Regulatory T Cells, Th17 Effector Cells and Cytokine Microenvironment in Inflammatory Bowel Disease and Coeliac Disease

NICOLA EASTAFF - LEUNG B.Sc. (Hons.)

Discipline of Pathology, School of Medical Sciences
University of Adelaide;
The Basil Hetzel Institute for Medical Research and the
Department of Gastroenterology and Hepatology, The
Queen Elizabeth Hospital

August 2009

A thesis submitted to the University of Adelaide as the requirement for the degree of Doctor of Philosophy

CHAPTER 1

LITERATURE REVIEW

1.1 Overview

Inflammatory bowel disease and coeliac disease affect the quality of life of thousands of Australians, with approximately 61,000 Australians affected with inflammatory bowel disease and 200,000 Australians with coeliac disease (Access-Economics, 2007; Bernstein and Shanahan, 2008). IBD is managed by anti-inflammatory and immunosuppressive drugs and by surgery, however relapses are common, with increased risk of intestinal malignancies in both Crohn's disease and ulcerative colitis (Hwang and Varma, 2008). Treatment of coeliac disease involves complete removal of gluten from the diet for life. However there is an increased risk of intestinal T cell lymphomas in those who are non-compliant to a gluten-free diet, and in those with refractory disease which is not controlled by a gluten-free diet (Freeman, 2008). Evidence of an aberrant immune response to harmless stimuli in inflammatory bowel disease (Duchmann *et al.*, 1995) and an exaggerated T cell mediated response in coeliac disease (Verbeek *et al.*, 2009) has prompted the investigation of regulatory and effector cells in these gastrointestinal diseases.

1.2 Immunobiology of the Gastrointestinal Tract

1.2.1 Morphology of the Gastrointestinal Tract

The gastrointestinal tract consists of four basic layers: mucosa, submucosa, muscularis propria and serosa. The serosa is a loose connective tissue covered by mesothelium that lines the peritoneal cavity. Adjacent to this layer lies the muscularis propria which comprises two sublayers of smooth muscle that are responsible for peristalsis (Burkitt, 1993). The submucosa comprises loose connective tissue with blood vessels, nerves, lymphatics, lymphoid tissue and glands.

The mucosa, which lines the gastrointestinal tract, collectively consists of epithelium, lamina propria and muscularis mucosae which is a thin layer of smooth muscle overlying the submucosa (Junqueira and Carneiro, 2003). The epithelium lines the intestinal surface, being exposed to the lumen, and invaginations form glands or crypts in the lamina propria. Its functions include the provision of a selectively permeable barrier between the contents of the lumen and the underlying tissues of the body and absorption of the products of digestion and water (Junqueira and Carneiro, 2003). The lamina propria, a layer of loose connective tissue, contains blood vessels, lymphatics, nerves, smooth muscle cells and various cells including lymphocytes, plasma cells, eosinophils and macrophages, which are involved in immunological defence. The specific features of the mucosa differ depending on the intestinal region.

The absorptive surface of the small intestine is increased 600-fold by mucosal folds (plicae circulares), finger-like projections of mucosa called villi (**Figure 1.1**) and by microvilli on absorptive epithelial cells (Junqueira and Carneiro, 2003). The epithelium also includes goblet cells, enteroendocrine cells, microfold (M) cells, lymphocytes in the intercellular spaces, and Paneth cells at the base of the crypts. Goblet cells produce mucus to protect and lubricate the small intestine (Burkitt, 1993), and Paneth cells secrete the anti-bacterial enzyme lysozyme (Junqueira and Carneiro, 2003). The duodenum contains Brunner's glands located in the submucosa that produce an alkaline secretion that neutralises acid entering from the stomach.

There are no villi in the large intestine, and the mucosa contains tubular glands that are specialised for water absorption. In addition, goblet cells produce mucus for lubrication (Burkitt, 1993). The large intestine is the major site of intestinal microbiota that aid in

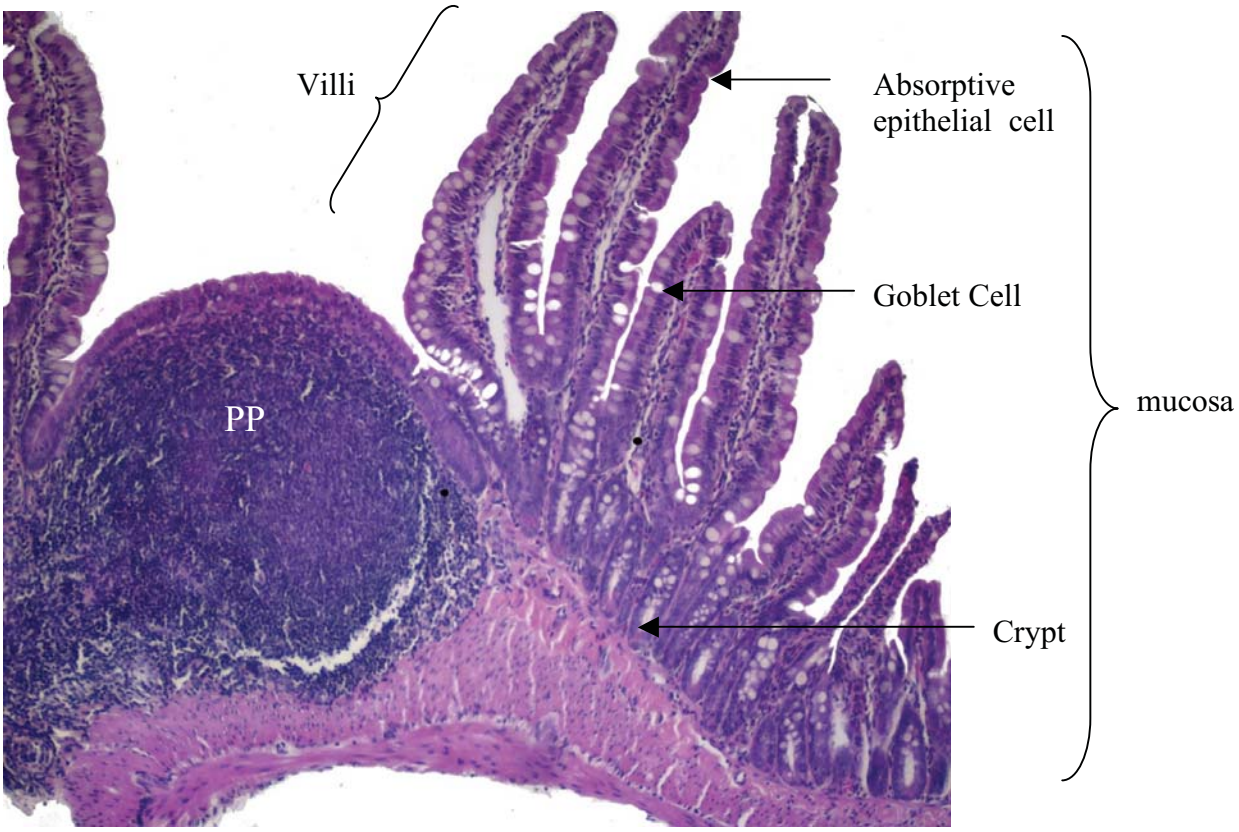

Figure 1.1 Histology of the small intestine. (Ileum, H&E, 100x) Within the small intestine are lymph nodules of Peyer's patches (PP), which contribute to the gut associated lymphoid tissue (GALT). The intestinal mucosa includes intestinal villi that are lined with columnar absorptive epithelial cells interspersed with goblet cells. Crypts are continuous with villi and contain Paneth cells at their base.

the processing of non-digestible dietary residue, and are involved in vitamin synthesis (Thompson-Chagoyan *et al.*, 2005). Intestinal bacteria are primarily of the following genera: *Bacteroides, Clostridium, Fusobacterium, Eubacterium, Ruminococcus, Peptococcus, Peptostreptococcus, Bifidium Escherichia* and *Lactobacillus* (Guarner and Malagelada, 2003). These bacteria maintain a commensal symbiotic relationship in the intestine and assist in the digestion of certain carbohydrates, with short chain fatty acids (SCFA) resulting from the fermentation process. SCFAs are a source of energy and nutrients and also aid in the absorption of calcium, magnesium and iron (Guarner and Malagelada, 2003; Sears, 2005).

The gut associated lymphoid tissue (GALT) includes the mesenteric lymph nodes, Peyer's Patches (large lymphoid aggregates situated in the lamina propria and submucosa of the distal small intestine, **Figure 1.1**), large lymphocyte aggregates in the large intestine, lymphocyte-filled villi and scattered T lymphocytes (Junqueira and Carneiro, 2003; Garside *et al.*, 2004; Abbas and Lichtman, 2005). The cells of the GALT are responsible for immune responses within the gastrointestinal tract and the GALT is recognised as the largest lymphoid organ in the human body.

1.2.2 Antigen Sampling

The gastrointestinal tract is constantly exposed to food and microbial antigens. In a state of health, an inflammatory immune response is avoided due to well-regulated mechanisms within the GALT. Ingested antigens are constantly sampled in the gastrointestinal tract by the GALT. Antigens however must pass through specialised anatomical structures for passage through the intestinal epithelium to the underlying lamina propria. There are three pathways by which antigen uptake can occur (**Figure**

1.2). Firstly, soluble antigen can be processed by intestinal epithelial cells acting as non-professional antigen-presenting cells that present antigen on MHC molecules to scattered T cells in the lamina propria (Mayer and Shao, 2004). Antigen also passes through intestinal epithelial cells to be processed by underlying macrophages (Garside and Mowat, 2001). The second route is via highly specialised antigen-presenting cells known as dendritic cells. Dendritic cells in the lamina propria extend their processes between tight junctions of the epithelial cells to sample antigen directly from the lumen whilst still maintaining the integrity of the epithelial layer (Dubois *et al.*, 2005; Johansson and Kelsall, 2005). The third pathway is via the microfold cell (M cell) located in the epithelium overlying Peyer's Patches. These cells are specialised endocytic epithelial cells that ingest particulate matter but not soluble protein (Chehade and Mayer, 2005). Antigen collected by M cells is delivered to the subepithelial dome of the Peyer's patch, which are rich in dendritic cells and lymphocytes. Antigen presenting cells carrying antigen travel via the lymphatic system to the mesenteric lymph nodes (Mayer and Shao, 2004). The presentation of antigen by dendritic cells, macrophages and other antigen presenting cells to T cells results in initiation of the immune response.

1.2.3 T cell activation

The interaction between antigen presenting cells, such as dendritic cells, and T lymphocytes is a fundamental immunological process that provides for selectivity and specificity of adaptive immunity. T lymphocytes originate from bone marrow progenitor cells that migrate and mature in the thymus. Naïve T cells, which have not previously encountered antigens, circulate through the blood stream to lymphoid organs such as to the GALT (Abbas and Lichtman, 2005).

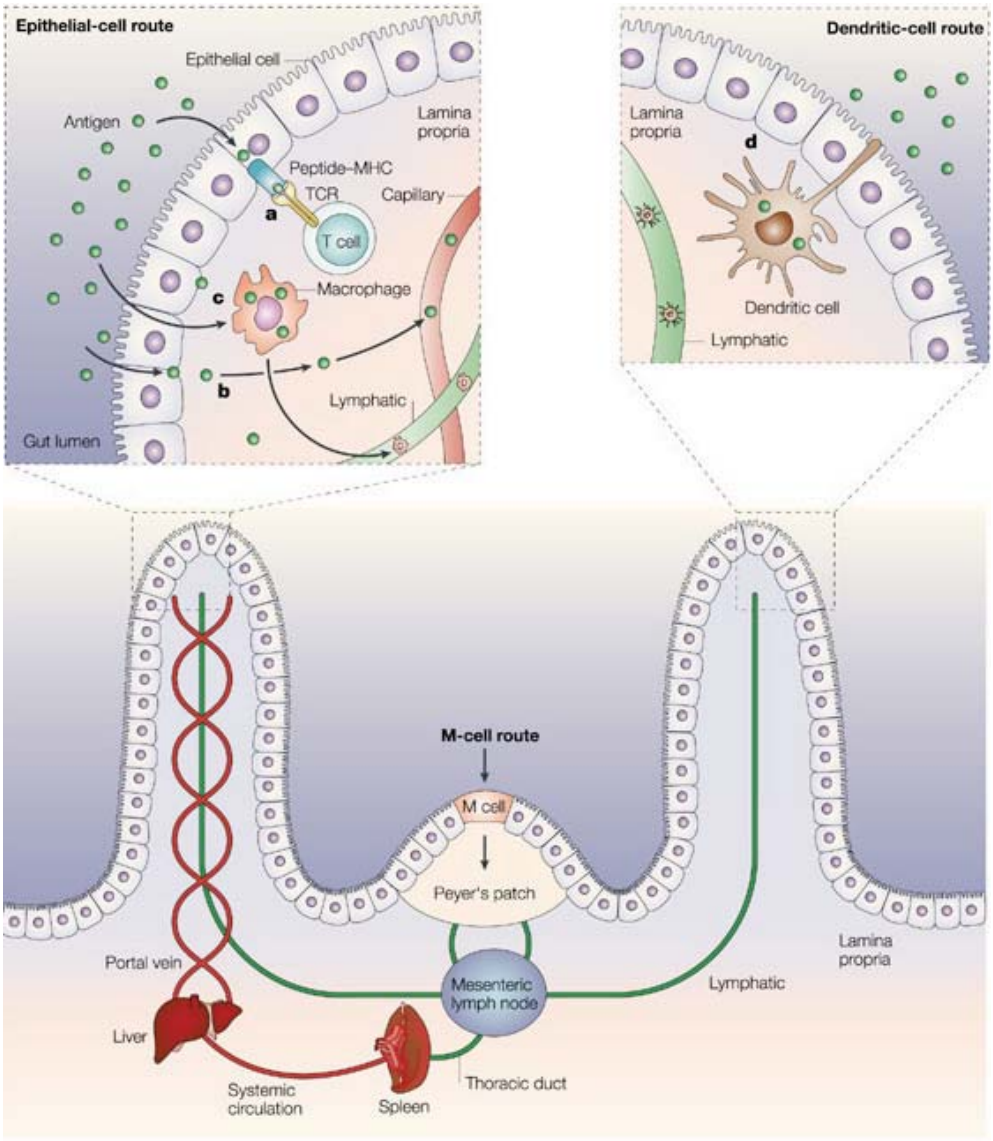

Figure 1.2. Pathways of antigen uptake in the gastrointestinal tract. There are three routes by which antigen can pass from the gut lumen and penetrate the epithelial barrier to enter the lamina propria. 1) via intestinal epithelial cells 2) via dendritic cells, and 3) via M-cells overlying the Peyer's Patch (Mayer and Shao, 2004). Published with permission.

T cell activation occurs when antigen-presenting cells present antigen to a T cell in the presence of adequate co-stimulation. Dendritic cells are the most potent activators of naïve T cells (Abbas and Lichtman, 2005). Dendritic cells are derived from hemopoietic bone marrow progenitor cells and undergo a complex maturation process to become mature antigen presenting cells. Immature dendritic cells act as 'antigen-capturing' cells that constantly sample antigen from the surrounding environment via pattern recognition receptors including toll-like receptors. Following contact with antigen, dendritic cells mature and develop the ability to activate T cells through their expression of co-stimulatory molecules and the production of cytokines (Blanco *et al.*, 2008). Dendritic cells express two co-stimulatory, CD80 and CD86 (also known as B7.1 and B7.2, respectively) (Banchereau *et al.*, 2000). T cell activation requires the presentation of antigen by the major histocompatibility complex (MHC) of the antigen presenting cell to the T cell receptor (MHC class II molecules for CD4 T cells, and MHC class I molecules for CD8 T cells). Co-stimulatory molecules are required for T cell activation with the binding of the CD28 molecule with its ligands, CD80 and CD86 (Vincenti, 2008). These two signals trigger the transcription and synthesis of cytokines and cytokine receptors, including interleukin-2 (IL-2) and the IL-2 receptor (CD25) which are crucial for the clonal expansion and differentiation of T cells (Abbas and Lichtman, 2005).

Upregulation of other cytokine receptors augments responses to various cytokines in the micro-environment to induce the activation of transcription factors and promote the differentiation of particular T cell subsets (Ansel *et al.*, 2003). These cytokines are derived from antigen presenting cells, mast cells, natural killer (NK) cells, eosinophils, basophils, γδ T cells and also from T cells (Corthay, 2006). Proliferation and

differentiation of effector cells results in antigen clearance, and consequent decline of the T cell response with absence of antigen. Without survival signals of antigen presentation, co-stimulation and proinflammatory cytokines, these T cells undergo apoptosis, allowing re-establishment of equilibrium within the system (Abbas and Lichtman, 2005). Activation of T cells however does not inherently confer an inflammatory response. For example, tolerogenic dendritic cells have been described that have a limited ability to activate effector cells, producing low levels of proinflammatory cytokines and instead activate regulatory T cells that mediate tolerance (Rutella and Lemoli, 2004).

1.3 Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) refers to a group of chronic inflammatory disorders that primarily affect the gastrointestinal tract, with the two main forms being Crohn's disease and ulcerative colitis. The Australian Crohn's and Colitis Association (ACCA) reported that in 2005 approximately 61,000 individuals were living with IBD in Australia (28,000 with CD and 33,000 with UC), and that the total financial cost of IBD in 2005 was estimated at $500 million dollars (Access-Economics, 2007) . In the United States, approximately 3 to 10 new cases of IBD are reported per 100,000 individuals per year, and the incidence in Europe is as high as 1 in 200 in individuals of Northern European descent (Kumar, 2005). IBD is predominantly a disease of the developed world (principally North America, Europe and Australia), however an increase is being observed in developing regions such as Asia (Bernstein and Shanahan, 2008). The highest prevalence of IBD is evident in persons of Jewish background with a 3 to 5 times greater risk (Kumar, 2005), followed by Anglo-Saxons, African Americans, Asians and Hispanics (Baumgart and Carding, 2007).

Crohn's disease and ulcerative colitis are both considered autoimmune in nature, in which environmental factors act to precipitate disease in genetically predisposed individuals. These diseases affect women and men equally. Disease onset peaks bimodally in the late teens to early 20s or between 50-70 years of age (Baumgart and Carding, 2007). Symptoms appear intermittently with periods of remission interrupted by disease relapses that may last for weeks or months. Diagnosis involves a range of haematological and radiological tests, in addition to endoscopy with biopsy collection for pathological diagnosis (Kumar, 2005).

1.3.1 Crohn's Disease

Crohn's disease (CD) is characterised by transmural inflammation of the gastrointestinal tract that results in thickening and scarring of the gut wall especially in the small intestine. This inflammation may occur anywhere in the gastrointestinal tract, however, it predominates in the distal ileum (Levison, 2008). The clinical symptoms of CD include abdominal pain, diarrhoea, rectal bleeding, weight loss, fatigue, fever and vomiting (Kumar, 2005). Complications of CD include fluid and electrolyte disorders, anaemia, osteoporosis, fistula formation, fissuring, bowel perforation, intestinal obstruction, adhesions, intussusception, malabsorption and an increased risk of malignancy (Cotran, 1999). Extraintestinal manifestations are often observed including various types of arthritis (including migratory polyarthritis and ankylosing spondylitis), sacroiliitis, iritis (inflammation of the eye), erythema nodosum (skin lesions of the shins and calves), aphthous ulcers and primary sclerosing cholangitis (Kumar, 2005).

In the early stages of disease, superficial mucosal ulcers are common, with oedema of the intestinal wall and irregular mucosal texture (Kumar, 2005). With progression of

disease, this ulceration deepens, causing fissures. Deep fissures divide the swollen oedematous mucosa into segments giving the mucosa a typical 'cobblestone' appearance (**Figure 1.3**), and can result in the formation of fistulae (Levison, 2008). Thickening and fibrosis of the intestinal wall causes stricture formation. Areas of inflammation are commonly segregated by normal tissue forming 'skip lesions'. Histologically, CD is characterised by chronic transmural inflammation that is focal and non-uniform. Lymphocyte aggregates are distributed throughout the intestinal wall (Xavier and Podolsky, 2007). Crypt abscesses may be located in the mucosa and granulomas are common (Sell, 2001).

CD can be treated with a variety of drugs, commonly used in combination. Traditionally corticosteroids, such as prednisolone, are used, however relapses are common. Long-term use of corticosteroids can result in dependency or the development of tolerance to treatment (Rutgeerts *et al.*, 2009). Corticosteroids also have severe and irreversible side effects such as an increased risk of osteoporosis (Ali *et al.*, 2009). Immunosuppressive agents, such as azathioprine and 6-mercaptopurine, are commonly used to maintain remission in CD, however, these are also associated with an increased risk of infection, non-Hodgkin's lymphoma, hepatosplenic T cell lymphoma and death in a small number of cases (Sandborn, 2008). Cyclosporine-A (a calcineurin inhibitor) is used in severe cases that do not respond to corticosteroids (Domenech, 2006). Anti-TNF-α therapies such as infliximab (a chimeric monoclonal IgG1 against TNF-α) are highly effective in treating CD. TNF-α is a proinflammatory cytokine that acts upon effector cells to promote differentiation, proliferation and upregulates adhesion molecules (Rutgeerts *et al.*, 2009). Infliximab has demonstrated efficacy in inducing mucosal healing and maintenance of health (Hanauer *et al.*, 2002).

Figure 1.3. **Comparison of histologic and endoscopic appearance in health and IBD.** Examples of cobblestoning and thickening of the intestinal wall in CD can be seen in the histology and endoscopic images. Typical examples of ulceration, crypt distortion and pseudo polyps are evident in UC. (Bayless, 2008).

However side effects to Infliximab have been reported in 10-15% of patients, including shortness of breath, urticaria, myalgias, joint pain, fever and in serious cases anaphylaxis (Moss *et al.*, 2008).

Surgery is used to treat complications such as perforation, haemorrhage, fistula formation and intestinal obstruction. Colectomy and ileostomy may be required in patients with extensive disease or in those who do not respond to treatment (Merkle, 2005). It has been estimated that 70-90% of CD sufferers will require surgery over their lifetime (Hwang and Varma, 2008).

1.3.2 Ulcerative Colitis

Ulcerative colitis (UC) is characterised by inflammation which is confined to the large intestine, and predominantly affects the mucosa. Inflammation typically involves the rectum and extends proximally for a variable distance rather than forming skip lesions (Cotran, 1999). A study of 1186 UC patients in Copenhagen found that 44% had distal colitis, 36% had disease that extended to the transverse colon and 18% had pancolitis that affected the whole large intestine (Cottone *et al.*, 2008).

The clinical features of UC include abdominal pain, with an increased frequency of bloody diarrhoea containing pus and mucus (Kumar, 2005). Weight loss and weakness also occur due to malabsorption and anaemia (Merkle, 2005). Complications of UC include gastrointestinal perforation, toxic megacolon, stricture formation, anaemia and a higher incidence of colorectal carcinoma than in CD. The risk of colorectal carcinoma is 20 to 30 times higher in patients suffering with severe and extensive disease for 10 years or more than the general public (Kumar, 2005). Extra-intestinal manifestations are

similar to CD, including arthritis sacroillitis, iritis, erytheda nodosum, and aphthous ulcers (Kumar, 2005).

In the early stage of disease, there is visible reddening and granularity of the mucosa due to shallow areas of inflammation (Porth, 2005). This inflammation becomes confluent and leads to the haemorrhagic ulceration with purulent exudate that is stereotypical of UC (Merkle, 2005). Pseudopolyps are also common (**Figure 1.3c**), in which areas of regenerating mucosa form small polyp-like projections (Porth, 2005). Continued inflammation results in scarring and thickening of the large intestine wall and its progressive dilation (Levison, 2008).

Histopathologically, significant congestion and oedema of the mucosa are observed. There is an increased infiltrate of predominantly mononuclear cells, including activated T and B lymphocytes, plasma cells and eosinophils; however no granulomata are present (Levison, 2008). Neutrophils infiltrate crypts and cause crypt abscesses which may become necrotic and ulcerate (Porth, 2005). In severe cases, this ulceration extends to the submucosa and sometimes deepen exposing the muscularis propria to the gut lumen (Kumar, 2005).

The treatment of UC is similar to CD, and include the use of corticosteroids, aminosalicylates and immunomodulators to control inflammation. Most patients will experience at least one relapse in a 10-year period, and approximately 30% will require surgery due to unresponsiveness to treatment. Total colectomy is performed typically if a patient is unresponsive to corticosteroids and still having 5-8 bowel movements a day,

with blood in the stool and a C-reactive protein level of greater than 45 mg/mL (Hwang and Varma, 2008).

1.3.3 Pathogenesis of Inflammatory Bowel Disease: Environmental Factors

The incidence of IBD is highest in developed regions of the world such as Australia, Europe and North America, whereas lower rates are observed in developing regions including Asia, Africa and South America (Baumgart and Carding, 2007). The incidence of IBD is, however, increasing rapidly in developing countries at a rate that suggests the involvement of environmental factors in its pathogenesis (Koloski *et al.*, 2008).

The hygiene hypothesis is one theory that attempts to explain this phenomenon. This theory suggests that high levels of hygiene in developed countries limits the exposure of the immune system to micro-organisms at a young age and therefore inappropriate immune responses occur to these when exposed later in life (Koloski *et al.*, 2008). Improved hygiene conditions includes access to clean water, hot water, smaller family sizes, and refrigeration of food (Feeney *et al.*, 2002), Lack of exposure to soil-borne helminths through improved sanitation is also thought to play a role in the development of IBD. Helminths stimulate a Th2 response and suppress the Th1 response associated with IBD. They also encourage the development of regulatory T cells that mediate immune tolerance (Ruyssers *et al.*, 2008), and do so in order to control and evade the host immune system (Weinstock and Elliott, 2009). Treatment of these parasitic infections exacerbates IBD (Buning *et al.*, 2008), and the observation of this inverse relationship has prompted the use of helminths in clinical trials aimed at reducing the inflammation typical of IBD and improving clinical outcome (Summers *et al.*, 2005).

Helminths however, can contribute to malnutrition and affect physical and cognitive growth in children. The induction of hyporesponsiveness by helminth infections also allows co-infection with other parasites such as malaria, which commonly occurs with helminth infections in tropical regions where malaria is endemic (van Riet *et al.*, 2007).

Diet, specifically the Western diet with high consumption of sugar, meat and fast foods has been implicated as a risk factor of IBD (Asakura *et al.*, 2008). The increased incidence of IBD in Japan is hypothesised to be related to the change in dietary trends following the onset of economic growth in the 1960s, with the incorporation of more animal fats, proteins, wheat products and reduced rice consumption in the diet (Asakura *et al.*, 2007).

Other associations with IBD include oral contraceptive use, smoking, appendectomy and non-steroidal anti-inflammatory drug (NSAID) use. There is an increased risk of developing CD, and to a lesser extent UC, in women taking oral contraceptives. The risk is greater with increasing duration of use and for those prescribed the older, high dose oestrogen pill (Cornish *et al.*, 2008). There is also an increased risk of CD but not UC in women undergoing hormone replacement therapy (Cornish *et al.*, 2008). Smoking has an interesting relationship with IBD, as it has been found to decrease the risk of UC yet increases the risk of CD (Garcia Rodriguez *et al.*, 2005). There is a positive association between appendectomy before the age of 20 years in CD but having had an appendectomy has a protective effect in UC (Firouzi *et al.*, 2006). Some studies have found a positive link between NSAID use and IBD (Forrest *et al.*, 2004), with others showing that neither aspirin nor NSAIDs increase the risk of IBD. However an association between paracetamol and IBD has been reported (Garcia Rodriguez *et al.*,

2005). Childhood vaccination, in particular the measles vaccination, (Thompson *et al.*, 1995), the incidence of gastroenteritis and diarrhoea in infancy (Garcia Rodriguez *et al.*, 2006), and the absence of breast feeding (Klement *et al.*, 2004) have also been suggested to influence the development of IBD. Other disease groups with an increased risk of IBD are those already suffering irritable bowel syndrome (IBS), diabetes and rheumatoid arthritis (Garcia Rodriguez *et al.*, 2005). A psychological link has also been identified, with an association with both UC and CD in those suffering depression and anxiety (Graff *et al.*, 2009).

1.3.4 Pathogenesis of IBD: Microbial Factors

The intestinal microbiota plays an important role in the development of IBD, and animal studies support the involvement of microbial factors. Animal models of IBD do not develop disease in germ-free conditions, but do so when exposed to a conventional environment (Taurog *et al.*, 1994). Experimental colitis is also alleviated by the use of broad spectrum antibiotics (Sartor, 2004). In humans, disease commonly occurs in regions of the GIT exposed to high levels of bacteria, such as the ileum and colon (Gersemann *et al.*, 2008). Also, the diversion of faeces via a colostomy reduces inflammation in the distal bowel of CD, whereas restoration can induce inflammation (Fiocchi, 2005). IBD is proposed to be the result of an aberrant immune response to commensal organisms which prevents the resolution of inflammation (Duchmann *et al.*, 1995). Bacterial flagellin has been indicated as a dominant antigen in CD, with high levels of flagellin-specific immunoglobulins detected in CD but not UC (Sitaraman *et al.*, 2005). Flagellin-specific CD4 T cells have also been shown to induce severe colitis when transferred into SCID mice (Lodes *et al.*, 2004).

The possibility of a pathogenic bacterial infection has been closely investigated with a number of potential candidates. The *Mycobacterium avium* subspecies *paratuberculosis* is highly expressed in CD compared to UC and control groups (Sanderson *et al.*, 1992). However it is suggested that rather than being a pathogen it may be an opportunistic bacteria that colonises the damaged mucosa of individuals with CD (Packey and Sartor, 2008). Variations in the concentrations of intestinal *Escherichia coli* (*E. coli*) have also been observed, with elevated levels of *E. coli* found in the terminal ileum, mesenteric lymph nodes, and granulomata of CD patients (Packey and Sartor, 2008). Overexposure to psychotrophic bacteria, *Listeria monocytogenes*, *Yersinia enterocolitica*, and *Clostridium botulinum*, which are capable of surviving the low temperatures in refrigerators, may also be important, as these bacteria have been detected in the lesions of CD patients (Hugot *et al.*, 2003).

Studies of the intestinal microbiota in patients with IBD have revealed an altered composition in IBD with a decrease in diversity and absence of certain commensal anaerobic bacteria, such as the *Bacteroides*, *Eubacterium* and *Lactobacillus* species (Ott *et al.*, 2004). The composition and function of intestinal bacteria are controlled by diet and environmental factors and may explain the geographical distribution of IBD. Prebiotics, such as fructo-oligosaccharides and galacto-oligosaccharides, are foods that stimulate the growth and/or activity of intestinal bacteria (Steed *et al.*, 2008). Intestinal bacteria convert such indigestible fibers and starches into short chain fatty acids (SCFA) which maintain pH, osmotic balance and provide energy to intestinal colonocytes (Nishikawa *et al.*, 2009). A diet lacking indigestible fiber has reduced SCFA production, and this deficiency may affect the composition of intestinal bacteria and contribute to IBD. Inflammation in the dextran sulphate induced animal model of IBD,

is significantly reduced following the ingestion of indigestible fibers, such as inulin (Videla *et al.*, 2001) or resistant starch (Moreau *et al.*, 2003). The use of certain probiotics (benign living microorganisms such as *Lactobacillus acidophilus*) also results in a reduction of inflammation in animal models of IBD (Cherbut *et al.*, 2003), and in human clinical trials of UC but not CD (Vanderpool *et al.*, 2008).

1.3.5 Pathogenesis of IBD: Genetic Factors

The greatest independent risk factor for IBD is a positive family history of IBD. Between 2-20% of individuals diagnosed with IBD have a family history of the disease. In 75-80% of cases, family members are diagnosed with the same disease, however, in the remaining 20%, family members can present with mixed diagnoses in which one family member is diagnosed with CD and another with UC (Cho, 2008). Concordance studies in twins provide the strongest evidence for the involvement of genetic factors in the pathogenesis of IBD, particularly for CD. These studies have shown a concordance risk in dizygotic twins of 7% for CD and 3% for UC, and 37.3% for CD and 10% for UC in monozygotic twins (Baumgart and Carding, 2007).

The genetic predisposition to CD and UC is multi-factorial. Genome wide association studies have identified more than 30 loci associated with CD and UC. This range of genes includes NOD2 (CARD15), DLG5, OCTN1, OCTN2, TLR4, CARD4 (NOD1), IL23R, IRGM, PTGER4, ATG16L1, HLA-DQR (Henckaerts *et al.*, 2008). NOD2 (CARD15) in CD, and IL-23R in both CD and UC are the best understood in IBD.

Certain polymorphisms of the NOD2 (CARD15) gene on chromosome 16 have been associated with susceptibility to CD, but not to UC (Hugot *et al.*, 2001; Ogura *et al.*,

2001). NOD2 encodes nucleotide-binding oligomerization domain protein 2, which plays an important role in signalling in the innate immune system. Activation of NOD1 and NOD2 in turn activates the NF-κB pathway, which results in the production of a variety of pro-inflammatory cytokines (Kucharzik *et al.*, 2006). NOD2 is present in antigen presenting cells, Paneth cells and intestinal epithelial cells and is believed to be a crucial intracellular sensor of bacteria within the intestinal system (Lala *et al.*, 2003). It is unclear how the polymorphisms of the NOD2 gene are involved in the pathogenesis of IBD, however abnormal recognition and processing of bacteria may initiate the inflammatory process (Kucharzik *et al.*, 2006). Uncontrolled growth of *Listeria monocytogenes* occurs following its inoculation into $NOD2^{-/-}$ mice, suggesting that NOD2 plays a role in the antibacterial response (Kobayashi *et al.*, 2005). A decrease in α-defensins, an anti-microbial peptide, has also been reported in CD patients with NOD2 polymorphisms (Wehkamp *et al.*, 2005).

Early genetic studies uncovered an association between UC and specific polymorphisms of the genes for the human leukocyte antigen (HLA), with the most consistent being HLA-DR1 and HLA-DR2 (Brant, 2009). These genes encode the MHC class II molecules that present antigenic derived peptides on the surface of antigen presenting cells leading to activation of CD4 T cells. HLA-DR1 is also associated with CD, and the combination of HLA-DR1 and NOD2 polymorphisms predict disease specific to the ileum (Brant, 2009). The HLA risk alleles however, only contributed to a 3 to 4-fold increased risk compared to a 20-fold risk in homozygotes for the NOD2 risk allelles (Brant, 2009). Both CD and UC are also associated with certain polymorphisms of the gene for the interleukin-23 receptor (IL-23R) (Duerr *et al.*, 2006; Cummings *et al.*, 2007; Raelson *et al.*, 2007; Rioux *et al.*, 2007; Abraham and Cho, 2008). IL-23R is

expressed by CD4, CD8, natural killer (NK), NKT and by memory T cells (Cho, 2008). Most importantly, IL-23 is critical in the development and life span of the proinflammatory Th17 effector cell, a new key player in autoimmune inflammatory conditions (Wilson *et al.*, 2007). Other genes in IBD have been identified that are associated with the Th17 effector cell, namely IL-12β, JAK2, STAT3 and CCR6 (Anderson *et al.*, 2009), suggesting an important role of the Th17 cell in IBD.

1.3.6 Pathogenesis of IBD: Immunological Factors

A range of immunological factors involving both the innate and adaptive immune systems are associated with IBD. These factors may independently or collectively contribute to the dysfunctional immune response observed in these diseases. Components of the innate immune system shown to be dysfunctional in IBD include alterations in both tight junctions between epithelial cells and epithelial permeability (Clayburgh *et al.*, 2004). The epithelial surface of the gastrointestinal tract provides a physical barrier to prevent pathogenic organisms in the luminal contents from entering the underlying lymphoid tissue. The intestinal mucosal epithelium consists of epithelial cells attached to each other by tight junctions. This barrier is selectively permeable, allowing the absorption of nutrients and the limited sampling of luminal antigens (Chichlowski and Hale, 2008), via transcellular (through cells of the epithelium) and paracellular (between cells) routes. During inflammation, transcellular and paracellular permeability are increased, however paracellular permeability is believed to be the cause, rather than the result, of inflammation (Chichlowski and Hale, 2008). The surface of the epithelium is also covered by a layer of mucus and glycoproteins. This provides an additional physical barrier to the luminal contents, as micro-organisms bind to this layer and are then passed in the stool (Plevy, 2002). Ulcerative colitis patients

have a lowered expression of the gene encoding mucin (MUC2), the structural component of the colonic mucus layer (Moehle *et al.*, 2006), and mice deficient in MUC2 spontaneously develop colitis (Van der Sluis *et al.*, 2006). Other proposed perturbations of the innate immune response involved in IBD pathogenesis include irregular function of antigen-presenting cells whereby antigen presenting cells incorrectly recognise commensal bacteria as a pathogenic antigen (Hart *et al.*, 2005) and the altered recognition of commensal bacteria as antigenic by epithelial toll-like receptors (Pierik *et al.*, 2006),

The adaptive immune system is proposed to play a key role in IBD pathogenesis, with commensal microbial factors potentially initiating and preventing the resolution of an aberrant immune response. Patients with IBD have an abnormal cytokine profile lending support to the theory that dysregulation of the immune system contributes to disease. CD has been typically associated with an excessive T helper (Th)-1 response due to the presence of Th1 cytokines interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and IL-12 (Torres and Rios, 2008). In UC, there is a modified Th2 response with the presence of IL-5 and IL-13 but not IL-4 (Fuss *et al.*, 2004). More recently a new effector cell, the Th17 cell that produces the proinflammatory cytokine IL-17, has been proposed to be involved in the pathogenesis of IBD. Under normal conditions, the immune system remains in a constant state of controlled inflammation with a homeostatic balance between immune activation and tolerance maintained by regulatory cells. These regulatory T cells have been shown to abrogate disease in animal models of IBD (Sakaguchi *et al.*, 1995; Mottet *et al.*, 2003).

1.4 Coeliac Disease

Coeliac disease is characterized by chronic inflammation of the small intestine with nutrient malabsorption, and is the result of an abnormal immune response following ingestion of gluten-containing grains (Kumar, 2005). Coeliac disease was first described in 1887, although it was not until the 1950s that wheat, barley and rye were shown to trigger this disease and their omission from the diet would reverse the illness (Niewinski, 2008). Coeliac disease is typically diagnosed in early childhood between 9 to 24 months of age, or in adults between 30 to 40 years of age. In children the ratio between females and males is 1:1, however in adults the female to male ratio is 1.5:1 (van Heel and West, 2006). The clinical featuress vary greatly, and include classical symptoms such as diarrhoea, weight loss and failure to thrive in infants, or non-classical symptoms such as infertility, bleeding, anaemia and osteoporosis (Cotran, 1999). Other common symptoms include constipation, bloating, flatulence and fatigue (Porth, 2005). There is no cure for coeliac disease, however, the permanent avoidance of dietary gluten reverses malabsorption and improves general well being in the majority of those diagnosed with coeliac disease.

The prevalence of coeliac disease in developed countries ranges from 0.5-2%, but unlike IBD its occurrence in underdeveloped countries is similar to that of the developed world (Cataldo and Montalto, 2007). The prevalence of coeliac disease was previously underestimated in third world countries due to the lack of commercial serological tests. Recent serological screening in Northern Africa revealed that the Saharawi people exhibit the highest prevalence of coeliac disease in the world with 5.6% of the population affected (Cataldo and Montalto, 2007). It is suggested that in the general population 80% of coeliac disease remains undiagnosed, which is known as ‘the iceberg effect’ (van Heel and West, 2006). With the greater availability of sensitive endomysium or IgA tissue transglutaminase antibody assays, more cases are becoming apparent that would, in the

past, have gone unrecognized (Martin, 2008). However, a definitive diagnosis of coeliac disease involves positive serology in combination with an abnormal intestinal biopsy and a clinical response to a gluten-free diet with the return of normal intestinal features (Setty *et al.*, 2008).

Pathological changes of the small intestine in response to gluten, involve a "flattening" of the villi, crypt hyperplasia and increased intraepithelial lymphocytes (**Figure 1.4**). This is most pronounced in the duodenum and proximal jejunum as these areas are exposed to the highest concentrations of gluten (Kumar, 2005). The macroscopic appearance of the duodenum is recognized to be abnormal at endoscopy in approximately 50% of patients. There are four endoscopic markers of coeliac disease in additon to abnormal villi (**Figure 1.5**). These are scalloped duodenal folds, a mosaic mucosal pattern, layering of duodenal folds, and a micronodular mucosa (Spada *et al*., 2008).

Histologically, there are three main features of coeliac disease (Stepniak and Koning, 2006). Villus atrophy occurs where there is a loss of the microvillus brush border as well as flattening of the villi, which significantly reduces the surface area for absorption (Kumar, 2005). Crypt hyperplasia results from an increase in enterocyte production and mitotic activity in the crypts, in an attempt to replace damaged enterocytes (Levison, 2008). Chronic inflammatory cells are increased in the lamina propria, including an increase in plasma cells, lymphocytes, macrophages, eosinophils and mast cells (Kumar, 2005).

At highest risk of developing coeliac disease are those already suffering an autoimmune disease such as type 1 diabetes, thyroiditis, hepatitis and cardiomyopathy, and also those with Down's syndrome, William's and Turner's syndrome (Martin, 2008). The most significant complications associated with coeliac disease are lymphomas and small-intestinal adenocarcinomas that may develop in those not adhering to a gluten-free diet.

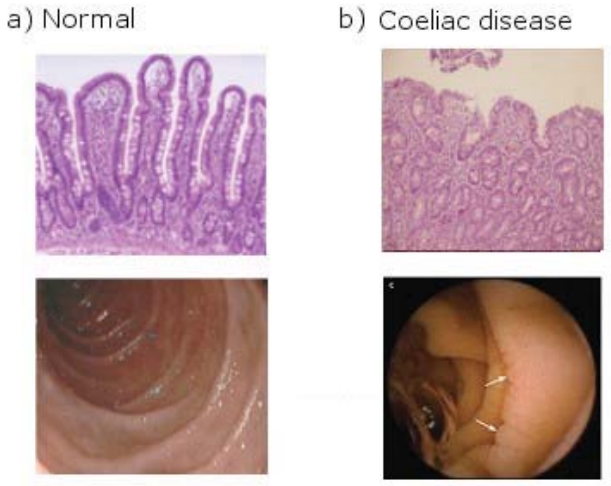

Figure 1.4 Endoscopic and histological images of small intestine. Normal endoscopic and histologic appearance (with intact villi) is demonstrated in a). The endoscopic image from a coeliac disease patient b), illustrates villous atrophy in the histological image, and fissures in the intestinal mucosa at endoscopy (Ersoy *et al.*, 2009). Published with permission.

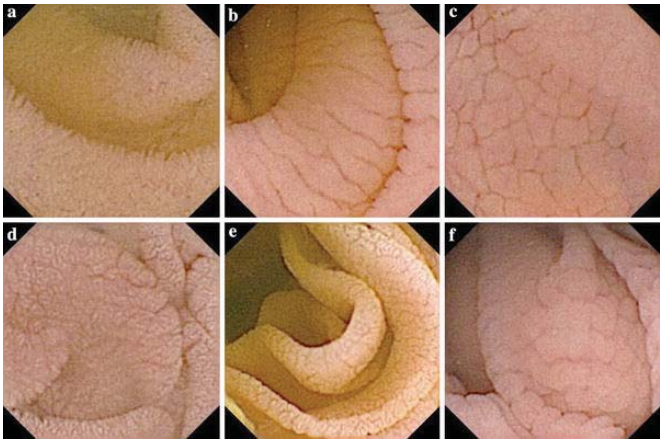

Figure 1.5 Capsule endoscopy of the duodenum. a) normal villus, b) scalloping of duodenal folds, c) mosaic mucosal pattern, d) abnormal villi, e) layering of duodenal folds, and f) micronodular mucosa (Ersoy *et al.*, 2009). Published with permission.

The main neoplasm is enteropathy-associated T cell lymphoma (EATL) (Swinson *et al*., 1983). Patients also have a 50 to 100-fold increased risk of developing esophageal squamous cell carcinoma compared to the general population (Kumar, 2005). The risk of developing EATL is reduced in those following a strict gluten-free diet for more than 5 years (Holmes *et al*., 1989). Coeliac patients with refractory disease, in whom the intraepithelial lymphocyte infiltration is not controlled by a gluten-free diet (Freeman, 2008), are at highest risk of developing EATL. In refractory disease, uncontrolled malabsorption can continue despite steroid and immunosuppressive therapy (Freeman, 2008). In patients who lapse from their gluten-free diet, steroids such as prednisolone are used, in addition to azathioprine, however unlike IBD, cyclosporine does not control this disease (Fraser and Ciclitira, 2001).

1.4.1 Pathogenesis of Coeliac Disease: Environmental Factors

Ingestion of gluten containing food products is the major environmental factor involved in coeliac disease. Gluten is present in wheat, barley and rye, which are phytogenetically related cereals. The glycoproteins gliadin and glutenin in gluten contain high levels of glutamine and proline which are highly resistant to proteolytic digestion by pepsin and chemotrypsin (Martin, 2008). Subsequently, large polypeptides, as long as 50 amino acids in length, accumulate in the proximal small intestine (Bethune and Khosla, 2008; Niewinski, 2008).

Gliadin is the alcohol-soluble component of the gluten protein and is composed of four fractions, α, β, γ and δ. (Ferranti *et al.*, 2007). A 33 amino acid α-gliadin fragment (33-mer) has been implicated as the reactive component of gluten in coeliac disease. This 33-mer is resistant to proteolytic digestion, and has the highest specificity for

deamidation by the proteolytic enzyme, tissue transglutaminase (Mowat, 2003; Ciccocioppo *et al.*, 2005). Once deamidated, these gliadin peptides become immunoreactive (Anderson *et al.*, 2000; Ferranti *et al.*, 2007).

Breast-feeding plays a partially protective role in the development of coeliac disease. It has been suggested that the late weaning of children in developing countries may account for milder symptoms of coeliac disease, and for a later onset of disease (Cataldo and Montalto, 2007). The risk of coeliac disease is reduced in infants breast-fed while gluten-containing foods are introduced. Large amounts of gluten at the time of solid food introduction increases the risk of coeliac disease, especially between 4 to 6 months of age (Ivarsson *et al.*, 2002).

1.4.2 Pathogenesis of Coeliac Disease: Genetic Factors

Genetic factors are more prominent in coeliac disease than IBD with a 70-90% concordance rate for monozygotic twins and a 10-fold risk in first degree relatives of affected patients (Fraser and Ciclitira, 2001). Coeliac disease, like IBD, is a polygenic disease, with a number of genes contributing to its pathogenesis. However it is unusual that one gene, HLA-DQ2 contributes to approximately 40-50% of genetic susceptibility (Hourigan, 2006). Approximately 97% of coeliac disease patients have genetic markers on chromosome 6p21, with 90-95% expressing the HLA-DQ2 (DQA1*05/DQB1*02) heterodimer with the remaining 5%–10% expressing HLA-DQ8 (DQA1*0301/DQB1*0302) (Hourigan, 2006). Variations in the HLA-DQ2 molecule also exist, for example, individuals homozygous for HLA-DQB1*02 have the greatest risk of developing coeliac disease. However, expression of HLA-DQ2 does not necessarily confer disease as 30% of healthy Caucasians also express HLA-DQ2 (van

Heel and West, 2006). Other genes investigated in coeliac disease are those encoding tight junction proteins. Dysfunction of tight junction proteins may allow α-gliadin antigens to travel from the gut lumen through the epithelial barrier to the underlying immune complexes thus activating an immune response (Wapenaar *et al.*, 2008). Genome wide association studies in British, Irish and Dutch cohorts also identified risk variants in the IL-2/IL-21 region (Hunt *et al.*, 2008). Interestingly, IL-2 is important in the maintenance of the CD4+ CD25+ Foxp3+ regulatory T cell (Fontenot *et al.*, 2005), and IL-21 has recently been identified as an important cytokine in the development of the Th17 effector cell (Fantini *et al.*, 2007; Yang *et al.*, 2008).

1.4.3 Pathogenesis of Coeliac Disease: Immunological factors

Coeliac disease results from an aberrant immune response due to the combination of genetic, immunological and environmental factors. Antibodies to both gliadin (anti-gliadin antibodies, AGA) and tissue transglutaminase (anti-tissue transglutaminase antibodies, tTGA) are present in the serum of coeliac disease subjects (Falini *et al.*, 2008), and α-gliadin specific T cells have been isolated from the intestinal mucosa of coeliac disease patients (Nilsen *et al.*, 1995).

A two-signal model of coeliac disease has been proposed to explain the involvement of both the innate and adaptive immune systems (**Figure 1.6**). The innate immune system is activated by toxic-gliadin peptides that act directly on epithelial cells to induce the production of IL-15 that is independent of the HLA DQ2/8 molecule (Brandtzaeg, 2006). IL-15 promotes the expansion of intestinal epithelial lymphocytes (IELs) and the expression of natural killer (NK) receptors. The NK expressing IELs target and induce apoptosis in enterocytes, resulting in damage to the epithelial layer. IL-15 increases the

permeability of tight junctions, and combined with damage to the epithelial barrier, allows passage of toxic gliadin peptides into the lamina propria, stimulating an adaptive response (Stepniak and Koning, 2006; Bernardo *et al.*, 2008).

The adaptive immune system is activated by deamination of glutamine to glutamate by tissue transglutaminase, which exposes negatively charged amino acids (Ferranti *et al.*, 2007). This modified gliadin sequence binds tightly in the antigen cleft of the DQ2 molecule (Ferranti *et al.*, 2007). Genetically predisposed individuals expressing the HLA-DQ2 genes have class II MHC molecules that recognise, and have preference for the negatively charged α-gliadin antigens. These bind at key positions of the core peptide-binding groove and are presented to T cells resulting in their activation and subsequent inflammation (Mowat, 2003; Dubois and van Heel, 2008).

Coeliac disease has been traditionally described as a Th1 mediated disease, due to high levels of the Th1 cytokine IFN-γ being expressed after exposure to gluten (Nilsen *et al.*, 1998). T-bet, the transcription factor for Th1 effector cells is over-expressed in the mucosa of untreated coeliac patients not complying to a gluten-free diet (Monteleone *et al.*, 2004). Th1 cytokines, IFN-α and IL-18 are also increased, however IL-12 is present in low levels (Salvati *et al.*, 2002). These cytokines affect the epithelial barrier, with TNF-α shown to disrupt tight junctions and paracellular permeability (Rodriguez *et al.*, 1995), and IFN-γ increasing transcellular permeability (Terpend *et al.*, 1998), exposing the underlying immune complex to the gliadin antigen.

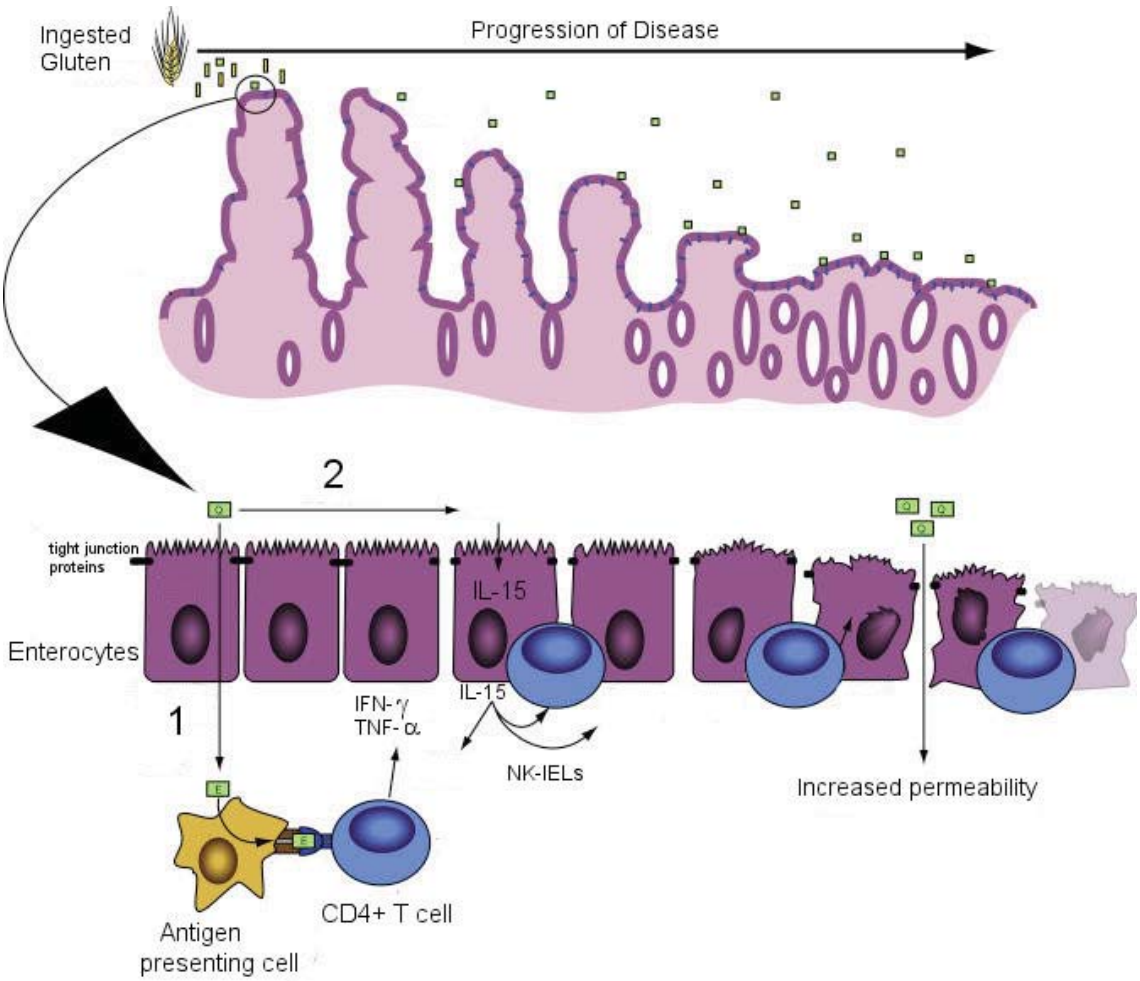

Figure 1.6 The two-signal immune response to gliadin in coeliac disease. 1) Ingested gluten sends stress signals to intestinal epithelial cells resulting in the production of IL-15, and the subsequent expansion of NK-intestinal epithelial lymphocytes (NK-IELs). This results in damage to the epithelial barrier and passage of more toxic gliadin antigen into the lamina propria. 2) Gluten is ingested and passes into the lamina propria where it is deamidated by tissue transglutaminase. These immunogenic peptides bind to HLA-DQ2 on the surface of APCs and presentation of these peptides to T cells results in a cell mediated response. (Bethune and Khosla, 2008). Published with permission.

1.5 Effector Cells

Both IBD and coeliac disease involve an aberrant immune response resulting in various forms of inflammation in the gastrointestinal tract. Only in coeliac disease is the pathogenic antigen known, whereby gluten peptides activate an immune response. In CD, microbial agents have been proposed to activate the immune system inappropriately, however the involvement of a pathogenic antigen in UC is unknown. In healthy individuals and in the majority of the population, innocuous antigens do not result in inflammation. A greater understanding of the cells involved in inciting inflammation and mediating tolerance can therefore provide us with insight into the pathogenesis of these diseases.

1.5.1 Th1 and Th2 Effector Cells

The T helper 1 (Th1) and T helper 2 (Th2) effector cell paradigm has been used to explain the adaptive immune response since its conception in 1989 (Mosmann and Coffman, 1989). Under this theoretical framework, naïve T cells were believed to differentiate into Th1 or Th2 effector cells upon presentation of antigen from an APC and adequate co-stimulation. Th1 cells are involved in cell-mediated immunity, and respond to cell based pathogens such as viruses and some bacteria, secreting primarily Interleukin (IL)-12, IL-18 and interferon (IFN)-γ (Abbas and Lichtman, 2005). Th1 cell differentiation is controlled by the master transcription factor T-bet which is activated via signal transducer and activator or transcription (STAT) 1 and STAT4 signalling by the self regulating Th1 cytokines IFN-γ and IL-12 (Korn *et al.*, 2007).

Th2 effector cells are essential for humoral immunity and control extracellular pathogens such as helminths through the secretion of IL-4, IL-5 and IL-13. IL-4

promotes the differentiation of Th2 cells by activating STAT6 signalling thereby promoting expression of the master transcription factor, GATA binding protein 3 (GATA3). Expression of GATA3 induces chromatin remodelling of the Th2 cytokine gene loci, whereby these cells acquire the Th2 phenotype (Nakayama and Yamashita, 2008). Th1 and Th2 cells reciprocally suppress each other, as IFN-γ and IL-12 inhibit the differentiation of Th2 cells, whilst IL-4 suppresses the formation of Th1 cells (Abbas and Lichtman, 2005). The Th1/Th2 paradigm, however, does not explain all inflammatory conditions and the recent discovery of a $CD4^+$ effector T cell that produces the proinflammatory cytokine interleukin-17 (IL-17) adds a new effector cell to this theory.

1.5.2 The Th17 Effector Cell – A Distinct Effector Cell Lineage

Originally, abnormal Th1 activity (and the Th1 associated cytokines IL12 and IFN-γ) was implicated in the pathogenesis of autoimmune disease, such as CD, and a Th2-like response was implicated in the pathogenesis of UC and allergy (MacDermott and Stenson, 1988; Niessner and Volk, 1995). However, this paradigm has been updated following the discovery of Th17 cells, an effector cell lineage distinct from Th1 and Th2 effector cells (Infante-Duarte *et al.*, 2000). The discovery of Th17 was made following research in animal models of autoimmunity, specifically experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA). These diseases had both been explained by an excessive Th1 response and were treated successfully using neutralising antibodies to IL-12, the predominant Th1 cytokine (Annunziato *et al.*, 2007). However, IL-23 shares a p40 subunit with IL-12 (Oppmann *et al.*, 2000). Depletion of IL-12, but not IL-23, still resulted in inflammation, whereas anti-IL23p19 reversed active colitis in animals (Oppmann *et al.*, 2000; Elson *et al.*,

2007). In mouse models, IL-23 is crucial for the establishment of experimental colitis, rather than the Th1 cytokine, IL-12 (Hue *et al.*, 2006; Yen *et al.*, 2006). IL-23 (reviewed in Chapter 4) has since been shown to be involved in promoting the development of Th17 cells which are characterised by the production of the proinflammatory cytokine IL-17 (Aggarwal *et al.*, 2003).

1.5.3 The IL-17 Cytokine Family

The IL-17 family of cytokines includes IL-17a, IL-17b, IL-17c, IL-17d, IL-17e and IL-17f. IL-17a (previously known as CTLA-8) and IL17f are the most homologous, sharing 50% of their protein structure, and both are produced by Th17 cells, whereas other IL-17 members have non-T cell origins (Ouyang *et al.*, 2008). Only IL-17a and IL-17f have pro-inflammatory properties affecting the secretion of other cytokines, chemokines, and metalloproteinases (Korn *et al.*, 2007). The focus of this literature review is on IL-17a (herein referred to as IL-17), as it is predominantly secreted by Th17 cells (Bettelli *et al.*, 2007). CD4 effector cells are not the only cell type that produces IL-17, as CD8 T cells, γδ T cells, NKT cells and neutrophils also produce low levels of IL-17a and IL-17f (Weaver *et al.*, 2007). Whereas T cells only express IL-17, the IL-17 receptor (IL-17R) is expressed ubiquitously in all tissues. mRNA for IL-17R has been found in the lungs, kidney, liver and spleen. It is also detectable in epithelial cells, fibroblasts, lymphocytes, myelomonocytes and marrow stromal cells (Ouyang *et al.*, 2008).

1.5.4 Biological Function of Th17 Effector Cells

Upon activation, the Th17 effector cell produces predominantly IL-17a and smaller quantities of IL-17f, in addition to IL-6, granulocyte macrophage colony stimulating

factor (GM-CSF) and tumour necrosis factor-α (TNF-α) (Bettelli *et al.*, 2007). The Th17 cell is said to bridge the gap between the innate and adaptive immune systems due to its ability to activate neutrophils, monocytes, dendritic cells and T cells. It does this through a number of mechanisms inducing proinflammatory mediators such as GM-CSF, granulocyte colony stimulating factor (G-CSF), keratinocyte chemoattractant (KC), and through chemokine and cytokine activity (Gaffen, 2008). IL-17 induces a range of proinflammatory chemokines (CXCL1, CXCL2, CXCL5, CXCL8, CXCL10) (Ouyang *et al.*, 2008) and cytokines (including IL-6, IL-8, IL-21, IL-22 and TNFα) (Annunziato *et al.*, 2008) as well as a range of factors involved in tissue remodelling, such as matrix metalloproteases (MMP1, MMP3, MMP9, MMP13), and anti-microbial factors such as β-defensins and mucins (Kolls and Linden, 2004; Bettelli *et al.*, 2007; Gaffen, 2008). The proinflammatory actions of the Th17 cell are important in clearing extracellular bacteria that are not covered by the Th1 and Th2 immune response. Its importance is evident in animal models, in which mice deficient in IL-17R are highly susceptible to extracellular pathogens such as the yeast *Candida albicans*, and the bacteria *Klebsiella pneumoniae* (Gaffen, 2008). IL-23$^{-/-}$ mice also have an increased mortality due to infection compared to wild type mice (Happel *et al.*, 2005).

1.5.5 IL-17 and Autoimmunity

High levels of the Th17 cytokine, IL-17, occurs in a range of human autoimmune and allergic diseases, with elevated IL-17 levels present in the synovial fluid and peripheral blood of patients with rheumatoid arthritis (Hwang and Kim, 2005), in the cerebrospinal fluid of multiple sclerosis patients (Matusevicius *et al.*, 1999; Lock *et al.*, 2002), in bronchial lavage of asthma patients (Bullens *et al.*, 2006), psoriasis (Wilson *et al.*, 2007), systemic lupus erythematosus (Wong *et al.*, 2000), and periodontal disease

(Gaffen and Hajishengallis, 2008). The Th17 cell has also been linked to the pathogenesis of IBD (Fujino *et al.*, 2003; Nielsen *et al.*, 2003).

The role that IL-17 plays in rheumatoid arthritis (RA) is the most documented, with early animal models of collagen induced arthritis demonstrating the importance of IL-17 in inflammation. In mouse studies, intra-articular injection of IL-17 into the joint resulted in inflammation and damage similar to human rheumatoid arthritis (Lubberts *et al.*, 2000). In human studies, high levels of IL-17 are found in the synovium and sera of RA patients (Hwang and Kim, 2005). Despite the implication of Th1 effector cells in autoimmune disease, Th1 cytokines are not found in excess within the RA joint (Ulfgren *et al.*, 1995; Stamp *et al.*, 2004). However, the damage apparent in RA is induced by the chemokine and cytokine network closely tied to the Th17 cell. Proinflammatory mediators such as IL-6 control the influx of mononuclear cells and also protect T cells from apoptosis (Mitsuyama *et al.*, 2006). IL-17 exacerbates damage to the extracellular matrix by activating matrix-metalloproteases and inhibiting repair by proteoglycans and collagens (Chabaud *et al.*, 2000).

Over expression of IL-17 has also been implicated in diseases of airway inflammation such as asthma, where both the serum (Wong *et al.*, 2000) and bronchial lavage of asthma patients have shown elevated IL-17 levels (Molet *et al.*, 2001). Originally the Th2 effector cell was associated in the pathogenesis of asthma and other allergic diseases, however, a role for the Th17 cell is now becoming evident. Studies in mice demonstrated the capacity for both the Th2 and Th17 cell to induce airway inflammation. However, inflammation resulting from Th17 cells was resistant to steroid treatment (McKinley *et al.*, 2008), suggesting distinct lung pathologies driven by each

effector cell type. The ability of the immune system to maintain tolerance to self-antigen and avoid autoimmunity yet remain vigilant towards pathogens, demonstrates the exquisite specificity of the immune response. The critical role of maintaining tolerance and regulating the immune response by suppressing the proliferation and effector function of other T cells is performed by regulatory T cells.

1.6 Regulatory T cells

1.6.1 Tolerance

Under normal conditions, the immune system remains poised and prepared to mount an immune response against invading pathogens, however it must also remain tolerant to self-antigens and to non-threatening foreign antigens such as ingested food and resident gut bacteria. There are two main forms of tolerance whereby unnecessary immune activation is avoided. Central tolerance involves thymic selection of self-reactive T cells and their clonal deletion. The process of positive and negative selection during the maturation phase in the thymus eliminates the majority of T cells, with only 2-4% of thymocytes in mice leaving the thymus as mature T cells (Janeway, 2001). T cells undergo the process of clonal deletion by negative selection, whereby developing T cells expressing high affinity receptors for self antigens are deleted (Cotran, 1999). Positive selection also occurs, whereby developing lymphocytes expressing receptors that interact weakly with self antigens, receive a signal enabling them to survive (Janeway, 2001). However, clonal deletion within the thymus does not eliminate all self-reactive T cells, and some manage to enter the periphery. In the periphery, there is continual positive and negative selection that is likely to be sustained by repeated contact with MHC/self peptide complexes (Janeway, 2001). In the absence of co-stimulation, T cells become anergic and are unable to respond, even to further

stimulation such as secondary T cell receptor stimulation and co-stimulatory signals (Macian *et al.*, 2004). Anergic cells are characterised by a decrease in IL-2 production which therefore prevents them from proliferating and differentiating (Janeway, 2001).

Peripheral tolerance and control of self-reactive T cells is mediated by specialised regulatory cells that actively suppress the immune system. The concept of regulatory cells, or suppressor cells as they were formerly known, was first demonstrated in the 1970's (Gershon and Kondo, 1970), however it was not until the 1990s when adequate cell surface marker technology enabled the cells with this capacity to be investigated more thoroughly. It is now commonly accepted that regulatory cells maintain tolerance within the immune system. Through these mediators of peripheral tolerance, the immune system is capable of distinguishing between self and non-self in addition to innocuous and pathogenic antigens.

1.6.2 Regulatory T cells

Regulatory T cells were originally defined by their ability to secrete suppressive cytokines, such as IL-10, transforming growth factor β (TGF-β) and IL-4. Regulatory cell populations include the Tr1 cell, Th3, $CD8^+$, iNKT, γδT and $CD4^+$ $CD25^+$ $Foxp3^+$ T cell (Piccirillo and Shevach, 2004; Paust and Cantor, 2005). It has been suggested that there may in fact be an overlap between these Treg subsets in terms of their phenotype and cytokines involved (Piccirillo and Shevach, 2004). This review will focus on the $CD4^+$ $CD25^+$ $Foxp3^+$ Treg.

1.6.3 $CD4^+$ $CD25^+$ Regulatory T Cell (Treg)

In the last 10 years, particular interest has focused on a subpopulation of $CD4^+$ T cells that express the IL-2 receptor α chain (CD25), the $CD4^+$ $CD25^+$ T cell (Treg), which constitute 5-10% of the total population of T cells in mice. In 1995, Sakaguchi and colleagues, reported that the adoptive transfer of T cells, minus the $CD4^+$ $CD25^+$ T cell population, into athymic nude mice resulted in lethal autoimmune disease. In three months, the mice lacking $CD4^+$ $CD25^+$ T cells developed inflammatory lesions including gastritis, thyroiditis, adrenalitis and insulitis. Co-transfer of normal lymphocytes, including the $CD4^+$ $CD25^+$ population, prevented histologic and serologic autoimmune development and abrogated disease (Sakaguchi *et al.*, 1995). Mottet *et al* (2003) found that $CD4^+$ $CD25^+$ T cells but not $CD4^+$ $CD25^-$ T cells were able to cure intestinal inflammation (Mottet *et al.*, 2003), demonstrating the ability for $CD4^+$ $CD25^+$ T cells to reverse established colitis, rather than only prevent disease onset. A single transfer of $1x10^6$ $CD4^+$ $CD25^+$ T cells into mice with established colitis improved clinical status, survival rate and intestinal pathology. Within 10 weeks of the transfer, the histological abnormalities were almost completely resolved (Mottet *et al.*, 2003).

1.6.4 Foxp3

Expression of the transcription factor, Forkhead box p3 (Foxp3) is a defining feature of the $CD4^+$ $CD25^+$ regulatory T cell (Nielsen *et al.*, 2004). There are no cell surface markers that uniquely distinguish the $CD4^+$ $CD25^+$ regulatory T cell from conventional T cells, as CD25 is also apparent in non-regulatory T cells and expressed transiently by cells after activation (Nelson and Willerford, 1998). CTLA-4 and GITR, which are commonly expressed by Tregs, are also induced upon TCR ligation of non-suppressive T cells (Bruder *et al.*, 2004).

Foxp3 was originally identified as the gene mutated in scurfy in mice and IPEX (immunodysregulation polyendocrinology and enteropathy X-linked syndrome) in humans. The symptoms of IPEX manifest as gastritis, protracted diarrhoea, dermatitis, insulin dependent diabetes, thyroiditis and anaemia, with death commonly occurring in the first year of life (Coffer and Burgering, 2004). These diseases are both caused by an X-linked recessive mutation of the Foxp3 gene resulting in a lack of $CD4^+$ $CD25^+$ T cells and an over proliferation of activated $CD4^+$ T cells (Coffer and Burgering, 2004).

Foxp3 is a member of the forkhead/winged helix transcription factor family. Forkhead transcription factors are commonly associated with regulation of development, and binds DNA functionally activating or inhibiting gene transcription (Yagi *et al.*, 2004). Analysis of Foxp3 expression revealed increased expression in lymphoid organs such as the thymus and spleen, but more specifically by $CD4^+$ T cells (Hori and Sakaguchi, 2004). The mutation in IPEX and scurfy results in a truncated protein that lacks the DNA binding domain, and results in the inability of Foxp3 to correctly function as a transcription factor (Coffer and Burgering, 2004).

The connection between the autoimmune diseases, scurfy and IPEX, and the lack of functional Foxp3 suggested an involvement of Foxp3 in immune regulation. Further investigation of the connection between Foxp3 and regulatory T cells revealed Foxp3 expression was confined to one group of regulatory cells, the $CD4^+$ $CD25^+$ T cell subset (Hori and Sakaguchi, 2004). Retroviral transfer of Foxp3 into $CD4^+$ $CD25^-$ T cells resulted in the $CD4^+$ $CD25^-$ T cell acquiring a regulatory T cell phenotype similar to the naturally occurring $CD4^+$ $CD25^+$ T cell. These cells expressed cell surface molecules evident in naturally occurring Tregs such as CD25, CTLA-4, GITR and CD103. They

were also capable of preventing experimentally induced IBD and gastritis (Chen *et al.*, 2003; Hori and Sakaguchi, 2004). It has been suggested that Foxp3 is in fact the master regulatory gene for regulatory T cell development and function (Fehervari and Sakaguchi, 2004).

Similar findings have been reported in human studies, however, Foxp3 is also expressed by both CD4 and to a much lesser extent in CD8 populations. Foxp3 is expressed by the $CD4^+$ $CD25^+$ T cells but in particular the $CD4^+$ $CD25^{high}$ T cells. In addition, the presence of Foxp3 correlates with the suppressive action of these cells (Walker *et al.*, 2003). However, in humans, Foxp3 is also transiently expressed in activated $CD4^+$ $CD25^-$ T cells. Cells that transiently express Foxp3 had different cytokine profiles to the traditional Treg, were not able to suppress IFN-γ production from target cells, and did not maintain their Foxp3 expression over time (Wang *et al.*, 2007). This expression of Foxp3 therefore does not confer suppressive activity which requires a stable expression of Foxp3 (Roncarolo and Gregori, 2008). In human studies, this may pose a problem, as Foxp3 alone as a marker may not be an accurate measure of regulatory phenotype particularly in patients with active disease (Pillai *et al.*, 2007).

1.6.5 Suppressive Mechanisms of Tregs

The mechanisms by which $CD4^+$ $CD25^+$ T cells regulate the immune response remain unclear. Traditionally *in vitro* assays suggested their suppressive activity is dependent upon cell-to-cell contact and independent of suppressive cytokines. They also require stimulation through the T cell receptor in order to activate suppressive abilities. Once activated, suppressive function is antigen non-specific and does not require further engagement of the T cell receptor (Read and Powrie, 2001; Thornton *et al.*, 2004). A

key response of the $CD4^+$ $CD25^+$ T cell is the inhibition of IL-2 transcription in the effector cell population, which together with IL-2 depletion, results in the inability of the responder cell to proliferate and differentiate (Fontenot and Rudensky, 2004; Hori and Sakaguchi, 2004).

A number of mechanisms of Treg function have been proposed to explain the potency of Tregs at maintaining tolerance (Vignali *et al.*, 2008). Tregs may in fact suppress via inhibitory cytokines. The dependence upon cell-to-cell contact was based upon *in vitro* experiments that showed Tregs could not control effector cell proliferation when separated by a permeable membrane (Thornton and Shevach, 1998). However, *in vivo* studies suggest Treg function may be dependent upon IL-10 and TGF-β (Joetham *et al.*, 2007). TGF-β is crucial for the differentiation of induced Tregs in murine models (Vignali *et al.*, 2008), and induced Tregs produce high levels of IL-10, a well known suppressive cytokine (Levings *et al.*, 2001). Alternatively, suppression may occur via the induction of apoptosis in a target cell. Human Tregs have been shown to express granzyme A, a serine protease that induces apoptosis (Grossman *et al.*, 2004). The expression of granzyme B was upregulated in mouse Tregs and mice deficient in granzyme B had Tregs with reduced suppressive capacity (Vignali *et al.*, 2008).

Tregs may alter the metabolic environment, particularly with respect to IL-2, which is necessary for the division of effector cells. High levels of the IL-2 receptor (CD25) on Tregs may deplete the IL-2 in the microenvironment needed for effector cell survival, thereby resulting in effector cell apoptosis (Pandiyan *et al.*, 2007). The interaction between the Treg and dendritic cell may be another means by which Tregs confer suppression, particularly in the gut where sentinel dendritic cells play a key role in

regulating the immune response. Dendritic cells are responsible for the presentation of antigen and differentiation of effector cells via the production of specific cytokines. Tregs may reduce the dendritic cell's capacity for activating effector cells by down-regulating co-stimulatory molecules (Cederbom *et al.*, 2000). Dendritic cells are also effective at inducing Tregs, and this process is dependent upon TGF-β and retinoic acid (Coombes *et al.*, 2007).

1.6.6 Natural and Induced Regulatory T Cells

There are two subtypes of $Foxp3^+$ Tregs that have similar function with variation existing in their origin. Natural Tregs develop in the thymus by the normal means of T cell maturation before entering the periphery as $CD4^+$ $CD25^+$ $Foxp3^+$ T cells. Induced Tregs on the other hand develop as a consequence of activation and are induced from $CD4^-$ $CD25^-$ precursors in peripheral lymphoid organs (Horwitz *et al.*, 2008). These induced Tregs have a similar phenotype and function as natural Tregs but demonstrate different functional requirements (Horwitz *et al.*, 2008). IL-2 and TGF-β are required for the generation of only the induced Treg, however IL-2 and TGF-β are needed for the continued maintenance of both induced and natural Tregs (Fontenot *et al.*, 2005; Marie *et al.*, 2005). Natural Tregs stably express Foxp3, however induced Tregs can lose Foxp3 expression in the absence of IL-2 and TGF-β (Zheng *et al.*, 2007). Induced Tregs also produce TGF-β and IL-10, and can promote the induction of Tregs from $CD4^+$ $CD25^-$ T cells (Zheng *et al.*, 2007). The stability of Tregs is also affected by proinflammatory cytokines such as IL-6, in which the suppressive capacity of natural Tregs is inhibited. Induced Tregs, however, are resistant to IL-6 alterations to suppressive function and consequently have a reduced IL-6 receptor expression than the natural Treg (Zheng *et al.*, 2008). The ability to distinguish between natural and induced

Tregs in human clinical samples is difficult, and the reference to Tregs in human studies may refer to both natural and Treg populations.

1.6.7 Human Tregs

Human $CD4^+$ $CD25^+$ $Foxp3^+$ T cells retain a number of similarities to their mouse counterparts. Like murine Tregs, human Tregs are anergic to stimulation of their T cell receptor in the absence of IL-2, their suppressive abilities are dependent upon cell to cell contact and independent of suppressive cytokines. Human Tregs also produce IL-10, TGF-β, low levels of IFN-γ and no IL-4 or IL-2 (Baecher-Allan *et al.*, 2004). Several significant differences however, do exist. For example, rather than forming a distinct population consisting of approximately 10% of $CD4^+$ T cells in flow cytometry profiles as described in mouse studies, human $CD4^+$ $CD25^+$ T cells only comprise 1-2% of T cells (Baecher-Allan *et al.*, 2005). From closer investigation, it appears the $CD4^+$ $CD25^{high}$ Treg cells subset describes a more homogeneous Treg population in humans (Baecher-Allan *et al.*, 2004). Separation of the $CD4^+$ $CD25^+$ population into $CD4^+$ $CD25^{high}$ and $CD4^+$ $CD25^{low}$ T cells, and stimulating these in co-culture with $CD4^+$ $CD25^-$ clearly defined human Tregs in the $CD25^{high}$ population. The $CD4^+$ $CD25^{low}$ population showed a strong proliferative response from stimulation and showed no capacity for suppression. The $CD4^+$ $CD25^{high}$ population on the other hand did not proliferate in response to stimulation, and are capable of suppressing proliferation of $CD4^+$ $CD25^-$ cells (Baecher-Allan *et al.*, 2005). Human Tregs are therefore best described as $CD4^+$ $CD25^{high}$ (Baecher-Allan *et al.*, 2005).

1.6.8 Tregs in Autoimmunity

A considerable body of research has focused on Tregs in a variety of disease conditions. These range from rheumatoid arthritis (Sarkar and Fox, 2008), diabetes (Walker, 2008), systemic lupus erythromatosis (La Cava, 2008), transplant tolerance (Cobbold, 2008) and many forms of cancer (Piersma *et al.*, 2008). Early Treg research showed resolution of murine colitis with the transference of regulatory T cells (Sakaguchi *et al.*, 1995; Mottet *et al.*, 2003), instigating the investigation of regulatory T cells in inflammatory bowel disease (reviewed in Chapter 3). However, findings in human studies have been variable and inconclusive. This is likely to be due to insufficient markers for Tregs, the lack of adequate antibodies, inadvertent exclusion of potential regulatory cells due to poor gating strategies and a general lack of standardisation in measuring these cells.

The role of Tregs in autoimmune disease has been of particular importance, as a deficiency in number or function of these cells may be involved in the pathogenesis of disease. Rheumatoid arthritis and multiple sclerosis are two main autoimmune diseases with a focus in Treg involvement. Multiple sclerosis, a chronic de-myelinating inflammatory disorder of the central nervous system, is thought to result from self-reactive cells attacking components of the myelin sheath (Venken *et al.*, 2006). Reports have emerged of a decrease in suppressive function of $CD4^+$ $CD25^+$ T cells derived from the peripheral blood of multiple sclerosis patients (Viglietta *et al.*, 2004; Huan *et al.*, 2005; Venken *et al.*, 2007). A decrease in $CD4^+$ $CD25^+$ $Foxp3^+$ cells were also reported in the peripheral blood of multiple sclerosis patients (Huan *et al.*, 2005; Venken *et al.*, 2006).

Elevated levels of $CD4^+$ $CD25^+$ cells from the synovial fluid of rheumatoid arthritis patients have been reported by a number of investigators, with numbers in peripheral blood equal to that of the control group (van Amelsfort *et al.*, 2004; Liu *et al.*, 2005; Mottonen *et al.*, 2005). Measurement of $CD4^+$ $CD25^{high}$ cells, which more closely represent the Treg population, produced similar findings whereby the numbers of these cells were elevated in the synovial fluid yet remained unchanged in the peripheral blood (Cao *et al.*, 2003; Cao *et al.*, 2004), or exhibited decreased numbers compared to the control group (de Kleer *et al.*, 2004; Lawson *et al.*, 2006). The $CD4^+$ $CD25^{high}$ cells from RA patients were also found to be functional with equal or greater suppressive capacity than control cells (de Kleer *et al.*, 2004; Cao *et al.*, 2006; Lawson *et al.*, 2006). Measurement of Foxp3 using real time PCR of RNA extracted from $CD4^+$ $CD25^{high}$ indicated high levels of $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells in the synovial fluid of RA patients (de Kleer *et al.*, 2004).

However, the consistent finding of increased Treg markers at the site of disease, with these cells maintaining a suppressive capacity *in vivo*, does not rule out the involvement of perturbed regulatory T cells in autoimmune disease. High numbers of $CD4^+$ $CD25^{high}$ T cells that retain regulatory abilities have also been demonstrated in the intestinal mucosa of patients with inflammatory bowel disease (Makita *et al.*, 2004). Makita et al (2004), however, suggested that high levels of proinflammatory cytokines and upregulation of co-stimulatory molecules in the inflamed mucosa of IBD patients may abolish the regulatory abilities of these cells at the site of disease (Makita *et al.*, 2004). Furthermore, the increase in regulatory T cells may reflect their accumulation in order to control elevated effector cell populations. Therefore the study of regulatory T cell numbers in autoimmune disease also requires the investigation of effector cells.

1.7 Summary

While extensive literature exists on Tregs and Th17 as individual components of the immune system, very few studies have investigated the relationship between these two cell types in human disease. The growing information relating the Treg and Th17 cell in a reciprocal manner suggests an important area of research, in which disease may be the result of an imbalance between these cell types. Currently, the use of medication to eliminate effector cells to control inflammation introduces significant side effects including an increased risk of infection and malignancy. An understanding of the relationship between Tregs and Th17 cells in health and disease may provide new treatment strategies in the future.

1.8 Specific Aims:

The general hypothesis of this thesis was that IBD and coeliac disease are the result of insufficient regulatory T cells and an excessive Th17 immune response.

The specific aims of this PhD were:

1. To measure Treg numbers in the peripheral blood and relative expression of Foxp3 in intestinal biopsies of IBD and coeliac patients.

2. To measure Th17 numbers in the peripheral blood and IL-17a relative expression in intestinal biopsies of IBD and coeliac patients

3. To determine the relationship between Tregs and Th17 cells in peripheral blood and intestinal biopsies of IBD and coeliac patients.

4. To investigate the intestinal cytokine microenvironment and correlate these cytokines levels with relative expression of Tregs and Th17 cells.

CHAPTER 2

MATERIALS,

METHODS

AND

SUBJECT INFORMATION

2.1 Materials

2.1.1 Antibodies

Table 2.1 Antibodies used in Flow Cytometry

Specificity	Clone	Isotype	Conjugate	Source (catalogue number)
CD3	UCHT1	IgG_1	PE-Cy5	eBioscience, SDG, USA (15-0038)
CD4	RPA-T4	Mouse IgG_1	FITC and PE	BD Biosciences, NJ, USA (11-004973 and 12-004973)
CD8	HIT8a	Mouse IgG_1	PE-Cy5	BD Biosciences, NJ, USA (555730)
CD25	B1.49.9	Mouse IgG_{2a}	PE-Cy5	Beckman Coulter, CA, USA (IM-2646)
CD127	hIL-7R-M21	Mouse IgG_1	PE	BD Biosciences, NJ, USA (557938)
IL-17	eBio64DEC17	Mouse IgG_1	PE	eBioscience, SDG, USA (12-7179)
Foxp3	PCH101	Rat IgG_{2a}	PE	eBioscience, SDG, USA (12-4776)

Table 2.2 Isotype controls used in Flow Cytometry

Specificity	Isotype	Conjugate	Source (catalogue number)
IgG_{2a}	IgG_{2a} rat	PE	eBioscience, SDG, USA (12-4321)
IgG_{2a}	IgG_{2a} mouse	PE-Cy5	eBioscience, SDG, USA (15-4714)
IgG_1	IgG_1 mouse	PE	eBioscience, SDG, USA (12-4714)
IgG_1	IgG_1 mouse	FITC	eBioscience, SDG, USA (11-4714)
IgG_1	IgG_1 mouse	PE-Cy5	eBioscience, SDG, USA (15-4714)

2.1.2 Reagents

Table 2.3 Reagents used in flow cytometry

Reagent	Source
BD Falcon FACS tubes	BD Biosciences, NJ, USA
Lymphoprep	Axis-Sheild, Oslo, Norway
Mouse serum	Sigma Aldrich, MO, USA
Non-fat dry milk powder	Nestle, Vervey, Switzerland
Paraformadehyde	Sigma Aldrich, MO, USA
Rat serum	Sigma Aldrich, MO, USA
Saponin	Sigma Aldrich, MO, USA
Sodium Azide	Sigma Aldrich, MO, USA

Table 2.4 Reagents used for Tissue Culture

Reagent	Source
Brefeldin A	Sigma Aldrich, MO, USA
Cryo-tube vials	Nunc, Thermo Fisher Scientific, Denmark
Dimethyl sulfoxide (DMSO)	Sigma Aldrich, MO, USA
Foetal Calf Serum (FCS)	Gibco®, Invitrogen, Vic, Australia
Ionomycin	Sigma Aldrich, MO, USA
Isopropyl alcohol	Sigma Aldrich, MO, USA
Mr Frosty	Nalgene Labware, Denmark
Phorbol 12-myristate 13-acetate (PMA)	Sigma Aldrich, MO, USA
RPMI-1640	Gibco®, Invitrogen, Vic, Australia
Trypan Blue	Gibco®, Invitrogen, Vic, Australia

Table 2.5 Reagents used for Molecular Biology

Reagent	Source
Agarose Powder	Progen Industries, QLD, Australia
Biorad Experion Standard sensitivity kit	Biorad, CA, USA
Chloroform	Ajax, Vic, USA
Experion Electrode Cleaner	Biorad, CA, USA
Ethidium Bromide	Sigma Aldrich, MO, USA
Power SYBR Green Master mix	Applied Biosystems, CA, USA
Qiazol	Qiagen, Vic, Australia
Qiaquick DNA Purification Kit	Qiagen, Vic, Australia
Quantitect Reverse Transcription Kit	Qiagen, Vic, Australia
RNase Inhibitor	Qiagen, Vic, Australia
RNase Zap	Ambion, TX, USA
RNAlater	Ambion, TX, USA
RNeasy mini lipid extraction kit	Qiagen, Vic, Australia

Table 2.6 Real time RT-PCR primers

Gene of Interest	Sense (5'- 3')	Anti-sense (5'- 3')	Primer Bank ID
β-Actin	AAGAGCTACGA GCTGCCTGAC	GTAGTTTCGTGG ATGCCACAG	4501885a1
Foxp3	GAAACAGCACAT TCCCAGAGTTC	ATGCCCAGCG GATGAG	31982943a1
IL-17	CAATCCCACGAA ATCCAGGATG	GGTGGAGATTC CAAGGTGAGG	4504651a1
IL-1β	CAGCTACGAAT CTCCGACCAC	GGCAGGGAAC CAGCATCTTC	10835145a2
IL-6	AAATTCGGTACA TCCTCGACGG	GGAAGGTTCAG GTTGTTTTCTGC	10834984a1
IL-21	CATGGAGAGGAT TGTCATCTGTC	CAGAAATTCAGG GACCAAGTCAT	11141875a1
IL-23	GGACAACAGTC AGTTCTGCTT	CACAGGGCTA TCAGGGAGC	7706702a1
TGF-β	CAAGCAGAGTA CACA CAGCAT	TGCTCCACTTTT AACTTGAGCC	10863873a1
T-bet	CAAGGGGGCG TCCAACAAT	TCTGGCTCTC CGTCGTTCA	7019549a1
GATA3	TCACAAAATGAA CGGACAGAACC	GGTGGTCTGAC AGTTCGCAC	4503929a1
RORC	GTAACGCGGC CTACTCCTG	GTCTTGACCA CTGGTTCCTGT	758420a1
Vα24	GATATACAGCAA CTCTGGATGCA	GGCAGACAGACT TGTCACTGGAT	758420a2

2.1.3 Buffers and Solutions

Phosphate Buffered Saline (PBS) 10x, pH 7.4

Compound	**Quantity**
NaCl	80g
KCl	2.0g
Na_2HPO_4	14.4g
KH_2PO_4	2.4g
dH_20	800ml
Dilute to 1x working solution and autoclave	

PBS Azide 10x

Compound	**Quantity**
10x PBS	1L
Sodium Azide	1g
Dilute to 1x working concentration and autoclave Final 1x PBS contains 0.01% sodium azide	

FACS Fixative

Compound	Quantity
Glucose	10g
Formaldehyde	13ml
PBS azide 1x	Up to 500 ml
Adjust to pH 7.3 Protect from light and store at 4°C	

4% Paraformaldehyde solution

Compound	Quantity
Paraformaldehyde	4.0g
PBS azide (Sterile)	100ml
Dissolve paraformaldehyde by heating solution at 56°C for an hour under a fume hood. Stir with a magnetic stirrer overnight until solids are fully dissolved. Store at 4°C protected from light, for no longer than 2 weeks.	

0.1% Saponin Solution

Compound	Quantity
Saponin	1g
PBS azide (Sterile)	1L
Dissolve saponin in PBS azide at RT with a magnetic stirrer overnight. Ensure all solids are fully dissolved. Store at 4°C for one month	

Complete Media: RPMI-1640 + 20% FCS

Compound	**Quantity**
RPMI-1640 with L-glutamine 1x liquid	1L
Foetal Calf Serum (FCS), heat inactivated and filter sterilised	200ml

Freezing Media: Complete Media + 20%DMSO

Compound	**Quantity**
Complete Media	100ml
Dimethy Sulfoxide (DMSO)	20ml

1M EDTA

Compound	**Quantity**
$Na_2EDTA_2H_2O$	186.1g (1M)
dH_20	Up to 1L
Adjust pH to 8 with 10M NaOH	

50X TAE Buffer

Compound	**Quantity**
Tris Base	193.8g (1.6M)
Sodium Acetate	65.6g (800nM)
EDTA	14.9g (40.27mM)
DH_2O	Up to 100ml
Adjust to 7.2 and autoclave Dilute to 1X working solution	

50X TBE Buffer

Compound	**Quantity**
Tris Base	54g (89mM)
Boric Acid	27.5g (444mM)
1M EDTA	10mL (100mM)
dH_20	Up to 1L
Dilute to 0.5x working concentration	

DEPC-treated water

Compound	**Quantity**
Diethlypyrocarbonate (DEPC)	1ml (0.1%v/v)
dH_20	1L
Stir for 3 hours in fume hood. Leave solution at room temperature overnight. Autoclave to inactivate DEPC prior to use	

Ethidium Bromide Stain

Compound	**Quantity**
10mg/ml Ethidium Bromide	50ul (1.25 μg/ml)
dH_20	Up to 400ml

6X loading dye

Compound	**Quantity**
50X TAE	100μl
Glycerol	2.5ml
Bromophenol Blue	1.2ml
dH_20	Up to 5ml

2.2 Methods

2.2.1 Sample collection

Peripheral blood samples were collected from patients attending the Queen Elizabeth Hospital (QEH) Department of Gastroenterology and Hepatology by QEH blood collection staff. Whole blood (10mls) was collected from each volunteer in a lithium-heparin tube to prevent coagulation (See section 2.3 for patient details).

2.2.2 Isolation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep^{TM} reagent following the protocol provided by the manufacturer. Collected whole blood was diluted with an equal volume of sterile PBS. 10mls of diluted blood was carefully overlayed onto 7mls of Lymphoprep^{TM} in a 30ml sterile centrifuge tube. Samples were centrifuged at 400g for 30 minutes at room temperature in a swing out motor with no brake. After centrifugation, the mononuclear interface layer was carefully removed using a Pasteur pipette, washed twice in sterile PBS and stored on ice.

2.2.3 Cryogenic Storage of PBMCs

Separated PBMCs were pelleted by centrifugation in a sterile 10ml falcon tube, chilled on ice and resuspended at a concentration of $1x10^7$ cells/ml in complete media (RPMI-1640 plus 20% FCS). Freezing media (complete media plus 20% DMSO) kept on ice was added drop wise to the cells while gently swirling the tube until a 1:1 ratio of cell suspension and freezing media was achieved. The tube was carefully placed on ice and further mixing and agitation avoided. 1ml of the cell suspension was carefully pipetted into 1.5ml cryovials, placed into a pre-cooled 4°C Mr Frosty (Nalgene, Denmark) filled

with isopropanol and transferred to a -70°C Freezer. After 12 hours, the cryovials were transferred to liquid nitrogen for long-term storage.

2.2.4 Thawing of Cryogenically Frozen Samples

Cryovials were transferred from liquid nitrogen to a 37°C water bath on dry ice. Frozen PBMC aliquots were thawed quickly by immersion and after thawing the outside of the cryovial wiped with 70% ethanol to prevent contamination. Warm complete medium (37°C) was added drop wise to the cell suspension and 1 drop per second, and transferred to a sterile 10ml tube containing 7ml of complete media. Cells were washed twice by centrifugation at 300g for 7 minutes. Cell pellets were resuspended in RPMI-1640 with 10% FCS. Cells to be used for intracellular flow cytometry were 'rested' overnight in RPMI-1640 with 10% FCS at 37°C (5% CO_2) incubator for 12-18 hours prior to permeabilisation to increase cell viability (Maecker *et al.*, 2005; Maecker *et al.*, 2005). Cell viability was assessed using 0.2% trypan blue exclusion, and cell numbers determined using a haemocytometer. Cells suspensions were accepted with cell viability > 80%.

2.2.5 Flow cytometry – General methods

$1x10^6$ cells were aliquoted into BD Falcon FACS tubes and incubated on ice with 5% mouse serum in PBS for 30 minutes. Without washing, saturating quantities of anti-human mouse antibodies conjugated to phycoerythrin (PE), fluorescein isothiocyanate (FITC) or phycoerythrin-cyanin-5 (PE-Cy5) were added to the cell suspension and incubated at 4°C in the dark. Surface staining was carried out prior to intracellular labelling. All wash steps involved the addition of approximately 4 mls of cold FACS wash followed by centrifugation at 400g for 4 minutes to pellet cells before decanting of

supernatant. The labelled cells were washed three times in FACS wash and then fixed with 50µl of FACS fixative. Tubes were stored overnight at 4°C in the dark and read the following day. Isotype matched control antibodies were used for each patient sample to determine non-specific staining.

Antibody labelling of cells was determined on a FACScan flow cytometer (BD Biosciences, CA, USA) and analysed using BD Cell Quest software (BD Biosciences, CA, USA). Lymphocyte gating was established based on forward and side scatter characteristics allowing exclusion of cell debris and monocytes from the mononuclear cell fraction (**Figure 2.1**), and 300,000-500,000 events were analysed within this gate. Compensation adjustments were made before each run using individually and combined PE, PE-Cy5 and FITC labelled cells to ensure there was no 'spill-over' from one channel into another thus avoiding false positives or negatives.

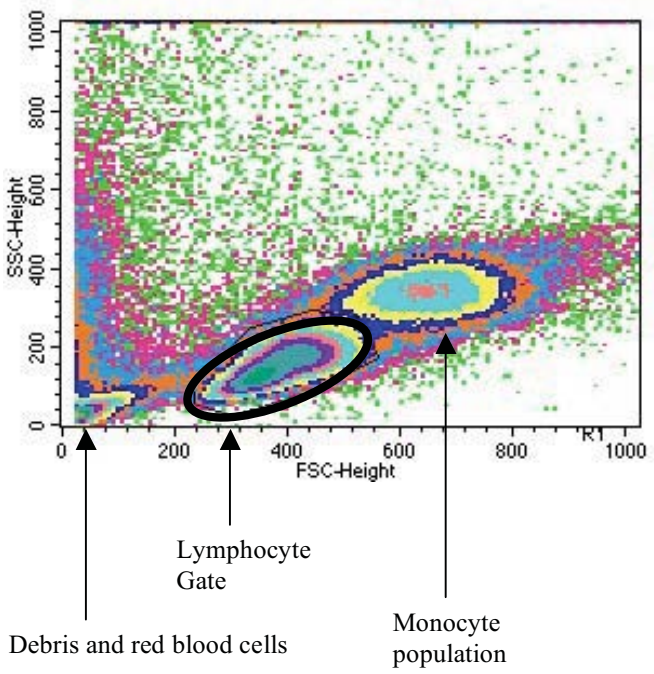

Figure 2.1. Lymphocyte gating for flow cytometry. Lymphocytes were gated based on forward and side scatter properties, in order to exclude monocyte populations and cell debris from flow cytometric analysis

2.2.6 Intestinal Biopsy Collection

Intestinal biopsies were collected from patients undergoing endoscopy within the QEH Department of Gastroenterology and Hepatology. Samples collected for RNA extraction were collected in a sterile cryovial containing RNALater (Ambion, USA) to prevent RNA degradation. Samples in RNAlater (Ambion, USA) were then stored at -20°C until needed.

2.2.7 RNA Extraction

Approximately 30mg of intestinal tissue was homogenized in 1mL QIAzol reagent (Qiagen, Melbourne, Australia) using the TissueRuptor (Qiagen, Vic, Australia). RNA was extracted using the RNeasy Lipid Tissue mini kit according to the manufacturer's instructions (Qiagen, Vic, Australia), as follows. Homogenised samples were incubated at room temperature (RT) for 5 minutes before adding 1ml of chloroform and shaken vigorously for 15 seconds. Tubes were incubated for a further 3 minutes before centrifuging at 5000g for 15 minutes at 4°C. After centrifuging, the upper aqueous phase containing RNA was carefully removed and added to 600ul of 70% ethanol and vortexed. This solution was then transferred to the RNeasy mini spin column in a 2ml tube and centrifuged for 15 seconds at 8000g and the flow through discarded. 700µl of RNeasy RW1 buffer was added and centrifuged for 15s at 8000g, followed by centrifugation with 500µl of the RNeasy RPE buffer for 15 seconds and then for 2 minutes, while discarding flow through after each step. The column with the washed RNA bound to it was then transferred to a new tube and 30µl of RNAse free water was added and centrifuged for 1 minute at 8000g to elute the RNA. This flow through was collected and placed into the column again for a final centrifuge of 1 minute at 8000g.

The spin column was then discarded and RNase Inhibitor (Qiagen, Vic, Australia) added to prevent degradation RNA was stored at –70°C until required.

2.2.8 Nucleic Acid Quantification

RNA quantification was conducted using 2μL of RNA samples on a NanoDrop ND-1000 (Nanodrop Technologies, DE, USA). Purity of RNA was determined where the ratio of the absorptions at 260nm vs 280nm was approximately 2. Samples with ratios of 260nm vs 230nm less than 1.8 were rejected due to the presence of organic contaminants.

2.2.9 Experion Virtual Electrophoresis RNA Gel

RNA integrity was determined for each patient sample using the BioRad Experion standard sensitivity automated electrophoresis system (BioRad, CA, USA), following the manufacturers protocol. Twelve patient samples at a time were analysed on a standard sensitivity chip. Prior to loading patient samples, the electrodes of the Experion Electrophoresis station were cleaned with Experion Electrode Cleaner and the chip was primed with gel stain. Denatured patient RNA (1μl) and 5μl of loading buffer was used for each well, with 1μl of ladder and 5μl loading buffer added to the ladder well. The chip was carefully checked for bubbles before running the standard sensitivity analysis. Samples were accepted if 28S ribosomal RNA bands were present with intensity approximately twice that of the 18S RNA band. Samples with smeared ribosomal bands suggested that the RNA degradation, and these samples were omitted from further experiments (**Figure 2.2**).

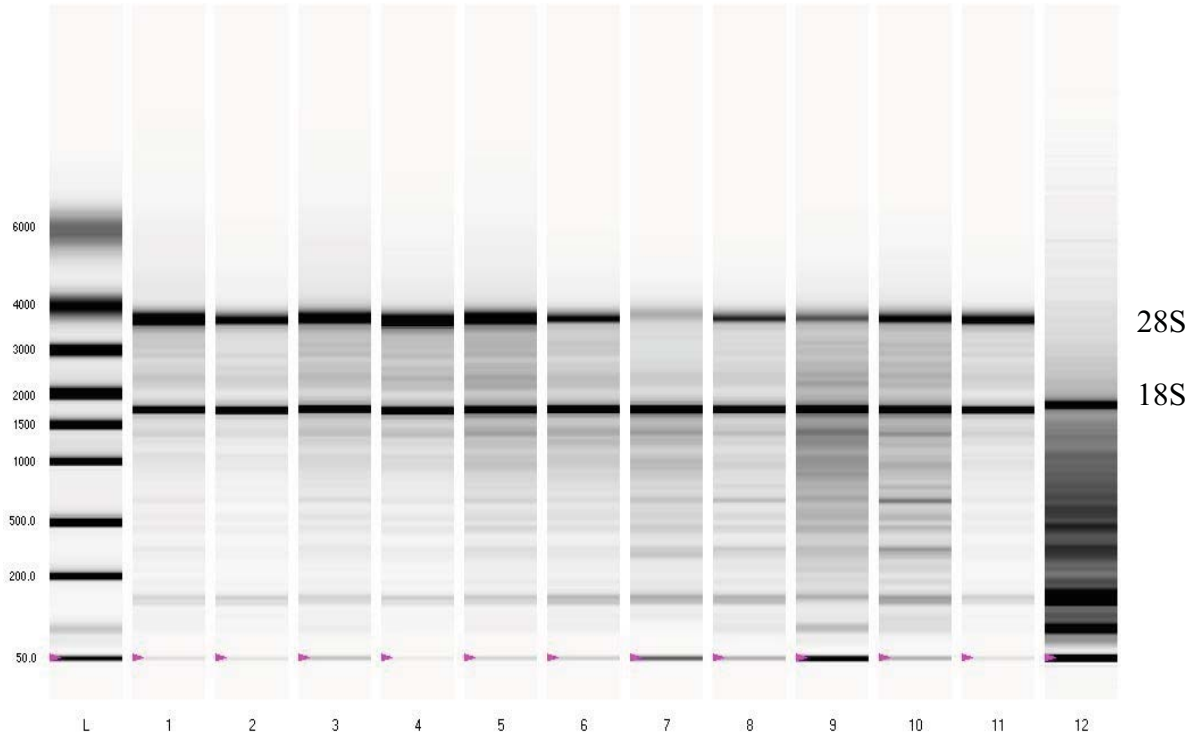

Figure 2.2. RNA capillary electrophoresis. The integrity and purity of RNA samples extracted from intestinal biopsies were measured using the Biorad Experion, an automated electrophoresis system. RNA samples were accepted if the 28S ribosomal RNA band was present with approximately twice the intensity of the 18S RNA band. Twelve samples were run on each chip (1-12), in addition to a molecular weight ladder (L). In this example, sample 7 and sample 12 were omitted from further real-time PCR analysis due to evidence of RNA degradation.

2.2.10 Conversion of mRNA to cDNA

Messenger RNA was converted to cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Vic, Australia). First, 1μg of RNA was incubated with the gDNA wipeout buffer for 2 minutes at 42°C, to eliminate any contaminating genomic DNA. The purified RNA was then added to 6μl of reverse transcription master mix and incubated at 42°C for 15 minutes followed by a 3-minute incubation at 95°C.

2.2.11 Real Time Reverse Transcription Polymerase Chain Reaction

Real time reverse transcription polymerase chain reaction (real time RT-PCR) was performed for a range of target genes with β-actin as a housekeeping gene. Real time RT-PCR primers were selected based on information provided by Primer Bank, and primer sequences are shown in **Table 2.6**. All primers were designed to span one intron to avoid genomic DNA contamination, and PCR products sizes ranged between 100-250 base pairs to guarantee primer efficiency. Double stranded DNA was detected by SYBR Green dye that intercalates into double stranded DNA producing a fluorescent signal (Arya *et al.*, 2005) using the Corbett Rotorgene 3000 (Corbett Research, Australia). Ct values were obtained for each sample indicating the number of cycles required for the fluorescent signal to reach threshold (**Figure 2.3**)

In each experiment, duplicates of 25μl reactions with 50ng of samples (1μl) were amplified using the Power SYBR Green Master mix with Amplitaq Gold (Applied Biosystems, CA, USA). The real time thermal PCR profile consisted of 1 cycle of 95°C for 10 minutes to activate the Amplitaq Gold enzyme, followed by 40 cycles of 95°C for 15 seconds (denature) and 60°C for 60 seconds (anneal/extend). At the end of 40 cycles, a melt curve of the PCR product was generated to determine contamination, non-specific product accumulation or primer dimer formation (**Figure 2.4**).

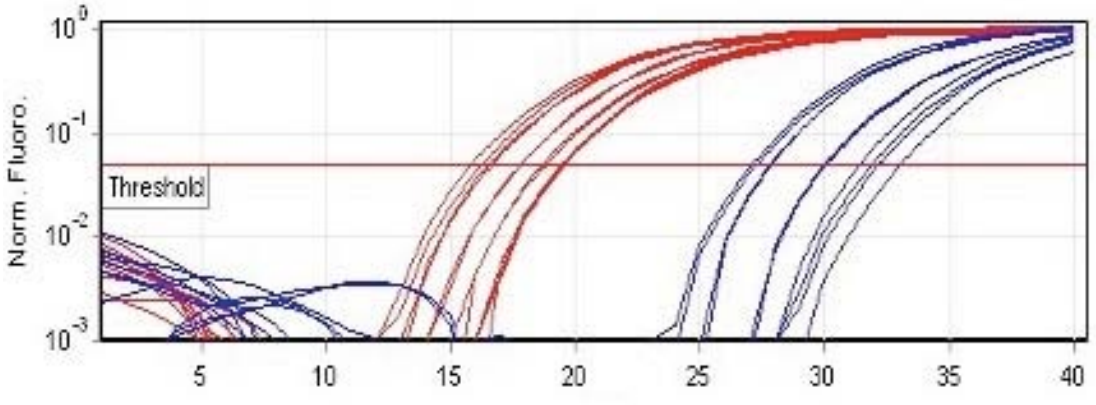

Figure 2.3 Example of amplification plots for β-actin (red) and Foxp3 (blue). Fluorescent emission data collected from 5 patient duplicate samples during PCR amplification was measured at a fixed arbitrary threshold level to determine Ct values. Ct values describe the number of cycles required for fluorescent signal to reach threshold, and are indirectly proportional to nucleic acid quantity.

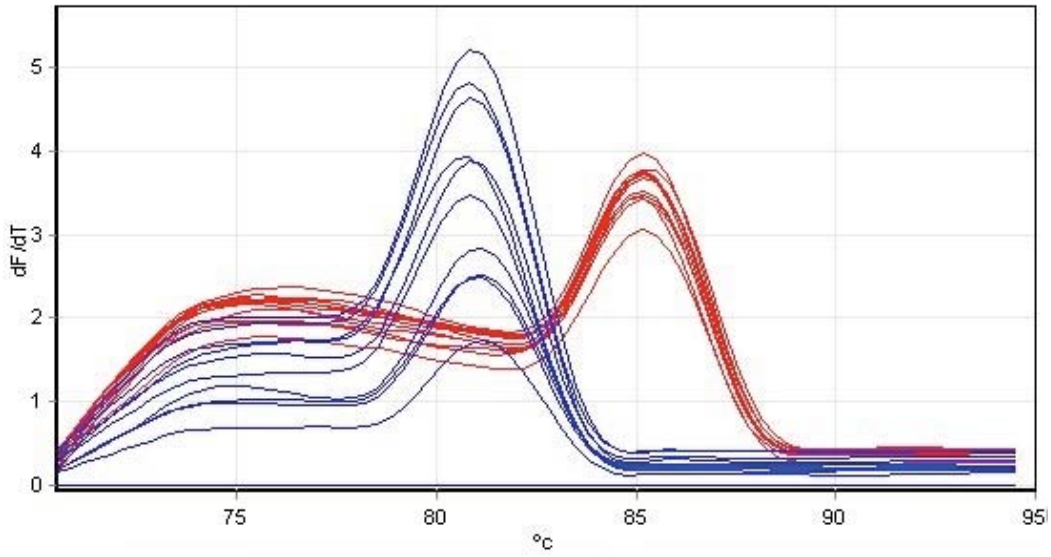

Figure 2.4 Melt curve analysis of β-actin (red) and Foxp3 (blue) PCR products. Melt analysis following the PCR reaction allows measurement of the temperature required for DNA dissociation. The presence of a single peak indicates a single PCR product, which is confirmed with gel electrophoresis.

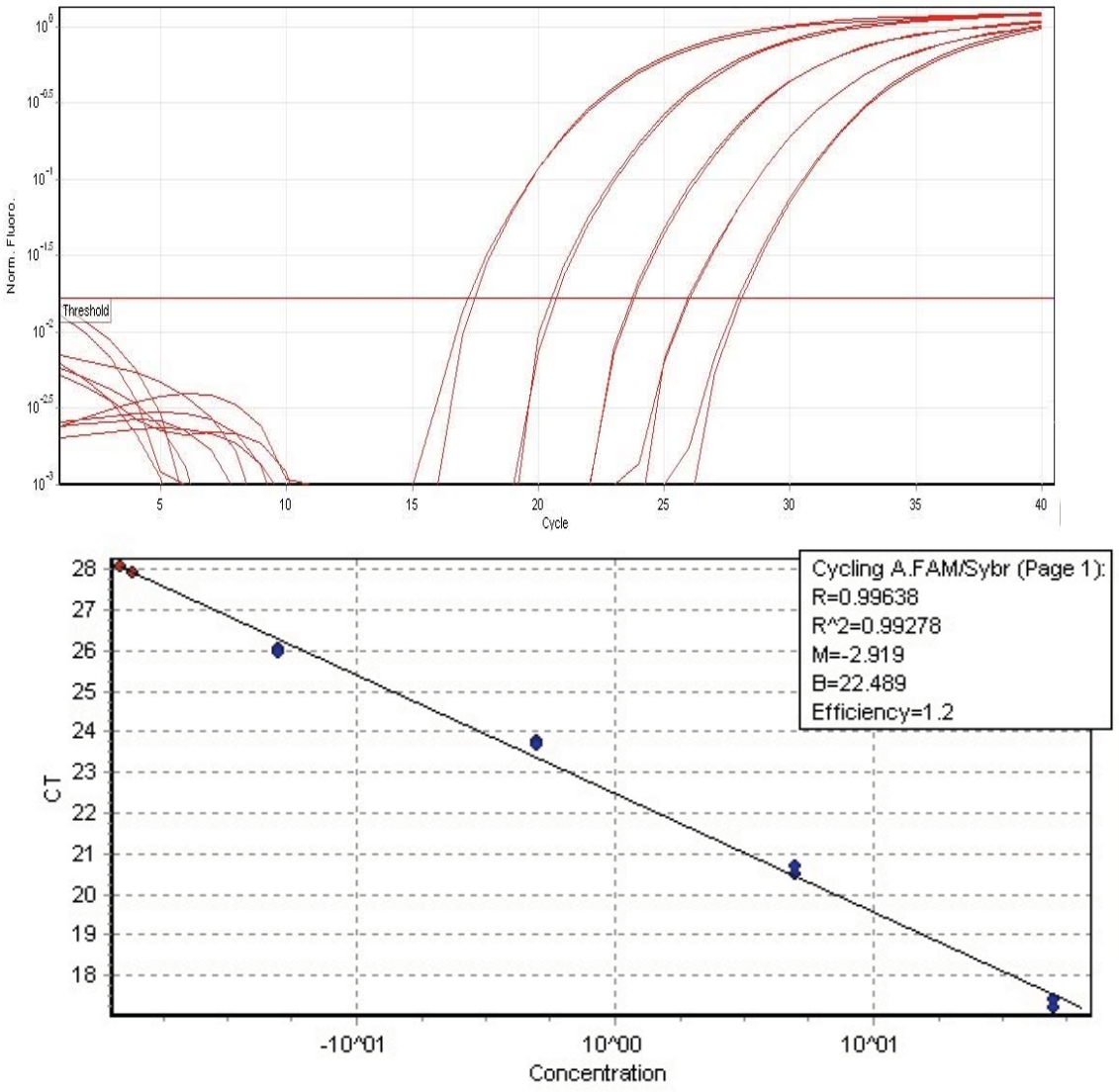

Figure 2.5. Primer efficiency calculations. The amplification efficiency of each primer pair was determined by using a 10x dilution series and calculating a linear regression based on the Ct values obtained. These graphs demonstrate the efficiency of β-actin primers, calculated with an efficiency of 1.2. The efficiencies of all primer pairs were incorporated into the Q-gene ΔCt calculations that do not assume equal efficiency of different primers.

2.2.12 Real Time RT-PCR Calculations

Real time RT-PCR results were calculated from Ct values for the target gene and β-actin. Q-gene software, utilising the ΔCt method, was used to calculate normalised mean expression whilst taking into consideration varying primer efficiencies (Simon, 2003). The efficiency of each primer pair was calculated from a ten times dilution series and linear regression calculations (**Figure 2.5**).

2.2.13 Agarose gel electrophoresis

All real time RT-PCR products were run on an electrophoresis gel to confirm the presence of a single PCR product and size of the product. A 2% agarose gel was made by dissolving 2g of molecular grade agarose in 100ml of 0.5x TBE and heated in a microwave on high for 2 minutes until dissolved. The solution was allowed to cool to approximately 50°C and then 10µl of 1mg/ml of ethidium bromide was added to the agarose solution and poured into a Perspex tray with appropriate well forming comb. Gels were allowed to set for 30 minutes before loading samples. Total PCR products were mixed with 2.5µl of 6x loading buffer, and 10µl of sample plus loading buffer was loaded per well, with 4µl PUC19 used as a DNA ladder for each gel. Electrophoresis of PCR products was carried out at 100 volts until the dye front had migrated to at least two thirds of the length of the gel.

2.2.14 Real time RT-PCR product purification

PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Vic, Australia). A total of 100µl of PCR sample was added to 500µl of Buffer PB and added to a QIAquick spin column in a collection tube. The QiaQuick column was centrifuged for 1 minute at 13,000 rpm and flow through discarded. To wash the bound DNA on the

spin column, 0.75ml of Buffer PE was added to the column and centrifuged to 1 minute at 13,000 rpm. The flow through was discarded and centrifugation repeated for an additional minute to remove residual ethanol. The QIAquick column was then placed in a clean sterile microcentrifuge tube and DNA eluted by adding 50µl of Buffer EB to the centre of the QIAquick column membrane. The column was allowed to stand for 1 minute at RT and then centrifuged for 1 minute to collect the eluted purified PCR product.

2.2.15 Sequencing

Real time RT-PCR products were sequenced at the DNA Sequencing Facility, Department of Haematology, Flinders Medical Centre, Bedford Park, South Australia. PCR products and primers were required, with 10µl of 10ng/µl of purified PCR product and 2µl of 5µM of each primer pair sent for sequencing. PCR products were confirmed using the NCBI Human blast database.

2.2.16 Statistics

Flow cytometry data were converted to absolute T cell numbers and results were expressed as cells/ml of whole blood. This was calculated as the product of target cell frequencies from flow cytometric analysis and the absolute lymphocyte count determined from complete blood exams (CBE) during routine blood examinations (SA Pathology, South Australia, Australia). Data was analysed using GraphPad Prism 4 Software (San Diego, CA). Differences between the disease and control groups were assessed using the Mann Whitney Ranked Sums Test. Comparison of paired samples was analysed using a paired samples T-test. All data were expressed as mean ± standard error of the mean. Statistical significance was considered for $p<0.05$.

2.3 Subject Information

2.3.1 Human Ethics Approval

In total, 117 patients from the QEH Gastroenterology and Hepatology Department were enlisted in our study. Informed consent was obtained from all patients before collection of samples. This study was approved by TQEH Ethics of Human Research Committee, and carried out according to the National Statement on Ethical Conduct in Research Involving Humans (1999) of the National Health and Medical Research Council of Australia and was in accord with the Declaration of Helsinki.

2.3.2 Clinical History of Enrolled Subjects

Control Subjects:

Thirty volunteers were considered eligible to represent a control group for this study. The control group consisted of sixteen females and thirteen males, with an average age of 41.4 ± 3.1 years. Individuals were eligible if screened negative for autoimmune disease markers such as rheumatoid factor, anti-nuclear antibodies, anti-neutrophil cytoplasmic antibodies and thyroid peroxidase antibodies (SA Pathology, Adelaide, South Australia). Of the thirty total individuals, twelve were healthy volunteers, and the remaining were patients of the QEH Gastroenterology and Hepatology Department with non- IBD or coeliac related illness, such as reflux (7), esophagitis (3), diverticulitis (2), polyps (3), constipation (1) and cysts (2). Seventeen patients undergoing endoscopic procedures for non-IBD or coeliac disease related illness donated intestinal biopsy samples for this study. Four patients were diagnosed with dyspepsia, two patients with reflux, five with gastritis, two with esophagitis, two with intestinal polyps, and one with anaemia. One patient underwent endoscopy for a routine bowel cancer screening.

Crohn's Disease Subjects:

Thirty-four Crohn's disease subjects were enlisted from the QEH Department of Gastroenterology and Hepatology. This group consisted of twenty females and fourteen males, with an average age of 36.6 ± 2.5 years. All of these patients were in a state of disease inactivity based upon clinical diagnosis and C-reactive protein levels (CRP<10). These patients donated blood during routine check-ups. Investigation into the individual case histories of these thirty-four patients revealed that six of these patients underwent intestinal resections with more than two resections required for each patient. Four patients underwent colectomy, two patients had a colostomy inserted, and six patients showed signs of fistula formation. One of these patients died from bowel perforation and one from ileum carcinoma. In regard to additional illness with possible association to CD, one patient was diagnosed with tuberculosis, two patients suffered osteoporosis, two with diabetes, two with cancer and two female patients had recurrent miscarriages. Biopsy samples were collected from ten CD patients after informed consent. Unlike the CD patients that provided blood samples, these CD patients were in various states of disease activity. Two patients were in disease remission based on clinical diagnosis from histological reports, the remaining eight patients were in a state of disease activity.

Ulcerative Colitis Subjects:

Twenty-nine UC subjects were enlisted through the QEH Department of Gastroenterology and Hepatology and donated blood samples for this study. This group consisted of nine females and twenty males. All of these patients were in a state of disease inactivity based upon clinical diagnosis and CRP levels. Investigation into individual patient clinical notes showed that two of these patients had undergone a total colectomy, three had appendectomies and four had a current history of cancer. One was

diagnosed with tuberculosis, two patients had diverticulitis, one with esophagitis, three patients suffered osteoarthritis and one had intestinal polyps. Fourteen UC subjects undergoing endoscopic procedures donated biopsy samples after collection of informed consent. Five of these patients were in a state of disease inactivity, five were diagnosed in a state of mildly active disease, and four were in a state of moderate disease. Three of these patients also presented with polyps.

Coeliac Disease Subjects:

Twenty-four coeliac disease subjects were recruited for this study through the QEH Department of Gastroenterology and Hepatology and donated blood samples. The majority of this cohort was female, with eighteen females and six males. The average age was 45.77 ± 3.6 years. Of these twenty-four patients, twelve were compliant to a gluten-free diet for more than two years, with six compliant to a gluten free diet for less than two years. Four patients were non-compliant to a gluten-free diet and two were diagnosed with refractory sprue with symptoms not controlled by a gluten free diet. In regard to illnesses with association to coeliac disease, three patients were also diagnosed with diabetes, three with osteoporosis, two suffered from dermatitis, two presented with gastrointestinal polyps and two had other gastrointestinal illnesses such as gastritis. Three were diagnosed with cancer, three previously had tonsillectomies, three had appendectomies, six suffered from depression and anxiety, and one woman suffered recurrent miscarriages. Fifteen coeliac disease patients undergoing endoscopic procedures donated biopsy samples for this study after collection of informed consent. Of these fifteen patients, nine were currently compliant to a gluten-free diet, two were non-compliant, 2 were diagnosed with refractory sprue, and one patient had enteropathy associated T cell lymphoma.

CHAPTER 3

FOXP3^{+} REGULATORY T CELLS

IN

INFLAMMATORY BOWEL DISEASE

AND

COELIAC DISEASE

3.1 Introduction

The intestinal mucosa remains in a state of controlled inflammation with an equilibrium maintained between protective immunity and tolerance to self-antigen/tissues (Duchmann *et al.*, 1995). Regulatory T cells (Tregs) are a population of $CD4^+$ T cells that regulate immune responses in the gut and limit the proliferation of effector cells (Sakaguchi *et al.*, 1995). Animal studies have demonstrated that T lymphocyte mediated inflammation plays an important role in murine colitis. This has been demonstrated through the adoptive transfer of T cells depleted of $CD4^+$ $CD25^+$ T cells into athymic nude mice, which results in multi-organ autoimmune disease, while the co-transfer of $CD4^+$ $CD25^+$ T cells inhibits disease (Sakaguchi *et al.*, 1995; Mottet *et al.*, 2003). These early studies suggest that within normal animals reside self reactive T cells capable of inducing autoimmune disease. Regulatory T cells restrain pathological self reactive T cells and therefore prevent autoimmunity and contribute to self tolerance (Sakaguchi *et al.*, 2008)

Tregs are identified by expression of the high affinity IL-2 receptor α-chain (CD25), however, this is also an activation marker on T cells (Nelson and Willerford, 1998). Expression of CD25 on activated T cells, however, does not confer suppressive capacity. Unlike activated T cells that lose their CD25 expression with the abrogation of stimulation, CD25 expression on the regulatory T cell is stable (Kuniyasu *et al.*, 2000). Tregs are also characterised by the expression of a range of other activation markers, such as glucocorticoid-induced TNFR (GITR), OX40, L-selectin and cytotoxic T-lymphocyte antigen-4 (CTLA-4) (Jonuleit and Schmitt, 2003). CD45 isoforms have also been implicated as markers of Tregs. CD45RO is expressed by T cells positively

selected in the thymus, which converts to CD45RA in the periphery. After antigen recognition this returns to the CD45RO isoform (Seddiki *et al.*, 2006). Two populations of Tregs exist, those expressing CD45RA and those expressing CD45RO. These cell populations both express the transcription factor Foxp3 and have equivalent suppressive activity (Seddiki *et al.*, 2006).

Foxp3 is constitutively expressed by Tregs and is necessary for their development and function (Fontenot *et al.*, 2003; Hori and Sakaguchi, 2004). Unlike the *in vitro* induction of CD25 expression, stable transfection of T cells with Foxp3 is sufficient to confer the phenotype and suppressive activity of the Treg (Hori *et al.*, 2003). The importance of Foxp3 to the development of the Treg lineage is evident in diseases resulting from mutations of the Foxp3 gene. These include IPEX (immunodysregulation polyendocrinology and enteropathy X-linked syndrome) in humans and scurfy in mice. This mutation results in a lack of Tregs and is characterised by excessive effector T cell proliferation and infiltration into peripheral tissues that leads to systemic inflammation and death (Bennett *et al.*, 2001; Brunkow *et al.*, 2001; Wildin *et al.*, 2001). Activated T cells also express Foxp3 transiently, however, this does not confer suppressive activity (Allan *et al.*, 2007; Wang *et al.*, 2007). Stimulated Foxp3 expressing $CD4^+$ $CD25^-$ T cells do not suppress the proliferation of autologous $CD4^+$ T cells, however, stimulated $CD4^+$ $CD25^{high}$ T cells are potent suppressors (Wang *et al.*, 2007). These findings indicate the importance of using a clearly defined Treg phenotype in human studies, thus avoiding contamination with activated T cells.

The poorly defined phenotype and lack of standardisation in measuring Tregs has been an obstacle in the investigation of human Treg. Prior to the availability of an anti-human Foxp3 antibody, Treg were measured in a number of ways. Early research

utilised Treg phenotypes from animal studies and used $CD4^+$ $CD25^+$ dual positivity to identify Tregs, however, this categorisation also included activated T cells (Allan *et al.*, 2007). Further definition of human Tregs improved the identification of these cells as residing in the brightest of the CD25 population, in which $CD4^+$ $CD25^{high}$ identify human Treg (Baecher-Allan *et al.*, 2005). With the introduction of intracellular staining for Foxp3 using the Foxp3 antibody (clone: PCH101, sBioscience 2005), a more accurate phenotypic definition of Tregs was possible. The addition of Foxp3 to the $CD4^+$ $CD25^{high}$ phenotype allowed for greater specificity and also the exclusion of activated T cells.

Tregs have been investigated in the peripheral blood and intestinal biopsies of IBD patients to determine if the uncontrolled inflammation present in the gut mucosa is associated with a deficiency of Treg as revealed in IPEX and scurfy. However, the interpretation of these findings has been limited by the inconsistent use of markers to identify Treg. Maul *et al* (2005) reported that numbers of circulating $CD4^+$ $CD25^{high}$ Tregs were decreased in Crohn's disease patients during active disease, whilst Treg numbers increased during disease remission. In contrast, an increase in $CD4^+$ $CD25^{high}$ T cells was demonstrated in the inflamed mucosa of IBD patients. No change in $CD4^+$ $CD25^{high}$ Treg numbers were observed in ulcerative colitis patients (Maul *et al.*, 2005). Takahashi (2006) employed the $CD4^+$ $CD45RO^+$ $CD25^+$ phenotype to define Tregs. These cells were decreased in active ulcerative colitis but increased in active Crohn's disease (Takahashi *et al.*, 2006). This study also reported an inverse relationship between the percentage of Tregs in UC patients with clinical activity, with fewer Tregs correlating to increased disease activity. Furihata (2006) investigated Treg numbers in the peripheral blood of UC patients before and after colectomy. They defined Tregs as $CD4^+$ $CD25^+$ $CD45RA^+$, and demonstrated that these cells were significantly lower in

UC when compared to controls prior to colectomy, and increased after colectomy (Furihata *et al.*, 2006).

The measurement of Tregs at the site of disease has consistently shown an increase of Tregs in the intestinal mucosa of IBD patients (Makita *et al.*, 2004; Maul *et al.*, 2005; Yu *et al.*, 2007; Sitohy *et al.*, 2008). In ulcerative colitis, increased numbers of $CD4^+$ $Foxp3^+$ T cells were identified in the mucosal lymphoid tissue (Yu *et al.*, 2007) and increased $Foxp3^+$ $CD25^+$ in the lamina propria (Sitohy *et al.*, 2008). Impairment of Treg suppressive function has also been investigated in both IBD and coeliac disease. No functional defect has been described in Tregs extracted from peripheral blood and intestinal tissue IBD patients (Makita *et al.*, 2004; Kelsen *et al.*, 2005; Maul *et al.*, 2005; Yu *et al.*, 2007), however, impaired suppressor activity appears to be a characteristic feature of coeliac disease (Pignata *et al.*, 1985; Granzotto *et al.*, 2008). Regulatory T cells have only recently been investigated in coeliac disease with high Foxp3 expression in circulating $CD4^+$ $CD25^+$ T cells of untreated coeliac disease patients (Frisullo *et al.*, 2009). An increase in regulatory T cells has been linked to the development of cancer (Beyer and Schultze, 2006). This increase in Tregs could explain the increased risk of malignancies in those non-compliant to a gluten-free diet and for individuals affected by IBD. Increased numbers of Foxp3 expressing cells were also described in the small intestine of coeliac disease patients (Tiittanen *et al.*, 2008), however no changes in Foxp3 expression was reported in the peripheral blood of children with coeliac disease (Kivling *et al.*, 2008).

The need to identify a specific cell surface marker for Tregs has emerged due to the limitation of intracellular Foxp3 as a phenotypic marker, which cannot be detected without killing cells. This is particularly necessary for functional assays that use the

$CD4^+$ $CD25^+$ phenotype, which does not accurately identify a homogenous Treg population. In 2006, a potential cell surface marker of Tregs was identified, with CD127 shown to be inversely correlated with Foxp3 (Liu *et al.*, 2006; Seddiki *et al.*, 2006). Differential gene expression analyses of $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^-$ T cells revealed that CD127 is expressed at approximately a 2.4 fold lower level in $CD4^+$ $CD25^+$ cells compared with $CD4^+$ $CD25^-$ cells. Indeed, $CD127^-$ $CD25^+$ T cells possess potent immunosuppressive activity, indicating that CD127 expression may be used to identify a population of functional Tregs (Liu *et al.*, 2006). CD127 is the alpha chain of the IL-7 receptor that binds IL-7 with low affinity. This enables IL-7 to homeostatically regulate the size of the peripheral T cell pool (Palmer *et al.*, 2008). Whilst IL-7 and IL-2 are critical for the survival and development of non-Tregs, only IL-2 is required for the homeostatic control of Tregs due to expression of CD25, the IL-2 receptor (Seddiki *et al.*, 2006). Depletion of $CD127^+$ cells therefore purifies Treg populations by eliminating contaminating activated T cells and allows the use of these cells for clinical Treg based therapy (Peters *et al.*, 2008).

This chapter describes the different phenotypic definitions of Tregs reported in the literature regarding human autoimmune disease; $Foxp3^+$, $CD4^+$ $CD25^+$ $Foxp3^+$, $CD4^+$ $CD25^{high}$ $Foxp3^+$, and $CD4^+$ $CD25^+$ $CD127^{low}$. These cell phenotypes were examined in a healthy control group and compared to patients with Crohn's disease, ulcerative colitis and coeliac disease to determine whether a deficiency of these cells is a feature of these gastrointestinal diseases.

3.2 Aims and Hypotheses:

The general hypothesis of this chapter is that IBD and coeliac disease are associated with a reduced number of immunosuppressive regulatory T cells.

Aims:

1) To measure and compare total $Foxp3^+$ cell numbers from the peripheral blood of IBD, coeliac and control subjects.
2) To measure and compare $CD4^+$ $CD25^+$ $Foxp3^+$ cell numbers from the peripheral blood of IBD, coeliac and control subjects.
3) To measure and compare $CD4^+$ $CD25^{high}$ $Foxp3^+$ cell numbers from the peripheral blood of IBD, coeliac and control subjects.
4) To investigate $CD4^+$ $CD25^+$ $CD127^{low}$ as a useful cell phenotype for identifying Tregs.
5) To measure and compare Foxp3 relative expression in the intestinal biopsies of control, CD, UC and coeliac disease subjects

3.3 Methods

3.3.1 Subjects

IBD patients were recruited from inpatients or outpatients attending the Department of Gastroenterology and Hepatology at The Queen Elizabeth Hospital (TQEH), Woodville, South Australia. Informed consent was obtained from each participant prior to commencement (See Chapter 2 for details). Peripheral blood was collected from thirty-four CD, twenty-nine UC and twenty coeliac subjects. These patients were diagnosed clinically and all were in a state of disease inactivity on the basis of on clinical notes and C-Reactive Protein levels (CRP<10). Coeliac disease patients were predominantly maintaining long-term gluten-free diets for more than 1 year. The mean ± SEM age of CD, UC and coeliac patients was 37.7 ± 2.8, 51.4 ± 4.0 and 49.8 ± 6.0 years, respectively. The control group comprised of twenty-eight subjects that had non-inflammatory disorders or were healthy volunteers (n=28), with a mean ± SEM age of 46.8 ±3.1 years.

Intestinal biopsies were obtained from an additional subset of IBD patients in various states of disease activity. Biopsies were collected from ten CD, fourteen UC, fifteen coeliac disease and eighteen control subjects at colonoscopy. Of the total twenty-four IBD biopsies collected, six were from patients with moderate disease activity, eight had mild disease activity and ten were in a state of inactive disease, based on global colonoscopic appearance and histologic reports (SA Pathology, Adelaide, South Australia). Control subjects had non-inflammatory disorders or were undergoing colon cancer screening. Biopsy samples were collected and stored in RNA*later* (Ambion, TX, USA) at -20°C to prevent RNA degradation prior to extraction.

Jurkat cells transduced with a lentivirus to stably over express full length human Foxp3 were generated by the Barry Lab, and were used as a positive Foxp3 control.

3.3.2 Flow cytometry

Intracellular labeling of Foxp3 (PCH101) followed the eBioscience specified methods; $1x10^6$ PBMCs were aliquoted into FACS tubes (BD Biosciences, USA), and were incubated with saturating quantities of CD4 (20µl) and CD25 (20µl) directly conjugated antibodies for 30 minutes at 4°C in the dark. Cells were washed twice in cold FACS wash. After decanting, the cell pellet was resuspended by pulse vortexing. Freshly prepared eBioscience Foxp3 Fixation/Permeabilisation buffer (1ml) was added to each sample and pulse vortexed again. Samples were incubated at 4°C for 30 minutes in the dark, and then washed twice with 2ml of eBioscience 1x Permeabilisation buffer. Prior to intracellular antibody labeling, cells were blocked with 2µl of normal rat serum in approximately 100µl total volume (2%) and incubated for 15 minutes. Without washing, either 20µl of the anti-human Foxp3 (clone: PCH101, eBioscience, CA, USA) antibody was added to the sample or 20µl rat IgG2a isotype control added to a corresponding control tube and samples incubated for 30 minutes at 4°C in the dark. Samples were washed twice in eBioscience Flow Cytometry Staining Buffer and stored at 4°C in the dark until run on the flow cytometer. Quantification was carried out using a BD FACScan (BD Bioscience, USA), where between 300,000 and 500,000 events were collected per sample. Lymphocytes were gated based on their forward and side scatter properties (See Chapter 2, **Figure 2.1**) and data were analyzed with Cell Quest (BD Bioscience, USA) analysis program.

3.3.3 Real-time RT-PCR analysis for Foxp3

Total RNA was isolated from intestinal biopsies using the RNeasy Lipid Minikit (Qiagen, Vic, Australia). RNA gel electrophoresis was performed to assess RNA quality. One microgram of RNA was reverse transcribed to obtain complimentary DNA (cDNA) using Qiagen Quantitect Reverse transcription kit (Qiagen, Vic, Australia). Gene specific primers were designed to span an intron of the genomic sequence. Quantitative real time RT-PCR was performed using a Corbett Rotorgene RG-3000 (Corbett Research, Australia), and all reactions were carried out using Power SYBR green master mix (Applied Biosystems, CA, USA). Expression of Foxp3 mRNA was normalised to β-actin. PCR products were purified and sequenced to confirm product (See Chapter 2 for details).

3.3.4 Statistics

Flow cytometry results were converted from percentage of PBMCs to absolute cell numbers as described in Chapter 2. Real time RT-PCR results were converted to normalised mean expression using Qgene software applying the ΔCT method. All data are expressed as mean ± SEM. Statistical significance between the patient cohorts and control group was evaluated using the 2-tailed Mann-Whitney ranked sums test. Data were analysed using Graphpad Prism software. Statistical significance was achieved when $p<0.05$.

3.4 Results

3.4.1 Quantification of Peripheral Foxp3$^+$ Cells

In order to determine whether low levels of Foxp3$^+$ cells exist in the peripheral blood of IBD and coeliac disease patients, Foxp3 was measured using intracellular flow cytometry of patient peripheral blood mononuclear cells. Specificity of the Foxp3 (PCH101) antibody was confirmed using Foxp3 expressing Jurkat cells as a positive control (donated by the Barry lab), and 86.5% of these cells expressed Foxp3 (**Figure 3.1**). The pre-peak of Foxp3 seen in **Figure 3.1** may indicate the contamination of non-Foxp3 Jurkat cells that were not transduced, or a small proportion of the tranduced cells may have silenced the expression cassette. Foxp3 positivity is therefore determined to be within the dominant second peak.

Representative flow cytometric plots show that 1.5% of PBMCs were Foxp3$^+$ in a control patient with minimal non-specific staining seen in the isotype control (**Figure 3.2)**. The proportion of Foxp3$^+$ cells among patient PBMCs ranged from 0.2 - 3.4% in the control group, 0.2 - 6.8% in the CD patients, 0.4 - 7.5% in UC patients and 0.3 - 4.1% in coeliac disease patients. The absolute number of Foxp3$^+$ cells (mean ± SEM per ml of whole blood) for the control, CD, UC and coeliac groups were 4.2 ± 0.5 x10^4, 4.7 ± 0.7 x10^4, 3.5 ± 6.4 x10^3, and 4.01 ± 0.6 x10^4 cells/ml respectively (**Figure 3.3**). There was no statistically significant difference in the means between these groups.

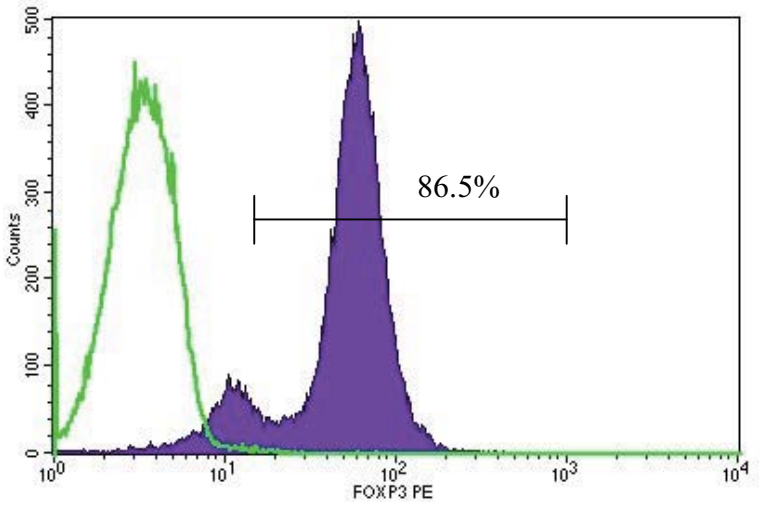

Figure 3.1 Foxp3 labelling of Jurkat cells constitutively expressing Foxp3. Foxp3 expressing Jurkat cells were labeled with a PE conjugated anti-human Foxp3 antibody (PCH101). The purple graph indicates cells stained with anti-Foxp3, with the green line showing its corresponding isotype control (rat IgG2a-PE).

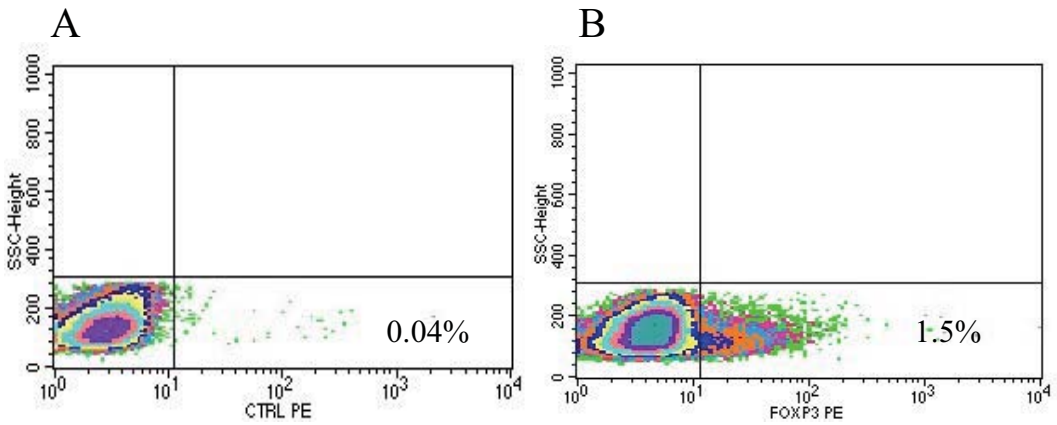

Figure 3.2 Detection of Foxp3 in peripheral blood using flow cytometry. PBMCs isolated from the peripheral blood of patient cohorts were intracellularly labelled with (A) isotype matched control (rat IgG2a-PE) and (B) Foxp3-PE (PCH101). Analysis of $Foxp3^{+}$ cells in a representative control patient indicated that 1.51% of lymphocytes were $Foxp3^{+}$.

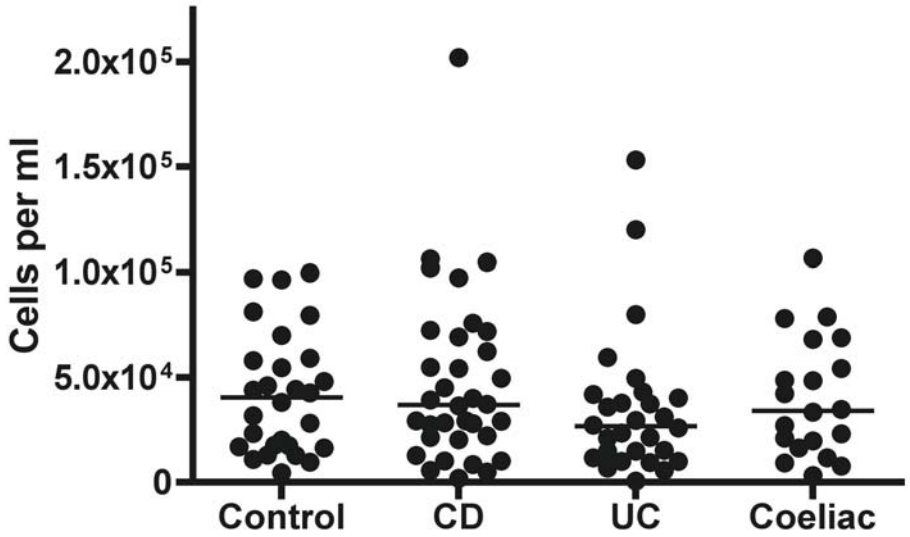

Figure 3.3 Quantification of Foxp3$^+$ cells in the peripheral blood of control, Crohn's disease, ulcerative colitis and coeliac subjects. Percentages of positive cells obtained via flow cytometry were converted to an absolute number using individual patient lymphocyte counts. Each point represents an individual subject, with the horizontal line representing the group median. No significant differences in Foxp3$^+$ numbers were observed among these groups.

3.4.2 Quantiification of Peripheral $CD4^+$ $CD25^+$ $Foxp3^+$ Cells

To determine whether a deficiency of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells existed in IBD and coeliac disease, three-colour flow cytometry was utilised with directly conjugated monoclonal antibodies to CD4, CD25 and Foxp3. Representative three-colour flow cytometric plots from a healthy control (**Figure 3.4b**) revealed that 15.1% of lymphocytes were $CD4^+$ $CD25^+$, of which 12.2% were also positive for Foxp3. Isotype matched controls were used for each patient sample to set quadrants and gates (**Figure 3.4a**).

The proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ cells for the patient groups ranged from 0.2 – 2.7% in the control group, 0.1 – 4.7% in CD, 0.01 – 2.8% in UC and 0.07 – 2.8 in the coeliac disease group (**Figure 3.5**). The mean ± SEM absolute number of $CD4^+$ $CD25^+$ $Foxp3^+$ (Cells/ml whole blood) for the control, CD, UC and coeliac groups were 25.9 ± 0.4 $x10^3$, 29.4 ± 0.5$x10^3$, 19.6 ± 0.3$x10^3$, 26.1 ± 0.4$x10^3$ cells/ml respectively. There was no statistical difference between the means of these groups.

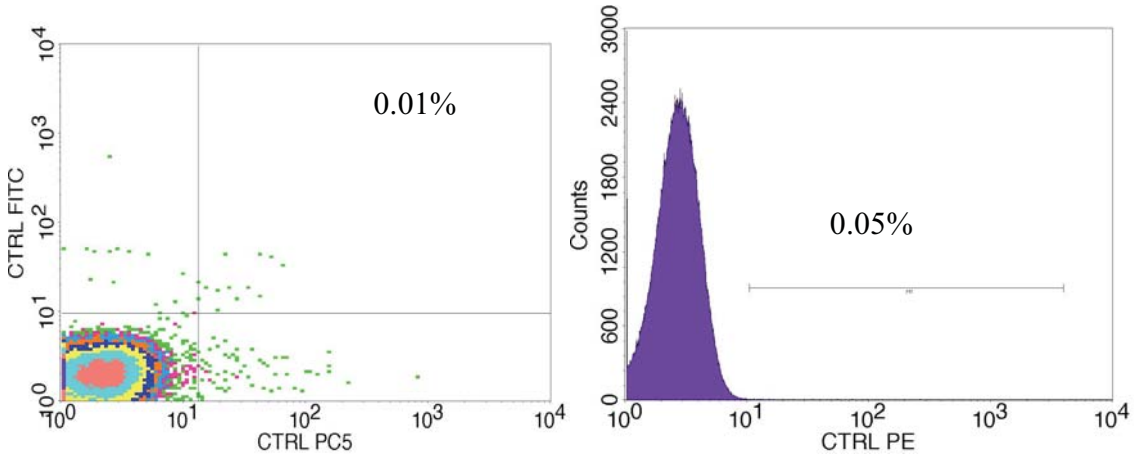

Figure 3.4a Isotype matched controls for CD4-FITC, CD25 PE-Cy5 and Foxp3-PE. All gates and quadrants were set based on negative staining of isotype controls.

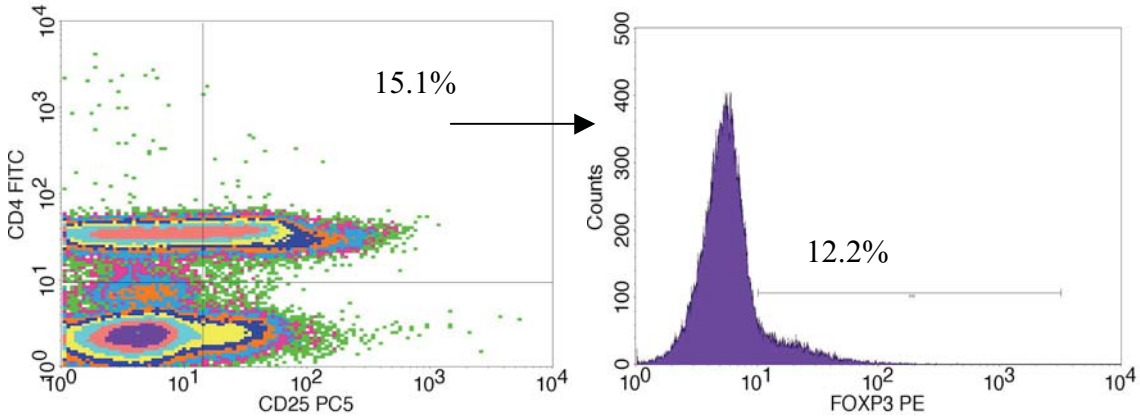

Figure 3.4b Detection of $CD4^+$ $CD25^+$ $Foxp3^+$ using multi-colour flow cytometry. PBMCs isolated from the peripheral blood were labeled with CD4-FITC, CD25-PeCy5, and Foxp3-PE. Gates were set based on isotype controls. Analysis of a representative control patient indicated that 12.2% of $CD4^+$ $CD25^+$ cells were also Foxp3+.

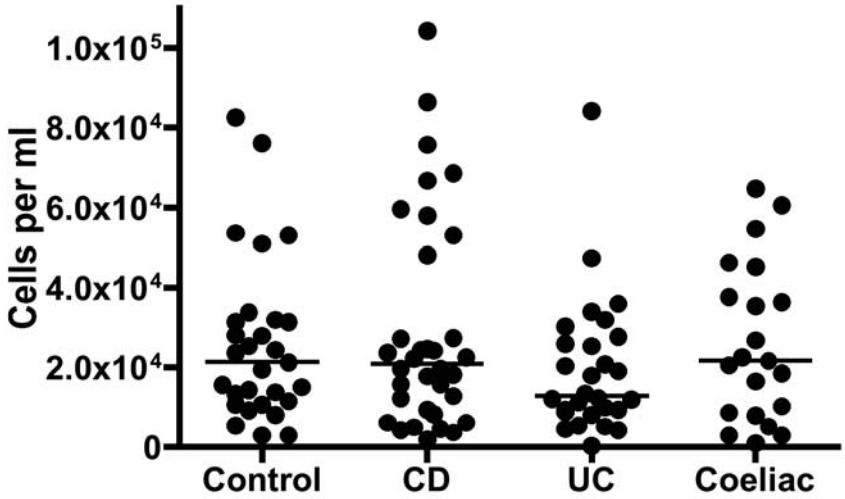

Figure 3.5 Quantification of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in IBD, coeliac disease and control subjects. Percentages of positive cells, obtained via flow cytometry were converted to an absolute number using individual patient lymphocyte counts. Each point represents an individual subject, with the horizontal line representing the group median. No statistically significant difference in $CD4^+$ $CD25^+$ $Foxp3^+$ numbers was observed among these groups

3.4.3. CD4+ CD25^{high} gating strategy

To confirm the expression of Foxp3 in the CD25^{high} CD4^{+} T cell population, CD25 cells were divided into CD4^{+}, CD25^{-}, CD25^{intermediate} and CD25^{high} (**Figure 3.6**) and Foxp3 levels measured. For the purpose of this study, CD25^{high} was defined as the top 0.5% of CD4^{+} T cells expressing CD25. The percentage of Foxp3^{+} cells in each CD25 gate is shown in a representative group of healthy controls (**Figure 3.7**). The highest proportion of Foxp3^{+} cells was detected in the CD25^{high} population, with up to 90% of cells Foxp3^{+}, compared to less than 10% expression in the CD4^{+} and CD25^{-} gates.

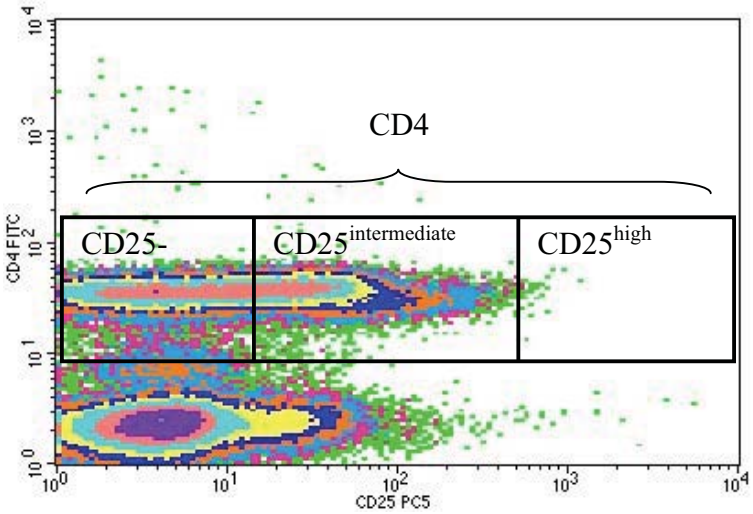

Figure 3.6. CD4 CD25 Gating. Representative flow cytometric density plot showing the division of $CD4^+$ cells into $CD4^+$, $CD25^-$, $CD25^{intermediate}$ and $CD25^{high}$ populations. PBMCs were isolated from a control patient and labeled with CD4-FITC, CD25-PeCy5.

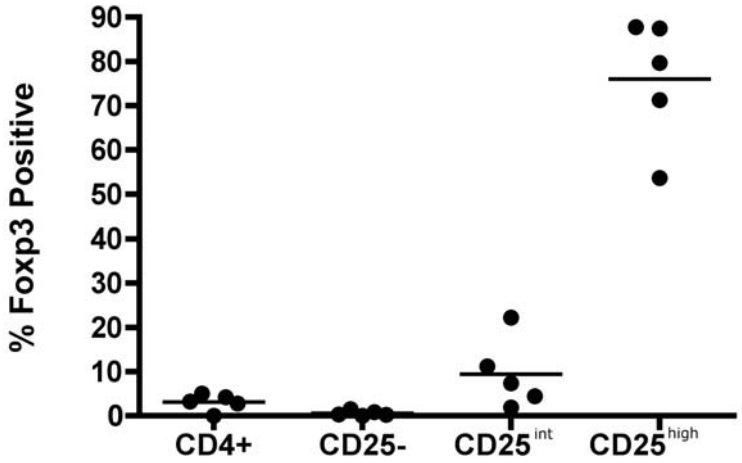

Figure 3.7 Expression of Foxp3 in $CD4^+$, $CD25^-$, $CD25^{intermediate}$ and $CD25^{high}$ populations. PBMCs extracted from five healthy control samples were labeled with CD4-FITC, CD25-PeCy5, and Foxp3-PE. Percentage of $Foxp3^+$ cells were measured n $CD4^+$ CD25-, $CD25^{intermediate}$ and $CD25^{high}$ gates. Each point represents an individual and the horizontal line indicates the median value.

3.4.4 Quantification of Peripheral CD4+ CD25^{high} Foxp3+

We defined the phenotype of T_{reg} cells as $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells, as only $CD4^+$ $CD25^{high}$ T cells are consistently Foxp3 positive and highly suppressive, while $CD4^+$ $CD25^{intermediate}$ T cells can include activated T cells that transiently express lower levels of Foxp3 and lack suppressive activity (Allan *et al.*, 2007; Wang *et al.*, 2007). Stringent staining and gating strategies described in section **3.4.3** were used to accurately measure Treg numbers in patient cohorts. $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells were measured in the control, CD, UC and coeliac groups using three-colour flow cytometry.

A representative density plot and histogram of the raw flow cytometry data are seen in **Figure 3.8**, showing the results from both a control subject and a CD patient. The CD patient sample indicated that 43.1% of $Foxp3^+$ cells were within the $CD4^+$ $CD25^{high}$ population, compared to 85.6% of the $CD4^+$ $CD25^{high}$ cells in the control group. The proportion of $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells among PBMCs ranged from 0.12 – 0.5% in the control group, 0.01 – 0.5% in CD, 0.003 – 0.5 in UC and 0.04 – 0.5% in coeliac disease patients. The mean absolute $CD4^+$ $CD25^{high}$ $Foxp3^+$ cell numbers (mean ± SEM per ml of whole blood) for the patient groups are described in **Figure 3.9**. The mean ± SEM of circulating $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells (per ml whole blood) for the control, CD, UC and coeliac groups were 8.08 ± 0.38 x 10^3, 5.87 ± 0.33x10^3, 5.15 ± 0.30x10^3, 6.05 ± 0.35x10^3 cells/ml, respectively. The CD and UC subject groups had statistically decreased numbers of $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells compared to the controls, p=0.02 and p=0.006 respectively. A trend towards decreased $CD4^+$ $CD25^{high}$ $Foxp3^+$ in coeliac disease patients was observed, however just failed to attain statistical significance (p=0.07).

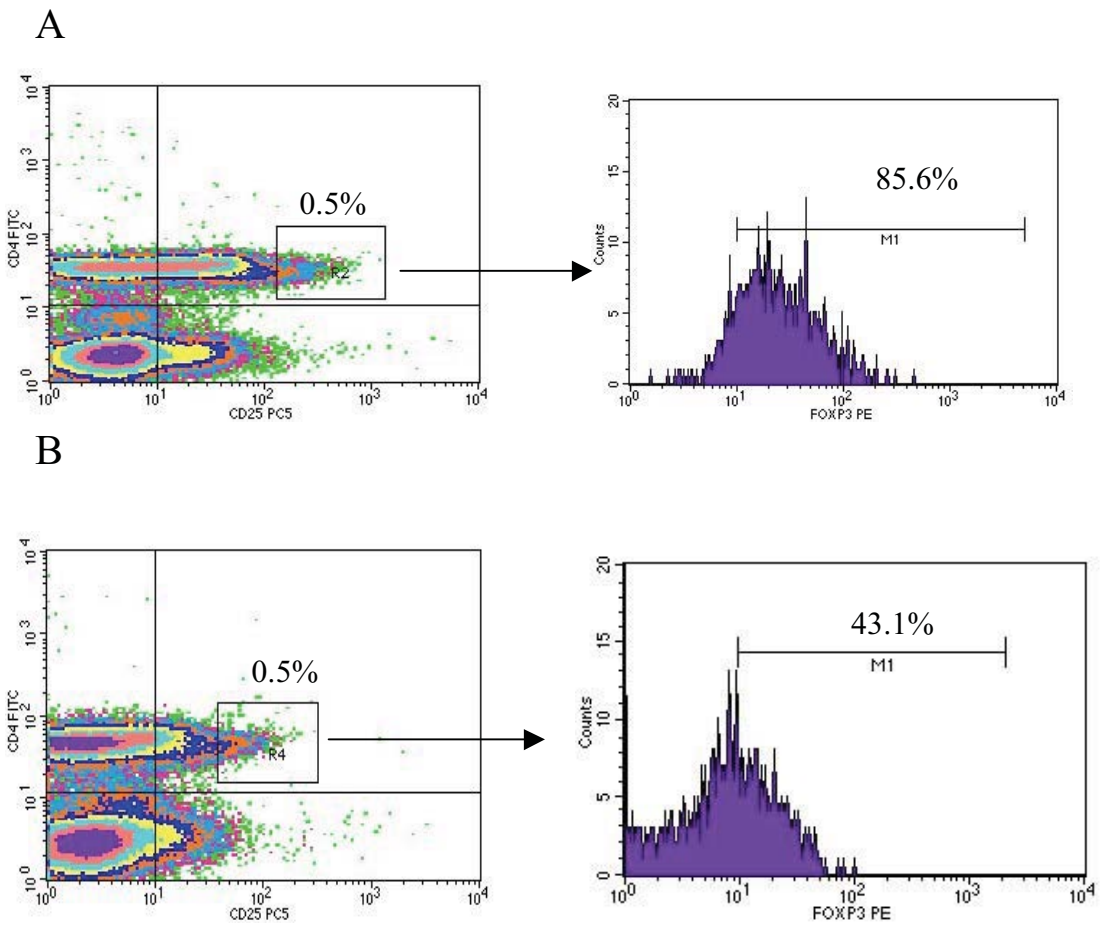

Figure 3.8 Detection of $CD4^+$ $CD25^{high}$ Foxp3+ T cells using three-colour flow cytometry. PBMCs isolated from the peripheral blood of patient cohorts were stained for CD4-FITC, CD25-PE Cy5 and Foxp3-PE. Gates were set based on isotype control staining. Although the mean fluorescent intensity of CD4+ CD25+ staining varies between patients, the $CD25^{high}$ population is fixed at the 0.5% brightest CD25+ cells. Representative $CD4^+$ $CD25^{high}$ flow cytometric density plots with corresponding Foxp3 frequency histograms for (A) a control subject and (B) a CD patient. 85.59% of the control $CD4^+$ $CD25^{high}$ cells are positive for Foxp3 compared to 43.1% in the CD patient. Gates and quadrants were based on isotype-matched controls (See **Figure 3.4a**).

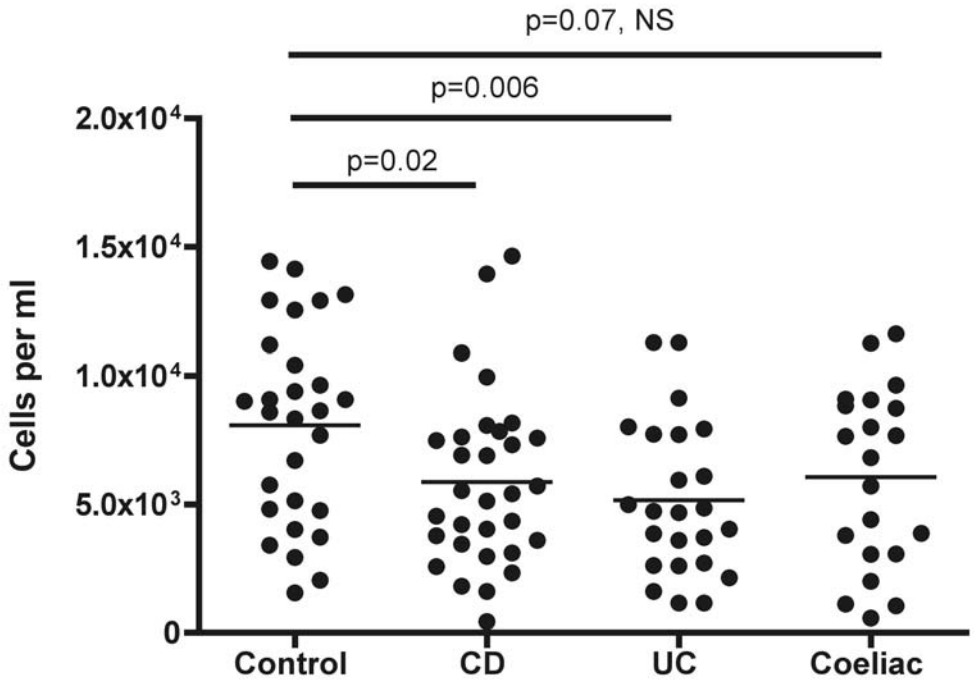

Figure 3.9 Quantification of regulatory T cells in IBD and coeliac disease. Absolute numbers of Tregs were calculated using patient lymphocyte counts and the frequency of $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells determined by flow cytometry. Comparisons of $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells are demonstrated in the control, CD, UC and coeliac groups. Each point represents an individual patient, with the horizontal line representing the group median. Statistical significance was observed between the control and IBD groups, where $CD4^+$ $CD25^{high}$ Foxp3 numbers were decreased in CD and UC as compared to the control group. No significant difference was shown in the coeliac disease patients when compared to the control group.

3.4.5 Evaluation of CD127^{low} as a marker of Tregs

During the course of this project, CD127^{low} in conjunction with CD4^{+} and CD25^{+} was proposed as an alternative strategy for measuring Treg, without requiring intracellular labelling of cells (Seddiki *et al.*, 2006; Shen *et al.*, 2009). As CD4^{+} CD25^{high} Foxp3^{+} cells were decreased in IBD patients, we investigated CD4^{+} CD25^{+} CD127^{low} T cells to confirm a similar trend. A subgroup of thirty-six patients was measured for both CD4+ CD25^{high} Foxp3^{+} and CD4^{+} CD25^{+} CD127^{low} cells. This group consisted of eleven controls, ten CD, six UC and nine coeliac subjects. The gating strategy for measuring CD4^{+} CD25^{+} CD127^{low} followed published protocols (Liu *et al.*, 2006; Seddiki *et al.*, 2006) and was demonstrated in a representative control patient (**Figure 3.10**). The accumulated results for these two Treg phenotypes were compared to determine whether the trends within patient groups were identical. The group medians of CD4^{+} CD25^{high} Foxp3^{+} T cells ranged between 0.2 - 0.3% of the lymphocyte population (**Figure 3.11**). CD4^{+} CD25^{+} CD127^{low} cells, however, ranged between 2 – 3% (**Figure 3.12**) indicating that this phenotype measured a ten-fold greater population of CD4^{+} CD25^{+} T cells than the CD4^{+} CD25^{high} Foxp3^{+} phenotype.

As the CD4^{+} CD25^{+} CD127^{low} phenotype contained a population of T cells that are both CD25^{intermediate} and CD25^{high}, we reanalysed our data to ascertain whether CD4^{+} CD25^{high} CD127^{low} more closely resembled the CD4^{+} CD25^{high} Foxp3^{+} Treg population that we found to be decreased in our IBD cohorts. The graphical representation of these cell numbers in our patient cohorts revealed that the trends for this cell phenotype differed from that of the CD4^{+} CD25^{high} Foxp3^{+} phenotype (**Figure 3.13**).

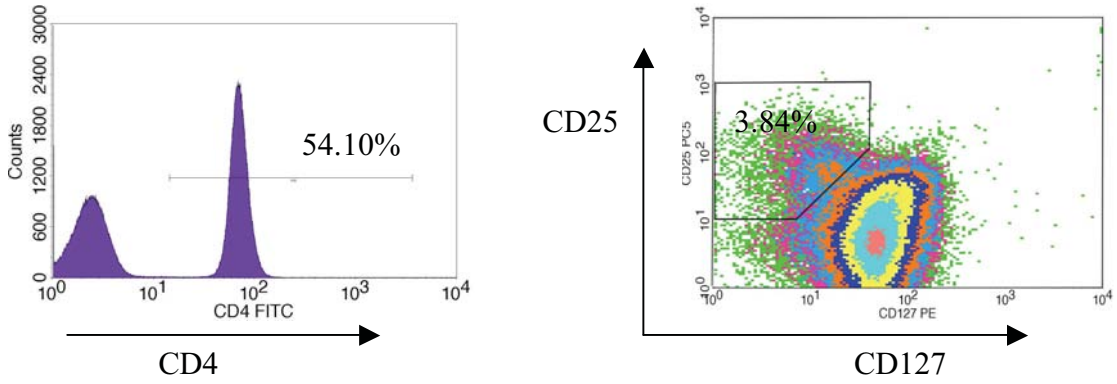

Figure 3.10 Detection of $CD4^+$ $CD25^+$ $CD127^{low}$ Tregs using multi-colour flow cytometry. Isolated PBMCs from peripheral blood were labelled with CD4-FITC, CD25-PeCy5 and CD127-PE. $CD4^+$ lymphocytes were gated and percentage of $CD25^+$ $CD25^{low}$ determined for the CD4+ population. In this representative sample, 3.84% of $CD4^+$ cells were $CD25^+$ $CD127^{low}$. This corresponded to 2.1% of circulating lymphocytes.

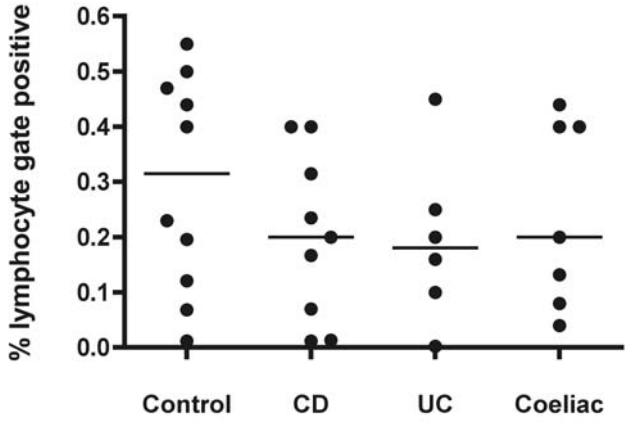

Figure 3.11. Quantification of CD4$^+$ CD25^{high} Foxp3$^+$ T cells. Percentages of CD4$^+$ CD25^{high} Foxp3$^+$ T cells were measured in the same group of patients described in **Figure 3.12**. A trend towards decreased CD4$^+$ CD25^{high} Foxp3$^+$ T cells was observed for the IBD and coeliac patient cohorts compared to the control group. However, this was not statistically significant.

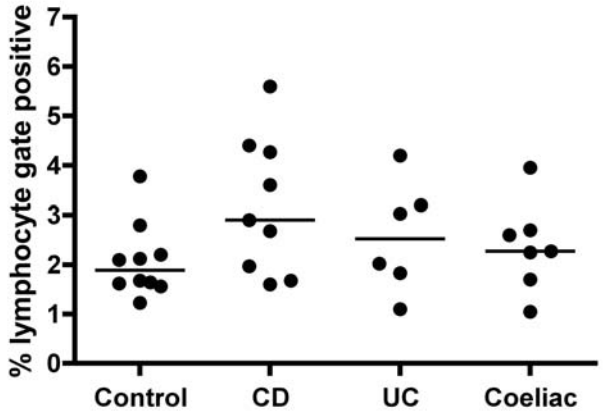

Figure 3.12. Quantification of CD4$^+$ CD25$^+$ CD127^{low} T cells. Percentages of cells positive for CD4$^+$ CD25$^+$ CD127^{low} were obtained via flow cytometry for subgroups of patient cohorts. Each point represents an individual patient, with horizontal lines representing the group median. No statistical differences were observed among the groups, however a trend towards increased cell numbers was observed for our patient cohorts when compared to the control group

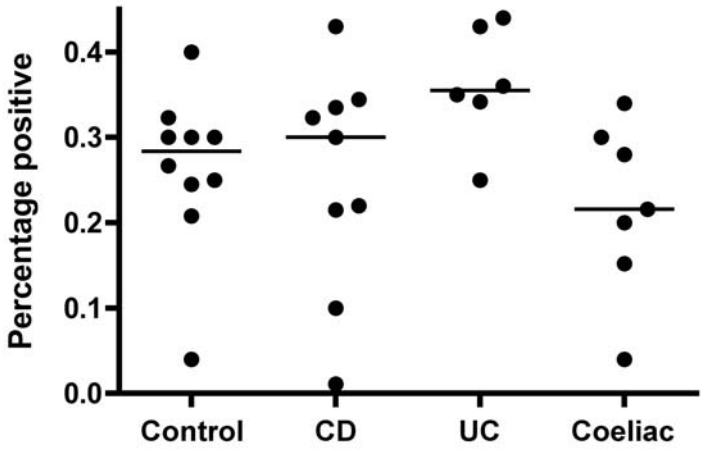

Figure 3.13 Quantification of $CD4^+$ $CD25^{high}$ $CD127^{low}$. Percentages of $CD4^+$ $CD25^{high}$ $CD127^{low}$ were measured in the patients described in **Figures 3.11** and **3.12**. Despite measuring a smaller Treg population than $CD4^+$ $CD25^+$ $CD127^{low}$, the trends revealed for this cell phenotype in our patient cohorts differed significantly from the $CD4^+$ $CD25^{high}$ $Foxp3^+$ Treg phenotype.

3.4.6 Relative Expression of Foxp3 in the Intestinal Mucosa

In order to analyse samples of intestinal mucosa for the presence of Treg, the expression of the Treg specific transcription factor Foxp3 was determined by real time RT-PCR. The ability to measure Foxp3 mRNA with high sensitivity was confirmed using various dilutions of Foxp3 expressing Jurkat cells. This demonstrated that the sensitivity of real time RT-PCR was sufficient to detect as little as 1000 Foxp3+ cells (**Figure 3.14 and Figure 3.15**). A 10-fold increase in Foxp3 expression was observed in CD patients ($p = 0.0007$) and coeliac disease patients ($p = 0.0011$) compared to controls, while a 100-fold increase in Foxp3 expression was observed in UC patients ($p < 0.0001$)(**Figure 3.16**). Therefore, despite a deficiency in the number of T_{reg} in the peripheral blood of IBD patients, the Treg transcritption factor, Foxp3, was present at considerably higher concentration in the intestinal mucosa, compared to that of control subjects. Unfortunately biopsy samples were not collected from patients who donated peripheral blood samples and therefore direct correlations between peripheral Treg numbers and Foxp3 expression in intestinal biopsies were not possible.

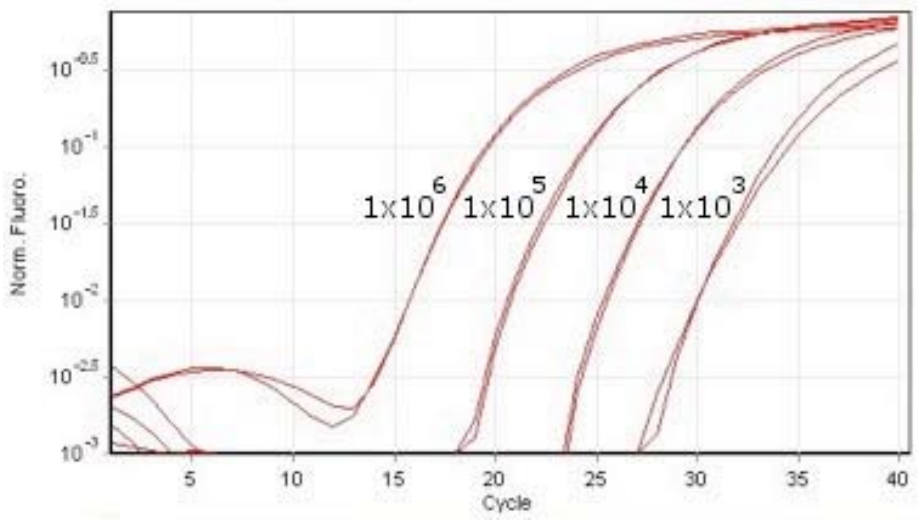

Figure 3.14 Determination of real time RT-PCR sensitivity using Foxp3 expressing Jurkats. Foxp3 expressing Jurkats were serially diluted from $1x10^6$ to $1x10^1$ cells. Real time RT-PCR quantitation data demonstrated Foxp3 could be measured accurately in as little as $1x10^3$ Foxp3 expressing Jurkat cells.

A B

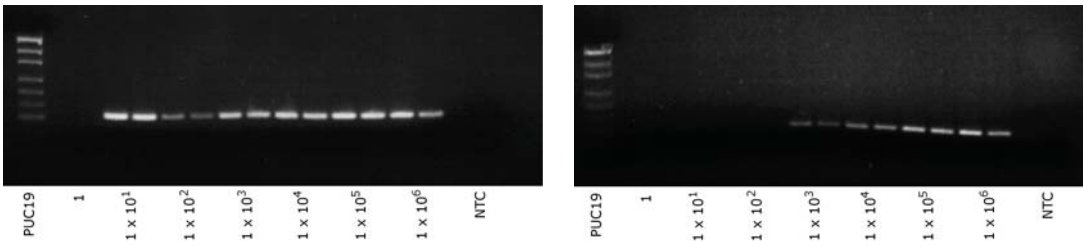

Figure 3.15. **Agarose gel electrophoresis of (A) β-actin (B) and Foxp3 real time RT-PCR products from Foxp3 expressing Jurkat cells.** The number of Foxp3+ cells ranged from 1 to $1x10^6$ cells. Beta actin was detectable at cell numbers greater than $1x10^1$ and Foxp3 was detectable at cell numbers greater than $1x10^3$.

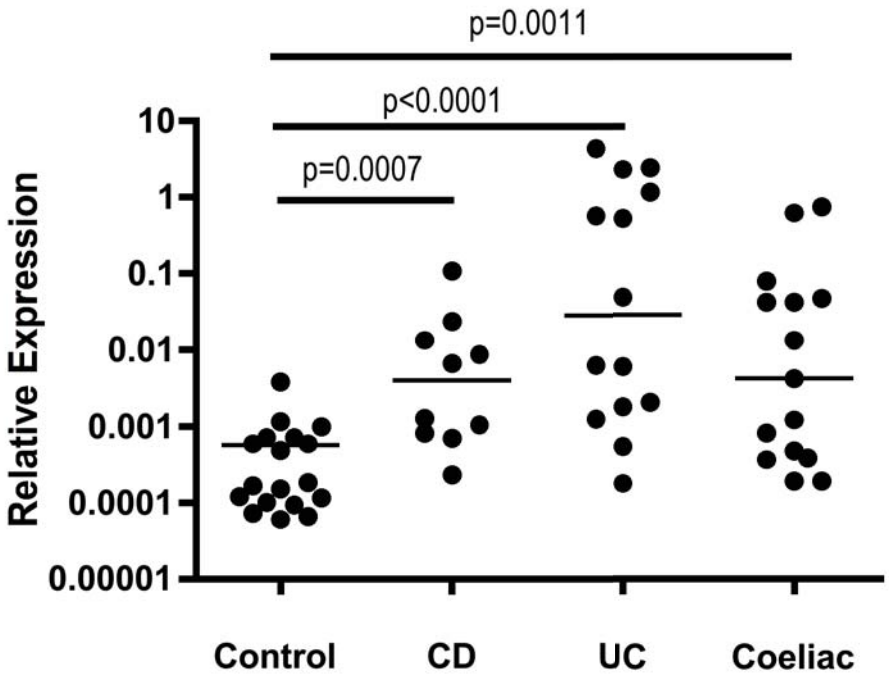

Figure 3.16 Foxp3 expression in the intestinal mucosa of IBD, coeliac and control patients. RNA extracted from intestinal biopsies of IBD, coeliac and control patients was quantified using real time RT-PCR, and normalised to β-actin expression. Foxp3 expression was significantly increased in CD, UC and coeliac patients compared to the control group. Each patient is represented with a point, with horizontal bars indicating the group median.

3.4.7 Regulatory T cells and Disease Activity

To determine whether disease activity contributed to increased Foxp3 expression, each patient's clinical status was tracked and overlayed onto Foxp3 relative expression graphs. IBD patients were allocated to categories of disease inactivity (remission), mild disease activity and moderate disease activity based on SA Pathology histological reports (**Figure 3.17**). Disease activity did not correlate with Foxp3 expression in CD, with patients identified as suffering moderate disease activity expressing variable Foxp3 levels. UC patients with mild disease exhibited low Foxp3 expression, and patients in a state of inactive disease demonstrated high and low Foxp3 expression. A sub-group of patients with moderate disease showed high Foxp3 levels, however, this was not the case for all patients with moderate disease activity.

Coeliac disease patients were subdivided into those maintaining a strict gluten-free diet, those non-compliant to a gluten-free diet and individuals diagnosed with refractory disease whereby inflammation was not controlled by a gluten-free diet (**Figure 3.18**). Patients non-compliant to a gluten-free diet expressed high levels of Foxp3, and refractory cases demonstrated low Foxp3 expression. Those maintaining a strict gluten free diet showed variable Foxp3 levels with both high and low expression.

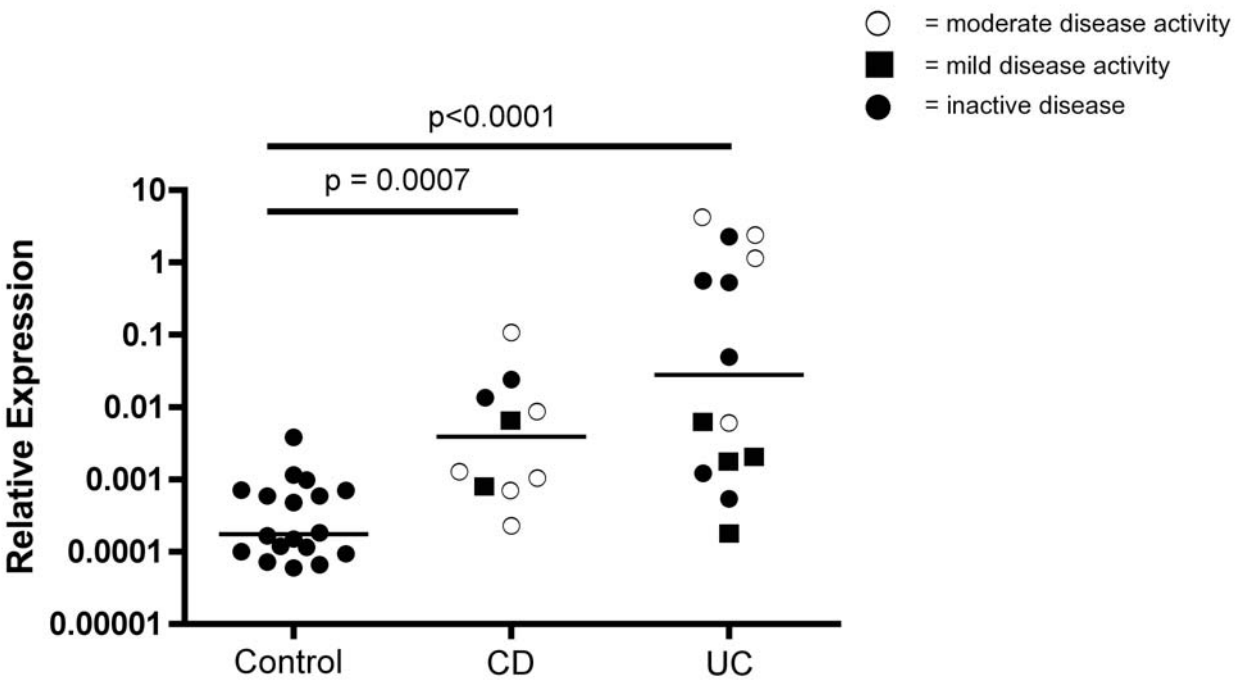

Figure 3.17 Foxp3 Expression and Disease Activity in IBD. Disease activity of IBD patients were overlayed onto the graph of Foxp3 relative expression. IBD patients were categorised as suffering moderate disease activity, mild disease activity and inactive disease. UC patients with mild disease activity exhibited low Foxp3 expression, with moderate disease generally demonstrating higher levels of Foxp3 expression. No relationship between disease activity and CD was observed.

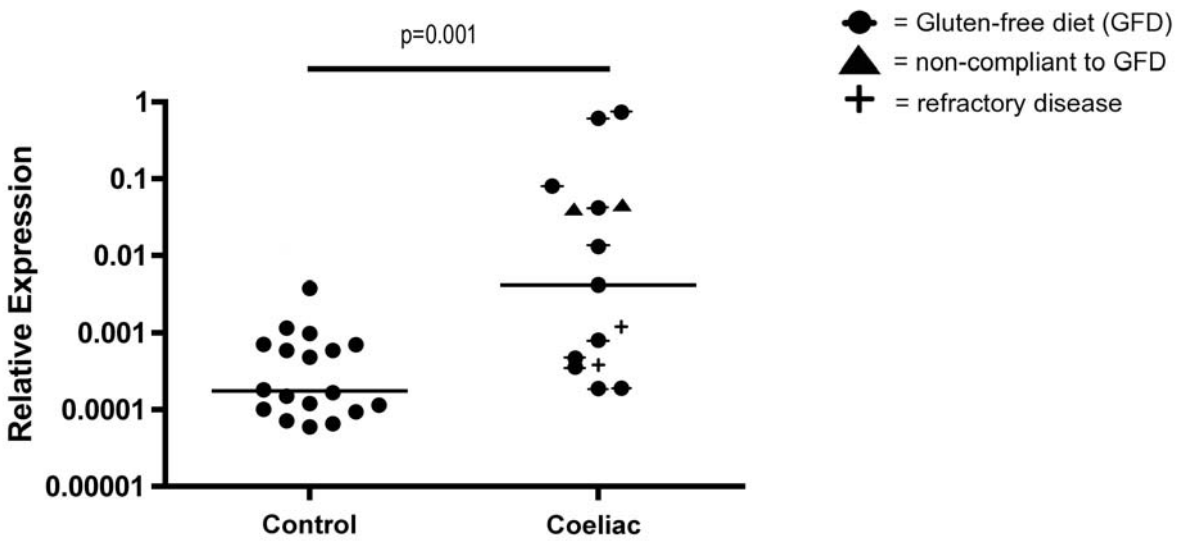

Figure 3.18 Foxp3 Expression and Disease Activity in Coeliac Disease. Disease activity of coeliac disease patients were overlayed onto the graph of Foxp3 relative expression Coeliac disease patients were identified as those maintaining a gluten-free diet, those non-compliant to a gluten-free diet and those with refractory disease. No relationship between Foxp3 expression and disease activity was observed.

3.5 Discussion:

The discovery of a Foxp3 defect and a regulatory T cell deficiency in the diseases scurfy and IPEX has served as a basis for considering the role of Tregs in health and disease. However, a lack of consensus in studies of human disease suggests methodological inconsistencies may be confounding results. As the confirmation of Treg involvement in human autoimmune diseases could provide new areas of therapeutic treatment and non-invasive diagnostics, a refined and reproducible protocol for Treg enumeration in humans would provide significant advances in the measurement and treatment of these diseases.

Human Tregs have been identified previously by a range of cell phenotypes including $Foxp3^+$, $CD4^+$ $CD25^+$ $Foxp3^+$, and more recently $CD4^+$ $CD25^{high}$ $Foxp3^+$. The data presented herein show that $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells are significantly decreased in the CD and UC groups compared with the control group. However, there was no change in $Foxp3^+$ and $CD4^+$ $CD25^+$ $Foxp3^+$ T cell numbers between the groups. This may have been due to the inclusion of activated T cells. The decreased numbers of $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells in CD and UC may permit the uncontrolled inflammation characteristic of IBD. However, a causal link between inflammation and Treg deficiency is difficult to determine, and it is unclear whether the deficiency observed was the cause or a consequence of the disease.

Neither Foxp3 nor CD25 alone are adequate markers of Tregs due to inclusion of activated human T cells (Wang *et al.*, 2007). The combination of Foxp3 with CD4 and $CD25^{high}$ gating is the optimal measure of human Tregs, excluding contamination of

activated T cells (Baecher-Allan *et al.*, 2001). CD127 is a potential alternative cell surface marker of Tregs, as it is expressed at low levels on the surface of Tregs, but at higher levels on other T cells (Seddiki *et al.*, 2006). In this study, the comparison of $CD4^+$ $CD25^{high}$ $Foxp3^+$ with $CD4^+$ $CD25^+$ $CD127^{low}$ within patient groups revealed different trends in the cell numbers for these Treg phenotypes. The $CD4^+$ $CD25^{high}$ $Foxp3^+$ phenotype may have been highly conservative describing approximately 0.2 – 0.3% of lymphocytes. The $CD4^+$ $CD25^+$ $CD127^{low}$ phenotype conversely describes a larger T cell pool constituting 2 – 3% of lymphocytes. The trends in numbers of these cells accumulated from patient cohorts also varied significantly with phenotype. The $CD4^+$ $CD25^{high}$ $Foxp3^+$ phenotype showed a decrease in these cells in IBD patients compared to controls, however the $CD4^+$ $CD25^+$ $CD127^{low}$ phenotype was increased in the IBD and coeliac groups. The $CD4^+$ $CD25^+$ $CD127^{low}$ phenotype includes both $CD25^{high}$ and $CD25^{intermediate}$ cells and may contain a larger heterogenous population. Using the markers $CD127^{low}$ with $CD4^+$ $CD25^{high}$ resulted in a T cell population with similar cell numbers as the $CD4^+$ $CD25^{high}$ $Foxp3^+$ phenotype, however the trends in these 2 cell populations remained unequal. Therefore the $CD4^+$ $CD25^{high}$ $CD127^{low}$ phenotype does not measure the same Treg population that we revealed to be decreased in IBD.

Seddiki *et al* (personal communication, 2006) demonstrated the utility of $CD127^{low}$ as a marker for Tregs in combination with CD45RA isoforms. A decrease in $CD4^+$ $CD25^+$ $CD45RA^+$ $CD127^{low}$ T cells was found in both CD and UC, but with an increase in $CD4^+$ $CD25^+$ $CD45RA^-$ $CD127^{low}$ T cells. This suggested a decrease in naïve Tregs and increased memory Tregs in IBD. The percentage of cells positive for the $CD4^+$ $CD25^+$ $CD45RA^+$ $CD127^{low}$ phenotype closely resembles the levels of $CD4^+$ $CD25^{high}$ $Foxp3^+$

in the lymphocyte pool. Therefore the use of $CD45RA^+$ as an additional marker may identify a more homogenous Treg population.

In the current study, analysis of Treg in IBD and coeliac disease was particularly informative as circulating Treg in the peripheral blood were enumerated, enabling meaningful comparisons between patient groups. Such an approach is less common in animal studies, in which cells are described in terms of their frequency of cells studied. However, in the clinical setting, patient lymphocyte subpopulations are enumerated using percentages from flow cytometric data and the total lymphocyte number from haematology results. For example, absolute lymphocyte counts is employed in clinical settings with HIV patients in which accuracy in measuring T cell numbers is crucial, as these results provide important information in classifying the levels of immunodeficiency, prognosis and treatment (O'Gorman and Zijenah, 2008).

Despite the decrease in Treg demonstrated in the peripheral blood of IBD patients, a significant increase of Foxp3 mRNA was observed in the intestinal mucosa of IBD patients. This may have indicated the active recruitment of Tregs to the site of inflammation in the intestinal mucosa where they are most needed to control inflammation (Yu *et al.*, 2007). Alternatively, the increased expression of Foxp3 may have been accounted for by activated T cells transiently expressing Foxp3 that do not exhibit suppressive activity (Allan *et al.*, 2007). An increase in Foxp3 at the site of disease is apparent in IBD (Makita *et al.*, 2004; Maul *et al.*, 2005; Yu *et al.*, 2007; Sitohy *et al.*, 2008), coeliac disease (Vorobjova *et al.*, 2009) and also other diseases such as rheumatoid arthritis (Cao *et al.*, 2003; Cao *et al.*, 2004). Interestingly, T_{reg} isolated from gut associated lymphoid tissue and peripheral blood of IBD patients are functionally suppressive *ex vivo* (Yu *et al.*, 2007), (Maul *et al.*, 2005). The suppressive

capacity of Tregs isolated from rheumatoid arthritis patients has also been confirmed (de Kleer *et al.*, 2004; Cao *et al.*, 2006; Lawson *et al.*, 2006). However, the ability of these cells to maintain suppressive function may be hampered *in vivo* in the environment of the disease.

Recent research has indicated that human T_{reg} express ROR-γt and IL-17 and lose suppressive function in the presence of high levels of IL-1β and IL-6 (Koenen *et al.*, 2008; Beriou *et al.*, 2009). Hence, the prolonged exposure of T_{reg} to these inflammatory cytokines may promote their conversion to Th17 cells (Koenen *et al.*, 2008; Beriou *et al.*, 2009). This process may account for the deficit in T_{reg} numbers observed in the peripheral blood of IBD patients. Additionally, the presence of high levels of IL-6 in the microenvironment blocks the suppressive capacity of $CD4^+$ $CD25^+$ Tregs in mice (Pasare and Medzhitov, 2003), suggesting that despite high Treg numbers in the intestinal mucosa these may not have been functionally active.

3.5.1 Summary

In summary, we have shown that $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells are deficient in IBD patients. Other phenotypes used in earlier research such as $CD4^+$ $CD25^+$ showed no difference in the numbers of these cells. This implied that activated T cells were likely to be present in increased numbers in IBD patients. Tregs express a range of markers that are shared with activated T cells, including Foxp3, which is transiently expressed by activated T cells. Gating on the $CD25^{high}$ population, eliminates activated T cells that have an intermediate expression of CD25 and that may also express Foxp3. CD127 may provide an alternative marker for Foxp3, however, its effectiveness may rely on the use of $CD45RA^+$ as an additional cell marker in IBD. The increase of Foxp3 in the

intestinal mucosa of IBD and coeliac patients suggests the recruitment of Tregs, or an increase in transiently expressing Foxp3^{+} effector cells, at the site of disease. However, the confirmation that Tregs isolated from patient intestinal tissue retains suppressive abilities *ex vivo* suggests that either the intestinal environment is not conducive to Treg function, or that Tregs are over-powered by proinflammatory effector cells at the site of disease. In order to further investigate the involvement of Treg in IBD, the contribution of the effector cell and cytokine microenvironment must also be examined.