitis by suppressing inflammatory cytokines (TNFα, IL-12) or promoting the production of immune-regulatory cytokines (TGFβ, IL10) (Khan, Blennerhasset et al. 2002; Hunter, Wang et al. 2005). Our studies indirectly support other studies, suggesting exposure to helminths or their products can prevent or reverse colitis in animal models of IBD. Clinical trials are already underway exploiting helminths as therapeutic agents to treat immune-mediated diseases. Principle characteristics for an ideal therapeutic helminth include, not multiplying within the host, have no pathogenic potential, self-limiting colonisation in humans and does not alter behaviour in patients with depressed immunity. *T. suis* is shown to be a safe and effective therapy in early studies of IBD (Summers, Elliott et al. 2005; Reddy and Fried 2009).

The use of probiotics in improving health in patients with UC have also been demonstrated (Venturi, Gionchetti et al. 1999; Guslandi, Mezzi et al. 2000; Kruis, Frič et al. 2004; Tursi, Brandimarte et al. 2004; Furrie, Macfarlane et al. 2005; Karimi, Peña et al. 2005). One study depicting probiotics having anti-inflammatory properties such as, improving appearances of chronic inflammation, decreasing TNFα and improving histology in TNBS models of rat colitis (Sheil, McCarthy et al. 2004; Furrie, Macfarlane et al. 2005; Peran, Sierra et al. 2007). Our findings support these studies demonstrating microbial-induced TLR signalling is beneficial for epithelial wound repair. Further studies should be carried out in SOCS3 knockdown IEC, to ascertain whether reciprocal findings are attained, i.e. SOCS3 knockdown promoting TLR-induced epithelial repair.

The influence of commensal microflora on IEC occurs in an environment that is highly regulated. Cessation in this controlled network of interactions is associated with the onset of IBD and cancer. Levels of TNFα are elevated in serum and intestinal mucosa of patients with IBD, with neutralization of TNFα associated with improved health, particularly in patients with CD (Stillie and Stadnyk 2009, Van Dullemen *et al.,* 1995, Sandborn *et al.,* 2004). The fifth chapter of this study involved assessing the effects of IEC-derived SOCS3 upon cytokine responses following TLR stimulation. SOCS3 was shown to enhance microbial-induced TNFα mRNA expression. However, only mild increases in secreted TNFα protein were observed. Studies have suggested SOCS3 acts as an adaptor that facilitates ubiquitination and subsequent degredation of the associated signalling molecule (Krebs and Hilton 2001; Babon, Sabo et al. 2008; Linossi and Nicholson 2012). Therefore, although the TNFα is transcribed (mRNA) and translated (protein) SOCS3 may degrade TNFα before it is secreted in the supernatant. Another plausible explanation could be that TNFα binds to its receptor,

and is therefore no longer detectable in the supernatant. Western blotting has already been used to show SOCS3 blocks insulin signalling by ubiquitin mediated degradation of insulin receptor substrates 1 (IRS1) and IRS2 (Rui, Yuan et al. 2002). Further studies would also use this technique to investigate ubiquitination of TNFα following TLR ligation.

Our studies provide further evidence that SOCS3 plays a pivotal role in normal homeostasis and epithelial repair by demonstrating an impact on microbial induced intestinal epithelial responses. Moreover, the findings also support the role of anti-TNFα therapies such as infliximab, adalimumab and certolizumab being beneficial for mucosal healing and treating patients with IBD (Rutgeerts, Van Assche et al. 2004; Ghosh 2005; Assche, Vermeire et al. 2010). These findings also suggest a role of modulating mucosal SOCS3 levels as a therapy, reducing secondary TNFα levels. Further studies should investigate the effect of TLR ligation on TNFα expression in SOCS3 knockdown IEC to determine whether observations are reciprocal to findings in SOCS3 overexpressing IEC.

The mechanism by which IEC are sheltered from incessant immune activation by commensal flora is of great importance. Failure of neonates to correctly downregulate responses to LPS appear to radically contribute to the development of NEC (Jilling, Simon et al. 2006; Lin and Stoll 2006). Other studies have shown that following birth, neonates downregulate the expression of IEC TLR4 becoming "hyporesponsive" to LPS (Levy 2007). Our findings also support other studies by indicating IEC become hyporesponsive to microbes by downregulating TLR4 expression to LPS. Further studies should investigate the hyporesponsive nature of other TLR, such as TLR2, TLR3 and TL5 following ligation with their respective ligands.

LPS induces expression of SOCS3 in macrophages (Baetz, Frey et al. 2004), data within this study also showed similar findings but in an IEC model. Furthermore, results also indicated pre-treatment with LPS attenuated LPS-induced SOCS expression making IEC hyporesponsive to LPS. An interesting observation in this experiment was the time-course expression profile of SOCS3 following LPS stimulation, with results depicting an oscillatory pattern upon TLR4 ligation. Other researchers have also suggested SOCS3 behaves in a wavelike motion following TLR4 ligation (Yoshiura, Ohtsuka et al. 2007). Future studies would investigate the oscillatory behaviour of SOCS3 further by observing SOCS3 protein expression following LPS stimulation. Furthermore, it would also be interesting to determine whether activation of other TLR signalling pathways exhibit this SOCS3 oscillatory behaviour.

Regulation of cytokine expression is important as they effect mucosal homeostasis, with dysregulated expression of cytokines implicated in the onset of intestinal diseases (Planchon, Martins et al. 1994; Ghosh 2005). Differentiated IEC which had formed monolayers were used as a model of normal homeostasis, whereas proliferating IEC were used as a model to signify loss of barrier integrity, characteristic of IBD. TLR4 stimulation was shown to upregulate SOCS3 and TNFα in proliferating IEC; emulating observations in IBD. This indicates improper barrier integrity, absent in IBD elevates expression of SOCS3 and TNFα following microbial stimulation.TLR4 ligation was shown to promote TGFβ expression in both normal and damaged models, supporting its role as an immuno-regulatory cytokine limiting expression of other cytokines following microbial challenge. Further studies would assess the effect of other TLR on SOCS3 and cytokine expression in our model of normal and damaged epithelium.

In conclusion, the work presented within this thesis suggests that SOCS3 does impact on TLR-induced intestinal epithelial responses associated with normal homeostasis and epithelial repair. Furthermore, our data supports other data indicating SOCS3 oscillates in expression, with overexpression impeding TLR-induced epithelial wound repair. The oscillatory expression of SOCS3 must be tightly regulated allowing microbial-induced epithelial repair. Aberrations in the oscillatory expression of SOCS3 are associated with pathological states, such as continual overexpression in IBD or silencing in cancer. SOCS3 overexpression is also shown to enhance the expression of TNFα, a pro-inflammatory cytokine further enhancing inflammation. Future studies should be carried out generating SOCS3 knockdown IEC allowing investigation of SOCS3 on TLR-induced IEC proliferation and epithelial wound repair. Additionally, primary or non-cancerous cell lines such as HIEC would also be used to generate SOCS3 overexpressing and knockdown IEC. The findings presented within this study provide a good foundation for future *in vivo* translational studies on how SOCS3 impacts on intestinal disease.

8. BIBLIOGRAPHY

Abreu, M., M. Fukata, et al. (2005). TLR Signaling in the Gut in Health and Disease 1, Am Assoc Immnol. **174:** 4453-4460.

Abreu, M. T. (2010). "Toll-like receptor signalling in the intestinal epithelium: how bacterial recognition shapes intestinal function." Nature Reviews Immunology **10**(2): 131-144.

Abreu, M. T., L. S. Thomas, et al. (2003). "TLR signaling at the intestinal epithelial interface." Journal of Endotoxin Research **9**(5): 322-330.

Adlerberth, I. and A. E. Wold (2009). "Establishment of the gut microbiota in Western infants." Acta Paediatrica **98**(2): 229-238.

Ahn, B. and H. Ohshima (2001). "Suppression of intestinal polyposis in ApcMin/+ mice by inhibiting nitric oxide production." Cancer Research **61**(23): 8357-8360.

Akazawa, T., T. Ebihara, et al. (2007). "Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells." Proceedings of the National Academy of Sciences **104**(1): 252-257.

Akira, S., M. Yamamoto, et al. (2003). "Toll-like receptors." Biochemical Society Transactions **31**(part 3).

Al Tawil, Y. and C. L. Berseth (1996). "Gestational and postnatal maturation of duodenal motor responses to intragastric feeding." The Journal of pediatrics **129**(3): 374-381.

Alam, M., T. Midtvedt, et al. (1994). "Differential cell kinetics in the ileum and colon of germfree rats." Scandinavian journal of gastroenterology **29**(5): 445-451.

Alexander, W. S. (2002). "Suppressors of cytokine signalling (SOCS) in the immune system." Nature Reviews Immunology **2**(6): 410-416.

Alexander, W. S. and D. J. Hilton (2004). "The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response." Annu. Rev. Immunol. **22**: 503-529.

Alexander, W. S., R. Starr, et al. (1999). "SOCS1 is a critical inhibitor of interferon γ signaling and prevents the potentially fatal neonatal actions of this cytokine." Cell **98**(5): 597-608.

Alexopoulou, L., A. C. Holt, et al. (2001). "Recognition of double-stranded RNA and activation of NF-kB by Toll-like receptor 3." Nature **413**(6857): 732-738.

Aman, M. J. and W. J. Leonard (1997). "Cytokine signaling: cytokine-inducible signaling inhibitors." Current Biology **7**(12): 784.

Andaloussi, A. E., A. M. Sonabend, et al. (2006). "Stimulation of TLR9 with CpG ODN enhances apoptosis of glioma and prolongs the survival of mice with experimental brain tumors." Glia **54**(6): 526-535.

Apetoh, L., F. Ghiringhelli, et al. (2007). "Toll-like receptor 4–dependent contribution of the immune system to anticancer chemotherapy and radiotherapy." Nature medicine **13**(9): 1050-1059.

Arias, C. F., P. Isa, et al. (2002). "Molecular biology of rotavirus cell entry." Archives of medical research **33**(4): 356.

Artis, D. (2008). "Epithelial-cell recognition of commensal bacteria and maintenance of immune homeostasis in the gut." Nature Reviews Immunology **8**(6): 411-420.

Artis, D., N. E. Humphreys, et al. (1999). "Tumor necrosis factor α is a critical component of interleukin 13–mediated protective T helper cell type 2 responses during helminth infection." The Journal of Experimental Medicine **190**(7): 953-962.

Babcock, A. A., M. Wirenfeldt, et al. (2006). "Toll-like receptor 2 signaling in response to brain injury: an innate bridge to neuroinflammation." Science Signalling **26**(49): 12826.

Babon, J. J., E. J. McManus, et al. (2006). "The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability." Molecular cell **22**(2): 205-216.

Babon, J. J., J. K. Sabo, et al. (2008). "The SOCS box domain of SOCS3: structure and interaction with the elonginBC-cullin5 ubiquitin ligase." Journal of molecular biology **381**(4): 928-940.

Babon, J. J., S. Yao, et al. (2005). "Secondary structure assignment of mouse SOCS3 by NMR defines the domain boundaries and identifies an unstructured insertion in the SH2 domain." Febs Journal **272**(23): 6120-6130.

Bamba, T., O. Kanauchi, et al. (2002). "A new prebiotic from germinated barley for nutraceutical treatment of ulcerative colitis." Journal of gastroenterology and hepatology **17**(8): 818-824.

Bancroft, A. J., D. Artis, et al. (2000). "Gastrointestinal nematode expulsion in IL-4 knockout mice is IL-13 dependent." European journal of immunology **30**(7): 2083-2091.

Bancroft, A. J., A. N. McKenzie, et al. (1998). "A critical role for IL-13 in resistance to intestinal nematode infection." The Journal of Immunology **160**(7): 3453-3461.

Barbosa, T. and M. Rescigno (2010). "Host-bacteria interactions in the intestine: homeostasis to chronic inflammation." Wiley Interdisciplinary Reviews: Systems Biology and Medicine **2**(1): 80-97.

Barnes, P. and M. Karin (1997). "Nuclear factor-{kappa} B--a pivotal transcription factor in chronic inflammatory diseases." The New England journal of medicine **336**(15): 1066.

Baumgart, D. C. and S. R. Carding (2007). "Inflammatory bowel disease: cause and immunobiology." The Lancet **369**(9573): 1627-1640.

Beatson, S., T. Minamino, et al. (2006). "Variation in bacterial flagellins: from sequence to structure." Trends in microbiology **14**(4): 151-155.

Belvin, M. P. and K. V. Anderson (1996). "A conserved signaling pathway: the Drosophila toll-dorsal pathway." Annual review of cell and developmental biology **12**(1): 393-416.

Ben-Neriah, Y. and M. Schmidt-Supprian (2007). "Epithelial NF- B maintains host gut microflora homeostasis." Nature immunology **8**(5): 479-481.

Bengmark, S. (1998). "Ecological control of the gastrointestinal tract. The role of probiotic flora." Gut **42**(1): 2-7.

Berg, H. C. and R. A. Anderson (1973). "Bacteria swim by rotating their flagellar filaments."

Bernet, M., D. Brassart, et al. (1994). "Lactobacillus acidophilus LA 1 binds to cultured human intestinal cell lines and inhibits cell attachment and cell invasion by enterovirulent bacteria." Gut **35**(4): 483-489.

Berseth, C. (1989). "Gestational evolution of small intestine motility in preterm and term infants." The Journal of pediatrics **115**(4): 646-651.

Bettelheim, K., A. Breadon, et al. (1974). "The origin of O serotypes of Escherichia coli in babies after normal delivery." J Hyg (Lond) **72**(1): 67-70.

Beutler, B. (2009). "TLRs and innate immunity." Blood **113**(7): 1399.

Bezirtzoglou, E. (1997). "The intestinal microflora during the first weeks of life." Anaerobe **3**(2): 173-177.

Biarc, J., I. S. Nguyen, et al. (2004). "Carcinogenic properties of proteins with pro-inflammatory activity from Streptococcus infantarius (formerly S. bovis)." Carcinogenesis **25**(8): 1477-1484.

Borriello, S., A. Welch, et al. (1988). Mucosal association by Clostridium difficile in the hamster gastrointestinal tract, Soc General Microbiol. **25:** 191-196.

Boutron, M., J. Faivre, et al. (1996). "Calcium, phosphorus, vitamin D, dairy products and colorectal carcinogenesis: a French case--control study." British Journal of Cancer **74**(1): 145.

Brackmann, S., G. Aamodt, et al. (2010). "Widespread but not localized neoplasia in inflammatory bowel disease worsens the prognosis of colorectal cancer." Inflammatory bowel diseases **16**(3): 474-481.

Brady, L., D. Gallaher, et al. (2000). "The role of probiotic cultures in the prevention of colon cancer." The Journal of nutrition **130**(2): 410-414.

Brandtzaeg, P., T. Halstensen, et al. (1989). "Immunobiology and immunopathology of human gut mucosa: humoral immunity and intraepithelial lymphocytes." Gastroenterology(New York, NY. 1943) **97**(6): 1562-1584.

Breynaert, C., S. Vermeire, et al. (2008). "Dysplasia and colorectal cancer in inflammatory bowel disease: a result of inflammation or an intrinsic risk?" Acta gastro-enterologica belgica **71**(4): 367-372.

Brown, J., H. Wang, et al. (2011). "TLR-signaling Networks An Integration of Adaptor Molecules, Kinases, and Cross-talk." Journal of dental research **90**(4): 417-427.

Buer, J. and R. Balling (2003). "Mice, microbes and models of infection." Nature Reviews Genetics **4**(3): 195-205.

Camuesco, D., L. Peran, et al. (2006). "Preventative effects of lactulose in the trinitrobenzenesulphonic acid model of rat colitis." Inflammatory bowel diseases **11**(3): 265-271.

Carey, R., I. Jurickova, et al. (2007). "Activation of an IL-6: STAT3-dependent transcriptome in pediatric-onset inflammatory bowel disease." Inflammatory bowel diseases **14**(4): 446-457.

Cario, E. and D. Podolsky (2000). "Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease." Infection and immunity **68**(12): 7010.

Carpenter, S. and L. O'Neill (2007). "How important are Toll-like receptors for antimicrobial responses?" Cellular Microbiology **9**(8): 1891-1901.

Challa, A., D. R. Rao, et al. (1997). "Bifidobacterium longum and lactulose suppress azoxymethane-induced colonic aberrant crypt foci in rats." Carcinogenesis **18**(3): 517-521.

Chassin, C., M. Kocur, et al. (2010). "miR-146a mediates protective innate immune tolerance in the neonate intestine." Cell host & microbe **8**(4): 358-368.

Chehade, M. and L. Mayer (2005). "Oral tolerance and its relation to food hypersensitivities." Journal of allergy and clinical immunology **115**(1): 3-12.

Chell, S., A. Kadi, et al. (2006). "Mediators of PGE< sub> 2</sub> synthesis and signalling downstream of COX-2 represent potential targets for the prevention/treatment of colorectal cancer." Biochimica et Biophysica Acta (BBA)-Reviews on Cancer **1766**(1): 104-119.

Chen, W. (2006). "Dendritic cells and (CD4+) CD25+ T regulatory cells: crosstalk between two professionals in immunity versus tolerance." Front Biosci **11**(3): 1360-1370.

Chen, Y., V. K. Kuchroo, et al. (1994). "Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis." Science (New York, NY) **265**(5176): 1237.

Chung, Y. C. and Y. F. Chang (2003). "Serum interleukin-6 levels reflect the disease status of colorectal cancer." Journal of surgical oncology **83**(4): 222-226.

Claud, E. C., L. Lu, et al. (2004). "Developmentally regulated IκB expression in intestinal epithelium and susceptibility to flagellin-induced inflammation." Proceedings of the National Academy of Sciences of the United States of America **101**(19): 7404-7408.

Claud, E. C. and W. A. Walker (2001). "Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis." The FASEB Journal **15**(8): 1398-1403.

Cliffe, L. J., N. E. Humphreys, et al. (2005). "Accelerated intestinal epithelial cell turnover: a new mechanism of parasite expulsion." Science **308**(5727): 1463-1465.

Coley, W. B. (1894). "Treatment of inoperable malignant tumors with the toxines of erysipelas and the bacillus prodigiosus." The American Journal of the Medical Sciences **108**(1): 50-66.

Comalada, M., M. Cardó, et al. (2003). "Decorin reverses the repressive effect of autocrine-produced TGF-β on mouse macrophage activation." The Journal of Immunology **170**(9): 4450-4456.

Coppa, G. V., S. Bruni, et al. (2004). "The first prebiotics in humans: human milk oligosaccharides." Journal of clinical gastroenterology **38**: S80-S83.

Crespo, J., A. Cayón, et al. (2001). "Gene expression of tumor necrosis factor α and TNF-receptors, p55 and p75, in nonalcoholic steatohepatitis patients." Hepatology **34**(6): 1158-1163.

Croker, B. A., H. Kiu, et al. (2008). SOCS regulation of the JAK/STAT signalling pathway. Seminars in cell & developmental biology, Elsevier.

Cummings, J., E. Beatty, et al. (2007). "Digestion and physiological properties of resistant starch in the human large bowel." British Journal of Nutrition **75**(05): 733-747.

Cummings, J., E. Pomare, et al. (1987). "Short chain fatty acids in human large intestine, portal, hepatic and venous blood." British Medical Journal **28**(10): 1221-1227.

Danese, S. and A. Mantovani (2010). "Inflammatory bowel disease and intestinal cancer: a paradigm of the Yin–Yang interplay between inflammation and cancer." Oncogene **29**(23): 3313-3323.

De Silva, N., M. Chan, et al. (1997). "Morbidity and mortality due to ascariasis: re-estimation and sensitivity analysis of global numbers at risk." Tropical Medicine & International Health **2**(6): 513-518.

deSchoolmeester, M. L., L. Martinez-Pomares, et al. (2009). "The mannose receptor binds Trichuris muris excretory/secretory proteins but is not essential for protective immunity." Immunology **126**(2): 246-255.

Dignass, A. (2001). "Mechanisms and modulation of intestinal epithelial repair." Inflammatory bowel diseases **7**(1): 68-77.

Dignass, A., D. Baumgart, et al. (2004). "The aetiopathogenesis of inflammatory bowel disease-immunology and repair mechanisms." Alimentary Pharmacology and Therapeutics **20**: 9-17.

Dimitriou, I. D., L. Clemenza, et al. (2008). "Putting out the fire: coordinated suppression of the innate and adaptive immune systems by SOCS1 and SOCS3 proteins." Immunological reviews **224**(1): 265-283.

Doyle, S. L. and L. A. O'Neill (2006). "Toll-like receptors: from the discovery of NFκB to new insights into transcriptional regulations in innate immunity." Biochemical pharmacology **72**(9): 1102-1113.

DuBois, R., A. Radhika, et al. (1996). "Increased cyclooxygenase-2 levels in carcinogen-induced rat colonic tumors." Gastroenterology **110**(4): 1259.

Eaden, J. (2004). "Colorectal carcinoma and inflammatory bowel disease." Alimentary Pharmacology & Therapeutics **20**(s4): 24-30.

Eaden, J., K. Abrams, et al. (2001). "The risk of colorectal cancer in ulcerative colitis: a meta-analysis." Gut **48**(4): 526-535.

Eaden, J. A. and J. F. Mayberry (2000). "Colorectal cancer complicating ulcerative colitis: a review." The American Journal of Gastroenterology **95**(10): 2710-2719.

Elliott, D. E., J. Li, et al. (2003). "Exposure to schistosome eggs protects mice from TNBS-induced colitis." American Journal of Physiology-Gastrointestinal and Liver Physiology **284**(3): G385-G391.

Elliott, D. E., R. W. Summers, et al. (2007). "Helminths as governors of immune-mediated inflammation." International journal for parasitology **37**(5): 457-464.

Ellmerich, S., M. Schöller, et al. (2000). "Promotion of intestinal carcinogenesis by Streptococcus bovis." Carcinogenesis **21**(4): 753-756.

Else, K. and M. deSchoolmeester (2003). "Immunity to Trichuris muris in the laboratory mouse." Journal of helminthology 77(02): 95-98.

Else, K., F. Finkelman, et al. (1994). "Cytokine-mediated regulation of chronic intestinal helminth infection." The Journal of Experimental Medicine **179**(1): 347-351.

Else, K., L. Hültner, et al. (1992). "Modulation of cytokine production and response phenotypes in murine trichuriasis." Parasite Immunology **14**(4): 441-449.

Encyclopædia Britannica, I. (2003). "Regions of the large intestine." Retrieved Aug 12, 2013, from http://media.web.britannica.com/eb-media/19/74319-004-68DBB5D6.jpg.

Endo, T. A., M. Masuhara, et al. (1997). "A new protein containing an SH2 domain that inhibits JAK kinases." Nature **387**(6636): 921.

Erridge, C., S. Duncan, et al. (2010). "The Induction of Colitis and Ileitis in Mice Is Associated with Marked Increases in Intestinal Concentrations of Stimulants of TLRs 2, 4, and 5."

Ewaschuk, J. B. and L. A. Dieleman (2006). "Probiotics and prebiotics in chronic inflammatory bowel diseases." WORLD JOURNAL OF GASTROENTEROLOGY **12**(37): 5941.

Fanaro, S., R. Chierici, et al. (2003). "Intestinal microflora in early infancy: composition and development." Acta Paediatrica **92**: 48-55.

Farhat, K., S. Riekenberg, et al. (2008). "Heterodimerization of TLR2 with TLR1 or TLR6 expands the ligand spectrum but does not lead to differential signaling." Journal of Leukocyte Biology **83**(3): 692-701.

Fava, F. and S. Danese (2011). "Intestinal microbiota in inflammatory bowel disease: Friend of foe?" World Journal of Gastroenterology: WJG **17**(5): 557.

Feil, W., E. Wenzl, et al. (1987). "Repair of rabbit duodenal mucosa after acid injury in vivo and in vitro." Gastroenterology **92**(6): 1973.

Feldman, M., L. S. Friedman, et al. (2010). Sleisenger and Fordtran's Gastrointestinal and Liver Disease-2 Volume Set: Pathophysiology, Diagnosis, Management, Expert Consult Premium Edition-Enhanced Online Features and Print E-Book, Elsevier Health Sciences.

Femia, A. P., C. Luceri, et al. (2002). "Antitumorigenic activity of the prebiotic inulin enriched with oligofructose in combination with the probiotics Lactobacillus rhamnosus and Bifidobacterium lactis on azoxymethane-induced colon carcinogenesis in rats." Carcinogenesis **23**(11): 1953-1960.

Firth, M. A., P. E. Shewen, et al. (2005). "Passive and active components of neonatal innate immune defenses." Animal Health Research Reviews **6**(2): 143.

Flynn, S., D. van Sinderen, et al. (2002). "Characterization of the genetic locus responsible for the production of ABP-118, a novel bacteriocin produced by the probiotic bacterium Lactobacillus salivarius subsp. salivarius UCC118." Microbiology **148**(4): 973-984.

Forchielli, M. L. and W. A. Walker (2005). "The role of gut-associated lymphoid tissues and mucosal defence." British Journal of Nutrition **93**(1): 41-48.

Franchimont, D., S. Vermeire, et al. (2004). "Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis." British Medical Journal **53**(7): 987.

Fukata, M. (2006). "Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: role in proliferation and apoptosis in the intestine." Gastroenterology **131**(3): 862-877.

Fukata, M. and M. Abreu (2007). "TLR4 signalling in the intestine in health and disease." Biochemical Society Transactions **35**: 1473-1478.

Fukata, M., A. Chen, et al. (2007). "Toll-like receptor-4 promotes the development of colitis-associated colorectal tumors." Gastroenterology **133**(6): 1869-1869. e1814.

Fukata, M., K. Michelsen, et al. (2005). "Toll-like receptor-4 is required for intestinal response to epithelial injury and limiting bacterial translocation in a murine model of acute colitis." American Journal of Physiology- Gastrointestinal and Liver Physiology **288**(5): 1055-1065.

Fukuda, M., O. Kanauchi, et al. (2002). "Prebiotic treatment of experimental colitis with germinated barley foodstuff: a comparison with probiotic or antibiotic treatment." International journal of molecular medicine **9**(1): 65.

Furrie, E., S. Macfarlane, et al. (2005). "Synbiotic therapy (Bifidobacterium longum/Synergy 1) initiates resolution of inflammation in patients with active ulcerative colitis: a randomised controlled pilot trial." Gut **54**(2): 242-249.

Fusunyan, R. D., N. N. Nanthakumar, et al. (2001). "Evidence for an innate immune response in the immature human intestine: toll-like receptors on fetal enterocytes." Pediatric research **49**(4): 589-593.

Garay, R. P., P. Viens, et al. (2007). "Cancer relapse under chemotherapy: why TLR2/4 receptor agonists can help." European journal of pharmacology **563**(1-3): 1.

Garcia Rodriguez, L., A. Gonzalez-Perez, et al. (2005). "Risk factors for inflammatory bowel disease in the general population." Alimentary Pharmacology & Therapeutics **22**(4): 309-315.

Gazouli, M., G. Mantzaris, et al. (2005). "Association between polymorphisms in the Toll-like receptor 4, CD14, and CARD15/NOD2 and inflammatory bowel disease in the Greek population." World J Gastroenterol **11**(5): 681-685.

Geier, M. S., R. N. Butler, et al. (2006). "Probiotics, prebiotics and synbiotics: a role in chemoprevention for colorectal cancer?" Cancer biology & therapy **5**(10): 1265-1269.

Geier, M. S., R. N. Butler, et al. (2007). "Inflammatory bowel disease: current insights into pathogenesis and new therapeutic options; probiotics, prebiotics and synbiotics." International journal of food microbiology **115**(1): 1.

Gewirtz, A. and J. Madara (2001). "Periscope, up! Monitoring microbes in the intestine." Nature Immunology **2**(4): 288-290.

Gewirtz, A. T. and J. L. Madara (2001). "Periscope, up! Monitoring microbes in the intestine." Nature immunology **2**(4): 288-290.

Ghosh, S. and M. Karin (2002). "Missing pieces in the NF-[kappa] B puzzle." Cell **109**(2): S81-S96.

Gibson, G., H. Probert, et al. (2004). "Dietary modulation of the human colonic microbiota: updating the concept of prebiotics." Nutrition research reviews **17**(02): 259-275.

Gibson, G. and M. Roberfroid (1995). "Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics." Journal of Nutrition **125**(6): 1401.

Giovannucci, E. (2002). "Modifiable risk factors for colon cancer." Gastroenterology Clinics of North America **31**(4): 925.

Goldin, B. R., L. J. Gualtieri, et al. (1996). "The effect of Lactobacillus GG on the initiation and promotion of DMH-induced intestinal tumors in the rat."

Grivennikov, S. I. and M. Karin (2010). "Dangerous liaisons: STAT3 and NF-κB collaboration and crosstalk in cancer." Cytokine & growth factor reviews **21**(1): 11-19.

Grölund, M.-M., O.-P. Lehtonen, et al. (1999). "Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after cesarean delivery." Journal of pediatric gastroenterology and nutrition **28**(1): 19-25.

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

Gupta, R. A. and R. N. DuBois (2001). "Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2." Nature Reviews Cancer **1**(1): 11-21.

Guslandi, M., G. Mezzi, et al. (2000). "Saccharomyces boulardii in maintenance treatment of Crohn's disease." Digestive diseases and sciences **45**(7): 1462-1464.

Hamilton, K. E., J. G. Simmons, et al. (2011). "Cytokine Induction of Tumor Necrosis Factor Receptor 2 Is Mediated by STAT3 in Colon Cancer Cells." Molecular Cancer Research **9**(12): 1718-1731.

Hanai, H., O. Kanauchi, et al. (2004). "Germinated barley foodstuff prolongs remission in patients with ulcerative colitis." International journal of molecular medicine **13**(5): 643-648.

Hanauer, S. B. (2006). "Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities." Inflammatory bowel diseases **12**(5): S3-S9.

Harmey, J. H., C. D. Bucana, et al. (2002). "Lipopolysaccharide-induced metastatic growth is associated with increased angiogenesis, vascular permeability and tumor cell invasion." International journal of cancer **101**(5): 415-422.

Harrell, L. and E. Chang (2006). "Intestinal water and electrolyte transport." Gastrointestinal and liver disease: pathophysiology/diagnosis/management. 8th ed. Philadelphia: WB Saunders Co: 2127-2146.

Hartsough, M. T. and K. M. Mulder (1997). "Transforming Growth Factor-[beta] Signaling in Epithelial Cells." Pharmacology & therapeutics **75**(1): 21-41.

Hashimoto, C., K. L. Hudson, et al. (1988). "The< i> Toll</i> gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein." Cell **52**(2): 269-279.

He, B., L. You, et al. (2003). "SOCS-3 is frequently silenced by hypermethylation and suppresses cell growth in human lung cancer." Proceedings of the National Academy of Sciences **100**(24): 14133-14138.

Heinrich, P. C., I. Behrmann, et al. (2003). "Principles of interleukin (IL)-6-type cytokine signalling and its regulation." Biochemical Journal **374**(Pt 1): 1.

Helmby, H., K. Takeda, et al. (2001). "Interleukin (IL)-18 promotes the development of chronic gastrointestinal helminth infection by downregulating IL-13." The Journal of Experimental Medicine **194**(3): 355-364.

Higgins, P. J. and H. Weiner (1988). "Suppression of experimental autoimmune encephalomyelitis by oral administration of myelin basic protein and its fragments." The Journal of Immunology **140**(2): 440-445.

Hill, D. A. and D. Artis (2009). "Intestinal bacteria and the regulation of immune cell homeostasis." Annual Review of Immunology **28**: 623-667.

Hoebe, K., X. Du, et al. (2003). "Identification of Lps2 as a key transducer of MyD88-independent TIR signalling." Nature **424**(6950): 743-748.

Hornef, M., B. Normark, et al. (2003). "Intracellular recognition of lipopolysaccharide by toll-like receptor 4 in intestinal epithelial cells." The Journal of experimental medicine **198**(8): 1225.

Horvat, S. and J. F. Medrano (2001). "Lack of< i> Socs2</i> Expression Causes the High-Growth Phenotype in Mice." Genomics **72**(2): 209-212.

Huang, B., J. Zhao, et al. (2007). "Listeria monocytogenes promotes tumor growth via tumor cell toll-like receptor 2 signaling." Cancer Research **67**(9): 4346-4352.

Imler, J.-L. and J. A. Hoffmann (2001). "Toll receptors in innate immunity." Trends in cell biology **11**(7): 304-311.

Ittmann, P. I., R. Amarnath, et al. (1992). "Maturation of antroduodenal motor activity in preterm and term infants." Digestive diseases and sciences **37**(1): 14-19.

Itzkowitz, S. H. (2002). "Cancer prevention in patients with inflammatory bowel disease." Gastroenterology Clinics of North America **31**(4): 1133.

Itzkowitz, S. H. and X. Yio (2004). "Inflammation and cancer IV. Colorectal cancer in inflammatory bowel disease: the role of inflammation." American Journal of Physiology-Gastrointestinal and Liver Physiology **287**(1): G7-G17.

Ivory, C. P., L. E. Wallace, et al. (2008). "Interleukin-10-independent anti-inflammatory actions of glucagon-like peptide 2." American Journal of Physiology-Gastrointestinal and Liver Physiology **295**(6): G1202-G1210.

Janeway Jr, C. A. and R. Medzhitov (2002). "Innate immune recognition." Science Signalling **20**(1): 197.

Jarnicki, A., T. Putoczki, et al. (2010). "Review Stat3: linking inflammation to epithelial cancer-more than a" gut" feeling?".

Jiang, D., J. Liang, et al. (2005). "Regulation of lung injury and repair by Toll-like receptors and hyaluronan." Nature medicine **11**(11): 1173-1179.

Jilling, T., D. Simon, et al. (2006). "The roles of bacteria and TLR4 in rat and murine models of necrotizing enterocolitis." The Journal of Immunology **177**(5): 3273-3282.

Jones, R. M., V. M. Sloane, et al. (2011). "Flagellin administration protects gut mucosal tissue from irradiation-induced apoptosis via MKP-7 activity." Gut **60**(5): 648-657.

Jones, S., R. Edwards, et al. (2004). "Inflammation and wound healing: the role of bacteria in the immuno-regulation of wound healing." The International Journal of Lower Extremity Wounds **3**(4): 201.

Judd, L. M., B. M. Alderman, et al. (2004). "Gastric cancer development in mice lacking the SHP2 binding site on the IL-6 family co-receptor gp130." Gastroenterology **126**(1): 196.

Kamura, T., S. Sato, et al. (1998). "The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families." Genes & development **12**(24): 3872-3881.

Kanauchi, O., K. Mitsuyama, et al. (2003). "Treatment of ulcerative colitis patients by long-term administration of germinated barley foodstuff: multi-center open trial." International journal of molecular medicine **12**(5): 701.

Kanauchi, O., T. Suga, et al. (2002). "Treatment of ulcerative colitis by feeding with germinated barley foodstuff: first report of a multicenter open control trial." Journal of gastroenterology **37**: 67.

Karimi, O., A. S. Peña, et al. (2005). "Probiotics (VSL# 3) in arthralgia in patients with ulcerative colitis and Crohn's disease: A pilot study." Drugs of Today **41**(7): 453-460.

Kato, I., S. Kobayashi, et al. (1981). "Antitumor activity of Lactobacillus casei in mice." Gann= Gan **72**(4): 517.

Kawai, T. and S. Akira (2007). TLR signaling. Seminars in immunology, Elsevier.

Kawai, T. and S. Akira (2009). "The roles of TLRs, RLRs and NLRs in pathogen recognition ARTICLE." International Immunology **21**(4): 317-337.

Khan, W., P. Blennerhasset, et al. (2002). "Intestinal nematode infection ameliorates experimental colitis in mice." Infection and immunity **70**(11): 5931-5937.

Kigerl, K. A., W. Lai, et al. (2007). "Toll-like receptor (TLR)-2 and TLR-4 regulate inflammation, gliosis, and myelin sparing after spinal cord injury." Journal of neurochemistry **102**(1): 37-50.

Kile, B. T., B. A. Schulman, et al. (2002). "The SOCS box: a tale of destruction and degradation." Trends in Biochemical Sciences **27**(5): 235-241.

Kim, D., M. A. Kim, et al. (2007). "A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity." Journal of Biological Chemistry **282**(20): 14975-14983.

Kim, T. K. and J. H. Eberwine (2010). "Mammalian cell transfection: the present and the future." Analytical and bioanalytical chemistry **397**(8): 3173-3178.

Kirkland, S. C. and K. Henderson (2001). "Collagen IV synthesis is restricted to the enteroendocrine pathway during multilineage differentiation of human colorectal epithelial stem cells." Journal of cell science **114**(11): 2055-2064.

Klein, R., S. Warman, et al. (1987). "Lack of association of Streptococcus bovis with noncolonic gastrointestinal carcinoma." American Journal of Gastroenterology **82**(6): 540-543.

Kluwe, J., A. Mencin, et al. (2009). "Toll-like receptors, wound healing, and carcinogenesis." Journal of Molecular Medicine **87**(2): 125-138.

Kolios, G., V. Valatas, et al. (2002). "Depletion of non specific esterase activity in the colonic mucosa of patients with ulcerative colitis." European Journal of Clinical Investigation **32**(4): 265-273.

Krebs, D. L. and D. J. Hilton (2001). "SOCS proteins: negative regulators of cytokine signaling." Stem Cells **19**(5): 378-387.

Kruis, W., P. Frič, et al. (2004). "Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine." Gut **53**(11): 1617-1623.

Kumar, H., T. Kawai, et al. (2009). "Toll-like receptors and innate immunity." Biochemical and Biophysical Research Communications **388**(4): 621-625.

Lamps, L. W., K. Madhusudhan, et al. (2003). "Pathogenic Yersinia DNA is detected in bowel and mesenteric lymph nodes from patients with Crohn's disease." The American journal of surgical pathology **27**(2): 220-227.

Landi, S., F. Gemignani, et al. (2006). "Polymorphisms within inflammatory genes and colorectal cancer." Journal of negative results in biomedicine **5**(1): 15.

Laqueur, G. L., H. Matsumoto, et al. (1981). "Comparison of the Carcinogenicity of Methylazoxymethanol-β-d-glucosiduronic Acid in Conventional and Germfree Sprague-Dawley Rats." Journal of the National Cancer Institute **67**(5): 1053-1055.

LeBouder, E., J. E. Rey-Nores, et al. (2003). "Soluble forms of Toll-like receptor (TLR) 2 capable of modulating TLR2 signaling are present in human plasma and breast milk." The Journal of Immunology **171**(12): 6680-6689.

Lee, J., J.-H. Mo, et al. (2006). "Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells." Nature cell biology **8**(12): 1327-1336.

Lee, J. W., E. H. Kim, et al. (2004). "Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum." KOREAN JOURNAL OF VETERINARY RESEARCH **5**(1): 41-48.

Lee, T., D. Wakelin, et al. (1983). "Cellular mechanisms of immunity to the nematode< i> Trichuris muris</i>." International journal for parasitology **13**(4): 349-353.

Lemaitre, B., E. Nicolas, et al. (1996). "The Dorsoventral Regulatory Gene Cassette< i> spätzle/Toll/cactus</i> Controls the Potent Antifungal Response in Drosophila Adults." Cell **86**(6): 973-983.

Leonard, J. N., R. Ghirlando, et al. (2008). "The TLR3 signaling complex forms by cooperative receptor dimerization." Proceedings of the National Academy of Sciences **105**(1): 258-263.

Letterio, J. J. and A. B. Roberts (1998). "REGULATION OF IMMUNE RESPONSES BY TGF-β*." Annual Review of Immunology **16**(1): 137-161.

Levy, O. (2007). "Innate immunity of the newborn: basic mechanisms and clinical correlates." Nature Reviews Immunology **7**(5): 379-390.

Li, Y., C. de Haar, et al. (2010). "Disease-related expression of the IL6/STAT3/SOCS3 signalling pathway in ulcerative colitis and ulcerative colitis-related carcinogenesis." Gut **59**(2): 227.

Li, Y., C. de Haar, et al. (2011). "New insights into the role of STAT3 in IBD." Inflammatory bowel diseases.

Li, Y., C. de Haar, et al. (2012). "SOCS3 in immune regulation of inflammatory bowel disease and inflammatory bowel disease-related cancer." Cytokine & growth factor reviews.

Li, Y., J. Deuring, et al. (2012). "IL-6-induced DNMT1 activity mediates SOCS3 promoter hypermethylation in ulcerative colitis-related colorectal cancer." Carcinogenesis **33**(10): 1889-1896.

Lievin, V., I. Peiffer, et al. (2000). "Bifidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity." British Medical Journal **47**(5): 646.

Lim, C. C., L. R. Ferguson, et al. (2005). "Dietary fibres as "prebiotics": implications for colorectal cancer." Molecular nutrition & food research **49**(6): 609-619.

Lin, P. W. and B. J. Stoll (2006). "Necrotising enterocolitis." The Lancet **368**(9543): 1271-1283.

Lin, Q., M. Li, et al. (2011). "The essential roles of Toll-like receptor signaling pathways in sterile inflammatory diseases." International immunopharmacology **11**(10): 1422-1432.

Linke, A., I. Goren, et al. (2009). "The suppressor of cytokine signaling (SOCS)-3 determines keratinocyte proliferative and migratory potential during skin repair." Journal of Investigative Dermatology **130**(3): 876-885.

Linossi, E. M. and S. E. Nicholson (2012). "The SOCS box—adapting proteins for ubiquitination and proteasomal degradation." IUBMB life.

Lotz, M., D. Gütle, et al. (2006). "Postnatal acquisition of endotoxin tolerance in intestinal epithelial cells." The Journal of Experimental Medicine **203**(4): 973-984.

Luo, J.-L., S. Maeda, et al. (2004). "Inhibition of NF-κB in cancer cells converts inflammation-induced tumor growth mediated by TNFα to TRAIL-mediated tumor regression." Cancer Cell **6**(3): 297-305.

Macfarlane, G., J. Cummings, et al. (1986). "Protein degradation by human intestinal bacteria." Microbiology **132**(6): 1647.

Macpherson, A., U. Khoo, et al. (1996). "Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria." British Medical Journal **38**(3): 365-375.

Macpherson, A. J., D. Gatto, et al. (2000). "A primitive T cell-independent mechanism of intestinal mucosal IgA responses to commensal bacteria." Science **288**(5474): 2222-2226.

Macpherson, A. J., M. B. Geuking, et al. (2005). "Immune responses that adapt the intestinal mucosa to commensal intestinal bacteria." Immunology **115**(2): 153-162.

Madsen, K. L., J. S. Doyle, et al. (1999). "Lactobacillus species prevents colitis in interleukin 10 gene–deficient mice." Gastroenterology **116**(5): 1107-1114.

Maloy, K. J. and F. Powrie (2011). "Intestinal homeostasis and its breakdown in inflammatory bowel disease." Nature **474**(7351): 298-306.

Man, S. M., N. O. Kaakoush, et al. (2011). "The role of bacteria and pattern-recognition receptors in Crohn's disease." Nature Reviews Gastroenterology and Hepatology **8**(3): 152-168.

Manco, M., L. Putignani, et al. (2010). "Gut microbiota, lipopolysaccharides, and innate immunity in the pathogenesis of obesity and cardiovascular risk." Endocrine reviews **31**(6): 817.

Manichanh, C., L. Rigottier-Gois, et al. (2006). "Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach." Gut **55**(2): 205-211.

Marine, J.-C., C. McKay, et al. (1999). "SOCS3 is essential in the regulation of fetal liver erythropoiesis." Cell **98**(5): 617-627.

Marine, J.-C., D. J. Topham, et al. (1999). "SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality." Cell **98**(5): 609-616.

Martini, F. H. (2006). Fundementals of Anatomy & Physiology. San Francisco, Pearson Education.

Mayer, L. (2000). "Mucosal immunity and gastrointestinal antigen processing." Journal of pediatric gastroenterology and nutrition **30**(1): S4-S12.

McCartney-Francis, N., W. Jin, et al. (2004). "Aberrant Toll receptor expression and endotoxin hypersensitivity in mice lacking a functional TGF-β1 signaling pathway." The Journal of Immunology **172**(6): 3814-3821.

McCole, D. F. and K. E. Barrett (2007). "Varied role of the gut epithelium in mucosal homeostasis." Current Opinion in Gastroenterology **23**(6): 647-654.

McCormack, S. A., M. J. Viar, et al. (1992). "Migration of IEC-6 cells: a model for mucosal healing." American Journal of Physiology-Gastrointestinal and Liver Physiology **263**(3): G426-G435.

Medzhitov, R. (2008). "Origin and physiological roles of inflammation." Nature **454**(7203): 428-435.

Mitsuyama, K., S. Matsumoto, et al. (2006). "STAT3 activation via interleukin 6 trans-signalling contributes to ileitis in SAMP1/Yit mice." Gut **55**(9): 1263-1269.

Miyake, K. (2007). Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors, Elsevier.

Miyanaka, Y., Y. Ueno, et al. (2007). "Clinical significance of mucosal suppressors of cytokine signaling 3 expression in ulcerative colitis." WORLD JOURNAL OF GASTROENTEROLOGY **13**(21): 2939.

Mizoguchi, E., A. Mizoguchi, et al. (2002). "Role of tumor necrosis factor receptor 2 (TNFR2) in colonic epithelial hyperplasia and chronic intestinal inflammation in mice." Gastroenterology **122**(1): 134-144.

Mollen, K., R. Anand, et al. (2006). "Emerging paradigm: Toll-like receptor 4-sentinel for the detection of tissue damage." Shock **26**(5): 430.

Moran, G. W., F. C. Leslie, et al. (2008). "Review: Enteroendocrine cells: Neglected players in gastrointestinal disorders?" Therapeutic advances in gastroenterology **1**(1): 51-60.

Moreau, M.-C. and V. Gaboriau-Routhiau (1996). "The absence of gut flora, the doses of antigen ingested and aging affect the long-term peripheral tolerance induced by ovalbumin feeding in mice." Research in immunology **147**(1): 49-59.

Morelli, L. (2008). "Postnatal development of intestinal microflora as influenced by infant nutrition." The Journal of nutrition **138**(9): 1791S-1795S.

Moro, G., I. Minoli, et al. (2002). "Dosage-related bifidogenic effects of galacto-and fructooligosaccharides in formula-fed term infants." Journal of pediatric gastroenterology and nutrition **34**(3): 291-295.

Morowitz, M. J., V. Poroyko, et al. (2010). "Redefining the role of intestinal microbes in the pathogenesis of necrotizing enterocolitis." Pediatrics **125**(4): 777-785.

Morr, M., O. Takeuchi, et al. (2002). "Differential recognition of structural details of bacterial lipopeptides by toll-like receptors." European journal of immunology **32**(12): 3337-3347.

Morris, A. P. and M. K. Estes (2001). "Microbes and microbial toxins: paradigms for microbial-mucosal interactions VIII. Pathological consequences of rotavirus infection and its enterotoxin." AMERICAN JOURNAL OF PHYSIOLOGY **281**(1): 303-310.

Motomura, Y., H. Wang, et al. (2009). "Helminth antigen-based strategy to ameliorate inflammation in an experimental model of colitis." Clinical & Experimental Immunology **155**(1): 88-95.

Mshvildadze, M., J. Neu, et al. (2010). "Intestinal microbial ecology in premature infants assessed with non–culture-based techniques." The Journal of pediatrics **156**(1): 20-25.

Munkholm, P. (2003). "Review article: the incidence and prevalence of colorectal cancer in inflammatory bowel disease." Alimentary Pharmacology & Therapeutics **18**(s2): 1-5.

Naka, T., T. Matsumoto, et al. (1998). "Accelerated apoptosis of lymphocytes by augmented induction of Bax in SSI-1 (STAT-induced STAT inhibitor-1) deficient mice." Proceedings of the National Academy of Sciences **95**(26): 15577-15582.

Nanthakumar, N. N., R. D. Fusunyan, et al. (2000). "Inflammation in the developing human intestine: a possible pathophysiologic contribution to necrotizing enterocolitis." Proceedings of the National Academy of Sciences **97**(11): 6043-6048.

Neu, J. and W. A. Walker (2011). "Necrotizing enterocolitis." New England Journal of Medicine **364**(3): 255-264.

Neurath, M. F., I. Fuss, et al. (1996). "Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance." The Journal of Experimental Medicine **183**(6): 2605-2616.

Newburg, D. S. (2005). "Innate immunity and human milk." The Journal of nutrition **135**(5): 1308-1312.

Newburg, D. S. and W. A. Walker (2007). "Protection of the neonate by the innate immune system of developing gut and of human milk." Pediatric research **61**(1): 2-8.

Nicholson, S. E., D. De Souza, et al. (2000). "Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130." Proceedings of the National Academy of Sciences **97**(12): 6493-6498.

Nicholson, S. E., T. A. Willson, et al. (1999). "Mutational analyses of the SOCS proteins suggest a dual domain requirement but distinct mechanisms for inhibition of LIF and IL-6 signal transduction." The EMBO Journal **18**(2): 375-385.

Nusrat, A., C. Delp, et al. (1992). "Intestinal epithelial restitution. Characterization of a cell culture model and mapping of cytoskeletal elements in migrating cells." Journal of Clinical Investigation **89**(5): 1501.

O'Toole, P. W. and M. J. Claesson (2010). "Gut microbiota: changes throughout the lifespan from infancy to elderly." International Dairy Journal **20**(4): 281-291.

Ogata, H., T. Chinen, et al. (2006). "Loss of SOCS3 in the liver promotes fibrosis by enhancing STAT3-mediated TGF- 1 production." Oncogene **25**(17): 2520-2530.

Onizawa, M., T. Nagaishi, et al. (2009). "Signaling pathway via TNF-{alpha}/NF-{kappa} B in intestinal epithelial cells may be directly involved in colitis-associated carcinogenesis." American Journal of Physiology- Gastrointestinal and Liver Physiology **296**(4): G850.

Orrhage, K. and C. Nord (1999). "Factors controlling the bacterial colonization of the intestine in breastfed infants." Acta Paediatrica **88**: 47-57.

Otte, J.-M. and D. K. Podolsky (2004). "Functional modulation of enterocytes by gram-positive and gram-negative microorganisms." American Journal of Physiology-Gastrointestinal and Liver Physiology **286**(4): G613-G626.

Otte, J., E. Cario, et al. (2004). "Mechanisms of cross hyporesponsiveness to Toll-like receptor bacterial ligands in intestinal epithelial cells." Gastroenterology **126**(4): 1054-1070.

Ouburg, S., R. Mallant-Hent, et al. (2005). "The toll-like receptor 4 (TLR4) Asp299Gly polymorphism is associated with colonic localisation of Crohn's disease without a major role for the Saccharomyces cerevisiae mannan-LBP-CD14-TLR4 pathway." Gut **54**(3): 439-440.

Ozinsky, A., D. M. Underhill, et al. (2000). "The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors." Proceedings of the National Academy of Sciences **97**(25): 13766-13771.

Penders, J., C. Thijs, et al. (2006). "Factors influencing the composition of the intestinal microbiota in early infancy." Pediatrics **118**(2): 511-521.

Peran, L., S. Sierra, et al. (2007). "A comparative study of the preventative effects exerted by two probiotics, Lactobacillus reuteri and Lactobacillus fermentum, in the trinitrobenzenesulfonic acid model of rat colitis." British Journal of Nutrition **97**(01): 96-103.

Peters, R. K., M. C. Pike, et al. (1992). "Diet and colon cancer in Los Angeles county, California." Cancer Causes and Control **3**(5): 457-473.

Piessevaux, J., D. Lavens, et al. (2008). "The many faces of the SOCS box." Cytokine & growth factor reviews **19**(5): 371-381.

Pirzer, U., A. Schönhaar, et al. (1991). "Reactivity of infiltrating T lymphocytes with microbial antigens in Crohn's disease." Lancet(British edition) **338**(8777): 1238-1239.

Podolsky, D. K. (1991). "Inflammatory bowel disease." New England Journal of Medicine **325**(13): 928-937.

Podolsky, D. K. (1997). "Healing the epithelium: solving the problem from two sides." Journal of gastroenterology **32**(1): 122-126.

Potten, C. S., C. Booth, et al. (2003). "The intestinal epithelial stem cell: the mucosal governor." International journal of experimental pathology **78**(4): 219-243.

Prescott, L., J. Harley, et al. (2002). Microbiology; 5th (Edn.), McGraw-Hill Edu. Publishing.

Qin, H., K. L. Roberts, et al. (2007). "Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia." The Journal of Immunology **179**(9): 5966-5976.

Rafter, J. (2003). "Probiotics and colon cancer." Best Practice & Research Clinical Gastroenterology **17**(5): 849-859.

Rakoff-Nahoum, S. and R. Medzhitov (2007). "Regulation of spontaneous intestinal tumorigenesis through the adaptor protein MyD88." Science **317**(5834): 124.

Rakoff-Nahoum, S. and R. Medzhitov (2008). "Role of toll-like receptors in tissue repair and tumorigenesis." Biochemistry (Moscow) **73**(5): 555-561.

Rakoff-Nahoum, S., J. Paglino, et al. (2004). "Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis." Cell **118**(2): 229-241.

Recillas-Targa, F. (2006). "Multiple strategies for gene transfer, expression, knockdown, and chromatin influence in mammalian cell lines and transgenic animals." Molecular biotechnology **34**(3): 337-354.

Reddy, B. S., T. Narisawa, et al. (1976). Effect of quality and quantity of dietary fat and dimethylhydrazine in colon carcinogenesis in rats. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, NY), Royal Society of Medicine.

Rees, C. M., S. Eaton, et al. (2010). "National prospective surveillance study of necrotizing enterocolitis in neonatal intensive care units." Journal of Pediatric Surgery **45**(7): 1391-1397.

Rescigno, M., G. Rotta, et al. (2001). "Dendritic cells shuttle microbes across gut epithelial monolayers." Immunobiology **204**(5): 572-581.

Rescigno, M., M. Urbano, et al. (2001). "Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria." Nature immunology **2**(4): 361-367.

Rhee, S., E. Im, et al. (2008). "Toll-like receptor 5 engagement modulates tumor development and growth in a mouse xenograft model of human colon cancer." Gastroenterology **135**(2): 518-528.

Rhee, S., A. Keates, et al. (2004). "MEK is a key modulator for TLR5-induced interleukin-8 and MIP3 {alpha} gene expression in non-transformed human colonic epithelial cells." Journal of Biological Chemistry **279**(24): 25179.

Rhee, S., H. Kim, et al. (2006). "Role of MyD88 in phosphatidylinositol 3-kinase activation by flagellin/Toll-like receptor 5 engagement in colonic epithelial cells." Journal of Biological Chemistry **281**(27): 18560.

Rigby, R., J. Simmons, et al. (2007). "Suppressor of cytokine signaling 3 (SOCS3) limits damage-induced crypt hyper-proliferation and inflammation-associated tumorigenesis in the colon." Oncogene **26**(33): 4833-4841.

Rijkers, G. T., S. Bengmark, et al. (2010). "Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research." The Journal of nutrition **140**(3): 671S-676S.

Roberts, A. W., L. Robb, et al. (2001). "Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3." Proceedings of the National Academy of Sciences **98**(16): 9324-9329.

Rock, F. L., G. Hardiman, et al. (1998). "A family of human receptors structurally related to Drosophila Toll." Proceedings of the National Academy of Sciences **95**(2): 588-593.

Rodriguez-Vita, J. and T. Lawrence (2010). "The resolution of inflammation and cancer." Cytokine & growth factor reviews **21**(1): 61-65.

Rollo, E. E., K. P. Kumar, et al. (1999). "The epithelial cell response to rotavirus infection." The Journal of Immunology **163**(8): 4442-4452.

Rowland, I., C. Rumney, et al. (1998). "Effect of Bifidobacterium longum and inulin on gut bacterial metabolism and carcinogen-induced aberrant crypt foci in rats." Carcinogenesis **19**(2): 281-285.

Rumi, G., R. Tsubouchi, et al. (2004). "Protective effect of lactulose on dextran sulfate sodium-induced colonic inflammation in rats." Digestive diseases and sciences **49**(9): 1466-1472.

Rutter, M., B. Saunders, et al. (2004). "Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis." Gastroenterology **126**(2): 451-459.

Ruyssers, N. E., B. Y. De Winter, et al. (2008). "Therapeutic potential of helminth soluble proteins in TNBS-induced colitis in mice." Inflammatory bowel diseases **15**(4): 491-500.

Saebo, A., E. Vik, et al. (2005). "Inflammatory bowel disease associated with< i> Yersinia enterocolitica</i> O: 3 infection." European journal of internal medicine **16**(3): 176-182.

Salaun, B., I. Coste, et al. (2006). "TLR3 can directly trigger apoptosis in human cancer cells." The Journal of Immunology **176**(8): 4894-4901.

Saleh, M. and G. Trinchieri (2010). "Innate immune mechanisms of colitis and colitis-associated colorectal cancer." Nature Reviews Immunology **11**(1): 9-20.

Salminen, S., C. Bouley, et al. (1998). "Functional food science and gastrointestinal physiology and function." British Journal of Nutrition **80**(1): 147.

Salminen, S., C. Bouley, et al. (2007). "Functional food science and gastrointestinal physiology and function." British Journal of Nutrition **80**(S1): 147-171.

Samatey, F. A., K. Imada, et al. (2001). "Structure of the bacterial flagellar protofilament and implications for a switch for supercoiling." Nature **410**(6826): 331-337.

Sanders, M. E. (1994). "Lactic acid bacteria as promoters of human health." Functional Foods: 294-322.

Sartor, R. B. (2006). "Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis." Nature Clinical Practice Gastroenterology and Hepatology **3**(7): 390.

Sasaki, A., H. Yasukawa, et al. (1999). "Cytokine-inducible SH2 protein-3 (CIS3/SOCS3) inhibits Janus tyrosine kinase by binding through the N-terminal kinase inhibitory region as well as SH2 domain." Genes to Cells **4**(6): 339-351.

Sase, M., J. J. Lee, et al. (2005). "Maternal steroid enhancement of fetal rabbit gastrointestinal motility." Journal of Obstetrics and Gynaecology Research **31**(3): 263-267.

Sase, M., I. Miwa, et al. (2005). "Ontogeny of gastric emptying patterns in the human fetus." Journal of Maternal-Fetal and Neonatal Medicine **17**(3): 213-217.

Schmitz, J., M. Weissenbach, et al. (2000). "SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130." Journal of Biological Chemistry **275**(17): 12848-12856.

Schneider, M. R., A. Hoeflich, et al. (2000). "Interleukin-6 stimulates clonogenic growth of primary and metastatic human colon carcinoma cells." Cancer letters **151**(1): 31-38.

Schottelius, A. J. and H. Dinter (2006). "Cytokines, NF-κB, microenvironment, intestinal inflammation and cancer." The Link Between Inflammation and Cancer: 67-87.

Seki, E., H. Tsutsui, et al. (2005). "Contribution of Toll-like receptor/myeloid differentiation factor 88 signaling to murine liver regeneration." Hepatology **41**(3): 443-450.

Sellon, R., S. Tonkonogy, et al. (1998). "Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice." Infection and immunity **66**(11): 5224.

Sfondrini, L., A. Rossini, et al. (2006). "Antitumor activity of the TLR-5 ligand flagellin in mouse models of cancer." The Journal of Immunology **176**(11): 6624-6630.

Shahani, K. M. and A. D. Ayebo (1980). "Role of dietary lactobacilli in gastrointestinal microecology." American Journal of Clinical Nutrition **33**(11, Supplement): 2448-2457.

Shaykhiev, R., J. Behr, et al. (2008). "Microbial patterns signaling via Toll-like receptors 2 and 5 contribute to epithelial repair, growth and survival." PLoS ONE **3**(1).

Sheil, B., J. McCarthy, et al. (2004). "Is the mucosal route of administration essential for probiotic function? Subcutaneous administration is associated with attenuation of murine colitis and arthritis." Gut **53**(5): 694-700.

Simon, G. and S. Gorbach (1984). "Intestinal flora in health and disease." Gastroenterology **86**(1): 174.

Slattery, M. (2000). Diet, lifestyle, and colon cancer. Seminars in gastrointestinal disease.

Smith, E. and G. Macfarlane (1996). "Enumeration of human colonic bacteria producing phenolic and indolic compounds: effects of pH, carbohydrate availability and retention time on dissimilatory aromatic amino acid metabolism." Journal of Applied Microbiology **81**(3): 288-302.

Smith, K., E. Andersen-Nissen, et al. (2003). "Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility." Nature immunology **4**(12): 1247-1253.

Smyth, M. J., G. P. Dunn, et al. (2006). "Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity." Advances in Immunology **90**: 1-50.

Snell, R. S. (2003). Clinical Anatomy. Baltimore, Lippincott Williams and Wilkins.

Sokol, H., B. Pigneur, et al. (2008). "Faecalibacterium prausnitzii is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients." Proceedings of the National Academy of Sciences **105**(43): 16731-16736.

Starr, R., T. A. Willson, et al. (1997). "A family of cytokine-inducible inhibitors of signalling." Nature **387**(6636): 917.

Steenholdt, C., L. Andresen, et al. (2009). "Expression and function of toll-like receptor 8 and Tollip in colonic epithelial cells from patients with inflammatory bowel disease." Scandinavian journal of gastroenterology **44**(2): 195-204.

Stephen, A. M. and J. Cummings (1980). "The microbial contribution to human faecal mass." Journal of Medical Microbiology **13**(1): 45-56.

Sternini, C., L. Anselmi, et al. (2008). "Enteroendocrine cells: a site of 'taste'in gastrointestinal chemosensing." Current opinion in endocrinology, diabetes, and obesity **15**(1): 73.

Stillie, R. and A. W. Stadnyk (2009). "Role of TNF receptors, TNFR1 and TNFR2, in dextran sodium sulfate-induced colitis." Inflammatory bowel diseases **15**(10): 1515-1525.

Strober, W., I. Fuss, et al. (2007). "The fundamental basis of inflammatory bowel disease." Journal of Clinical Investigation **117**(3): 514.

Sturm, A. and A. Dignass (2002). "Modulation of gastrointestinal wound repair and inflammation by phospholipids." BBA-Molecular and Cell Biology of Lipids **1582**(1-3): 282-288.

Sturm, A. and A. Dignass (2008). "Epithelial restitution and wound healing in inflammatory bowel disease." World Journal of Gastroenterology: WJG **14**(3): 348.

Sturm, A. and A. U. Dignass (2002). "Modulation of gastrointestinal wound repair and inflammation by phospholipids." Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids **1582**(1): 282-288.

Sudo, N., S.-a. Sawamura, et al. (1997). "The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction." The Journal of Immunology **159**(4): 1739-1745.

Sugawara, I., H. Yamada, et al. (2003). "Mycobacterial infection in TLR2 and TLR6 knockout mice." Microbiology and immunology **47**(5): 327.

Suzuki, A., T. Hanada, et al. (2001). "CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation." Journal of Experimental Medicine **193**(4): 471-482.

Swidsinski, A., V. Loening Baucke, et al. (2007). "Comparative study of the intestinal mucus barrier in normal and inflamed colon." Gut **56**(3): 343.

Szebeni, B., G. Veres, et al. (2008). "Increased expression of Toll-like receptor (TLR) 2 and TLR4 in the colonic mucosa of children with inflammatory bowel disease." Clinical & Experimental Immunology **151**(1): 34-41.

Takahashi, Y., N. Carpino, et al. (2003). "SOCS3: an essential regulator of LIF receptor signaling in trophoblast giant cell differentiation." The EMBO Journal **22**(3): 372-384.

Takeda, K. and S. Akira (2004). "Microbial recognition by Toll-like receptors." Journal of Dermatological Science **34**(2): 73-82.

Takeda, K., B. E. Clausen, et al. (1999). "Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils." Immunity **10**(1): 39-49.

Takeda, K., T. Kaisho, et al. (2003). "T OLL-L IKE R ECEPTORS." Annual Review of Immunology **21**(1): 335-376.

Takeuchi, O., K. Hoshino, et al. (1999). "Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components." Immunity **11**(4): 443-451.

Takeuchi, O., T. Kawai, et al. (2001). "Discrimination of bacterial lipoproteins by Toll-like receptor 6." International Immunology **13**(7): 933-940.

Tebbutt, N. C., A. S. Giraud, et al. (2002). "Reciprocal regulation of gastrointestinal homeostasis by SHP2 and STAT-mediated trefoil gene activation in gp130 mutant mice." Nature medicine **8**(10): 1089-1097.

Török, H.-P., J. Glas, et al. (2004). "Polymorphisms of the lipopolysaccharide-signaling complex in inflammatory bowel disease: association of a mutation in the Toll-like receptor 4 gene with ulcerative colitis." Clinical Immunology **112**(1): 85-91.

Tsan, M. (2007). "Review: pathogen-associated molecular pattern contamination as putative endogenous ligands of Toll-like receptors." Journal of Endotoxin Research **13**(1): 6.

Tsujii, M. and R. N. DuBois (1995). "Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2." Cell **83**(3): 493-501.

Tursi, A., G. Brandimarte, et al. (2004). "Low-dose balsalazide plus a high-potency probiotic preparation is more effective than balsalazide alone or mesalazine in the treatment of acute mild-to-moderate ulcerative colitis." Medical science monitor: international medical journal of experimental and clinical research **10**(11): PI126.

UK, C. R. (2013). "Cancer deaths by age." Retrieved Aug 12, 2013, from http://www.cancerresearchuk.org/cancer-info/cancerstats/mortality/age/uk-cancer-mortality-statistics-by-age.

van Aubel, R., A. Keestra, et al. (2007). "Ligand-induced differential cross-regulation of Toll-like receptors 2, 4 and 5 in intestinal epithelial cells." Molecular immunology **44**(15): 3702-3714.

Venturi, A., P. Gionchetti, et al. (1999). "Impact on the composition of the faecal ora by a new probiotic preparation: preliminary data on maintenance treatment of patients with ulcerative colitis." Aliment Pharmacol Ther **13**: 1103-1108.

Videla, S., J. Vilaseca, et al. (2001). "Dietary inulin improves distal colitis induced by dextran sodium sulfate in the rat." The American Journal of Gastroenterology **96**(5): 1486-1493.

Vijay-Kumar, M., J. Aitken, et al. (2008). Toll like receptor-5: protecting the gut from enteric microbes, Springer.

Vijay-Kumar, M., J. Aitken, et al. (2008). "Flagellin treatment protects against chemicals, bacteria, viruses, and radiation." The Journal of Immunology **180**(12): 8280.

Vijay-Kumar, M., C. Sanders, et al. (2007). "Deletion of TLR5 results in spontaneous colitis in mice." Journal of Clinical Investigation **117**(12): 3909-3921.

Vollaard, E. and H. Clasener (1994). "Colonization resistance." Antimicrobial agents and chemotherapy **38**(3): 409.

Waaij, V. (1989). "The ecology of the human intestine and its consequences for overgrowth by pathogens such as Clostridium difficile." Annual Reviews in Microbiology **43**(1): 69-87.

Wang, D. and R. N. DuBois (2009). "The role of COX-2 in intestinal inflammation and colorectal cancer." Oncogene **29**(6): 781-788.

Wang, Y., J. D. Hoenig, et al. (2009). "16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis." The ISME journal **3**(8): 944-954.

Wilson, A. and P. Gibson (1997). "Epithelial migration in the colon: filling in the gaps." Clinical Science **93**: 97-108.

Wilson, A. and P. Gibson (1997). "Short-chain fatty acids promote the migration of colonic epithelial cells in vitro." Gastroenterology **113**(2): 487-496.

Wollowski, I., G. Rechkemmer, et al. (2001). "Protective role of probiotics and prebiotics in colon cancer." American Journal of Clinical Nutrition **73**(2): 451S.

Wurm, F. M. (2004). "Production of recombinant protein therapeutics in cultivated mammalian cells." Nature biotechnology **22**(11): 1393-1398.

Yang, L. and Z. Pei (2006). "Bacteria, inflammation, and colon cancer.": **12**(042): 6741-6746.

Yasukawa, H., M. Ohishi, et al. (2003). "IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages." Nature immunology **4**(6): 551-556.

Yasukawa, H., A. Sasaki, et al. (2000). "Negative regulation of cytokine signaling pathways." Annual Review of Immunology **18**(1): 143-164.

Yonekura, K., S. Maki-Yonekura, et al. (2003). "Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy." Nature **424**(6949): 643-650.

Yoshimura, A., T. Naka, et al. (2007). "SOCS proteins, cytokine signalling and immune regulation." Nature Reviews Immunology 7(6): 454-466.

Yoshimura, A., H. M. M. Ohishi, et al. (2004). "Regulation of TLR signaling and inflammation by SOCS family proteins." Journal of Leukocyte Biology **75**(3): 422.

Yue, J., B. Sun, et al. (2004). "Requirement of TGF-β receptor-dependent activation of c-Jun N-terminal kinases (JNKs)/stress-activated protein kinases (Sapks) for TGF-β up-regulation of the urokinase-type plasminogen activator receptor." Journal of cellular physiology **199**(2): 284-292.

Zeng, H., A. Carlson, et al. (2003). "Flagellin is the major proinflammatory determinant of enteropathogenic Salmonella." The Journal of Immunology **171**(7): 3668.

Zeng, H., H. Wu, et al. (2006). "Flagellin/TLR5 responses in epithelia reveal intertwined activation of inflammatory and apoptotic pathways." American Journal of Physiology- Gastrointestinal and Liver Physiology **290**(1): G96.

Zhang, J.-G., A. Farley, et al. (1999). "The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation." Proceedings of the National Academy of Sciences **96**(5): 2071-2076.

Zhang, Z. and H. Schluesener (2006). "Mammalian toll-like receptors: from endogenous ligands to tissue regeneration." Cellular and molecular life sciences **63**(24): 2901-2907.

Zhou, R., H. Wei, et al. (2007). "Recognition of double-stranded RNA by TLR3 induces severe small intestinal injury in mice." The Journal of Immunology **178**(7): 4548-4556.

Zhu, J. and C. Mohan (2010). "Toll-like receptor signaling pathways—therapeutic opportunities." Mediators of inflammation **2010**.

Zippi, M., M. C. Colaiacomo, et al. (2006). "Mesenteric adenitis caused by Yersinia pseudotubercolosis in a patient subsequently diagnosed with Crohn's disease of the terminal ileum." WORLD JOURNAL OF GASTROENTEROLOGY **12**(24): 3933.

9. ABBREVIATIONS

AZO	Azoxymethane
BSA	Bovine Serum Albumin
CAC	Cancer associated colitis
CD	Crohns disease
CD	Ulcerative colitis
CIS	Cytokine inducible SH2
CMV	Cytomegalovirus
COX-2	Cyclo-oxygenase-2
CRC	Colorectal cancer
CXCL10	C-X-C motif chemokine 10
DAMPs	Damage-associated molecular patterns
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate-Buffered Saline
dsRNA	Double stranded RNA
DSS	Dextran sulphate sodium
ECACC	European Collection of Cell Cultures
ECMV	Encephalomyocarditis
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ES	Excretory/secretory
ESS	Extended SH2 subdomain
EV	Empty vector
FBS	Foetal bovine serum
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate

GBF	Germinated barley foodstuff
GI	Gastro-intestinal
GST	Glutathione *S*-transferase
HMGB1	High-mobility group protein B1
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
IELs	Intestinal epithelial lymphocytes
IFN-β	Interferon-β
IgA	Immunoglobulin A
IGF-1	Insulin-like growth factor-1
IL-1	Interleukin-1
IL-6R	IL-6 receptor
iNOS	Inducible nitric oxide synthase
IRAK	IL-1 receptor-associated kinase
IRES	Internal ribosome entry site
IRF	Interferon regulatory factor
JAK	Janus Kinase
KIR	Kinase inhibitory region
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MCS	Multiple cloning site
MEM	Minimal Essential Medium
MLN	Mesenteric lymph nodes
MyD88	Myeloid differentiation factor 88
NCBI	National Centre for Biotechnology Information

NEC	Necrotizing enterocolitis
NF-*k*B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NPT II	Neomycinphosphotransferase
OVA	Ovalbumin
PAMP	Pathogen associated molecular patterns
PGE_2	Prostaglandin E_2
Poly I:C	Polyinosinic:polycytidylic acid
PP	Peyer's patches
PRR	Pattern recognition receptor
QPCR	Quantitate polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RTK	Receptor tyrosine kinase
S3	SOCS3 vector
SH2	Src homology 2
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
TBST	Tris Buffered Saline-Tween
TGFα	Transforming growth factor-α
TGF-β	Transforming growth factor-β
Th-2	T-helper 2
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNBS	2,4,6-trinitrobenzene sulfonic acid
TNF	Tumour necrosis factor

TRAF6	TNF receptor-associated factor 6
TRIF	TIR domain-containing adaptor-inducing interferon-β