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

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CHAPTER 4

TH17 EFFECTOR CELLS AND T HELPER CELL TRANSCRIPTION FACTORS IN INFLAMMATORY BOWEL DISEASE AND COELIAC DISEASE

4.1 Introduction

Upon activation, T helper cells of the adaptive arm of the immune system proliferate and produce a complex array of potent cytokines to promote the clearance of pathogens (Abbas and Lichtman, 2005). Classically over the last 20 years, the Th1/Th2 paradigm has been used to describe the adaptive immune response, with cytokine profiles and function defining these T cell subsets (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989; O'Garra and Murphy, 1994; Murphy and Reiner, 2002). Th1 cell development is coordinated by the activation of transcription factors including signal transducer and activator of transcription (STAT)- 4 and T box expressed in T cells (T-bet). These cells characteristically secrete IL-12, IL-18 and IFN-γ, which are effective in coordinating responses directed against intracellular viral antigens. Th2 cells develop via the activation of the transcription factors STAT-6 and GATA binding protein (GATA)-3, and secrete IL-4, IL-5 and IL-13 to generate responses to clear extracellular pathogens (Abbas and Lichtman, 2005; Romagnani, 2006). Excessive Th1 and Th2 responses have been implicated in gastrointestinal disease, specifically Th1 responses in the pathogenesis of CD and coeliac disease, and Th2 responses in UC (Salvati *et al.*, 2002; Torres and Rios, 2008). However, the discovery of a distinct subset of CD4 T cells that produces the pro-inflammatory cytokine IL-17 has led to revision of the Th1/Th2 paradigm and the inclusion of the new effector cell lineage known as Th17 cells.

The discovery of the Th17 cell lineage resulted from the discovery of IL-23, a new IL-12 family member associated with a range of chronic inflammatory conditions including IBD (Cua *et al.*, 2003; Chen *et al.*, 2006; Hue *et al.*, 2006). Animal models of arthritis and multiple sclerosis, collagen induced arthritis (CIA) and experimental

autoimmune encephalomyelitis (EAE), were initially thought to be driven by an uncontrolled Th1 response and an excessive IL-12 production by these cells (Murphy *et al.*, 2003). Support for IL-12 involvement was demonstrated in experiments demonstrating disease ablation with the treatment of anti-IL-12 p40 neutralising antibodies (Oppmann *et al.*, 2000). The discovery of IL-23, however, caused a significant change to the understanding of autoimmune diseases. IL-23 is an inflammatory cytokine that shares a p40 subunit with IL-12, in addition to its unique p19 subunit (Oppmann *et al.*, 2000). Mice deficient in IL-23p19 did not succumb to EAE or CIA, although mice lacking IL-12p35 still exhibited exacerbated disease (Cua *et al.*, 2003; Murphy *et al.*, 2003). This suggested that IL-23, rather than IL-12 was the key cytokine implicated in the pathogenesis of autoimmune reactions. Indeed, animal models of IBD demonstrated that depletion of IL-12 did not affect the progress of inflammation (Hue *et al.*, 2006), and that treatment with anti-IL-23p19 antibodies abrogated established colitis (Elson *et al.*, 2007). Although IL-23 deficient mice succumb rapidly to infection (Mangan *et al.*, 2006), IL-23 does not induce T cell proliferation (Annunziato *et al.*, 2007). Mice deficient in IL-23 ($p19^{-/-}$), maintained a robust Th17 cell population with the presence of TGF-β, however these animals demonstrated an impaired immune response with the inability to clear pathogens and succumbed to infection (Mangan *et al.*, 2006). IL-23 is therefore not necessary for the differentiation or development of Th17 cells, but is essential for their protective function (Mangan *et al.*, 2006). IL-23 potentially promotes the proliferation of memory T cells (Boniface *et al.*, 2008) and contributes to its maintenance, stabilisation, development, or amplification of the Th17 phenotype, although the exact mechanisms await elucidatedation (Aggarwal *et al.*, 2003; Harrington *et al.*, 2005; Langrish *et al.*, 2005; Yen *et al.*, 2006).

Support of IL-23 involvement in IBD is the result of genome-wide association studies implicating multiple genes involved in IL-23 signalling. The strongest association for both CD and UC is with the IL-23 receptor (IL-23R) (Duerr *et al.*, 2006; Cummings *et al.*, 2007; Raelson *et al.*, 2007; Rioux *et al.*, 2007). The IL-23R is expressed by haematopoietic cells, NKT cells, eosinophils, dendritic cells, macrophages and memory T cells (Abraham and Cho, 2008). IL-23R$^+$ CD4$^+$ memory T cells that produce IL-17 have been identified in the peripheral blood of healthy individuals, with increased expression of IL-17 after stimulation of the T cell receptor (Wilson *et al.*, 2007). High levels of IL-23 have been reported in the mucosal tissue of both CD and UC patients, which also correlates with high levels of IL-17 (Kobayashi *et al.*, 2008). Additionally, a unique subset of dendritic cells has been found to induce the development of intestinal granulomata present in CD, and these cells may promote an environment conducive to Th17 development and/or maintenance by secreting large quantities of IL-23 (Mizoguchi *et al.*, 2007).

Retinoid acid-related orphan nuclear hormone receptor-γt, RORγ-t is the master transcription factor for the Th17 cell and the human orthalog of RORγ-t is RORC, the retinoic acid related orphan receptor C. (Ivanov *et al.*, 2006). The differentiation of the Th17 cell does not require the same transcription factor signalling as Th1 and Th2 cells, and therefore does not require T-bet, STAT1, STAT4, STAT6 or GATA3 (Harrington *et al.*, 2005; Veldhoen *et al.*, 2006). However, STAT3 is critical for Th17 cell development, as it is the major signal transducer for IL-6, IL-21 and IL-23. The upregulation of RORγ-t is known to be STAT3 dependent (Harris *et al.*, 2007; Yang *et al.*, 2007), and deletion of STAT3 prevents the development of Th17 cells (Mathur *et al.*, 2007). Th17 cells express pattern recognition receptors capable of recognising

extracellular bacteria and fungi and are potent activators of neutrophils to enable clearance of invading pathogens (Ye *et al.*, 2001). Although Th17 cells play a critical biological function in clearing extracellular pathogens (Gaffen, 2008), the inappropriate production of IL-17a by these cells is thought to contribute to the pathology of a range of inflammatory diseases. Elevated levels of IL-17a have been found in rheumatoid arthritis (Hwang and Kim, 2005), multiple sclerosis (Matusevicius *et al.*, 1999; Lock *et al.*, 2002), asthma (Bullens *et al.*, 2006) and systemic lupus erythematosus (Wong *et al.*, 2000). Increased expression of IL-17 has also been documented in IBD (Fujino *et al.*, 2003; Nielsen *et al.*, 2003; Annunziato *et al.*, 2007) suggesting that a Th17 cell driven immune response contributes IBD pathology (Bettelli *et al.*, 2006).

High levels of IL-17 have been described in the intestinal tissue of IBD patients (Nielsen *et al.*, 2003; Holtta *et al.*, 2008). However, Fujino *et al* were the first to identify T cells as a source of IL-17 in IBD, with elevated numbers of IL-17^{+} T cells and serum IL-17 in IBD patients compared to controls (Fujino *et al.*, 2003). The production of IL-17 was also found to be greater in lamina propria T cells than PBMCs from IBD patients (Kobayashi *et al.*, 2008). Production of IL-17 by T cells was significantly greater in UC than CD, whereas CD patients exhibited an increase in IFN-γ production in addition to IL-17 (Kobayashi *et al.*, 2008). The production of both IL-17 and IFN-γ may be explained by a Th17 cell with Th1-like qualities (Th1/Th17 cell) that has been identified in the gut mucosa of CD patients (Annunziato *et al.*, 2007). These Th17 cells were shown to express the Th17 transcription factor Rorγ-t, but also produced IFN-γ, particularly in the presence of IL-12. Additionally, both the Th17 and Th1/Th17 cells were less susceptible to suppression by regulatory T cells (Annunziato *et al.*, 2007). Animal models of autoimmune gastritis compared the effect of Th1, Th2

and Th17 on the intestinal mucosa. Th17 cells were associated with the greatest damage to the gastrointestinal tract, and these cells were also shown to be resistant to suppression by $Foxp3^+$ Tregs (Stummvoll *et al.*, 2008). Studies in human psoriasis have suggested that Th1 and Th17 effector cells may work synergistically, in which IFN-γ from the Th1 cell promotes trafficking of Th17 cells to sites of inflammation (Kryczek *et al.*, 2008).

Currently there is little information on the role of the Th17 in coeliac disease. High levels of Th1 cytokines, such as IFN-γ and transcription factor T-bet, are well documented (Monteleone *et al.*, 2004; Johrens *et al.*, 2005). However, IL-12, a characteristic Th1 cytokine, has not been found in the gut mucosa of coeliac disease patients. (Nilsen *et al.*, 1998; Monteleone *et al.*, 2004). Recent studies have indicated IL-21 may be involved in coeliac disease (Fina *et al.*, 2008; Fina *et al.*, 2008; Meresse *et al.*, 2008). Interestingly, this cytokine is not only produced by the Th17 cell, but IL-21 may also be involved in its differentiation (Fantini *et al.*, 2007; Fina *et al.*, 2008). IL-23 has also been linked to coeliac disease, with the finding that wheat gliadin induces the production of IL-23 in PBMCs isolated from coeliac disease patients (Harris *et al.*, 2008).

This chapter describes Th17 cell measurement in the peripheral blood by flow cytometry and measures the Th17 cytokine IL-17a in the intestinal biopsies of CD, UC, coeliac and control subjects. The Th17 associated cytokine IL-23 was also investigated, together with the involvement of Th1, Th2 and Th17 transcription factors in these diseases.

4.2 Aims and Hypothesis:

The general hypothesis of this chapter is that inflammation present in IBD and coeliac disease is the result of an excessive Th17 immune response.

Aims:

1) To characterise the production of IL-17 in stimulated PBMCs from a population of controls

To measure and compare:

2) Th17 numbers from the peripheral blood of IBD, coeliac disease and control subjects.
3) Th17 mRNA expression from intestinal biopsies of IBD, coeliac disease and control subjects.
4) IL-23 mRNA expression in intestinal biopsies of IBD, coeliac disease and control subjects.
5) Th1, Th2 and Th17 transcription factors in the intestinal mucosa of IBD, coeliac disease and control subjects.

4.3 Methods

4.3.1 Subjects

IBD patients were recruited from the Department of Gastroenterology at The Queen Elizabeth Hospital, Woodville, South Australia. Peripheral blood was collected, with written consent, from fourteen control, sixteen CD, sixteen UC and fifteen coeliac subjects. All IBD patients were diagnosed clinically and all were in a state of disease inactivity based on clinical assessment and C-reactive protein levels (CRP<10). Coeliac disease patients were predominantly on long-term gluten free diets. The mean age for these groups in years ± SEM were; control 35.5 ± 2.28y, CD 36.3 ± 3.9y, UC 47.5 ± 4.1y and coeliac 52.6 ± 3.4y. Intestinal biopsy samples were collected from an additional subset of patients undergoing endoscopy in the Department of Gastroenterology and Hepatology at the Queen Elizabeth Hospital. Biopsy samples were collected from fifteen control, eleven CD, fifteen UC and fifteen coeliac subjects. Samples were stored in RNALater (Ambion, USA) at -20C to prevent RNA degradation prior to extraction.

4.3.2 In vitro PMA/Ionomycin stimulation of PBMCs

PBMCs were isolated from patient whole blood and immediately cryopreserved (see chapter 2 for details). Samples were thawed and processed after the accumulation of ten patient samples and batch processed. Thawed PBMCs were rested overnight at 37°C, 5% CO_2 in RPMI-1640 supplemented with 10% FCS, to improve cell viability for permeabilisation (Lamoreaux *et al.*, 2006). PBMCs were stimulated prior to IL-17a intracellular flow cytometry. Mononuclear cells were cultured at 37°C, 5% CO_2 in RPMI-1640 culture medium supplemented with 10% FCS with PMA (50ng/ml) and

Ionomycin (1ug/ml). Brefeldin A (10ug/ml), a protein transport blocker that inhibits cytokine transport, was added in the last 4 hours to prevent excretion of cytokine. Brefeldin A was used due to its potency, effectiveness and reduced toxicity in comparison to monesin (Schuerwegh *et al.*, 2001). After 5 hours, cells were washed three times in PBS.

4.3.3 Flow Cytometry - Intracellular cytokine staining

$1x10^6$ cells were resuspended and labelled with surface markers, CD4, CD3 and CD8. The surface labelled cells were then fixed in a 4% paraformaldehyde solution for 10 minutes at RT and then washed 3 times in FACS wash. Cells were permeabilised in 2mls of 0.1% Saponin (in PBS) for 10 minutes at room temperature. Tubes were centrifuged at 400g without washing and the majority of the supernatant decanted, leaving approximately 200µl of saponin in the tube. As permeabilisation with saponin is reversible, prospectively every step contained 0.1% w/v saponin including the FACS wash and antibody incubation solutions. Due to the high levels of non-specific antibody binding after permeabilisation, cells were blocked with 50µl of a 5% w/v non-fat dry milk PBS saponin (0.1%) solution for 30 minutes at 4°C. Without washing, 20µl of IL-17a antibody (clone: eBio64DEC17, eBioscience, CA, USA) was added and incubated for 30 minutes in the dark at 4°C. Cells were washed three times in FACSwash with 0.1% saponin before examination by flow cytometer. Corresponding isotype controls were included for each patient sample. Quantification was conducted on a BD FACScan (BD Biosciences, USA), collecting between 300,000-500,000 events per sample. Lymphocytes were gated based on their forward and side scatter properties, and results were analysed using Cell Quest software (BD Biosciences, USA).

4.3.4 RNA extraction and cDNA production

Total RNA was isolated from intestinal biopsies using the RNeasy Lipid Minikit (Qiagen, Vic, Australia). RNA gel electrophoresis was performed to assess RNA quality and samples were accepted if 28S ribosomal RNA bands were present with intensity approximately twice that of the 18S RNA band. 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 their genomic sequence (See Chapter 2 for detailed methods).

4.3.5 Real time RT-PCR

Quantitative real time RT-PCR was performed using a Corbett Rotorgene RG-3000 (Corbett Research, Australia), with two replicates per sample, a non-template control and no-reverse transcription control for each experiment. All reactions were conducted using the Power SYBR green master mix (2x) solution (Applied Biosystems, CA, USA). A melt curve analysis and gel electrophoresis were performed to exclude the formation of non-specific PCR products. Expression of IL-17a and IL-23 mRNA was normalised to β-actin expression. Patient samples were excluded from statistical analysis if the target gene was below detectable levels. PCR products were purified using Qiaquick PCR purification kit (Qiagen, Vic, Australia) and sequenced at the Flinders University DNA Sequencing Facility (Department of Haematology, Flinders Medical Centre, SA, Australia). The PCR product sequences were confirmed using the National Center for Biotechnology Information (NCBI) basic local alignment search tool. Stimulated and unstimulated PBMCs were used as positive and negative controls.

4.3.4 Statistics

Flow cytometric results were converted to absolute cell numbers for each patient sample. Flow cytometry results are presented as mean ± SEM cells per ml of whole blood. Real time RT-PCR results were analysed using the ΔCT method with Q-gene software (Simon, 2003), taking into account individual primer efficiencies. Real time RT-PCR results are presented as normalised mean expression. The Mann-Whitney ranked sums test was employed via the GraphPad Prism software to determine statistical differences between disease and control groups.

4.4 Results

4.4.1 Production of IL-17 by stimulated PBMCs measured by flow cytometry

As the production of cytokines is low in resting cells (Rostaing *et al.*, 1999; Baran *et al.*, 2001), *in vitro* stimulation of PBMCs is required for cytokine detection using flow cytometry. Two signals are required to activate T cells to produce cytokine: protein kinase C (PKC) activation and the increase of cytosolic Ca^{2+} (Larsen, 1990). The stimulation of T cells with phorbol 12-myristate 13-acetate (PMA) and ionomycin (a Calcium ionophore) mimics this process, which activates PKC and allows the influx of Ca^{2+} (Larsen, 1990).

Optimal stimulation periods to measure IL-17a protein production from $CD4^+$ T cells were determined over a time course of various stimulation durations with PMA and ionomycin. A representation of $CD3^+$ IL-$17a^+$ flow cytometry data is illustrated in **Figure 4.1**. In this case, after 5 hours of stimulation 1.15% of lymphocytes were $CD3^+$ IL-$17a^+$. To determine the optimal duration of stimulation for IL-17a production in T cells, PBMCs ($2x10^6$) from a healthy control subgroup (n=6) were stimulated for 2, 5, 8, 16 and 24 hours (in the presence of brefeldin A in the last 4 hours) and then intracellularly labelled with anti-human mouse IL-17a-PE. IL-17a production by PBMCs was initially low at 2 hours and rapidly increased with peak production between 5 to 16 hours before decreasing by 24 hours. The optimal stimulation time using PMA/ionomycin for IL-17a production was 5 hours (**Figure 4.2**).

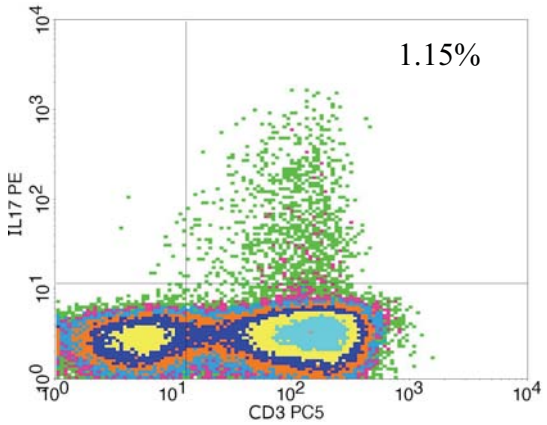


Figure 4.1. Detection of $CD3^+$ $IL\text{-}17a^+$ T cells using multi-colour flow cytometry. PBMCs isolated from the peripheral blood were stimulated with PMA/ionomycin and surface labelled with CD3-PeCy5 and intracellularly labelled with IL-17a-PE. Analysis of a representative patient indicated 1.15% of lymphocytes were $CD3^+$ IL-17a+.

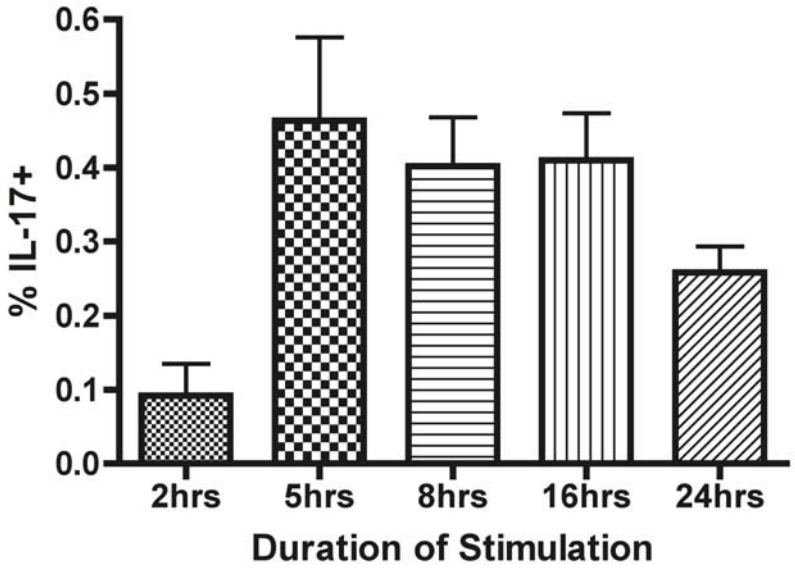


Figure 4.2 PBMC stimulation time course measuring IL-17a protein by flow cytometry. PBMCs isolated from the peripheral blood of 6 healthy controls were stimulated with PMA/Ionomycin for 2, 5, 8, 16 and 24 hours in the presence of brefeldin A in the last 4 hours. Cells were surface labelled with CD3, intracellularly labelled with IL-17a and $IL\text{-}17a^+$ cell numbers measured using flow cytometry.

4.4.2 Production of IL-17a by stimulated PBMCs measured by real time RT-PCR

The expression of IL-17a mRNA was also measured in stimulated PBMCs from four control samples using real time RT-PCR. RNA was extracted from PBMCs after stimulation at various time points and reverse transcribed to cDNA. The relative quantification method was used, whereby the expression of IL-17a was normalised to β-actin as a housekeeping gene. Ct values were calculated, which refer to the number of cycles required for the fluorescent signal to cross threshold level. Ct values for stimulated and unstimulated PBMCs (**Figure 4.3**) revealed IL-17a expression only in stimulated cells. Additional time points were included in this time course, at 1, 2, 3, 4, 5, 6, 10 and 24 hours to allow for increases in message prior to protein production. IL-17a mRNA expression was found to peak between 4 and 10 hours, with the highest amount shown at 4 hours (**Figure 4.4**).

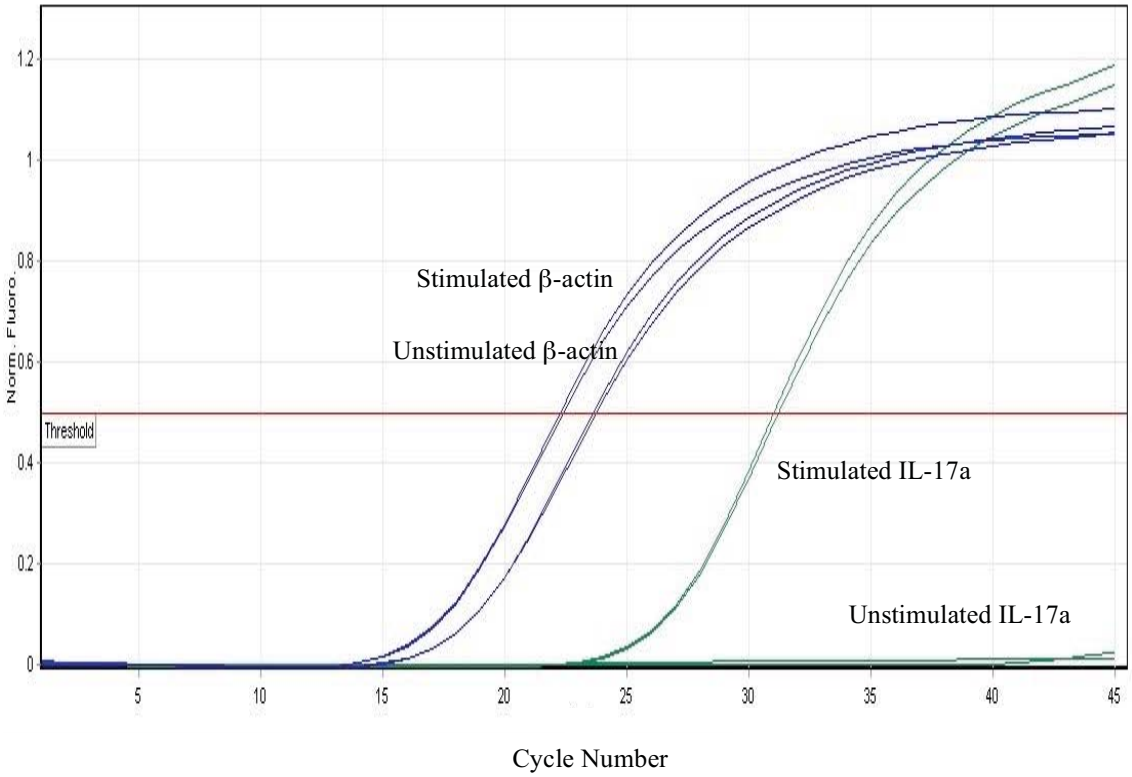


Figure 4.3. Identification of Ct values for stimulated and unstimulated PBMCs using real time RT-PCR. Only stimulated PBMCs expressed IL-17a, confirming stimulation was required for the production of IL-17a. β-actin however was detected in both stimulated and unstimulated cells.

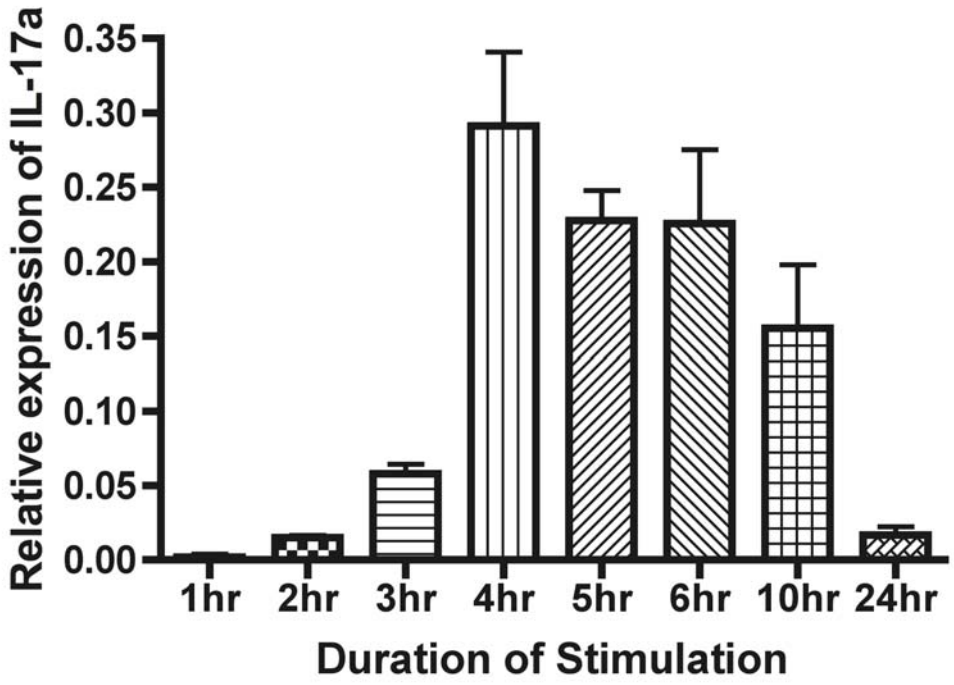
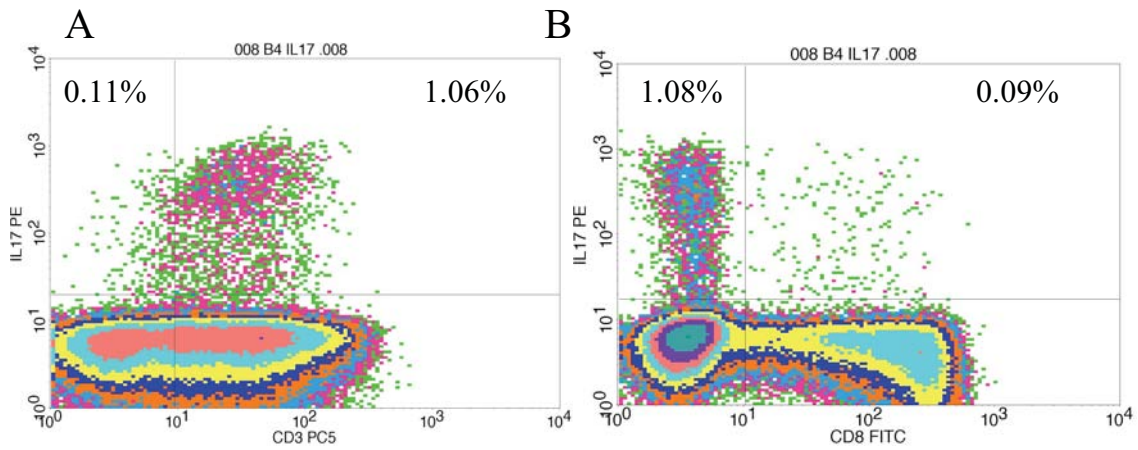


Figure 4.4 PBMC stimulation time course measuring IL-17a mRNA expression using real time RT-PCR. PBMCs were isolated from the peripheral blood of 4 healthy controls and stimulated with PMA/Ionomycin for 1, 2, 3, 4, 5, 6, 10 and 24 hours. Relative expression of IL-17a was measured using real time RT-PCR normalised to β-actin expression.

4.4.3 Investigation of CD8 cells producing IL17a

The activation of T cells is necessary to measure IL-17a production. However, in human T cells, the stimulation with PMA and ionomycin causes the rapid phosphorylation of CD4 in some patient samples and these CD4 molecules are internalised (Anderson and Coleclough, 1993; Daculsi *et al.*, 1998). In order to identify Th17 cells (defined as $CD4^+$ $IL\text{-}17a^+$) CD4 and IL-17a labelling is necessary. However, due to the down regulation of cell surface expression of CD4 after stimulation in some patients, a CD3 antibody was substituted for CD4. CD3 is a subunit of the T cell receptor complex and is expressed by all mature T cells but is not sensitive to activation. As CD3 antibodies label both CD4 and CD8 T cells, an investigation of CD8 T cells that express IL-17 was required before CD3 could be used as a substitute for CD4. **Figure 4.5A** and **Figure 4.5B** represents a patient sample that has been stimulated with PMA/Ionomycin and labelled for both $CD3^+$ $IL\text{-}17^+$ and $CD8^+$ $IL\text{-}17^+$. In a representative healthy patient, the proportion of $CD8^-$ T cells expressing IL-17 was approximately 1.08%. This was similar to the number of $CD3^+$ $IL\text{-}17a^+$ cells (1.06%), suggesting that most IL-17a producing PBMCs are CD4+. Negligible levels of $CD8^+$ $IL\text{-}17a^+$ cells were detected in a subgroup of control patients (**Figure 4.5C**), therefore the $CD3^+$ $IL\text{-}17a^+$ subset was considered equivalent to the $CD4^+$ $IL\text{-}17a^+$ subset.



C

Patient sample	% $CD3^+$ $IL\text{-}17a^+$	% $CD8^+$ $IL17a^+$	% $CD8^-$ $IL\text{-}17a^+$
B1	1.3	0.03	1.19
B2	0.69	0.03	0.61
B3	1.11	0.02	1.08
B4	1.06	0.09	1.08

Figure 4.5 Expression of IL-17a by $CD8^+$ and $CD3^+$ T cells. PBMCs isolated from the peripheral blood were labelled with IL-17a-PE, CD8-FITC and CD3-PeCy5 and measured using flow cytometry. Analysis of a representative patient sample indicates $CD3^+$ $IL\text{-}17a^+$ T cells (A) were equivalent to $CD8^-$ $IL\text{-}17a^+$ T cells (B). A summary of four patients revealed negligible amounts of IL-17a were produced by $CD8^+$ T cells, and the equivalence of $CD3^+$ $IL17a^+$ and $CD8^-$ $IL\text{-}17a^+$ (C).

4.4.4 IL-17a positive and negative controls

Intracellular cytokine staining is a powerful tool in measuring single cell cytokine production (Schuerwegh *et al.*, 2001; Maecker *et al.*, 2005). The labelling of intracellular cytokines for flow cytometry requires fixation and stabilisation and preservation of the outer cell membrane (eg. with paraformaldehyde solution) followed by permeabilisation (eg. with saponin solution). Fixation of the cell also cross-links intracellular proteins to prevent their leakage out of the cell following permeabilisation (Longobardi-Given, 1992). This process of fixation and permeabilisation allows the simultaneous measurement of both intracellular and cell surface markers, and provides valuable information describing specific cytokine producing cells. Permeabilisation allows the penetration of antibody into the cell membrane, cytosol and the membranes of the endoplasmic reticulum and golgi apparatus (Longobardi-Given, 1992). However antibody can become trapped within the cell, or bind non-specifically to intracellular structures of the permeabilised cell membrane (Arora, 2002). The appropriate controls for IL-17a antibody is therefore crucial to avoid false representation of positive staining. Careful blocking and washing steps were utilised to minimise non-specific staining and blocking with a 5% non-fat dry milk powder ensuring specificity of staining (Foster *et al.*, 2007).

To confirm positive staining of patient PBMCs with IL-17a antibody, isotype controls were utilised to show, in both stimulated and unstimulated cells, that non-specific binding was minimal. Three controls were used for each patient sample. These included two unstimulated controls and one stimulated control. Unstimulated cells were fixed and permeabilised followed by labelling with an isotype control (**Figure 4.6A**) and IL-

17a antibody (**Figure 4.6B**), which confirmed negligible non-specific staining in unstimulated PBMCs. Unstimulated PBMCs labelled with IL-17a also confirmed IL-17 was not produced by resting cells, and validated the stimulation protocols for IL-17a detection. Stimulated PBMCs were intracellularly labelled with an isotype matched control (**Figure 4.6C**) and IL-17a antibody following fixation and permeabilisation (**Figure 4.6D**). Negligible positive staining in the stimulated PBMC isotype control confirmed positive staining of IL-17 in stimulated cells was authentic and not due to increased non-specific binding of antibody due to the permeabilisation process.

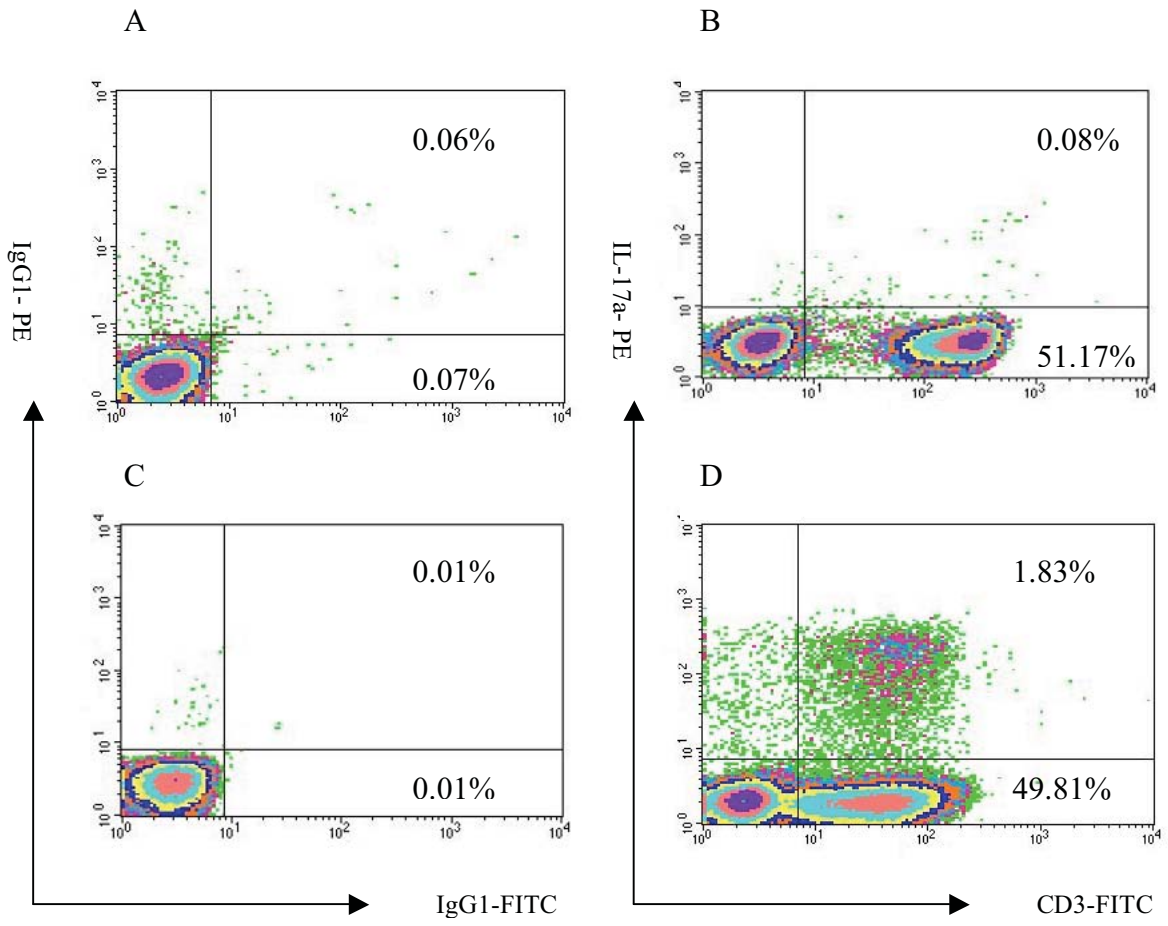


Figure 4.6 Expression of IL-17a by CD4 T cells. PBMCs extracted from peripheral blood were labelled with CD3-FITC, IL-17a-PE and with isotype matched controls. Three controls were used per patient to confirm the specific intracellular staining and the appropriate stimulation of PBMCs to produce IL-17a. The proportion of $CD3^+$ T cells that produce IL-17a (D) was validated by negligible non-specific staining in (A) unstimulated isotype control, (B) unstimulated CD3 and IL17 labelled cells, and (C) stimulated isotype control. Analysis of a representative ulcerative colitis patient indicated that negligible levels of non-specific staining were observed and 1.83% of lymphocytes were positive for the Th17 phenotype.

4.4.5 Quantification of Peripheral Blood Th17 Cells

Although IL-17 has been known as a proinflammatory cytokine since its identification in 1993 (Rouvier *et al.*, 1993), the concept that a $CD4^+$ T cell produces this cytokine is relatively new. High levels of the cytokine IL-17 have been documented in a range of inflammatory conditions including IBD (Fujino *et al.*, 2003; Nielsen *et al.*, 2003; Annunziato *et al.*, 2007). The measurement of $CD4^+$ $IL\text{-}17^+$ T cells, however has not been investigated extensively by flow cytometry in human disease. To determine whether an excessive Th17 response was associated with IBD and coeliac disease, PBMCs were isolated from patient peripheral blood and labelled with directly conjugated antibodies to CD3 and IL-17. Representative two-colour flow cytometric plots from a control, CD, UC and coeliac patient (**Figure 4.7**) demonstrate the method for enumerating Th17 cells. In a control subject (**Figure 4.7A**), 0.15% of lymphocytes were $CD3^+$ $IL17^+$. In CD and UC (**Figure 4.7B and 4.7C**), high levels of $CD3^+$ $IL\text{-}17^+$ T cells are illustrated with 1.2% and 1.87% of cells positive for Th17 cell markers, respectively. The coeliac patient also expressed a higher level of $CD3^+$ $IL\text{-}17^+$ cells than the control patient, with 0.84% of lymphocytes positive (**Figure 4.7D**). The proportion of $CD3^+$ $IL\text{-}17^+$ cells observed in the patient cohorts (**Figure 4.8**) ranged from 0.08 – 0.49% in the control group, 0.36 – 1.81% in CD, 0.31 – 2.58% in UC and 0.21 – 1.04% in the coeliac disease group. The absolute number of $CD3^+$ $IL\text{-}17^+$ cells (per ml of whole blood) was significantly higher in CD ($14.14 \pm 0.78 \times 10^3$ cells/ml, $p<0.005$) and UC patients ($16.72 \pm 2.9 \times 10^3$ cells/ml, $p<0.05$) than the control group ($7.41 \pm 0.78 \times 10^3$). Patients suffering from coeliac disease were observed to have an increase in Th17 cell numbers ($10.75 \pm 1.70 \times 10^3$ cells/ml) than the control group, although this was not statistically significant.

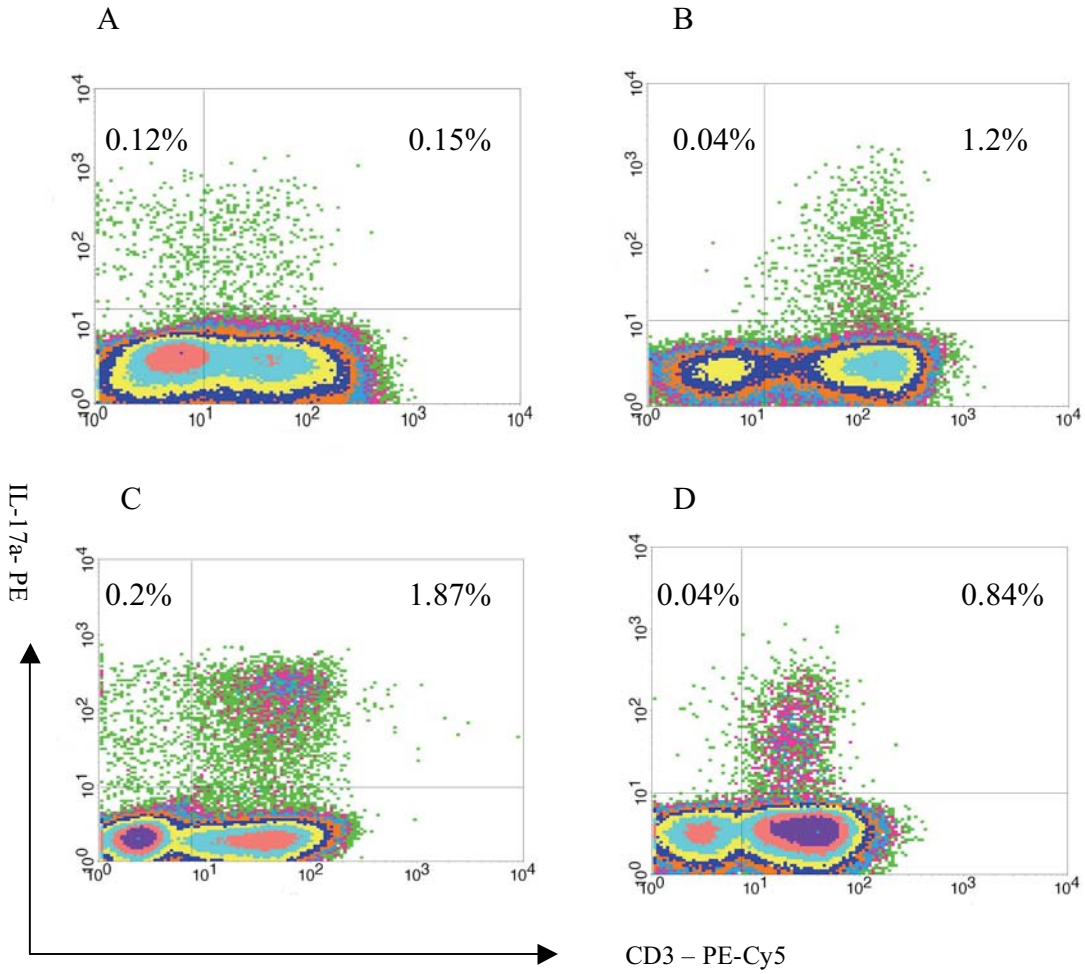


Figure 4.7 Detection of Th17 cells using multi-colour flow cytometry. PBMCs isolated from patient peripheral blood were labelled with CD3-PE-Cy5 and IL-17-PE. Analysis of representative patients indicates the percentage of lymphocytes positive for $CD3^+$ $IL\text{-}17^+$ cells are A) 0.15% in a control patient, B) 1.2% in a CD patient, C) 1.87% in a UC patient and D) 0.84% in a coeliac patient.

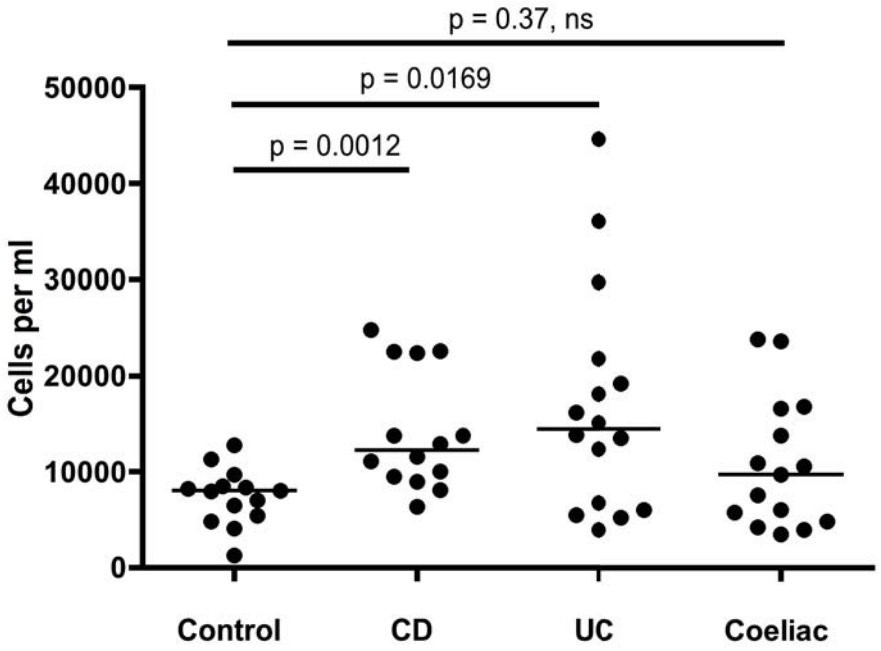


Figure 4.8 Quantification of $CD3^+$ $IL\text{-}17a^+$ T cells in IBD, coeliac disease and control subjects. Absolute numbers of $CD3^+$ $IL\text{-}17a^+$ T cells were calculated from the frequency of $CD3^+$ $IL\text{-}17a^+$ cells determined by flow cytometry and patient lymphocyte counts. Comparisons of $CD3^+$ $IL\text{-}17^+$ cells are illustrated in control, CD, UC and coeliac groups. Each point represents an individual patient with horizontal lines indicating the group median. A statistically significant increase of $CD3^+$ $IL\text{-}17a^+$ cells was observed in the IBD groups when compared to the control group.

4.4.6 Relative expression of IL-17 mRNA in the intestinal mucosa.

High levels of the pro-inflammatory cytokine IL-17 have been identified in a range of inflammatory diseases (Cua *et al.*, 2003; Chen *et al.*, 2006; Hue *et al.*, 2006). To investigate the association of IL-17a in IBD and coeliac disease, RNA was extracted from the intestinal biopsies of control and patient groups. Relative expression of IL-17 was measured using real time RT-PCR with β-actin as a housekeeper gene. Stimulated and unstimulated PBMCs were used as a positive and negative control as depicted in **Figure 4.6**. Relative expression of IL-17 was found to be significantly increased in CD, UC and coeliac disease (**Figure 4.9**). IL-17 relative expression was increased by 100-fold in the CD ($p=0.003$), 1000-fold in UC ($p=0.001$) and 10-fold in coeliac ($p=0.04$) subjects compared to the control subjects. Unfortunately biopsies samples were not collected from the same patients that donated blood samples, therefore direct correlations between IL-17 numbers in the peripheral blood and IL-17 mRNA expression in the intestines were not possible.

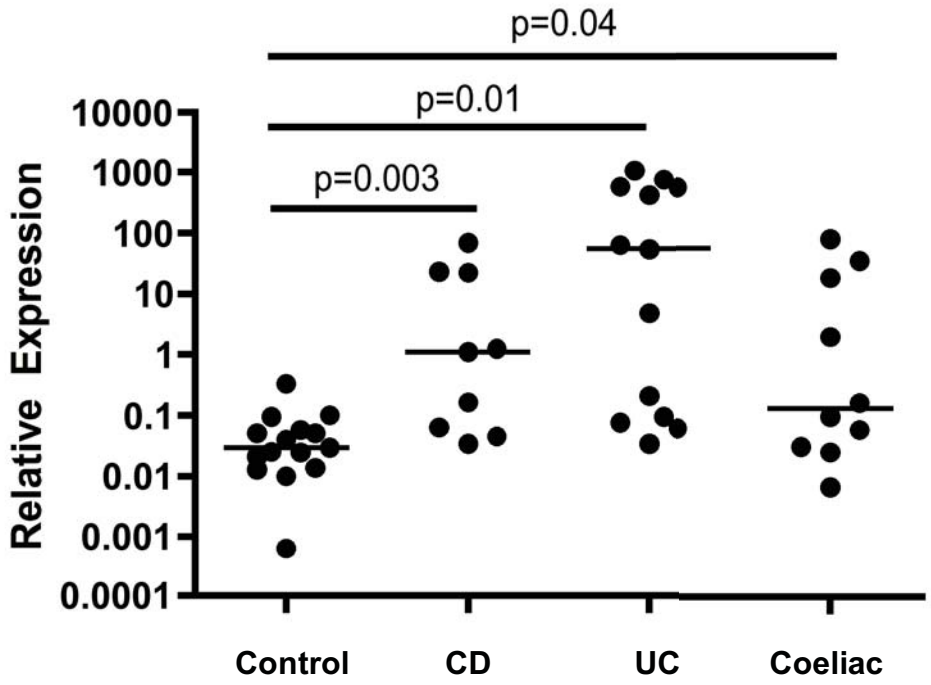


Figure 4.9 Relative expression of IL-17 mRNA in the intestinal mucosa of control, CD, UC and coeliac patients. RNA was extracted from the intestinal biopsies of IBD, coeliac and control patients. IL-17a mRNA was quantified using real time RT-PCR, and normalised to β-actin expression. A statistically significant increase in IL-17 mRNA expression was observed in the CD, UC and coeliac subjects when compared to the control subject. Each point represents an individual patient, with the horizontal line indicating the median

4.4.7 Disease activity and IL-17 expression in IBD and coeliac disease

The impact of disease activity upon IL-17 expression was investigated in both IBD and coeliac disease. IBD patients were divided into three categories; inactive disease, mild disease activity and moderate disease activity (**Figure 4.10**). Disease activity did not affect IL-17a expression in CD, with patients in remission demonstrating high levels of IL-17 while patients with moderately active disease exhibited both high and low IL-17a expression. Disease activity appeared to loosely predict IL-17a expression in UC with patients in moderately active disease states presenting with high IL-17a expression. Patients with inactive disease however expressed high levels of IL-17a, whereas the majority of patients with mild disease showed low IL-17a expression.

Coeliac disease patients were divided into those reportedly following a gluten-free diet, those who were non-compliant to a gluten free diet and individuals diagnosed with refractory disease (**Figure 4.11**). High levels of IL-17a were observed in patients non-compliant to a gluten free diet, however high levels were also observed in patients maintaining a strict gluten-free diet. Low levels of IL-17 were demonstrated in both patients with refractory disease.

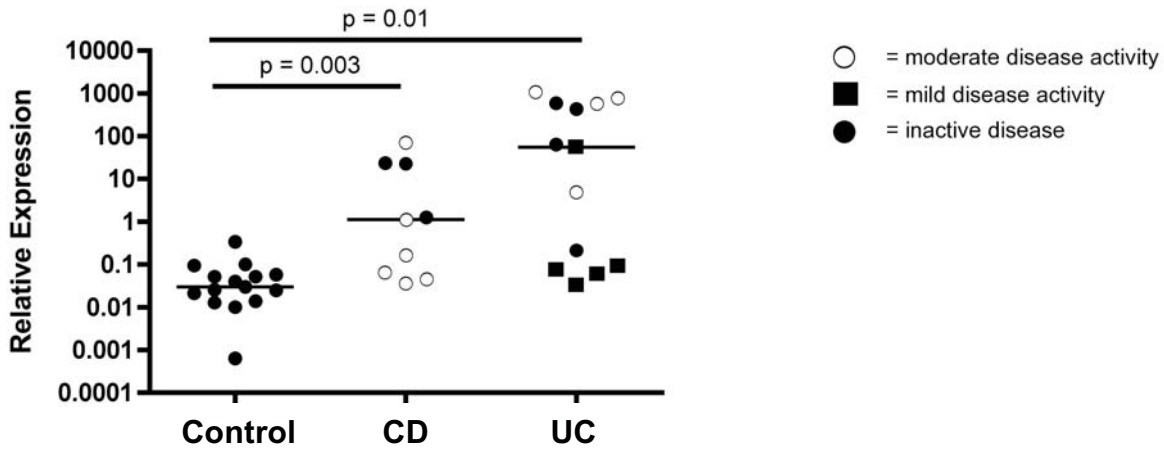


Figure 4.10 Disease activity and IL-17a expression in IBD. The disease activity of IBD patients was overlayed onto IL-17a relative expression graphs. IBD patients were allocated into groups with moderate disease activity, mild disease activity and inactive disease. UC patients with moderate disease activity demonstrated higher expression of IL-17a than the majority of those with mild disease. No relationship between disease activity and IL-17a expression was observed in CD.

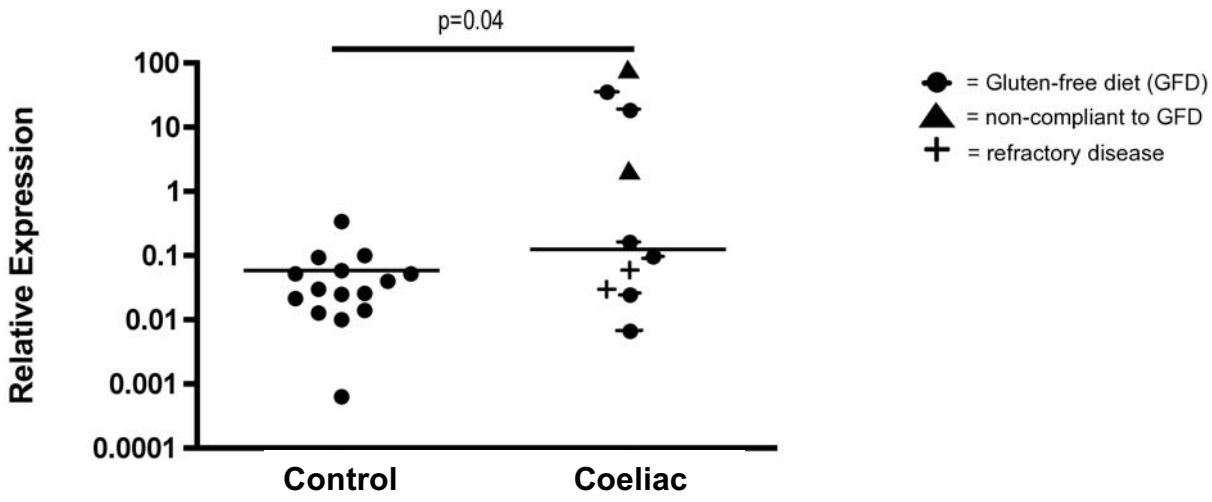


Figure 4.11 Disease Activity and IL-17a expression in coeliac disease. Coeliac disease patients were identified as those maintaining a gluten-free diet, those non-compliant to a gluten-free diet and those with refractory disease. No relationship between disease activity and IL-17 expression was observed.

4.4.8 Relative expression of IL-23 in the intestinal mucosa

IL-23 has been closely tied to the development and stabilisation of the Th17 phenotype (Aggarwal *et al.*, 2003; Harrington *et al.*, 2005; Langrish *et al.*, 2005; Yen *et al.*, 2006), and high levels of IL-23 have been described previously in IBD (Kobayashi *et al.*, 2008). To confirm whether the high expression of IL-17a is associated with high levels of IL-23, the relative expression of IL-23 was investigated in the intestinal biopsies of IBD, coeliac and control patients.

No significant increase was observed in IL-23 expression in the intestinal mucosa of IBD patients or coeliac patients compared to the control group (**Figure 4.12**). IL-23 was below detectable levels for three CD, five UC, three coeliac and two control patients, suggesting it was expressed in very low levels in the intestinal mucosa. IBD and coeliac patients however, exhibited much more variation in IL-23 expression than the control group. The CD patients that expressed greater levels of IL-23 coincidentally were those diagnosed with active disease. The two coeliac disease patients with higher IL-23 expression however were those compliant to a gluten-free diet.

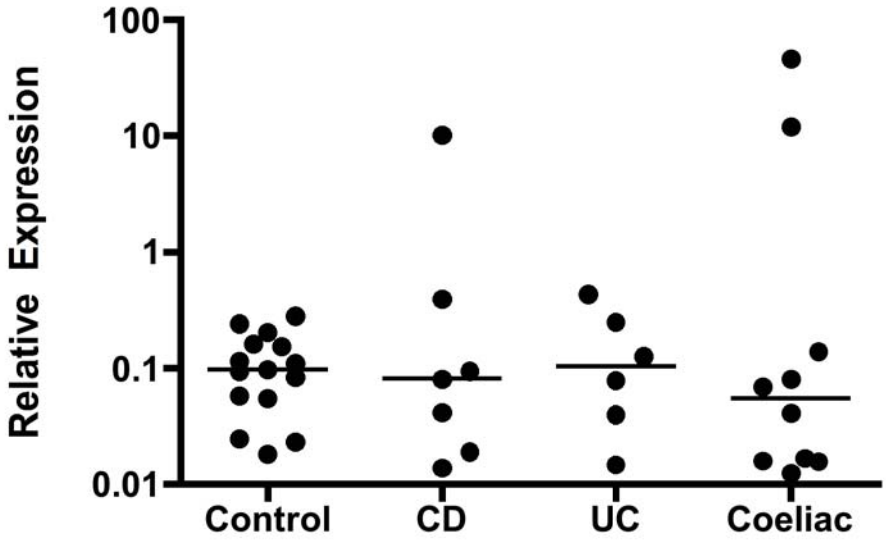


Figure 4.12 Relative expression of IL-23 in the intestinal mucosa of IBD, coeliac and control patients. RNA extracted from patient biopsies was converted to cDNA and IL-23 mRNA was measured using real time RT-PCR, and normalised to β-actin. IL-23 was below detectable levels for several patients, and was not found to differ in the remaining patient cohorts to the control patients.

4.4.9 Relative Expression of Effector Cell Transcription Factors in the Intestinal Mucosa

To confirm the involvement of specific T helper transcription factors in IBD and coeliac disease, the expression of transcription factors for Th1 cells (T-bet), Th2 cells (GATA-3) and Th17 cells (as measured by the human orthalog, RORC) was investigated in the intestinal biopsies of these patient cohorts and compared to control subjects. The relative expression of T-bet (**Figure 4.13**) was elevated in both CD ($p=0.03$) and also in UC patients ($p=0.025$). No significant change in T-bet expression was observed for coeliac disease patients. GATA3 expression was not found to be significantly increased in IBD or coeliac subjects (**Figure 4.14**). Despite the significant increase in IL-17a expression in IBD and coeliac disease, no significant differences were observed in RORC expression compared with controls (**Figure 4.15**). The IBD and coeliac disease cohorts however, exhibited greater variation in GATA3 and RORC expression than the control group that exhibited a consistently low expression of RORC.

A closer investigation of the patients with the highest IL-17a expression in the intestinal tissue revealed that in CD, these same patients also had the highest expression of T-bet, GATA3 and RORγ-t. This trend was not observed in UC or coeliac patients, where patients with high IL-17a expression expressed only low levels of T-bet, GATA3 and RORC.

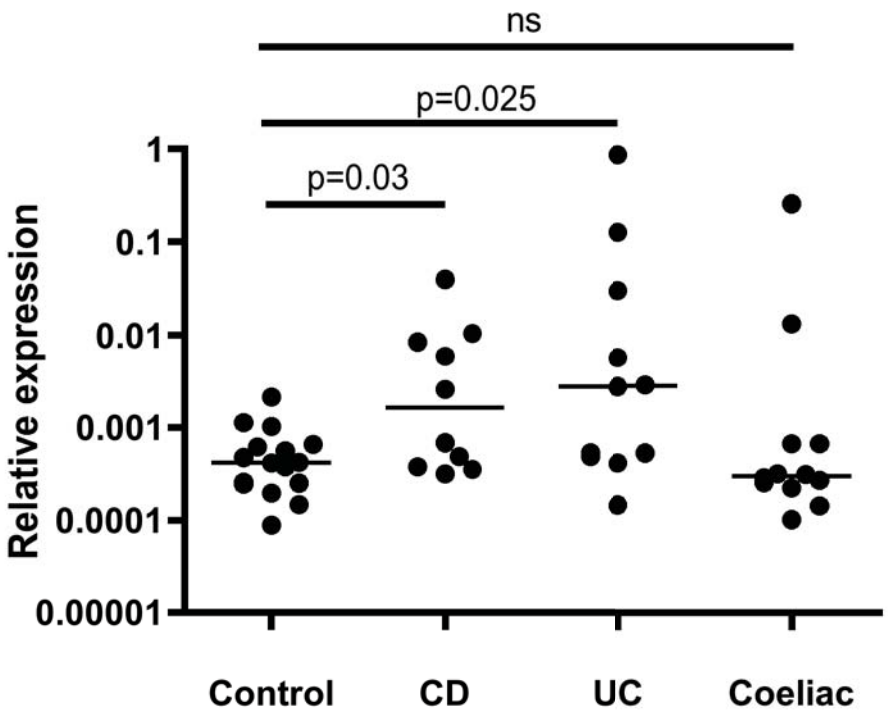


Figure 4.13 Relative Expression of T-bet in the intestinal mucosa of IBD, coeliac and control patients. RNA was extracted from intestinal biopsies of IBD, coeliac and control subjects. T-bet mRNA expression was quantified using real time RT-PCR, and normalised to β-actin expression. A significant increase in T-bet expression was demonstrated in CD ($p = 0.03$) and UC ($p = 0.025$) compared to the control subjects. No change in T-bet expression was observed in coeliac disease. Each point represents an individual subject, with horizontal lines indicating the median.

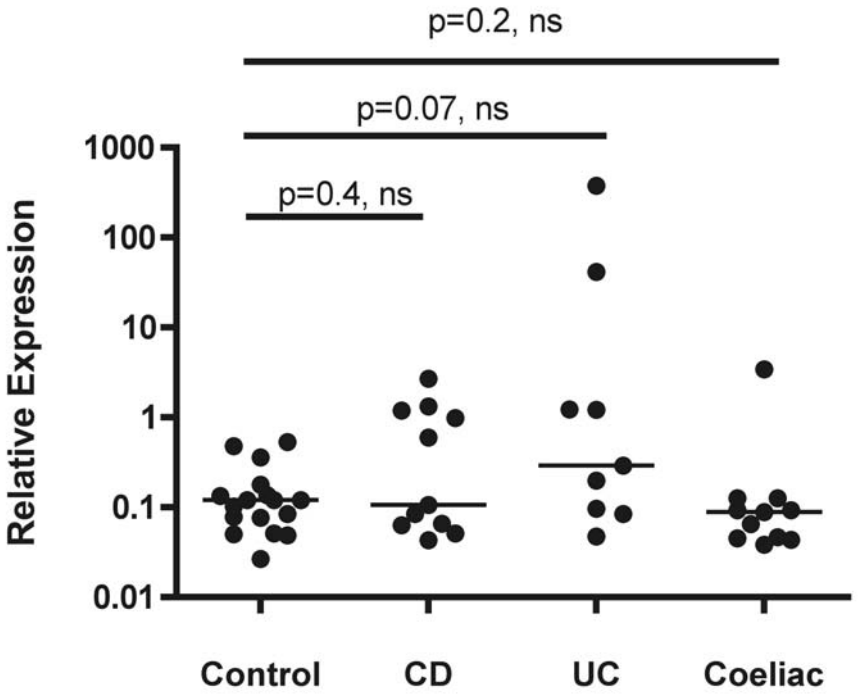


Figure 4.14 Relative Expression of GATA3 in the intestinal mucosa of IBD, coeliac and control patients. RNA extracted from intestinal biopsy samples were quantified for GATA3 relative expression using real time RT-PCR and normalised to β-actin expression. No significant alterations of GATA3 expression were observed in IBD or coeliac disease when compared to control subjects.

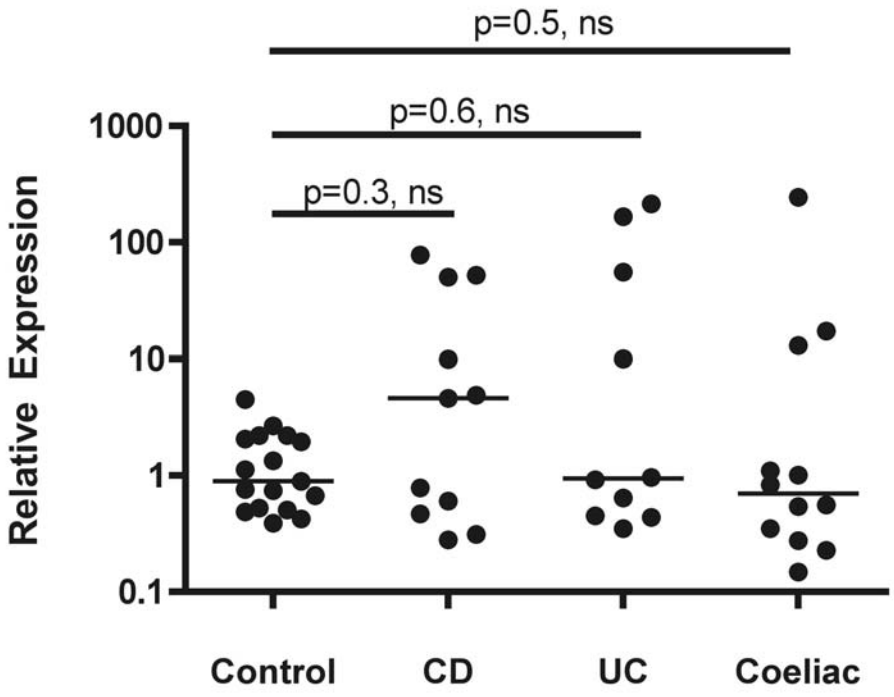


Figure 4.15 Relative Expression of RORC in the intestinal mucosa of IBD, coeliac and control patients. RORC relative expression was measured from the intestinal samples of IBD, coeliac disease and control subjects using real time RT-PCR. Gene expression was normalised to β-actin expression. No significant change in RORC expression was observed in IBD and coeliac disease subjects compared to control subjects.

4.5 Discussion

The current study has demonstrated that $CD4^+$ T cells exist in the peripheral blood that produce the proinflammatory cytokine IL-17a, moreover their measurement in the peripheral blood is dependent upon appropriate stimulation for cytokine production. Optimisation of the stimulation protocol for PBMCs isolated from the peripheral blood confirmed that PMA and ionomycin mimic T cell activation and is appropriate for IL-17a production. Stimulation for 5 hours results in optimal production of IL-17a, with the expression of IL-17a mRNA peaking at 4 hours of stimulation with PMA and ionomycin and rapidly declining after 10 hours. The stimulation of PBMCs to produce cytokine, however, results in the downregulation of CD4 surface expression. The use of CD3 as a substitute for CD4 was confirmed, as negligible amounts of IL-17a were produced by $CD8^+$ T cells. Intracellular labelling of IL-17a involved the fixation and permeabilisation of PBMCs after stimulation, and this procedure resulted in high levels of non-specific staining. After optimisation, a protocol using careful washing and a blocking step involving a 5% non-fat dry milk powder (Foster *et al.*, 2007) in conjunction with isotype matched controls, ensured the level of non-specific staining was negligible.

After optimisation of methods to measure IL-17 from PBMCs, $CD3^+$ $IL\text{-}17^+$ T cells were measured in IBD, coeliac disease and control subjects. High levels of IL-17 have been previously demonstrated in IBD, however investigation into Th17 cells in IBD or coeliac disease has been limited. It was found that significantly higher levels of $CD3^+$ $IL\text{-}17a^+$ T cells were present in the peripheral blood of IBD patients, and a trend towards elevated cell numbers was observed in some coeliac patients. The relative

expression of IL-17a was investigated in the intestinal biopsies of IBD and coeliac patients. High levels of IL-17a were detected in CD, UC and coeliac disease patients when compared with a control group, supporting observations that the proinflammatory cytokine IL-17a may have been associated with these gastrointestinal diseases (Fujino *et al.*, 2003; Nielsen *et al.*, 2003). IL-17a expression was greatest in UC with a 1000-fold increase in expression observed compared to the control group. A 100-fold and 10-fold increase was identified in CD and coeliac disease patients, respectively.

IL-23 has been linked with the development, stability and lifespan of the Th17 cell (Aggarwal *et al.*, 2003; Harrington *et al.*, 2005; Langrish *et al.*, 2005; Yen *et al.*, 2006). To investigate whether high levels of IL-23 were associated with IBD and coeliac disease, we measured the relative expression of this cytokine in the intestinal mucosa of patient cohorts. Despite the important role of IL-23 in the maintenance of Th17 cells, no increase in IL-23 was observed in IBD or coeliac disease patients. IL-23 was in fact below detectable levels in a number of IBD and coeliac patients. High levels of IL-17 in our patient groups may therefore not have been related to high levels of IL-23 in the intestinal mucosa. In both CD and UC a strong association has been reported from genome wide association studies with polymorphisms in the IL-23 receptor (IL-23R). Polymorphisms in the IL-23R of IBD patients may allow for the activation of the receptor with low levels of IL-23, and in turn result in the increased production and stabilisation of IL-17 in the absence of excessive IL-23. In fact, lamina propria T cells isolated from surgically resected specimens from IBD patients have shown increased expression of IL-23R (Kobayashi *et al.*, 2008).

Addition of the Th17 cell to the Th1/Th2 adaptive immunity paradigm has prompted reassessment of diseases previously categorised as Th1 or Th2. CD and coeliac disease have been categorised as Th1 disease, with UC a predominantly Th2 disease (Torres and Rios, 2008). However, inclusion of the Th17 cell prompts revision of this paradigm. Transcription factors driving the differentiation of Th1, Th2 and Th17 cells were investigated in patient cohorts to determine the involvement of these cell types in disease. A significant increase in the Th1 transcription factor, T-bet, was observed in both CD and UC, however an increase in T-bet was not observed in coeliac disease despite its characterisation as a Th1 disease. High levels of T-bet have been reported in coeliac disease in the past (Monteleone *et al.*, 2004). However, the notable absence of IL-12, a predominantly Th1 cytokine, has put question into the involvement of Th1 effector cells in coeliac disease (Salvati *et al.*, 2002). The increase in T-bet expression however, was only observed in patients with active coeliac disease and was not evident in patients on a gluten-free diet (Monteleone *et al.*, 2004). Significant variation in GATA3 and RORC relative expression was observed compared to the control group that consistently exhibited minimal variation of all three of these. However, there was no significant increase in expression of these transcription factors was observed in the patient cohorts.

Discovery of Th17 cells that produce the cytokine IFN-γ has been reported (Acosta-Rodriguez *et al.*, 2007; Chen *et al.*, 2007; Wilson *et al.*, 2007). These Th17 cells were also demonstrated to express both Th1 and Th17 transcription factors T-bet and RORγ-t (Annunziato *et al.*, 2007). Th1/Th17 cells have also been detected in the intestinal tissue of patients with active Crohn's disease with a reduced susceptibility to the suppression by autologous $Foxp3^+$ Tregs (Annunziato *et al.*, 2007). Th17 and Th17/Th1 cells

expressed IL-23R, but did not proliferate in response to IL-23. However, these cells were induced to produce IL-17 and IFN-γ in the presence of IL-12 (Annunziato *et al.*, 2007). This suggested that although previously described as Th1 diseases, CD and coeliac disease may be the result of Th1/Th17 effector cells that produce IL-17 or IFN-γ depending on the cytokine microenvironment. The Th2 cytokine IL-4, however, is not produced by Th17 cells (Annunziato *et al.*, 2007).

Treatment for IBD has currently focused on suppressing Th1 and Th2 cytokines, such as TNF-α, a crucial mediator of the Th1 response. Anti-TNF-α treatments, (eg. Infliximab) down regulate the production of TNF-α and IFN-γ in the mucosa and has a positive effect in 60% of CD patients (Ricciardelli *et al.*, 2008). Despite the usefulness of these medications, negative consequences can arise from the broad-spectrum approach of immune suppressor drugs, including an increased risk of infection and malignancy. These medications also do not treat all cases and relapse of disease is common (Hwang and Varma, 2008). The finding of high levels of Th17 cells in the peripheral blood of IBD patients, and in the intestinal biopsies of both IBD and coeliac disease patients suggests an association of the proinflammatory Th17 cell with intestinal inflammation in these diseases. The factors promoting Th17 cell differentiation require further exploration in IBD, in order to promote the development of more specific therapies to treat these diseases that target specific cell types without disarming the immune system.

4.5.1 Summary

In summary, this chapter has detailed the optimised techniques employed in measuring Th17 cells from the peripheral blood. From these optimised protocols the production of IL-17a by T cells was confirmed to require stimulation by PMA and Ionomycin for 5 hours, and strict blocking steps were required to avoid non-specific staining. Th17 cells were demonstrated to be elevated in the peripheral blood of IBD patients, and IL-17a mRNA expression was elevated in the intestinal mucosa of IBD and coeliac disease patients. In contrast, IL-23 expression did not vary between the patient cohorts and control subjects. The transcription factors for Th1 and Th2 cells were investigated and an increase in T-bet expression was observed in IBD. The increase in T-bet and also Th17 suggests the possible involvement of both Th1 and Th17 cells in IBD. To completely understand the involvement of Th17 cells in disease, the cytokine microenvironment must be considered that drives the differentiation of Th17 effector cells and allow for more specific treatment strategies.

CHAPTER 5:

THE CYTOKINE MICROENVIRONMENT

IN

INFLAMMATORY BOWEL DISEASE

AND

COELIAC DISEASE

5.1 Introduction

The differentiation of naïve T cells into Th1 and Th2 effector cells is well accepted, with established paradigms describing the cytokine driven differentiation of these cells. Antigen presenting cells such as macrophages and dendritic cells contribute to effector cell differentiation by producing large quantities of cytokines upon activation (Sanchez-Munoz *et al.*, 2008). The cytokine microenvironment is therefore critical in promoting the differentiation of T helper cells and this microenvironment is maintained in a finely tuned homeostatic state in the healthy gut. An imbalance of cytokines in the gut microenvironment, however, may drive the pathogenic inflammatory response that typifies IBD and coeliac disease.

Th17 effector cells have been investigated in a range of autoimmune diseases including IBD, and a Th17 immune response appears to be involved in autoimmune disease pathogenesis (Bettelli *et al.*, 2007). Mechanisms leading to the differentiation of Th17 effector cells require investigation in order to understand why these cells may predominate in certain diseases. Whilst a clearer understanding has been reached in murine systems, human Th17 differentiation appears to be more complex. Three independent research groups have identified IL-6 and TGF-β as central to Th17 differentiation in the mouse (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). These studies also confirmed that IL-23 is not required for Th17 differentiation, but may play a role in their maintenance (Bettelli *et al.*, 2006). The differentiation requirements of human Th17 cells differs from murine Th17 differentiation, with the suggested involvement of other cytokines. No conclusive cytokine combination has been agreed upon, however IL-6 and TGF-β alone have not been shown to induce

differentiation of human Th17 cells (Wilson *et al.*, 2007). A range of cytokines have been implicated in the differentiation of this effector cell, including, IL-6, TGF-β, IL-1β, IL-21 and IL-23. The differences in cytokine requirements could result from *in vitro* experimental variation, including the origin of cells used for differentiation (peripheral blood or cord blood) or different culture media.

Acosta-Rodriguez *et al* (2007) reported that IL-1β and IL-6 induced human Th17 cell differentiation, whereas TGF-β and IL-12 suppressed Th17 differentiation. They demonstrated IL-1β in particular was a potent inducer of human Th17 cells and these cells additionally expressed IFN-γ (Acosta-Rodriguez *et al.*, 2007). Manel *et al* (2008) proposed that TGF-β derived from serum in culture media could yield confounding results, and that true TGF-β free conditions (with neutralising antibody to TGF-β) did not increase Th17 cell differentiation. Under these 'serum-free' conditions, the cytokines determined to be required for human Th17 cell differentiation is TGF-β, IL-1β and IL-23. IL-6 and IL-21 were not found to play a significant role (Manel *et al.*, 2008).

Volpe *et al* (2008) titrated TGF-β into a human naïve T cell differentiation assay and revealed that TGF-β regulated IL-17 production in a dose dependent manner and was necessary together with IL-1β, IL-6 and IL-23, for the differentiation of the Th17 cell (Volpe *et al.*, 2008). Blockade of TGF-β with a neutralising antibody significantly decreased IL-17 production, as did neutralising antibodies to IL-6 and IL-1β (Volpe *et al.*, 2008). In 2008, Yang *et al* implicated IL-21 as another contender in Th17 differentiation, acting in conjunction with TGF-β to suppress IFN-γ expression and induce differentiation of Th17 cells (Yang *et al.*, 2008). This group suggested that IL-1β

and IL-6 or IL-23, induced IL-17 production from memory T cells. However IL-21 and TGF-β are required for Th17 differentiation from naïve T cells (Yang *et al.*, 2008). The role of IL-21, however, is unclear, as it has been shown to be involved in animal models of colitis (Fantini *et al.*, 2007), but not in EAE (Coquet *et al.*, 2008). Determining the combination of cytokines that support human Th17 differentiation may enable a greater understanding of the mechanisms by which they arise *in vivo*, and this subject requires further investigation. Preliminary findings suggest that IL-1β, IL-6, IL-21, IL-23 and TGF-β may all be involved in the development of Th17 cells from naïve T cells and memory cells.

IL-6 is produced by dendritic cells, macrophages, monocytes, mast cells, tumour cells, fibroblasts, endothelial cells, keratinocytes and activated T cells (Van Snick, 1990). IL-6 exerts its effect on cells by binding to the IL-6 receptor (IL-6R) on a target cell where it initiates intracellular signalling. The classic IL-6R is expressed only on hepatocytes, macrophages, neutrophils and some lymphocytes (Mitsuyama *et al.*, 2006). However a soluble IL-6R subunit is expressed ubiquitously, and can bind to a soluble form of the IL-6R, in a process termed 'trans-signalling' (Mudter and Neurath, 2007).

High levels of both IL-6 and the soluble IL-6R have been described in CD, UC and coeliac disease (Mitsuyama *et al.*, 1995; Reimund *et al.*, 1996; Fornari *et al.*, 1998; Brown *et al.*, 2002; Romaldini *et al.*, 2002). Serum IL-6 has also been shown to directly correlate with clinical activity of disease (Hyams *et al.*, 1993), and high levels were predictive of relapse in Crohn's disease (Van Kemseke *et al.*, 2000). In both CD and UC, IL-6 levels were found to be more predictive than C-reactive protein levels in measuring disease activity (Holtkamp *et al.*, 1995). HLA-DQ2 negative coeliac disease

patients have also demonstrated an increased frequency of genes relating to IL-6 production (Garrote *et al.*, 2005). The involvement and importance of IL-6 in Th17 differentiation is demonstrated clearly in IL-6 deficient mice, as these lack Th17 cells (Korn *et al.*, 2007). Furthermore the administration of a neutralising antibody to IL-6 halts murine Th17 cell differentiation (Veldhoen *et al.*, 2006). IL-6 also contributes to inflammation by enhancing T-cell survival and resistance to apoptosis, which results in T cell accumulation and lead to chronic inflammation (Atreya *et al.*, 2000). In addition, IL-6 promotes tumour growth and may be a causative factor behind the increased rate of colon cancers seen in IBD (Becker *et al.*, 2005).

IL-1β is also a known mediator of inflammatory immune responses. It is a pyrogenic cytokine, produced in response to infection, that increases body temperature resulting in fever (Church *et al.*, 2008). IL-1β induces expression of matrix metalloproteases, cytokines and chemokines, and increases expression of adhesion molecules in endothelial cells (Barksby *et al.*, 2007). It is produced by a range of cells including monocytes, macrophages, neutrophils and hepatocytes, epithelial cells and synovial fibroblasts (Arend *et al.*, 2008). High levels of IL-1β have been reported in rheumatoid arthritis (Firestein *et al.*, 1990; Ulfgren *et al.*, 2000) and in IBD (Woywodt *et al.*, 1994; Fukushima *et al.*, 1995; Reimund *et al.*, 1996; Dionne *et al.*, 1997). Unlike other characteristic proinflammatory cytokines, such as TNF-α, IL-1β is detected throughout the onset of rheumatoid arthritis, whereas TNF-α is only detected in the early stages of disease (Firestein *et al.*, 1990; Ulfgren *et al.*, 2000). IL-1β is also present in non-inflamed intestinal tissue of IBD patients in remission (Woywodt *et al.*, 1994; Fukushima *et al.*, 1995; Reimund *et al.*, 1996; Dionne *et al.*, 1997). Increased IL-1β disrupts intestinal epithelial tight junctions, increasing permeability of the epithelial

barrier through mechanisms independent of apoptotic pathways (Al-Sadi and Ma, 2007). Resolution of inflammation is attributed to the presence of a natural IL-1 receptor agonist (IL-1Ra), where a balance between IL-1 and IL-1Ra appears to determine the pathological effects of IL-1 (Arend, 2002). High levels of IL-1 are present in IBD patients, accompanied by low levels of IL-1Ra (Isaacs *et al.*, 1992). In contrast, untreated coeliac disease patients have normal IL-1Ra levels but high levels of IL-1β and IL-6 (Fornari *et al.*, 1998). The high levels of IL-1β in coeliac disease have been attributed to increased nitric oxide production as a result of gluten exposure (Beckett *et al.*, 1999). Exposure of PBMCs from coeliac disease patient to wheat gliadin increases production of IL-1β, IL-23 and TNF-α, and interestingly, IL-1β alone triggered IL-23 production in the absence of gluten exposure (Harris *et al.*, 2008).

IL-21 is produced by activated T cells and natural killer cells, and promotes additional cytokine production from CD4 and CD8 T cells (Fantini *et al.*, 2007). IL-21 is involved in critical B cell functions including immunoglobulin production, terminal differentiation of B cells to plasma cells and induces B cell apoptosis (Spolski and Leonard, 2008). IL-21 regulates the proliferation and effector functions of CD8 T cells, and maturation and functional development of NK and NKT cells (Spolski and Leonard, 2008). As described above, IL-21 is also a critical cytokine in Th17 development. Th1 and Th2 cells both produce IL-21 (Wurster *et al.*, 2002; Chtanova *et al.*, 2004), although highest IL-21 levels are produced by Th17 cells (Korn *et al.*, 2007; Nurieva *et al.*, 2007). IL-21 production is highly induced by IL-6, and induction of IL-21 leads to further upregulation of additional IL-21 production (Zhou *et al.*, 2007). IL-21 also induces expression of IL-23R, which is critical in maintenance and survival of Th17 cells (Korn *et al.*, 2007; Nurieva *et al.*, 2007). Elevated levels of IL-21 have been

described in mouse models of autoimmune disease, including animal models of systemic lupus erythematosus and murine diabetes (Spolski and Leonard, 2008). Elevated IL-21 levels have also been reported by western blot analysis in the gut mucosa of IBD patients (Monteleone *et al.*, 2005) and high expression of IL-21 mRNA demonstrated in duodenal biopsies of coeliac disease patients (Fina *et al.*, 2008). IL-21R positive cells were also increased in the inflamed mucosa of IBD patients and NKT cells derived from IBD intestinal samples exhibited more potent activity in response to IL-21 (Liu *et al.*, 2009).

TGF-β has typically been described as an immunosuppressive, anti-inflammatory cytokine associated with oral tolerance, a process by which systemic unresponsiveness to ingested antigens is attained (Weiner, 2001). Reduced TGF-β levels have been implicated in inflammatory gastrointestinal diseases including IBD (Marek *et al.*, 2002). However, as described previously, TGF-β may also be required for differentiation of proinflammatory Th17 cells. TGF-β is produced by neutrophils, natural killer cells, monocytes, macrophages, dendritic cells and mast cells (Wahl *et al.*, 2006). It is initially secreted in a latent form that undergoes conformational changes before its release as a mature, active form (Schmidt-Weber and Blaser, 2006). TGF-β has a range of functions that promotes both inflammation and tolerance. For example, TGF-β sustains inflammation by promoting cell proliferation, differentiation and initiation of leukocyte chemotaxis (Wahl *et al.*, 2006). However, TGF-β also exerts suppressive activity through its ability to inhibit Th1 and Th2 differentiation, and its promotion of apoptosis (Wahl *et al.*, 2006). TGF-β is critical in murine Th17 differentiation, as the absence of the TGF-β receptor in animal models precluded Th17 cell development and confers protection against experimental autoimmune encephalitis (EAE) (Veldhoen *et al.*,

2006). Intense debate is evolving over the involvement of TGF-β in human Th17 differentiation. Several groups have proposed that TGF-β appears to be a necessary factor for Th17 differentiation (Manel *et al.*, 2008; Volpe *et al.*, 2008; Yang *et al.*, 2008). However, it has been argued that TGF-β promotes Th17 differentiation by suppressing Th1 development, as Th17 cells are less susceptible to the suppressive properties of TGF-β (Annunziato *et al.*, 2008). TGF-β may therefore promote Th17 development by suppressing T-bet expression and the development of Th1 cells (Santarlasci *et al.*, 2009).

The aim of this chapter was to investigate the involvement of Th17 promoting cytokines in IBD. The expression of IL-6, IL-1β, IL-21 and TGF-β was investigated in the intestinal mucosa of IBD, coeliac disease and control patients, to determine if aberrant expression of these cytokines is a feature of these gastrointestinal diseases.

5.2 Aims and Hypothesis:

The general hypothesis of this chapter was that elevated Th17 levels in the peripheral blood and intestinal mucosa of IBD and coeliac disease patients is the result of a cytokine microenvironment that is conducive to Th17 development.

Aims:

Compared to a control group, the aims were to measure and compare

1) Relative expression of IL-1β in IBD and coeliac disease
2) Relative expression of IL-6 in IBD and coeliac disease
3) Relative expression of TGF-β in IBD and coeliac disease
4) Relative expression of IL-21 in IBD and coeliac disease

5.3 Methods

5.3.1 Subjects

IBD patients were recruited from the Department of Gastroenterology at The Queen Elizabeth Hospital, Woodville, South Australia. Intestinal biopsy samples were collected from patients undergoing endoscopy with the Department of Gastroenterology and Hepatology at The Queen Elizabeth Hospital. Biopsy samples were collected from seventeen control, eleven CD, twelve UC and fourteen coeliac subjects. Samples were stored in RNALater (Ambion, USA) at -20C to prevent RNA degradation prior to extraction.

5.3.2 RNA extraction and cDNA production

Total RNA was isolated from intestinal biopsies using the RNeasy Lipid Minikit (Qiagen, Vic, Australia). RNA gel electrophoresis was performed to assess RNA quality. RNA (1μg) was reverse transcribed to obtain complementary DNA (cDNA) using a Qiagen Quantitect Reverse transcription kit (Qiagen, Vic, Australia). Primers were designed to span an intron of the genomic sequence (See Chapter 2 for detailed methods).

5.3.3 Real time RT-PCR

Quantitative real time RT-PCR was carried out using a Corbett Rotorgene RG-3000 (Corbett Research, Sydney Australia), with two replicates per sample, a no-template control and no-reverse transcription control for each experiment. All reactions were conducted using Power SYBR green master mix (2x) solution (Applied Biosystems, CA, USA). A melt curve analysis and gel electrophoresis were performed to confirm

the formation of specific PCR products. Expression of IL-17 mRNA was normalised to β-actin expression. PCR products were purified using Qiaquick PCR purification kit (Qiagen, Vic, Australia) and sequenced at the Flinders University DNA Sequencing Facility (Department of Haematology, Flinders Medical Centre, SA, Australia). The PCR product sequences were confirmed using the National Center for Biotechnology Information basic local alignment search tool (BLAST).

5.3.4 Statistics

Real time RT-PCR results were analysed using the ΔCT method with Q-gene software (Simon, 2003), taking into account individual primer efficiencies. Real time RT-PCR results are presented as normalised mean expression. The Mann-Whitney ranked sums test was employed to determine any differences between mean normalised gene expression in disease and control groups. All statistical analyses were carried out using GraphPad Prism software.

5.4 Results

5.4.1 Relative expression of IL-1β in the intestinal mucosa

In order to measure levels of IL-1β in IBD and coeliac disease, RNA was extracted from intestinal biopsies and IL-1β mRNA was quantified using real time RT-PCR. IL-1β was detected in seventeen control, eleven CD, twelve UC and fourteen coeliac disease patients. The relative expression of IL-1β mRNA was significantly increased in CD ($p= 0.0005$) and UC ($p=0.0003$) patients when compared to the control group (**Figure 5.1**). An increase in IL-1β expression was observed in three coeliac disease patients, however no statistically significant increase was detected within the coeliac disease cohort.

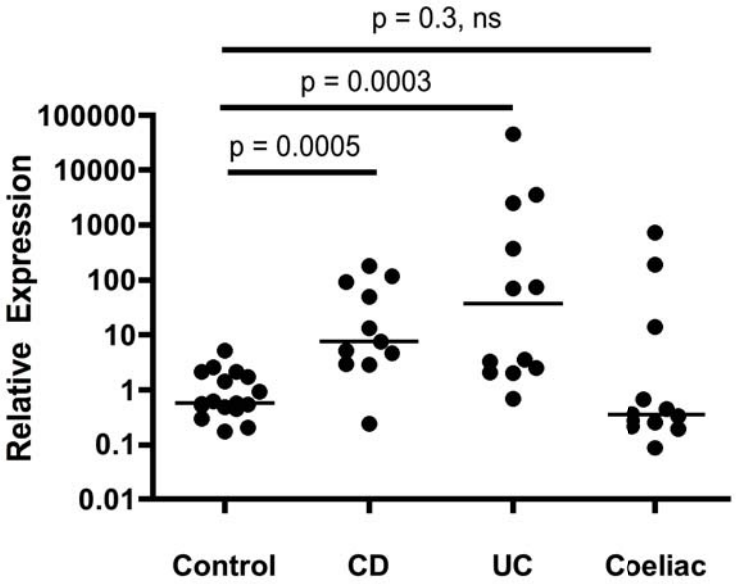


Figure 5.1. Relative expression of IL-1β mRNA in the intestinal mucosa of control, CD, UC and coeliac disease patients. RNA was extracted from the intestinal biopsies of IBD, coeliac disease and control subjects, and quantified using real time RT-PCR and normalised to β-actin. A statistically significant increase in IL-1β mRNA expression was observed in CD (p=0.0005) and UC (p=0.0003) patients when compared to the control group. No significant increase in IL-1β was observed in coeliac disease. Each dot represents an individual patient with the horizontal line indicating the median.

5.4.2 IL-1β expression and disease activity in IBD and coeliac disease

In order to determine the contribution of individual patient disease activity upon IL-1β expression, the disease status of each patient was investigated and overlayed upon IL-1β relative expression graphs. IBD patients were classified into categories of disease inactivity (remission), mild disease activity and moderate disease activity based on QEH hospital histology and pathology reports. IL-1β expression was highest in UC patients with moderate disease activity and lower in six out of eight patients with mild and inactive disease (**Figure 5.2**). There was no relationship between disease category and IL-1β expression in CD.

Disease activity in coeliac disease patients was separated into patients compliant to a gluten-free diet, those ingesting a normal diet (non-compliant to a gluten-free diet), and those diagnosed with refractory sprue, in which disease was not controlled by a gluten-free diet. No relationship was observed between disease activity and IL-1β expression. Three subjects demonstrated elevated IL-1β levels, however two of these subjects were reportedly compliant to a gluten-free diet. The subject with the highest IL-1β expression was non-compliant to a gluten-free diet, however the other subject non-compliant to a gluten-free diet exhibited low levels of IL-1β (**Figure 5.3**).

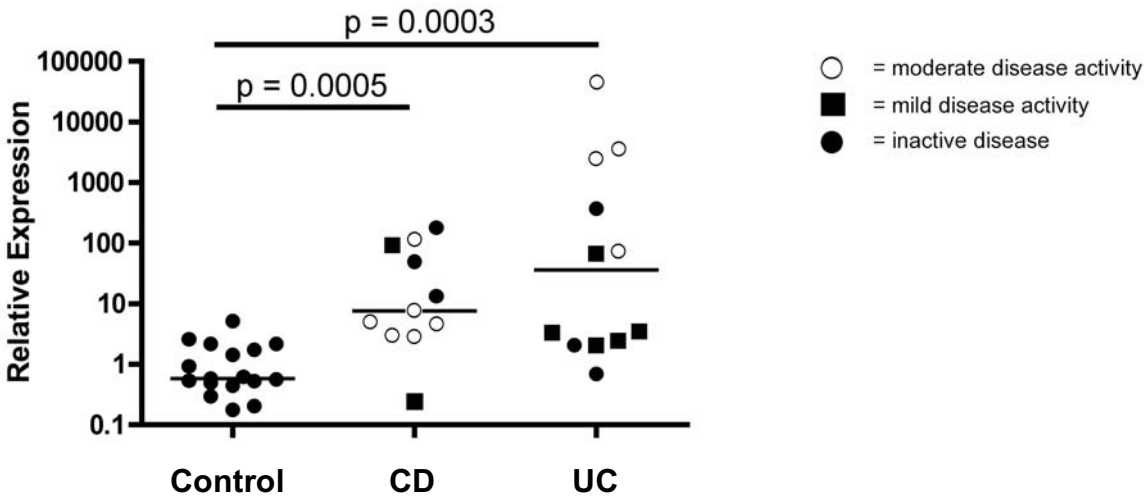


Figure 5.2 IL-1β relative expression and disease activity in IBD. The disease status of IBD patients was determined from histological pathology reports and overlayed onto IL-1β relative expression graphs. No relationship was observed between disease activity and IL-1β expression in CD. However disease activity appeared to be related to IL-1β expression in UC with higher IL-1β expression in those patients with moderate disease.

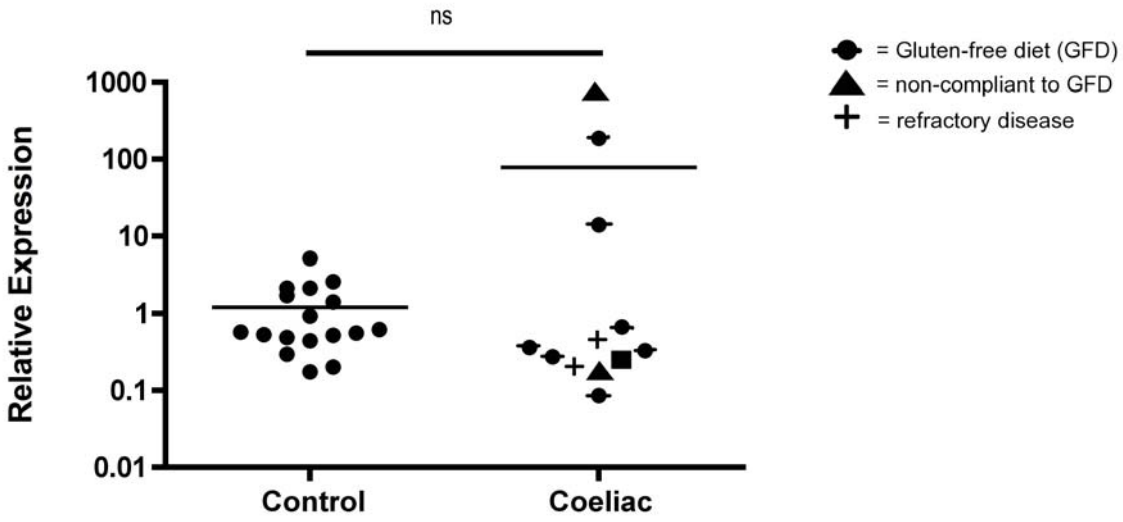


Figure 5.3 IL-1β expression and disease activity in coeliac disease. Coeliac subjects were separated into patients following a gluten-free diet, non-compliant to a gluten-free diet and with refractory disease, and overlayed upon IL-1β relative expression. No relationship was found between disease activity and IL-1β expression.

5.4.3 Relative expression of IL-6 in the intestinal mucosa

RNA extracted from the biopsies of IBD and coeliac disease patients were analysed for IL-6 expression using real time RT-PCR. IL-6 expression was measured in 17 control, eleven CD, twelve UC and twelve coeliac disease patients. Increased expression of IL-6 was demonstrated in CD ($p=0.007$) and UC patients ($p=0.003$) when compared to the control group (**Figure 5.4**). A significant decrease in IL-6 expression was observed in coeliac disease patients when compared to the control group ($p=0.005$).

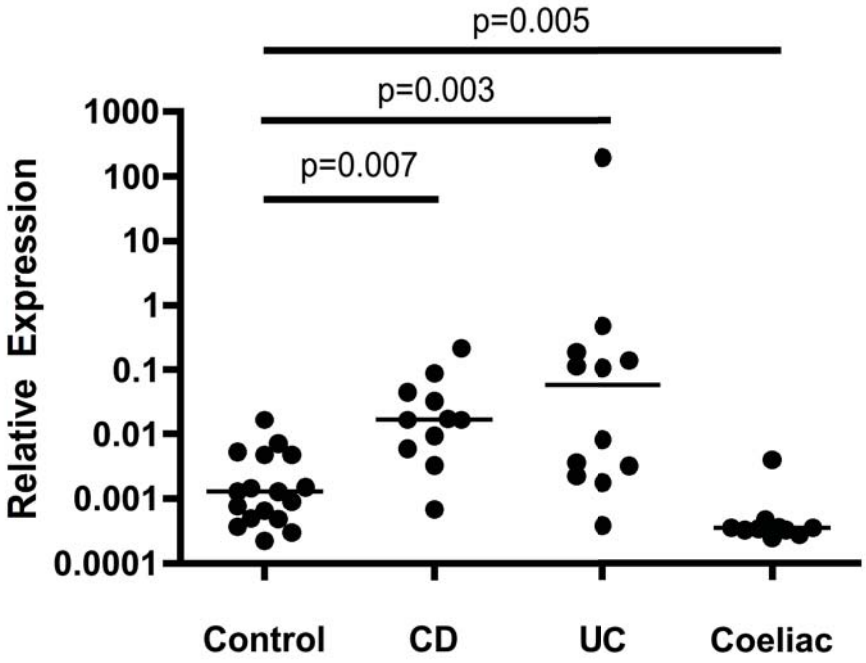


Figure 5.4. Relative expression of IL-6 mRNA in the intestinal mucosa of IBD, coeliac and control patients. RNA extracted from the intestinal biopsies of patient cohorts was quantified through real time RT-PCR and normalised to β-actin. The relative expression of IL-6 was significantly increased in CD (p=0.007) and UC (p=0.003) patients. IL-6 mRNA was significantly lower in coeliac disease patients compared to the control group (p=0.005). Individual patients are represented as a point, with the group median indicated with a horizontal line.

5.4.4 Disease activity and IL-6 expression in IBD and coeliac disease

The contribution of disease activity to relative expression of IL-6 was determined by investigating the disease status of each patient and overlaying disease activity on IL-6 relative expression graphs. Disease activity was determined from QEH histological and pathological reports, and IBD patients were categorised as either in inactive disease (remission), mild disease activity or moderate disease activity (**Figure 5.5**). No clear relationship was apparent between disease activity and IL-6 expression in CD, however in UC IL-6 expression was highest in UC patients with moderate disease activity. Coeliac disease patients were classified as ingesting a gluten-free diet, non-compliant to a gluten-free diet, and those diagnosed with refractory disease. IL-6 levels were significantly lower in coeliac disease patients than the control group, however one patient non-compliant to a gluten-free diet expressed high levels of IL-6 (**Figure 5.6**).

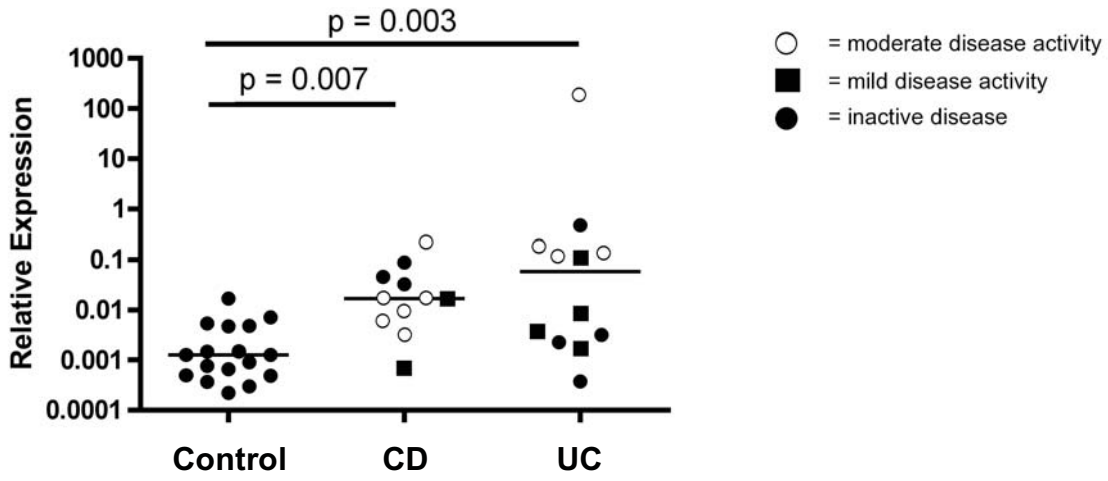


Figure 5.5 IL-6 relative expression and disease activity in IBD. The disease status of IBD patients was overlayed onto IL-6 relative expression plots. UC patients with moderate disease activity showed elevated IL-6 expression, however, no relationship was observed between disease activity and IL-6 expression in CD.

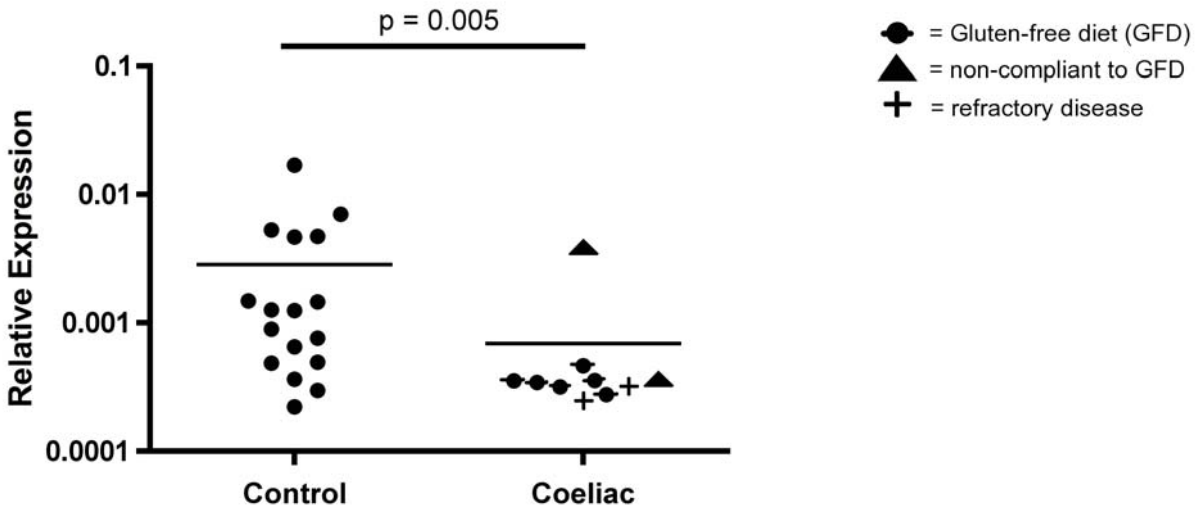


Figure 5.6 IL-6 relative expression and disease activity in coeliac disease. Coeliac disease patients were identified as compliant to a gluten-free diet, non-compliant to a gluten-free diet and those with refractory disease. No relationship between disease activity and IL-6 expression was observed for the coeliac disease cohort.

5.4.5 Relative expression of TGF-β in the intestinal mucosa

The involvement of TGF-β in IBD and coeliac disease was investigated through the extraction of RNA from biopsy samples and quantitation of TGF-β using real time RT-PCR. TGF-β was detectable in eleven control, ten CD, eleven UC and eleven coeliac disease patients (**Figure 5.7**). Expression of TGF-β was consistently low in control patients with expression maintained within tight limits. Increases in TGF-β expression were observed in CD, UC and coeliac disease groups, however, this was only found to be statistically significant in UC ($p=0.04$). The median TGF-β expression for UC patients was ten times greater than the control group, with several patients exhibiting up to a 10,000-fold increase in TGF-β. Several CD and coeliac disease patients also exhibited increased levels of TGF-β.

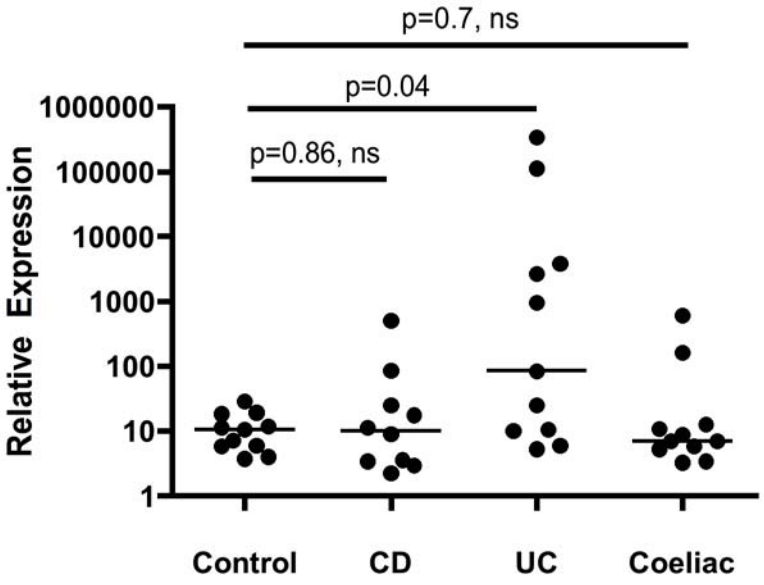


Figure 5.7 Relative expression of TGF-β in the intestinal mucosa of IBD, coeliac disease and control subjects. RNA was extracted from intestinal mucosal biopsies and expression of TGF-β was determined by real time RT-PCR and normalised to β-actin expression. A significant increase in TGF-β expression was evident only in UC. Each point represents an individual patient, with horizontal lines representing the group median.

5.4.6 Disease activity and TGF-β relative expression in IBD and coeliac disease

In order to determine the impact of disease activity upon TGF-β production in both IBD and coeliac disease, the disease activity of these patients was overlayed onto graphs of TGF-β relative expression (**Figure 5.8**). Relative expression of TGF-β was not predicted by disease activity in CD. In UC, highest TGF-β expression was observed in a sub-group of patients with mild disease activity. Intermediate levels of TGF-β were observed in UC patients with moderate disease activity. Levels of TGF-β were not predictive of disease activity in coeliac disease, with the highest TGF-β expression detected in a patient reportedly compliant to a gluten free diet and also by a patient non-compliant to a gluten-free diet (**Figure 5.9**).

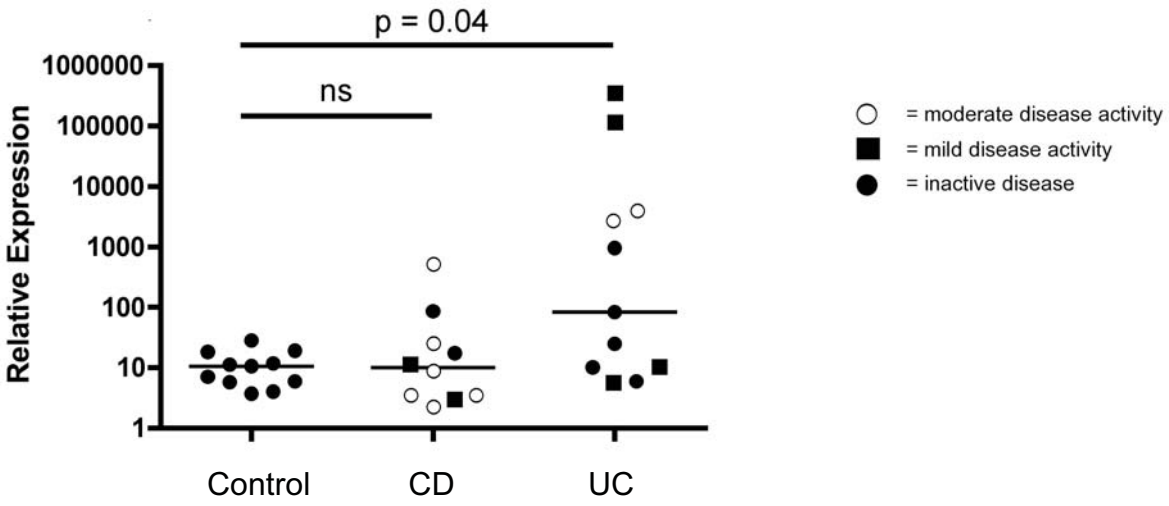


Figure 5.8 TGF-β relative expression and disease activity in IBD. The disease status of IBD patients was overlayed on TGF-β relative expression graphs. The relative expression of TGF-β did not reflect disease status in CD. In UC, high and low levels of TGF-β corresponded with mild disease activity, and an intermediate expression of TGF-β was expressed by patients with moderate disease activity.

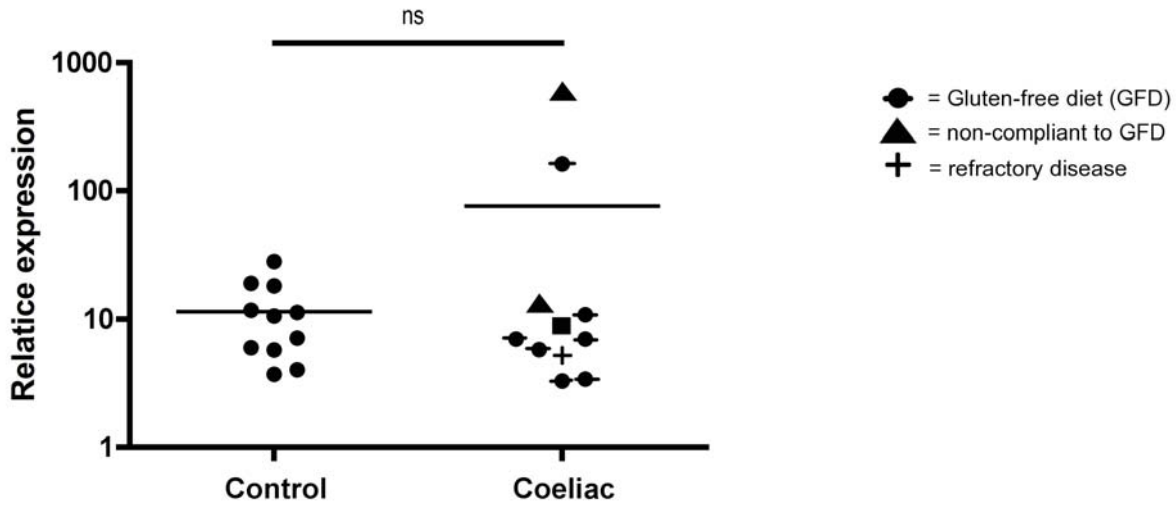


Figure 5.9. **TGF-β relative expression and disease activity in coeliac disease.** Coeliac disease patients were identified as compliant to a gluten-free diet, non-compliant to a gluten-free diet and those with refractory disease. No relationship between disease activity and TGF-β expression was observed in the coeliac disease cohort.

5.4.7 Relative expression of IL-21 in the intestinal mucosa

IL-21 expression was investigated in intestinal biopsies of IBD and coeliac disease patients using real time RT-PCR, and compared to a control group (**Figure 5.10**). IL-21 was detected in eight control, five CD, two UC and six coeliac disease patients, however, IL-21 was below detectable levels in the majority of patients. No significant changes in IL-21 levels were observed amoung IBD, coeliac and control patients. However, the small size of biopsy samples collected may have hindered efforts to measure IL-21 accurately.

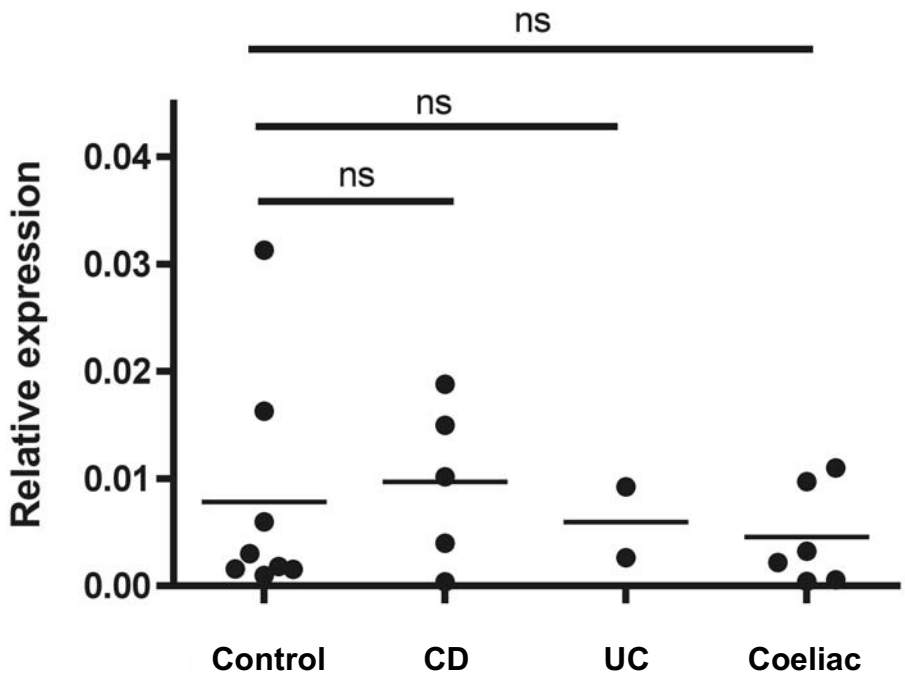


Figure 5.10 Relative expression of IL-21 in the intestinal mucosa of IBD, coeliac and control patients. RNA was extracted from patient biopsy samples and quantified using real time RT-PCR and normalised against β-actin. No statistical differences were observed. IL-21 was undetectable in the majority of samples.

5.4.8 Inter-relationship of IL-1β, IL-6, TGF-β and IL-17a

In order to determine whether the expression of Th17 promoting cytokines were associated with high IL-17a levels, individual patients were monitored with regard to their IL-17a, IL-6, IL-1β and TGF-β relative expression (**Figure 5.11**). Patients with CD and UC exhibiting high expression of IL-17a also had high levels of IL-1β and IL-6. This was not the case in coeliac disease, where only one of the three patients with the highest IL-17a expression exhibited high levels of IL-1β and IL-6. The remaining coeliac patients with high IL-17a expression exhibited low or undetectable levels of IL-1β and IL-6. A similar pattern was observed with TGF-β, where one coeliac patient with high levels of IL-17a revealed high levels of TGF-β. The remaining coeliac patients with high IL-17a expression demonstrated low or below detectable levels of TGF-β. An association between TGF-β and IL-17a relative expression was observed in both CD and UC patients. Although a significant increase in TGF-β was not observed in CD, patients with high TGF-β expression also exhibited high levels of IL-17a, IL-1β and IL-6. High expression of TGF-β appeared to also be associated with high levels of IL-17a in UC. However, two patients with the highest TGF-β expression expressed low levels of IL-17a. These two patients also expressed low levels of both IL-1β and IL-6. Unfortunately the sample sizes for these disease groups were too small for meaningful correlational analyses.

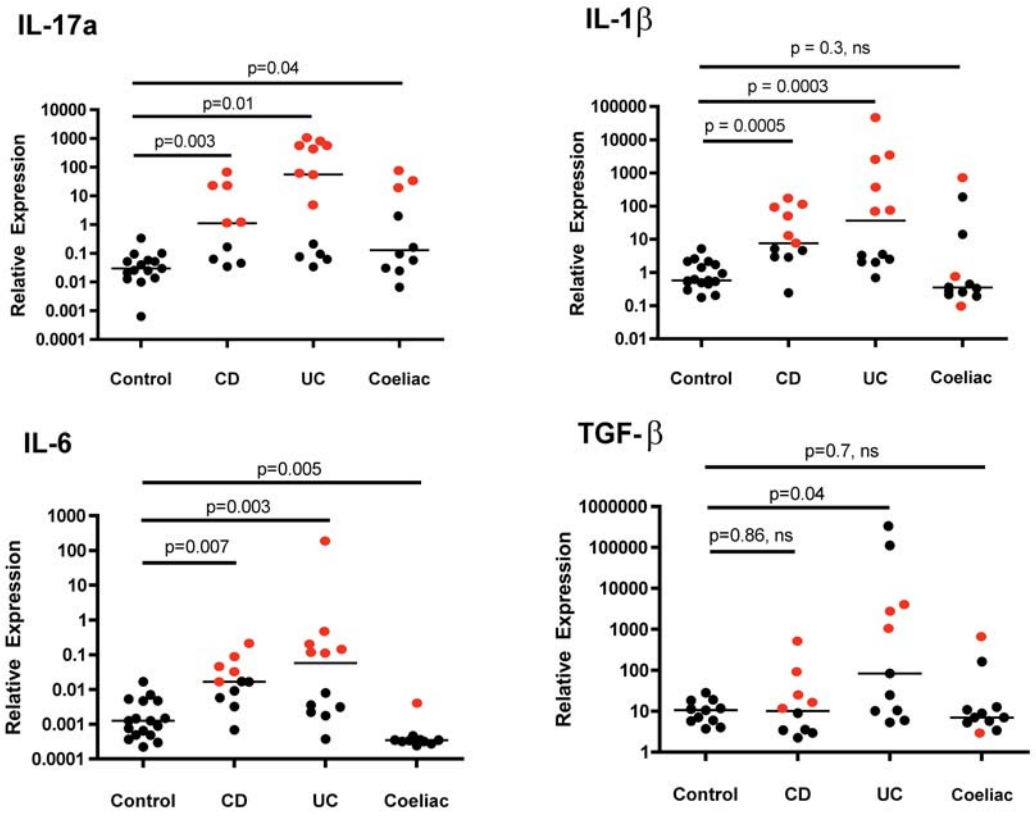


Figure 5.11. The inter-relationship of IL-17a, IL-1β, IL-6 and TGF-β relative expression from intestinal biopsies of IBD, coeliac and control subjects.

Relative expression of IL-17a from Chapter 4, was compared to the relative expression of IL-1β, IL-6 and TGF-β. Patients with high IL-17a expression are highlighted in red and monitored for levels of IL-1β, IL-6 and TGF-β. High expression of IL-17a correlated with high levels of IL-1β and IL-6 in IBD. Patients with high expression of TGF-β showed high levels of IL-17a. However, the highest TGF-β expression was associated with low levels of IL-17a, IL-6 and IL-1β. No relationship between IL-17a expression with IL-1β, IL-6 and TGF-β levels was observed in coeliac disease.

5.5 Discussion

Investigation of the Th17-promoting cytokine microenvironment was prompted by the discovery of high IL-17a expression in both IBD and coeliac disease in our disease cohorts (Chapter 4), and also from previous studies (Fujino *et al.*, 2003; Nielsen *et al.*, 2003; Annunziato *et al.*, 2007; Holtta *et al.*, 2008). We questioned whether the increased IL-17a expression observed was the result of an intestinal microenvironment that promoted development of Th17 cells. In IBD, we have demonstrated expression of IL-1β and IL-6 significantly higher than the control group, and a significant increase in TGF-β in UC patients. By monitoring the cytokine expression of individual patients, an association between IL-1β, IL-6, TGF-β and IL-17 was observed. IBD patients with high IL-17a expression concomitantly expressed high levels of the Th17 promoting cytokines, IL-1β, IL-6 and TGF-β. Despite finding high levels of IL-17a in coeliac disease patients (Chapter 4), the levels of Th17 promoting cytokines remained low, with no significant increase when compared to the control group.

High levels of IL-1β and IL-6 observed in the intestinal biopsies of both CD and UC patients may have promoted the development of Th17 cells from memory cells (Yang *et al.*, 2008). High numbers of memory cells resident in the gut may provide a large reservoir of Th17 precursors and this may be particularly relevant in IBD (Yen *et al.*, 2006). Proinflammatory cytokines are predominantly produced by antigen presenting cells, such as dendritic cells and macrophages, in response to the prevailing microbial environment of the gut (Quintana and Weiner, 2009). Animal studies have revealed an increased frequency of Th17 cells with the alteration of commensal bacteria, suggesting

a critical role of bacteria in limiting or promoting the development of Th17 cells (Zaph *et al.*, 2008).

Increased IL-1β levels found in intestinal biopsies from IBD patients in the current study may be linked to genetic factors in IBD. The proinflammatory nature of IL-1β requires that its production and release be tightly controlled. Unlike other cytokines that are controlled at the transcriptional level, IL-1β is also controlled through post-transcriptional mechanisms (Pan *et al.*, 2007). Two fundamental signals are thought to be required for the release of IL-1β. Firstly, toll-like receptors (TLRs) and their cytoplasmic counterpart, NOD-like receptors (NLRs) sense bacterial peptoglycans, and induce the transcription and storage of pro-IL-1β (Arend *et al.*, 2008; Church *et al.*, 2008). Secondly, pro-IL-1β is cleaved by the cysteine protease caspase-1 to release active IL-1β (Church *et al.*, 2008). NOD2 is an intracellular receptor for bacterial peptoglycans that has a dual effect in activating the transcription of pro-IL-1β, and inducing the release of active IL-1β indirectly through the activation of caspase-1 (Ferwerda *et al.*, 2008). Mutations in the NOD2 gene are associated with an increased risk of CD (Hugot *et al.*, 2001; Ogura *et al.*, 2001), and incorrect sensing by NOD2 may contribute to the increased production of IL-1β, and therefore the increased Th17 production evident in CD.

In the current study, low levels of IL-1β and IL-6 were detected in the intestinal biopsies of coeliac disease patients, despite high levels of IL-17. Levels of IL-6 were in fact significantly lower in coeliac disease than in the control group. This reduction in IL-6 mRNA may reflect the loss of IL-6 producing enterocytes secondary to villus atrophy. This suggests that high levels of IL-17a in the intestinal mucosa in coeliac

disease were unlikely to be associated with IL-6 mediated induction of Th17 cells and may, alternatively, be the result of an alternative pathway requiring IL-21 and TGF-β (Yang *et al.*, 2008). Whilst high levels of IL-21 in the intestinal biopsies of coeliac disease have been reported by other groups (Fina *et al.*, 2008), this was not demonstrated in the current study. Previous studies, however, have examined IL-21 expression in untreated coeliac disease patients, whereas the majority of the subjects in this study were compliant to a long-term gluten free diet. IL-21 mRNA levels were found to be low and below the detectable range for the majority of all patient cohorts. High levels of IL-21 previously reported in IBD were demonstrated using western blotting and densiometry scanning to measure levels of protein extracted from intestinal biopsies (Monteleone *et al.*, 2005). High levels of protein, however, may not correlate with mRNA expression. These previous studies also used much larger tissue samples from resected specimens compared to the pinch biopsies we obtained. In the current study, whereas expression of IL-21 may be variable in IBD, signalling through IL-21 may still represent a key mechanism in IBD pathogenesis. An increase in IL-21R in the intestinal biopsies of IBD patients has been demonstrated (Liu *et al.*, 2009), which may imply an increased sensitivity to low levels of IL-21 in the intestinal mucosa.

In the current study, expression of TGF-β in the intestinal mucosa did not vary between control, CD and coeliac disease. A significant increase in TGF-β expression, however, was found in UC patients, with half of these patients indicating a 100-fold to 10,000-fold increase in TGF-β. UC patients with the highest TGF-β expression concurrently expressed low levels of IL-17a, IL-1β and IL-6. UC patients with an intermediate expression of TGF-β exhibited high levels of IL-17a, IL-1β and IL-6. It is suggested that TGF-β could act in a suppressive or proinflammatory manner based on its

concentration, whereby at low concentrations, in combination with IL-6 and IL-21, it promotes expression of IL-23R (Zhou *et al.*, 2008). At high concentrations, TGF-β may inhibit the production of pro-inflammatory cytokines from T cells, and thereby inhibit generation of Th17 cells (Korn *et al.*, 2009). The role of TGF-β appears to be context dependent, with high TGF-β alone promoting immune homeostasis. However in an environment of high proinflammatory cytokines such as IL-1β and IL-6 the generation of Th17 cells ensues. This is confirmed in CD, whereby although a significant increase in TGF-β was not observed, patients with highest TGF-β expression concurrently expressed highest IL-17a, IL-1β and IL6 levels. High levels of TGF-β have also previously been reported in lamina propria mononuclear cells from UC patients but not in CD (Del Zotto *et al.*, 2003).

Cua and Kastelein (2006) have suggested a model to explain the dual nature of TGF-β in mice. This model proposes that homeostasis in the gut is maintained by high concentrations of TGF-β that induce Foxp3+ Tregs. In the case of infection, toll-like receptors trigger dendritic cells to produce large quantities of cytokine such as IL-6 and IL-23. The production of Th17 cells is preferential in this environment and protects the host immediately through its activation of neutrophils and other mechanisms. The continual production of high levels of TGF-β, IL-6 and IL-23 promote additional Th17 cell differentiation, and circulating naïve cells that arrive in this environment are driven to differentiate into Th17 cells. With the resolution of infection lower levels proinflammatory cytokines are produced and the microenvironment returns to a state of homeostasis with high levels of TGF-β and lower levels of Th17 promoting cytokines (Cua and Kastelein, 2006).

The control patients of our study however, expressed low levels of TGF-β, in conjunction with low levels of IL-1β, IL-6, IL-17a and also low Foxp3. We propose that Treg and Th17 cells remain in a state of balance within the gut, with changes in cytokine concentrations within the microenvironment promoting or suppressing the development of Th17 or Treg cells to control inflammation thus enabling protective immunological responses. The relapsing and remitting nature of IBD may be the result of a continual fluctuation of these cytokines and the inability to maintain a state of equilibrium. In IBD, varying TGF-β levels may contribute to both Th17 production and immunoregulation via the inhibition of antigen presenting cell function and potentially the generation of Treg. The variability of TGF-β in IBD may also be an additional contributing factor to the increased incidence of cancer seen in both diseases, as TGF-β has been shown to support tumour growth and metastasis by suppression of the immune system (Wakefield and Roberts, 2002).

5.5.1 Summary

In summary, we have demonstrated that the intestinal microenvironment in IBD is conducive to the continued generation and development of Th17 effector cells. IL-1β was significantly increased in IBD, but not coeliac disease, compared to the control cohort. Elevated IL-1β expression appeared to be associated with disease activity only in UC, with high levels demonstrated in UC subjects with moderate disease activity. Expression of IL-6 was higher in IBD patients and a lower expression of IL-6 was observed in coeliac disease patients compared to the control group. IL-6 expression appeared to be only associated with moderate disease activity in UC, as these subjects exhibited the highest levels of IL-6. Disease activity was not predictive of IL-6 expression in CD or coeliac disease. TGF-β expression was revealed to be elevated in

UC compared to the control cohort. No change in TGF-β expression was detected in CD or coeliac disease. Monitoring of IBD and coeliac disease subjects with high IL-17a expression revealed elevated levels of IL-1β and IL-6 in these IBD subjects. High TGF-β expression in UC was associated with low IL-1β, IL-6 and IL-17a. The involvement of a Th17 immune response in IBD may therefore be the result of multiple factors promoting the differentiation of these cells. The clear understanding of the cytokines that promote the development of these pro-inflammatory effector cells may therefore allow for more effective treatment for IBD.

CHAPTER 6

THE RELATIONSHIP BETWEEN REGULATORY T CELLS AND TH17 CELLS IN INFLAMMATORY BOWEL DISEASE AND COELIAC DISEASE

6.1 Introduction

The reciprocal relationship between Treg and Th17 cells was identified through research into the Th17 differentiation pathway. It has since become clear that Th17 cells and Treg differentiate along a similar pathway, and that this process can be influenced by specific cytokines to directly alter the balance of Treg and Th17 cells. The reciprocal differentiation of Treg and Th17 cells was first reported in mice, in which T cell activation with anti-CD3 in the presence of IL-6 and TGF-β was shown to generate Th17 cells (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). However, activation of T cells in the presence of TGF-β resulted in the exclusive differentiation of Tregs with functional suppressive abilities (Bettelli *et al.*, 2006).

Autoimmune disease has been in the past described as a Th1/Th2 imbalance, in which a Th1 response predominates in diseases such as CD, multiple sclerosis, diabetes and rheumatoid arthritis (Crane and Forrester, 2005). This paradigm however only partially explains these disease states and does not explain the inability of anti-IFN-γ antibodies to abrogate disease (Theofilopoulos *et al.*, 2001; Boissier *et al.*, 2008). The addition of Treg and Th17 cells to the Th1 and Th2 paradigm is now of great interest. Indeed, autoimmune disease has been proposed to be a consequence of an imbalance of Treg and effector T cells (Afzali *et al.*, 2007).

The balance between Treg and Th17 cells has only recently been examined in human diseases. At the commencement of this study we sought to investigate a Treg/Th17 imbalance as a novel concept. During the course of this PhD project other studies have been published that support this hypothesis in a range of other autoimmune conditions.

Patients with juvenile idiopathic arthritis were found to not only have elevated IL-17 in the joints, but a reciprocal relationship between the $Foxp3^+$ Treg cell and Th17 cell number within the joint was also observed (Nistala *et al.*, 2008). An imbalance was also reported in primary biliary cirrhosis, an organ-specific autoimmune disease. Increased Th17 cells and decreased Treg cells were detected in the peripheral blood of these patients, with increased serum levels of the Th17 promoting cytokines IL-1β, IL-6 and IL-23 (Rong *et al.*, 2009). The involvement of these polarized cells types has also been investigated in acute coronary syndrome (ACS). Atherosclerosis is a chronic inflammatory disease characterised by upregulation of Th1 cells that result in plaque rupture and the development of ACS (Cheng *et al.*, 2008). Further investigation of Treg/Th17 involvement revealed an increase in peripheral Th17 numbers in conjunction with decreased $Foxp3^+$ Tregs, and this imbalance may have caused plaque destabilisation and the onset of ACS (Cheng *et al.*, 2008).

The reciprocal relationship between Treg and Th17 cells is likened to the relationship between Th1 and Th2 cells, whereby each cell produces cytokines and conditions that promote their own development but limit the development of the other. For example, in the case of Treg/Th17 cells, IL-6 trans-signalling via the soluble IL-6 receptor promotes Th17 differentiation but inhibits the development of adaptive Tregs possibly by suppressing Foxp3 expression (Ichiyama *et al.*, 2008), and also interferes with the suppressive functioning of established naturally occurring Tregs (Pasare and Medzhitov, 2003; Dominitzki *et al.*, 2007). A number of factors contribute to the polarisation of Treg and Th17 cells. IL-2 is specifically required for the development and function of Foxp3, as $IL\text{-}2^{-/-}$ mice have significantly decreased Treg numbers and exhibit lymphoproliferative disease (Papiernik *et al.*, 1998; Wolf *et al.*, 2001). IL-2 is

not required for Foxp3 induction, but is critical for the maintenance of Tregs (Fontenot *et al.*, 2005). IL-2 also serves as a growth factor for Th1 and Th2 cells. However, IL-2 inhibits Th17 cell differentiation, and blockade of IL-2 with neutralising antibody increases Th17 differentiation (Kryczek *et al.*, 2007; Laurence *et al.*, 2007). Activation of antigen presenting cells by specific gut bacteria can also alter the Treg/Th17 balance, as animal studies have shown that Th17 differentiation was correlated with the presence of *Cytophaga-Flavobacter-bacteroidetes*. An absence of this bacteria decreased IL-17 production and increased Foxp3+ regulatory T cells (Ivanov *et al.*, 2008).

The vitamin A metabolite, retinoic acid, is another regulator of Treg cells and inhibitor of Th17 cells. Retinoic acid is formed by the digestion of vitamin A and provitamin A carotenoids, with dendritic cells in the GALT capable of producing retinoic acid from vitamin A (Kim, 2008). Although not critical in the differentiation process, retinoic acid inhibits IL-17 expression *in vitro* and *in vivo* (Elias *et al.*, 2008). Activated T cells revealed reduced RORγ-t expression when cultured with retinoic acid (Mucida *et al.*, 2007). Animals infected with *Listeria monocytogenes* and treated with retinoic acid exhibited a significant reduction in mucosal Th17 cells compared with mice treated with a retinoic acid inhibitor (Mucida *et al.*, 2007).

Retinoic acid also supports the differentiation of TGF-β dependent regulatory T cells. Treg cells generated with TGF-β and retinoic acid represent a stable Treg lineage and remained $Foxp3^+$ after stimulation, unlike Treg differentiated with TGF-β alone that lost their Foxp3 expression (Mucida *et al.*, 2007). Human Tregs induced in the presence of retinoic acid exhibit the stable expression of Foxp3 and suppressive ability, but also high expression of gut homing receptors necessary for migration to the small intestine

(Kang *et al.*, 2007). The addition of a retinoic acid receptor agonist significantly reduces the expression of Foxp3 by cells cultured with TGF-β, and Foxp3 expression is enhanced by the addition of retinoic acid (Mucida *et al.*, 2007)..

Evidence supporting the concept of a reciprocal relationship between Treg and Th17 cells signifies an important new direction for autoimmune disease research. A disturbance in Tregs and Th17 homestasis may result in a range of inflammatory conditions. The investigation of the relationship between Treg and Th17 cells in IBD and coeliac disease may provide a clearer understanding of these diseases and offer a new direction in therapeutic opportunities that aim to re-establish this balance. The purpose of this chapter was to investigate both Treg and Th17 cells in a subgroup of patients, and to determine whether disease was characterised by a inequality in these cell numbers.

6.2 Aims and Hypothesis

The general hypothesis of this chapter was that a decrease in Treg and concomitant increase in Th17 cells is involved in the pathologies seen in IBD and coeliac disease.

Aims:

To measure and characterise the relationship between Tregs and Th17 cells:

1) in healthy controls
2) in Crohn's disease
3) in ulcerative colitis
4) in coeliac disease

6.3 Methods

6.3.1 Subjects

Treg and Th17 cells were both measured in the peripheral blood of thirteen control, seventeen CD, fifteen UC and thirteen coeliac patients. All IBD patients were diagnosed clinically and all were in a state of disease inactivity based on clinical notes and C-reactive protein levels (CRP<10). Coeliac disease patients were predominantly ingesting long-term gluten free diets. The mean age ± SEM for these cohorts were; control (38.7 ± 3.6 y), CD (37.15 ± 4.1 y), UC (45.7 ± 3.7 y) and coeliac (46.2 ± 4.0 y).

6.3.2 Flow Cytometry

PBMCs isolated from whole blood were collected and labelled with directly conjugated antibodies for Treg and Th17 markers detailed in Chapter 3 and 4. Tregs and Th17 cells were identified as $CD4^{+}$ $CD25^{high}$ $Foxp3^{+}$ and $CD3^{+}$ $IL\text{-}17^{+}$, respectively. Quantification was conducted using a BD Facscan (BD Biosciences, USA) and analysed using the Cell Quest analysis program (BD Biosciences, USA).

6.3.3 Statistics

Flow cytometric results were converted to absolute cell number based on individual absolute lymphocyte counts. Patient paired Treg and Th17 cell numbers were compared using a paired samples T-test. Differences between patient cohorts were analysed using the Mann-Whitney ranked sums test. Group ratios were determined by dividing the number of Treg by the number of Th17 cells in each patient. All data are expressed as mean ± standard error of the mean. All statistical analyses were carried out using GraphPad Prism software.

6.4 Results

6.4.1 Peripheral Tregs and Th17 cells in control subjects

Treg and Th17 cells were both measured in PBMCs of control subjects (n=13) using flow cytometry. Absolute numbers of circulating Treg and Th17 cells were obtained from flow cytometric data using patient lymphocyte counts. The mean ± SEM numbers of Treg and Th17 (cells per ml of whole blood) were 7.6 ± 1.0 $x10^3$ cells/ml and 7.5 ± 0.98 $x10^3$ cells/ml, respectively (**Figure 6.1**). Treg and Th17 cell numbers did not differ significantly from each other.

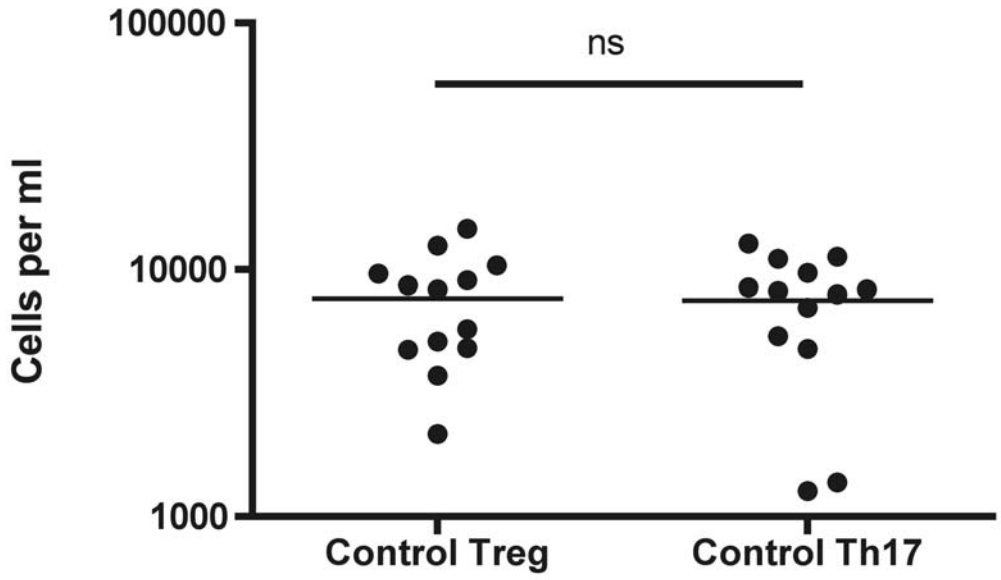


Figure 6.1. Treg and Th17 cells in control subjects. PBMCs extracted from the peripheral blood of a control group were analysed for both Treg and Th17 cells in the same patient using flow cytometry. No significant difference was observed between the Treg and Th17 numbers. Each individual patient is represented by a point, with group medians indicated by the horizontal line.

6.4.2 Peripheral Treg and Th17 cells in Crohn's disease

Treg and Th17 numbers were both measured from the PBMCs of CD patients using flow cytometry. The mean ± SEM Treg and Th17 cells (per ml of whole blood) in CD were 4.9 ± 0.62 $x10^3$ cells/ml and 18.2 ± 4.5 $x10^3$ cells/ml, respectively (**Figure 6.2**). A significant difference in Treg and Th17 cell numbers was observed ($p = 0.02$). Analysis of Treg and Th17 cell numbers comparing CD and control patients indicated a significant increase in Th17 cell numbers ($p = 0.007$) and a significant decrease in Treg numbers ($p = 0.03$) in CD compared to the control group (**Figure 6.3**).

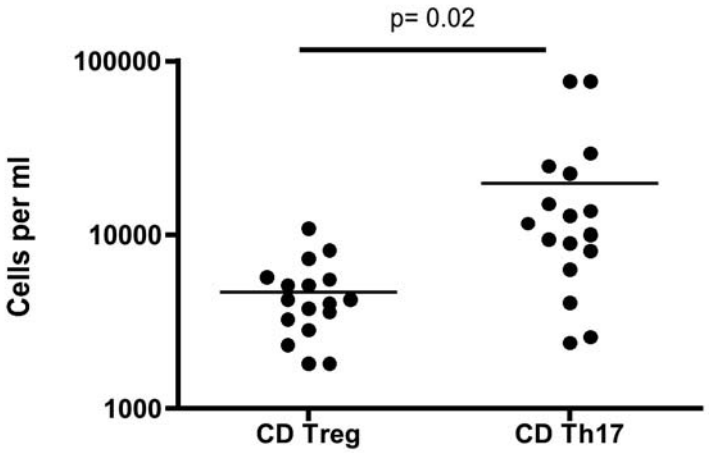


Figure 6.2. Treg and Th17 cells in CD. PBMCs isolated from the peripheral blood of CD patients were measured for both Treg and Th17 cells using flow cytometry. A disparity in Treg and Th17 cell numbers was observed between Treg and Th17 cells, with significantly higher Th17 cells observed with fewer Tregs. Individual patients are indicated by a point, with horizontal lines describing group medians.

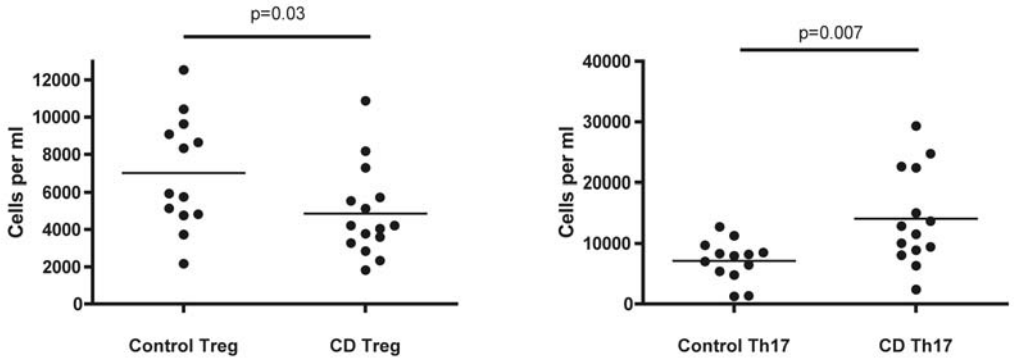


Figure 6.3. Quantification of Treg and Th17 cells in control and CD patients. Comparison of Treg and Th17 cell numbers obtained via flow cytometry of PBMCs in CD and control patients. A significant decrease in Treg cell numbers (p=0.03) and an increase in Th17 cell numbers (p=0.007) was observed in CD compared to the control group.

6.4.3 Peripheral Treg and Th17 in UC

Treg and Th17 cells were both measured in each patient using flow cytometry. Positive cell numbers were converted to absolute cell numbers using patient lymphocyte count information. The mean ± SEM Treg and Th17 cells (per ml of whole blood) in UC were 4.7 ± 0.6 $x10^3$ cells/ml and 17.1 ± 3.4 $x10^3$ cells/ml, respectively (**Figure 6.4**). An imbalance of Treg and Th17 cells was observed for the UC group (P=0.002) indicating a significant difference in Treg and Th17 cell numbers (**Figure 6.4**). Analysis between control and UC subjects (**Figure 6.5**) confirmed that UC patients had a significant increase in Th17 cells ($p = 0.03$) and a significant decrease in Treg ($p = 0.02$).

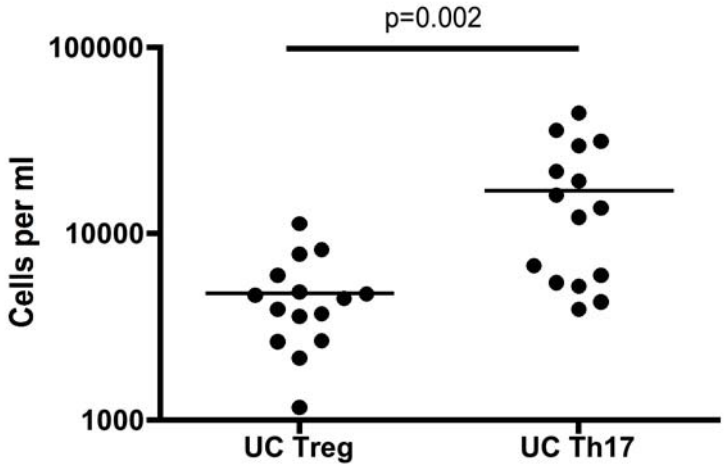


Figure 6.4. Treg and Th17 cells in UC. Treg and Th17 cell numbers were determined from flow cytometry of PBMCs extracted from patient peripheral blood. Elevated Th17 numbers were observed concomitant with decreased Treg numbers (p=0.002).

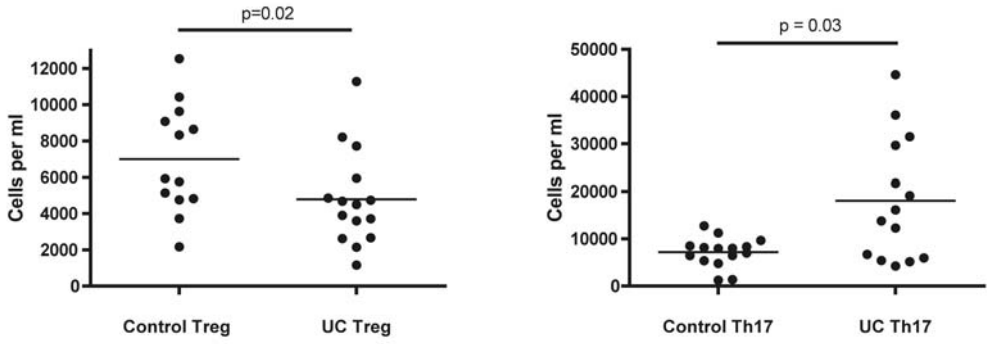


Figure 6.5. Comparison of Treg and Th17 cells in control and UC patients. Treg and Th17 cell numbers determined from flow cytometric analysis demonstrate a significant decrease in Treg and an increase in Th17 in UC patients compared to the control group.

6.4.4 Peripheral Treg and Th17 Cells in Coeliac Disease

Treg and Th17 cells were both measured in coeliac disease patients, and the numbers of these cells was compared within patients. Absolute cell counts were obtained from flow cytometric data and absolute lymphocyte counts. The mean ± SEM Treg and Th17 cells (per ml of whole blood) in coeliac disease were 5.46 ± 1.0 $x10^3$ cells/ml and 11.0 ± 2.02 $x10^3$ cells/ml, respectively (**Figure 6.6**). A significant difference in Treg and Th17 cell numbers was observed within coeliac disease patients ($p = 0.008$). Investigation into the numbers of Tregs and Th17 cells in coeliac disease compared to a control group indicated that these differences were not statistically significant (**Figure 6.7**).

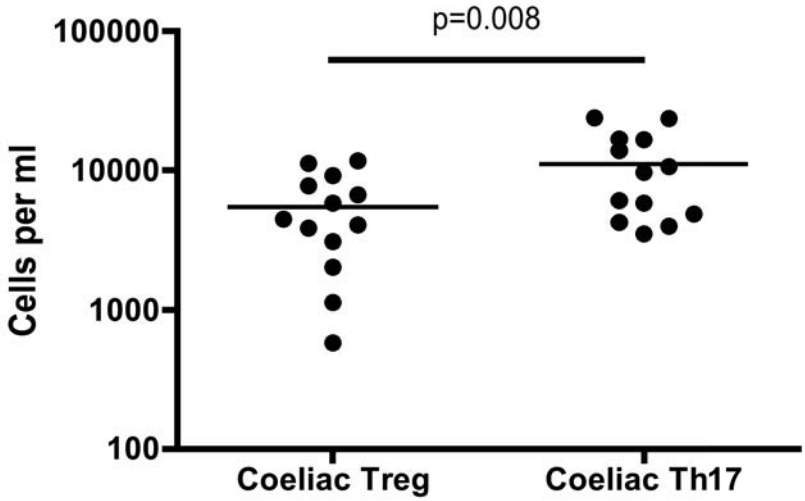


Figure 6.6. Treg and Th17 cells in coeliac disease. Treg and Th17 numbers were measured using flow cytometry of PBMCs extracted from the peripheral blood of coeliac disease patients. A decrease in Treg numbers and increased Th17 cells was observed in coeliac disease.

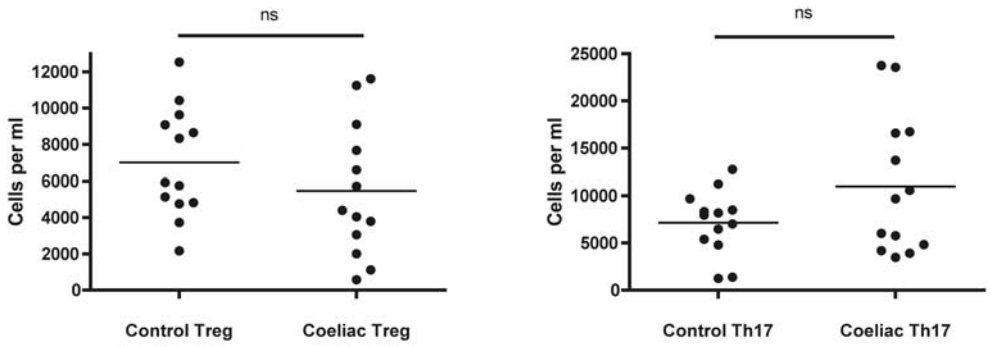


Figure 6.7. Comparison of Treg and Th17 numbers in coeliac and control patients. Treg and Th17 cell numbers as determined by flow cytometry, were compared in the control group and in coeliac disease patients. No significant differences were observed between these two groups.

6.4.5 The Ratio of Treg and Th17 Cells Within Patient Samples.

A decrease in Treg cell numbers and an increase Th17 cells was observed within CD, UC and coeliac patient groups. In order to further investigate this imbalance, the numbers of Treg and Th17 cells within each patient was investigated. A Treg/Th17 ratio was obtained for individual patients by dividing their absolute Treg cell number with their absolute Th17 cell number. The control group showed a near 1:1 ratio with an average Treg/Th17 ratio of 0.8 ± 0.004. The CD and UC group however, indicated an altered ratio of Treg and Th17 cells with average ratios of 0.55 ± 0.07 ($p=0.04$) and 0.35 ± 0.05 ($p=0.0002$) respectively (**Figure 6.8**). This suggests that within the patients of these groups, there are higher levels of Th17 cells to fewer Tregs, with a greater ratio difference observed in UC. Coeliac disease patients were not found to have altered Treg/Th17 ratio when compared to control patients, with an average ratio of 0.7 ± 0.01.

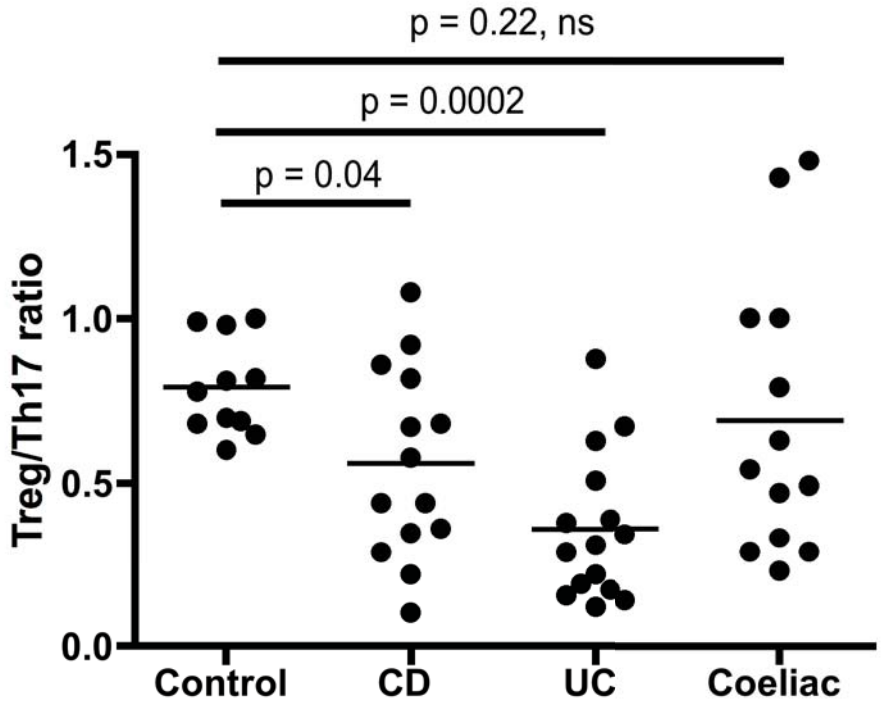


Figure 6.8. The ratio of Treg to Th17 cells within patient samples. Individual patient Treg and Th17 ratios was determined by dividing Treg by Th17 numbers acquired by flow cytometry for each patient. The control subjects demonstrated a near 1:1 ratio of Treg to Th17 cells. The CD and UC subjects, however, demonstrated a significantly lower ratio, indicating greater Th17 numbers to Treg in the peripheral blood of these individuals. The ratio of Treg to Th17 cells was not significantly altered within coeliac patients when compared to the controls.

6.5 Discussion

A developmental relationship between Treg and Th17 cells has only recently been identified, suggesting these cells differentiate from the same progenitor cell depending on cytokines in the microenvironment (Bettelli *et al.*, 2006; Veldhoen *et al.*, 2006). We, and others have demonstrated that the intestinal environment of IBD is characterised by an increase in the same proinflammatory cytokines that promote differentiation and development of the Th17 cell (Reimund *et al.*, 1996; Monteleone *et al.*, 2005; Kobayashi *et al.*, 2008). The link between Treg and Th17 cells implies that the environment in which they develop may result in the skewing of differentiation towards one cell type over the other. A range of other factors that promote the polarisation of Treg and Th17 cells, such as IL-2, retinoic acid, and the gut microbiota have also been identified. The relationship between Treg and Th17 cells has not yet been documented in either IBD or coeliac disease, and the finding that an imbalance exists has important therapeutic implications.

In the current study, equal Treg and Th17 cell numbers were found in the control group, with similar numbers of Treg and Th17 cells present in the peripheral blood. The balance of Treg and Th17 cell numbers, seen in our control cohort, suggests that a state of equilibrium exists between protective immunity and tolerance in health. In CD and UC, a discrepancy in Treg and Th17 numbers was observed with a significant increase in Th17 and a decrease in Treg cell numbers compared to the control group. In coeliac disease, an alteration in Treg and Th17 cell numbers was also revealed, with a trend towards increased Th17 cell numbers and decreased Treg numbers. However, an increase in Th17 and a decrease in Treg, compared to the control group was not

identified. This suggested that unlike the IBD patients, a decrease in Treg and an increase in Th17 cells in coeliac disease was only subtle, yet significant when regulatory and effector cells were compared together.

Control patients demonstrated a near 1:1 ratio of Treg to Th17 cells in the periphery, whereas the ratio of these cells in IBD was significantly decreased. This decreased ratio confirms an excess of Th17 cells in conjunction with a decrease in Treg in both CD and UC. An alteration in the Treg/Th17 ratio was greater in UC, where patients exhibited a greater number of Th17 cells to fewer Treg than the CD patients. Coeliac disease patients did not demonstrate a varied ratio of Treg to Th17 cells compared to the control group. A slight decrease in ratio was observed, however this was not statistically significant. These findings imply that an alteration in Treg and Th17 equilibrium may contribute to the pathogenesis of IBD, whereas other factors may predominate in coeliac disease. The factors that contribute to an imbalance between Treg and Th17 cell numbers therefore require further investigation.

The proinflammatory environment of IBD may promote the differentiation of Th17 cells in favor of Treg differentiation resulting in excessive and uncontrolled inflammation. We have shown an increase in IL-17 and Th17 promoting cytokines IL-1β and IL-6 in the intestinal biopsies of IBD patients (Chapter 4 and 5). High levels of IL-1β and IL-6 may promote the differentiation and development of Th17 cells and suppress the development of induced Treg cells in addition to impeding the functioning of mature Tregs (Pasare and Medzhitov, 2003; Dominitzki *et al.*, 2007; Ichiyama *et al.*, 2008). The functional capacity of Tregs may therefore be affected within this environment. Animal studies have shown that $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs can

differentiate into Th17 cells in the presence of IL-6 (Xu *et al.*, 2007). A subpopulation of human $CD25^{high}$ Foxp3+ regulatory T cells also co-expresses Foxp3 and RORγ-t, with the capacity to produce IL-17, when activated in the presence of IL-1β, IL-21 and IL-23 (Koenen et al., 2008; Voo et al., 2009). The deficiency of Tregs in the periphery and an increase in Th17 cells in IBD may therefore reflect the sequestration of Tregs to the gut and the hindering of their suppressive function by the proinflammatory environment.

Dendritic cells potentially play a critical role in the pathogenesis of IBD, through their ability to produce large quantities of cytokines that drive T cell differentiation (Kelsall and Leon, 2005). Dendritic cells of the gut related lymphoid organs also generate the production of retinoic acid, which is capable of suppressing Th1 and Th17 development and promoting Th2 and Treg development (Iwata *et al.*, 2003; Mucida *et al.*, 2007). Dendritic cells conditioned in an environment high in retinoic acid, induce less Th1 cells than conventional dendritic cells suggesting retinoic acid is an important factor in generating tolerance promoting dendritic cells (Wada *et al.*, 2009). Vitamin A, which is required for retinoic acid production, has been shown to be deficient in IBD patients (Main *et al.*, 1983; Bousvaros *et al.*, 1998), in addition to a deficiency in retinaldehyde dehydrogenase which is required for retinoic acid metabolism (Wada *et al.*, 2009). A synthetic retinoic acid with enhanced biological properties, AM80, has been shown to ameliorate EAE in mice in both early and late stages of disease (Klemann *et al.*, 2009). AM80 does not require retinaldehyde dehydrogenase for its metabolism, and a pilot clinical trial using AM80 to treat CD is currently underway in Japan (Wada *et al.*, 2009).

The imbalance of Treg and Th17 cells in IBD has implications for biological therapies that are demonstrated to affect these immunological pathways. Rapamycin and cyclosporin A are both immunosuppressive agents used to treat autoimmune disease, transplant rejection, and serious cases of IBD (Kountouras *et al.*, 2004). Cyclosporin A is a calcineurin inhibitor that inhibits IL-2 transcription, thereby depriving effector cells of necessary survival signals required for their differentiation and proliferation (Shibolet *et al.*, 2005). Cyclosporin A has been found to efficiently reduce Th17 production. However, due to the reduction of IL-2, cyclosporin A also inhibits the generation of Treg cells from naïve T cells (Kopf *et al.*, 2007). Rapamycin alternatively acts by binding to protein kinase and blocking the proliferation of cells in response to IL-2 and therefore does not deprive Tregs of IL-2. Rapamycin thus reduces Th17 cell numbers, but does not inhibit the development of Tregs from naïve T cells (Kopf *et al.*, 2007). The future of new therapies for autoimmune disease may lie in those that consider the suppression of Th17 effector cells whilst increasing tolerance through Treg numbers and function. The imbalance observed between Treg and Th17 cells in the peripheral blood may also serve as a non-invasive diagnostic tool and measure of treatment effectiveness.

6.5.1 Summary

In summary, this chapter has shown a discrepancy in Treg and Th17 cells numbers in IBD and coeliac disease. This suggests that the inflammation seen in these diseases may be the result of excessive Th17 cells concomitant with a Treg deficiency. The control group demonstrated a balance between Treg and Th17 cells in the peripheral blood with a near 1:1 ratio of these cells within individual patient samples. IBD patients in contrast

showed significantly greater numbers of Th17 cells concomitant with fewer Tregs. An imbalance between Treg and Th17 cells was also observed in coeliac disease, however this difference was not statistically significant when compared with the control group. The skewing of Treg and Th17 cells from a developmental origin suggest the involvement of excessive pro-Th17 cytokines, as confirmed in Chapter 5. The observation of a measurable imbalance in the peripheral blood may also offer a new diagnostic tool for IBD. Use of therapeutics that aim to re-establish a balance rather than just suppressing effector cell populations, such as retinoic acid therapy, may also be successful in the future.

CHAPTER 7

GENERAL

DISCUSSION

7.1 Discussion

The current study investigated regulatory T cells and Th17 cells in patients with IBD and coeliac disease, comparing them to control subjects. A decrease of T_{reg} and an increase in Th17 cells was demonstrated in the peripheral blood of IBD patients in disease remission, but no alteration of these cell numbers in the peripheral blood of coeliac disease patients. T_{reg} and Th17 cells had a near 1:1 ratio in the blood of control subjects, whereas a reduction in cell ratio was observed in IBD patients. We hypothesized that the inflammation in IBD is due to the inability of Treg to control the proinflammatory Th17 immune response. The elevated expression of Foxp3, the surrogate marker of Treg, in the intestinal mucosa may indicate potential sequestration of these cells from the periphery to the site of disease. Increased expression of IL-17a in the intestinal mucosa in IBD and coeliac disease patients suggests Th17 involvement in these diseases. IL-1β and IL-6 was also elevated in the intestinal mucosa of IBD patients, indicating a proinflammatory microenvironment that promotes the development of Th17 effector cells (Acosta-Rodriguez *et al.*, 2007; Yang *et al.*, 2008).

7.1.1 Regulatory T cells

Prior to the commencement of this study, no commercial Foxp3 antibody was available to measure Tregs by flow cytometry, and the markers of Treg were simply $CD4^+$ $CD25^+$ or $CD4^+$ $CD25^{high}$. With the introduction of the clone PCH101 (eBioscience, CA, USA), the optimisation of protocols for the measurement of Treg in the peripheral blood were required, and provided improved methods for the measurement of Tregs using multi-colour flow cytometry. The implementation of the $CD4^+$ $CD25^{high}$ $Foxp3^+$ phenotype to identify Treg not only incorporated a high percentage of $Foxp3^+$ cells, but also

potentially excluded enumeration of activated T cells that transiently express Foxp3 (Allan *et al.*, 2007; Wang *et al.*, 2007).

The current study found a lower number of $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells in the blood of IBD patients compared to the control group. No changes in Treg numbers were observed for coeliac disease patients. The decrease in Treg numbers in IBD is unlikely to be simply the result of immunosuppressive medication as evidenced by other studies. Infliximab, has been reported to expand regulatory T cell numbers in children with CD (Ricciardelli *et al.*, 2008). Regulatory T cell numbers have reportedly increased in myasthenia gravis patients after immunosuppressive therapy with prednisalone and azathoprine (Fattorossi *et al.*, 2005). Treatment of multiple sclerosis relapse with methylprednisolone also demonstrated a rapid increase in Treg number immediately after treatment (Braitch *et al.*, 2009). Decreased Treg numbers have been observed in patients treated with Cyclosporine A (Kopf *et al.*, 2007), however patients were omitted from the current study if they were prescribed this medication.

Previous studies have investigated the suppressor function of Tregs isolated from IBD and coeliac disease patients, with impaired function only observed in those with coeliac disease. Therefore, whilst no changes in Treg numbers were observed in coeliac disease, a Treg functional defect may exist. Measurement of Treg function requires the separation of these cells from mononuclear cell fractions without the use of the intracellular marker, Foxp3. We investigated the use of the cell surface maker CD127 to exclude activated T cells in the absence of Foxp3 and to isolate a homogenous Treg population. The IL-7 receptor (CD127) is downregulated on Foxp3 regulatory T cells and allows for the discrimination between Tregs and activated T cells (Liu *et al.*, 2006;

Seddiki *et al.*, 2006). However, in the current study, utilisation of the phenotype $CD4^+$ $CD25^+$ $CD127^{low}$ resulted in varied cell numbers and the absence of a statistically significant decrease of this Treg phenotype.

$CD4^+$ $CD25^+$ $CD127^{low}$ measured a larger number of cells, suggesting this phenotype captures a different population of regulatory cells than the $CD4^+$ $CD25^{high}$ $Foxp3^+$ phenotype. The use of $CD127^{low}$ in conjunction with $CD4^+$ and $CD25^+$ may comprise a more inclusive Treg population that does not exclude regulatory cells with lower CD25 expression. Indeed, murine studies have demonstrated that $CD25^{high}$ $Foxp3^+$ cells and $CD25^{low/-}$ $Foxp3^+$ cells have equal suppressive capabilities (Fontenot *et al.*, 2005). Using the markers of $CD4^+$ $CD25^+$ $CD127^{low}$ may therefore capture a heterogeneous regulatory T cell population that include both $CD4^+$ $CD25^{high}$ and $CD4^+$ $CD25^{intermediate}$ populations. Alternatively, using $CD127^{low}$ to remove activated T cells within the $CD4^+$ $CD25^{high}$ population measured a smaller population of regulatory cells similar to the numbers observed when using the $CD4^+$ $CD25^{high}$ $Foxp3^+$ phenotype. However, $CD4^+$ $CD25^{high}$ $CD127^{low}$ cells were increased in IBD patients compared to the control group and did not demonstrate a decrease in cell numbers as identified by the $CD4^+$ $CD25^{high}$ $Foxp3^+$ phenotype in the peripheral blood of IBD. The $CD4^+$ $CD25^+$ $CD127^{low}$ phenotype therefore does not represent the same Treg population that are decreased in number in the IBD patient cohorts of the current study.

Previous unpublished work by Seddiki *et al* (personal communication) divided the $CD4^+$ $CD25^+$ $CD127^{low}$ phenotype into $CD45RA^+$ and CD45RA- populations. Both the $CD45RA^+$ and $CD45RA^-$ Tregs have been demonstrated to possess equal suppressive function (Valmori *et al.*, 2005; Seddiki *et al.*, 2006), however Seddiki *et al* (personal

communication) reported that only the $CD4^+$ $CD25^+$ $CD45RA^+$ $CD127^{low}$ cell numbers were decreased in IBD patients. The number of $CD4^+$ $CD25^+$ $CD45RA^+$ $CD127^{low}$ in this previous study is comparable to the $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells measured in our patient cohorts. Seddiki *et al* (personal communication) also revealed that in comparison, $CD4^+$ $CD25^+$ $CD45RA^-$ $CD127^{low}$ in the same patient *s*amples were elevated in CD, and there was no change in $CD4^+$ $CD25^+$ $CD127^{low}$ T cells in IBD patients compared to the control group. This suggested that these IBD patients specifically had lower numbers of naïve $CD45RA^+$ Treg in the peripheral blood, but not $CD45RA^-$ Treg. The Treg population identified in the current study as deficient in IBD will include $CD45RA^+$ naïve Tregs that have not yet recognised antigen. However, in the current study the CD45 isoform status of Tregs was not measured in the patient cohorts. Inclusion of additional cell surface markers such as CD45RA may provide more information on the cells involved in the initiation of disease, and may also allow for the isolation of the cells that are specifically deficient in IBD for future suppressor function assays, which cannot be done using Foxp3 staining.

Interestingly, the $CD4^+$ $CD25^+$ $CD45RA^+$ naïve population of Tregs maintain stable expression of Foxp3 and suppressive function compared to the $CD45RA^-$ memory Treg that lose Foxp3 expression and suppressive abilities upon *in vitro* expansion (Hoffmann *et al.*, 2006). A deficiency in $CD45RA^+$ Tregs suggests the loss of a natural stable Treg population responsible for immune homeostasis in a healthy gut. The loss of this stable Treg population may have serious consequences in the environment of inflammation. Human $CD45RA^-$ Tregs, for example, have been shown to convert to IL-17 producing cells in the presence of IL-1β, IL-6, IL-21 and IL-23 and lose their ability to suppress other T cells (Koenen *et al.*, 2008; Voo *et al.*, 2009). A similar finding was

demonstrated with natural thymus-derived Tregs and induced Tregs that develop from $CD25^-$ precursors in peripheral lymphoid organs (Horwitz *et al.*, 2008). In animal studies, natural Tregs originating from the thymus lose Foxp3 expression and suppressive abilities when activated in the presence of IL-6 (Zheng *et al.*, 2008). IL-6 also converts murine natural Tregs into Th17 cells in the presence of TGF-β, whereas induced Tregs are unable to develop into Th17 cells (Xu *et al.*, 2007).

Unlike Th1 and Th2 cells that are believed to represent terminally differentiated end points, Treg and Th17 cells demonstrate a developmental plasticity. Evidence of Treg plasticity is apparent by the identification of Tregs that co-express both Treg and Th17 markers. A significant percentage of Treg derived from the peripheral blood of healthy blood donors express both Foxp3 and RORγ-t, and have the potential to secrete IL-17 (Voo *et al.*, 2009). Production of IL-17 by these $Foxp3^+$ RORγ-t^+ cells is driven by the cytokine milieu, with the involvement of similar cytokines required for differentiation of naive T cells into Th17 effector cell. Production of IL-17 by human Treg cells was enhanced by the addition of IL-6, IL-21 and IL-23 (Koenen *et al.*, 2008; Voo *et al.*, 2009). Beriou *et al*, 2009 identified a human memory cell Treg subset that was HLA-DR negative, and produced IL-17 in the presence of IL-1β and IL-6 whereas IL-17 production was inhibited by TGF-β. IL-17 production correlated with a decrease in suppressive activity, however, this loss of suppressive function was reversible with IL-2 (Beriou *et al.*, 2009). These memory Tregs also expressed the Th17-related chemokine, receptor CCR6 (Beriou *et al.*, 2009), which allows Th17 cells to migrate to areas of inflammation in response to a chemokine gradient (Hirota *et al.*, 2007).

The high levels of Foxp3 mRNA that we and others (Makita *et al.*, 2004; Maul *et al.*, 2005; Yu *et al.*, 2007; Sitohy *et al.*, 2008; Tiittanen *et al.*, 2008) have demonstrated within the intestinal mucosa of IBD patients could potentially represent a pathogenic population of T cells that not only express Foxp3 but also RORγ-t and produce IL-17. Increased numbers of Tregs are consistently present at sites of autoimmune inflammation including the synovial joints of rheumatoid arthritis patients (Cao *et al.*, 2003; Cao *et al.*, 2004; van Amelsfort *et al.*, 2004; Liu *et al.*, 2005; Mottonen *et al.*, 2005; Cao *et al.*, 2006). When removed from their tissue/cytokine microenvironment, these Tregs possess an equal or greater suppressive capacity than control Tregs (Cao *et al.*, 2003; Makita *et al.*, 2004; Maul *et al.*, 2005; Yu *et al.*, 2007). However *in vivo*, and under the influence of the proinflammatory cytokine environment, these cells may contribute to disease by producing IL-17 or by promoting Th17 differentiation. Unlike natural Tregs that suppress effector cells in a cell-contact dependent mechanism, peripherally induced Tregs suppress T cell proliferation by the production of IL-10 and TGF-β (Zheng *et al.*, 2004). TGF-β production by peripherally induced Tregs is capable of inducing the development of Tregs from $CD4^+$ $CD25^-$ T cells (Chen *et al.*, 2003; Fu *et al.*, 2004; Zheng *et al.*, 2007; Zheng *et al.*, 2008). However in a proinflammatory environment, TGF-β produced by these Tregs may assist in the differentiation of Th17 cells, and hence contribute to, rather than suppress, inflammation (Xu *et al.*, 2007).

7.1.2 Th17 Effector Cells

In the current study, Th17 cell numbers were significantly elevated in the peripheral blood of CD and UC patients compared to a control group, confirming previous studies in IBD (Fujino *et al.*, 2003; Nielsen *et al.*, 2003). No increase in Th17 cell numbers was detected in the peripheral blood of coeliac disease patients. However, IL-17a mRNA

was increased in intestinal biopsies from both IBD and coeliac disease patients. Elevated IL-17a in the peripheral blood and intestinal tissue indicates an exaggerated proinflammatory Th17 driven immune response. The pathological effect of the cytokine IL-17 has been demonstrated in a number of animal models of autoimmune disease. Transfer of IL-17a into RAG1 deficient mice (lacking mature T cells) caused severe colitis (Leppkes *et al.*, 2009). Furthermore, mice deficient in IL-17 are resistant to experimental autoimmune encephalitis and collagen induced arthritis (Nakae *et al.*, 2003), and treatment with IL-17 neutralising antibodies is capable of suppressing inflammation (Langrish *et al.*, 2005). High levels of IL-17 have also been reported in a range of human autoimmune diseases (Matusevicius *et al.*, 1999; Wong *et al.*, 2000; Lock *et al.*, 2002; Fujino *et al.*, 2003; Hwang and Kim, 2005; Bullens *et al.*, 2006). The high levels of IL-17 observed in the current study in the intestinal mucosa of both IBD and coeliac disease patients, and in the peripheral blood of IBD patients may therefore contribute to the pathologies observed in these diseases.

7.1.3 Effector Cell Transcription Factors

T-bet

To compare the involvement of Th1, Th2 and Th17 effector cells in the aetiology of IBD and coeliac disease, the defining transcription factors for these effector cells were measured in intestinal tissues of the patient cohorts. An increase in T-bet expression was identified in both CD and UC, however no significant changes were observed in T-bet expression for coeliac disease patients compared to the control group. Elevated T-bet expression has been documented previously in coeliac disease (Monteleone *et al.*, 2004). However this was found only in patients with active disease and not compliant to

gluten-free diet, unlike the coeliac disease cohort of the current study that was reportedly compliant to long-term gluten-free diets.

T-bet is known to regulate IFN-γ expression and is the crucial transcription factor for Th1 cells. In the current study, the finding of an increase in T-bet in both CD and UC was surprising as only CD is believed to be a Th1 disease, whereas UC is supposedly a Th2-like disease (Torres and Rios, 2008). The role of T-bet, however, may differ between these diseases, playing a pathogenic role in CD but a protective role in UC. For example, T-bet$^{-/-}$ mice develop spontaneous ulcerative colitis-like symptoms (Garrett *et al.*, 2007), yet T-bet$^{-/-}$ mice do not develop experimental autoimmune encephalitis (Yang *et al.*, 2009). T-bet is also upregulated by nicotine exposure (Kikuchi *et al.*, 2008), and has the capacity to inhibit a Th2, but not a Th1, response (Madretsma *et al.*, 1996). This may explain the protective effect of smoking in UC, but its deleterious effects in CD. High levels of T-bet in UC patients may therefore indicate the activation of a protective mechanism rather than the pathogenic pathway seen in CD. T-bet has also been suggested to inhibit Th17 differentiation in an IFN-γ independent manner, with mice lacking T-bet developing an exaggerated Th17 response that is additionally resistant to inhibition by IL-2 (Guo *et al.*, 2009).

Elevated levels of T-bet may alternatively have indicated the involvement of a Th1/Th17 cell in disease pathogenesis. T-bet expression has been demonstrated in Th17 cells under Th1 polarising conditions. These cells produced both IFN-γ and IL17 in response to exposure to IL-2 and IL-12, however natural Th1 cells maintained a stable phenotype under Th17 polarising conditions (Shi *et al.*, 2008). Elevated numbers of Th17 cells that produce both IFN-γ and IL-17 have been described previously in the

intestinal tissue of CD patients (Annunziato *et al.*, 2007). Cells expressing IFN-γ and IL-17 have higher levels of T-bet than cells expressing only IFN-γ (Yang *et al.*, 2009). The elevated levels of T-bet in both CD and UC patients in the present study, may therefore represent a Th1/Th17 cell that expresses T-bet and produces IFN-γ and IL-17 (Shi *et al.*, 2008). The additional measurement of IFN-γ in future studies could provide further information regarding the presence of cells that produce both IL-17 and IFN-γ and their role in IBD.

GATA3

In the current study, GATA3, the Th2 effector cell transcription factor, was not elevated in the UC cohort despite its characterisation as a Th2-like disease. GATA3 expression however was highly variable in UC patients, with a broad range of expression compared to the control group that expressed consistently low levels. In agreement with this finding was the observation that although allergic asthma was also considered a Th2 disease, a lack of significant increase in GATA3 expression has been described in asthma studies (Kaminuma *et al.*, 2009). GATA3 expression has been suggested to constrain Th17 mediated immune responses and reduce joint inflammation in mouse models of collagen induced arthritis (van Hamburg *et al.*, 2008; van Hamburg *et al.*, 2009). Hence the wide variation in GATA3 expression in UC may in fact have represented the attempt to control Th17 mediated pathology, rather than a polarised Th2 driven disease.

RORγ-t

An increase in the Th17 transcription factor, RORγ-t, was not observed in patient cohorts of the current study (as measured by RORC, the human ortholog of RORγ-t)

despite the increase in Th17 cells in the periphery of IBD patients and in the intestinal mucosa of IBD and coeliac patients. Upregulation of RORC has previously been reported in both CD and UC (Kobayashi *et al.*, 2008). However, these studies measured RORC in CD4+ T cells isolated from surgically resected patient specimens, whereas in the present study it was measured in significantly smaller biopsy samples, which included intestinal tissue in addition to T cells. RORγ-t is necessary for the differentiation of Th17 cells, however, RORγ-t is also expressed by immature $CD4^+$ $CD8^+$ thymocytes without conferring the ability to secrete IL-17 (Voo *et al.*, 2009), in foetal lymphoid tissue inducer cells (LTi) and LTi-like cells (Eberl and Littman, 2004). Recently a subpopulation of mucosal RORγ-t^+ cells expressing natural killer cell receptors and produce IL-17 has been described (Luci *et al.*, 2009; Sanos *et al.*, 2009). In addition, murine studies, have detected RORγ-t in $Foxp3^+$ Treg cells that co-exist with RORγ-t^+ IL-17^+ T cells. Foxp3 directly binds to RORγ-t, suggesting that dual expression of Foxp3 and ROR-γt may indicate a mechanism by which ROR-γt activity is regulated by Foxp3 (Lochner *et al.*, 2008). Therefore, although necessary for Th17 cell development, RORγ-t may not be a sufficient marker of this effector cell as it is also expressed by other cells that may or may not produce IL-17.

7.1.3 The Proinflammatory Cytokine Network in IBD

Having established an increase in Th17 cells in the peripheral blood of IBD patients and an increase in IL-17a expression in the intestinal mucosa of both IBD and coeliac disease patients, the expression of cytokines known to be involved in human Th17 differentiation and development was further investigated (Acosta-Rodriguez *et al.*, 2007; Manel *et al.*, 2008; Volpe *et al.*, 2008; Yang *et al.*, 2008). Elevated levels of IL-6 and IL-1β were demonstrated in IBD by real time RT-PCR of mRNA extracted from

intestinal biopsies of all patient cohorts and confirm previous studies in IBD (Woywodt *et al.*, 1994; Mitsuyama *et al.*, 1995; Reimund *et al.*, 1996; Fornari *et al.*, 1998; Brown *et al.*, 2002). The current study extends previous studies of cytokines in IBD by also measuring IL-17a expression in these same patients, and high levels of these cytokines additionally correlated to high IL-17a expression. In humans, IL-1β and IL-6 are thought to be the cytokines driving Th17 development from memory T cells in conjunction with IL-23, whereas differentiation of naïve cells requires IL-21 and TGF-β (Yang *et al.*, 2008). Low levels of IL-21 and IL-23, however, were detected in all patient cohorts of the current study, with elevated TGF-β evident in UC only. The large number of memory cells that reside in the gut may be a potential source of Th17 cells, especially in IBD in which an environment in connection with high levels of IL-1β and IL-6 exists.

The observation of elevated levels of IL-17a mRNA in intestinal samples of coeliac disease patients, yet low levels of IL-1β, IL-6, IL-21 and IL-23 is difficult to explain. Ingestion of gluten is known to trigger the production of IL-1β, IL-6, IL-21 and IL-23 (Romaldini *et al.*, 2002; Fina *et al.*, 2008; Harris *et al.*, 2008). However, inadvertent ingestion of small amounts of gluten via contaminated gluten-free products may have resulted in the more gradual accumulation of intestinal Th17 cells from both memory and naïve T cells. Alternatively, Th17 cells could differentiate and develop under different cytokine conditions in coeliac disease patients. High IFN-γ levels have been reported in the intestinal mucosa of coeliac disease patients (Troncone *et al.*, 1998), and the elevated IL-17 levels observed in the coeliac disease cohort may have represented a Th1/Th17 cell that produces both IFN-γ and IL-17.

Given that high levels of IL-1β and IL-6 may promote the development of Th17 cells from memory cells residing in the intestinal mucosa, the conditions that lead to the excessive production of IL-1β, IL-6 and TGF-β in IBD require investigation. Murine studies have linked the production of these cytokines to the recognition and phagocytosis of infected, apoptotic cells by dendritic cells (Torchinsky *et al.*, 2009). This suggests that pathogens with a tendency to initiate apoptosis may drive Th17 differentiation. IBD patients have indeed demonstrated a higher rate of apoptosis in endothelial progenitor cells and colonic lamina propria and epithelial cells (Souza *et al.*, 2005; Garolla *et al.*, 2009). Infectious agents have also demonstrated involvement in IBD (Hugot *et al.*, 2003; Packey and Sartor, 2008). However, it has become apparent that a range of associated risk factors involved in IBD may contribute to an environment that promotes Th17 cell pathology.

IL-1β

In the current study, levels of IL-1β were more than ten times higher than the control group in IBD patients. Coeliac disease patients, however, mostly expressed IL-1β levels consistent with the control group with three individuals demonstrating levels in a similar elevated range to the IBD patients. Investigation into the clinical status of these coeliac disease patients identified one of these patients as non-compliant to a gluten-free diet, however the remaining two individuals reportedly maintained a long-term gluten-free diet.

The proinflammatory effects of IL-1β have been well described in a range of inflammatory conditions including IBD (Barksby *et al.*, 2007), with IL-1β also demonstrated to be a potent inducer of Th17 cells that simultaneously produce IFN-γ

(Acosta-Rodriguez *et al.*, 2007). Exposure of peripheral blood monocytes to lipopolysaccharides (LPS), a component of the outer membrane of gram-negative bacteria, is a stimulus for IL-1β production (McAlindon *et al.*, 1998). These bacteria may gain access to the immune system via the gastrointestinal tract and result in the production of IL-1β. Coincidentally, high levels of *E. coli* and psychotrophic bacteria have been detected in intestinal samples of CD patients (Hugot *et al.*, 2003; Packey and Sartor, 2008).

A number of mechanisms along the IL-1β production pathway are known to be defective in IBD. IL-1β is released in an inactive form, requiring cleavage by caspase-1 for activation (Siegmund, 2002). Inactive IL-1β is produced by laminar propria mononuclear cells, however, mononuclear cells from IBD patients produce significantly higher quantities of IL-1β (Mahida *et al.*, 1989; Youngman *et al.*, 1993). Mononuclear cells from IBD patients also express higher levels of caspase-1 that releases the active form of IL-1β (McAlindon *et al.*, 1998). An association between NOD2 mutations and IL-1β production has been suggested in CD, with NOD2 signalling linked to the production of IL-1β in its active and inactive forms (Ferwerda *et al.*, 2008). Mice generated specifically with the same common NOD2 susceptibility allele described in CD patients, demonstrated an increased production and secretion of IL-1β in addition to hyper responsive NF-kβ activation (Maeda *et al.*, 2005). The over production of IL-1β in IBD may therefore be linked to known genetic susceptibilities and lead to the accumulation of Th17 cells in IBD.

IL-1β may provide a therapeutic target to minimise inflammation driven by a Th17 immune response. Trials of an IL-1R antagonist (Anakinra/Kineret) have been

undertaken in rheumatoid arthritis with some success, however, these results were deemed inferior to TNF-α blocking agents (Kalliolias and Liossis, 2008). There are however patient groups that are unresponsive to these therapeutics, suggesting that disease pathology is multifactorial and variable between patients

IL-6

In the current study, IL-6 mRNA expression was significantly higher in IBD patients than in the control group, with levels of IL-6 ten times higher in both CD and UC. IL-6 expression, however, was significantly lower in coeliac disease patients, than in the control group, suggesting an alteration of IL-6 production in coeliac disease. Human intestinal epithelial cells have been reported to produce IL-6 (Ng *et al.*, 2003). Damage to the epithelium due to an abnormal immune response to ingested gluten may affect the ability of these cells to produce IL-6. Conversely, IBD patients commonly express elevated levels of the IL-6 soluble receptor and elevated serum IL-6 (Mitsuyama *et al.*, 1995; Atreya *et al.*, 2000). High levels of the soluble IL-6 receptor have also been reported in rheumatoid arthritis, asthma and multiple sclerosis (Kotake *et al.*, 1996; Desgeorges *et al.*, 1997; Yokoyama *et al.*, 1997; Padberg *et al.*, 1999). Lamina propria macrophages and lamina propria T cells produce IL-6 in the inflamed gut (Atreya *et al.*, 2000), with levels of IL-6 directly correlating to disease activity (Hyams *et al.*, 1993). In addition to contributing to the generation of Th17 cells, IL-6 induces anti-apoptotic factors such as bcl-2 and bcl-xL via STAT3 pathways, and these allow the accumulation of T cells without resolution of inflammation (Atreya *et al.*, 2000). This may be of particular importance in IBD which is characterised by chronic inflammation. The anti-apoptotic effects of IL-6 may also be linked to the increase in tumour development observed in IBD patients. Inhibition of tumour growth with neutralising antibodies to

IL-6R has been successful in murine models (Becker *et al.*, 2005). IL-6 also contributes to the reciprocal development of Treg and Th17 cells via its ability to suppress Foxp3 expression whilst promoting Th17 development by upregulating IL-23R (Dominitzki *et al.*, 2007; Yang *et al.*, 2007; Ichiyama *et al.*, 2008).

IL-6 is clearly a target for therapeutic intervention with greater specificity than general immunosuppressive agents. Moreover it may reduce the risk of infections observed commonly with current therapies to treat IBD. Current treatments for IBD may already indirectly affect IL-6 pathways. Both corticosteroid and anti-TNF-α therapies suppress NF-kβ activation, which is crucial for the transcription of IL-6 and other inflammatory cytokines (Mitsuyama *et al.*, 2006; Mudter and Neurath, 2007). Anti-IL-6R antibodies have shown success at treating experimental colitis in mice (Atreya *et al.*, 2000), and anti-IL-6R therapy clinical trials have success in treating human rheumatoid arthritis patients in combination with methotrexate (Emery *et al.*, 2008). The first clinical trials with a humanised anti-IL-6R antibody were conducted in 2004, achieving positive results with 20% of treated patients entering remission and 80% reporting a clinical response (Ito *et al.*, 2004). These findings reinforce the need for a better understanding of the relationship between cytokine levels, effector cell numbers and disease symptoms.

TGFβ

In the present study, a significant increase in TGF-β mRNA expression was observed in UC compared to the control group, confirming previous studies that revealed an increase in TGF-β levels in UC but not CD (Del Zotto *et al.*, 2003). Extremely high levels of TGF-β exhibited by two UC patients was associated with low IL-17a, IL-6 and

IL-1β expression, whereas intermediate expression of TGF-β in both CD and UC patients was associated with high levels of these proinflammatory cytokines. Low levels of TGF-β were identified in the coeliac disease cohorts with high expression observed in only two patients. However, in coeliac disease this was not related to disease activity. IL-15 is a central cytokine promoting inflammation and intestinal damage in response to gluten ingestion in coeliac disease (Garrote *et al.*, 2008). IL-15 also inhibits TGF-β signalling and affects TGF-β mediated down regulation of the immune response (Campbell *et al.*, 2001; Benahmed *et al.*, 2007). Defective TGF-β signalling is also a hallmark of IBD (Hahm *et al.*, 2001) suggesting that despite being present in adequate quantities, TGF-β may be unable to control inflammation.

The variation in TGF-β expression in UC patients could be explained by the dual roles of TGF-β in both Treg and Th17 development. TGF-β function appears to be context dependent. During infection, antigen presenting cells produce large quantities of proinflammatory cytokines. In this environment TGF-β promotes differentiation of Th17 cells to assist in the clearance of pathogens. However, with the resolution of infection, these proinflammatory cytokines subside and the development of Treg cells is induced to re-establish homeostasis (Cua and Kastelein, 2006). Animal models have revealed that Foxp3 expression is upregulated and Treg differentiation is induced in environments of high TGF-β. However, at low levels of TGF-β, IL-6 and IL-21 synergise to promote upregulation of IL-23R and subsequent differentiation of Th17 cells (Zhou *et al.*, 2008).

TGF-β in combination with IL-2, is required for the differentiation of naive CD4+ CD25- T cells in the periphery, and TGF-β induces these cells to express Foxp3 (Chen

et al., 2003; Peng *et al.*, 2004; Zheng *et al.*, 2007; Zheng, 2008). These induced Tregs are also a source of TGF-β that promotes the development of additional induced Tregs, which inhibits the production of pro-inflammatory cytokines (Zheng *et al.*, 2004). However, as IBD pathology induces an environment of consistently high proinflammatory cytokines, TGF-β potentially aids the continual proliferation of Th17 cells (Cua and Kastelein, 2006). Therefore, instead of promoting tolerance, TGF-β may contribute to the ongoing inflammation characteristic of IBD.

TGF-β may also be involved in the increased cancer risk evident in IBD, and particularly in UC. An increase in Tregs has been described in the tumour microenvironment, and certain cancer cell lines have been demonstrated to produce high levels of TGF-β (Moo-Young *et al.*, 2009). TGF-β produced by these tumour cells is thought to promote the development of Tregs that suppress an anti tumour response by the immune system and hence allow continual tumour growth (Moo-Young *et al.*, 2009). Several studies of Tregs in the context of gastrointestinal cancer have confirmed elevated Treg numbers in the peripheral blood of gastrointestinal cancer patients, with high Treg numbers correlating with tumour progression and a poor prognosis (Xu *et al.*, 2009). Targeting TGF-β pathways to limit the Th17 immune response is difficult owing to the pleiotropic nature of TGF-β in both promoting and suppressing immune activation (Ouyang *et al.*, 2009).

IL-21 and IL-23

The current investigation into IL-21 and IL-23 in the intestinal mucosa of IBD and coeliac disease patients did not detect an increase in these cytokines. IL-21 and IL-23 levels were largely below the limits of detection in our patient cohorts, suggesting very

low levels of these cytokines were present. High IL-21 levels have been identified in coeliac disease patients prior to the commencement of a gluten-free diet (Fina *et al.*, 2008). In the present study, low levels of IL-21 in the coeliac disease patients may have reflected the effectiveness of a gluten-free diet at controlling the inflammatory response. High IL-21 levels have been reported in IBD, by western blot analysis indicating elevated levels of this protein (Monteleone *et al.*, 2005). Elevated IL-23 has also been demonstrated in IBD (Kobayashi *et al.*, 2008). However, these previous studies investigated IL-21 and IL-23 in resected specimens from IBD patients with moderate to severe disease. In the current study, these cytokines were measured in small pinch biopsy samples from individuals in varying disease activity. The absence of involvement of IL-21 and IL-23 may therefore have been a reflection the small biopsy samples utilised for mRNA extraction, and also the disease activity of the patient cohorts.

Signaling through IL-23, however, may still play a role in IBD, as genome-wide association studies have identified polymorphisms in the IL-23 receptor (IL-23R) in CD and UC. Moreover, increased IL-23R expression has been described previously in CD and UC (Langrish *et al.*, 2005; Schmidt *et al.*, 2005; Kobayashi *et al.*, 2008). Similarly, signaling through IL-21 may also be involved in IBD, with high levels of IL-21R present in intestinal biopsies from IBD patients, suggesting IBD patients may be sensitive to low levels of IL-21 in the intestinal mucosa (Liu *et al.*, 2009).

7.1.4 Other Factors affecting Treg/Th17 homeostasis

The measurement of Treg and Th17 cells within the peripheral blood of patient cohorts in the present study identified an inequality between the numbers of these cells in IBD.

IBD patients were characterised as having significantly higher Th17 cells concomitant with fewer Tregs in the peripheral blood, compared to the control group, demonstrating equal numbers of these two cell types. An disparity in Treg and Th17 cell numbers was further observed in coeliac disease patients with greater Th17 cell numbers to fewer Treg, however, these differences were not statistically significant when compared to Treg and Th17 numbers within the control group. The additional finding of elevated IL-1β, IL-6 and IL-17a expression in the intestinal mucosa of IBD patients suggested that a pro-Th17 cytokine microenvironment may have skewed the development of T cells towards a Th17 phenotype and away from Treg development. It is clear that the cytokine context within which T cells develop and differentiate is crucial to the maintenance of equilibrium. Fine-tuning of this environment may be influenced by a variety of factors including genetic predisposition, diet, intestinal microbe populations and the environment (Quintana and Weiner, 2009). The initiating events that result in a proinflammatory environment and the disturbance to the Treg/Th17 equilibrium requires further investigation.

Diet

The prevalence of IBD in developed regions of the world suggests the involvement of environmental factors in IBD pathogenesis. For example, the rapid increase in the incidence of IBD in Japan is proposed to be a reflection of the economic growth of Japan since the 1960's, with improved living standards and the influence of Western dietary habits, with the high consumption of animal meats, fats, refined sugars and dairy products (Asakura *et al.*, 2009). Japanese people have increased their intake of these products since the 1960's and concomitantly decreased their consumption of rice (Asakura *et al.*, 2008). Dietary intake may affect intestinal bacterial populations and

this imbalance may be involved in IBD pathogenesis. Investigation into the faecal samples of IBD patients has confirmed altered microbiota populations in IBD patients compared to controls, in addition there is a measurable decrease in beneficial faecal organic acids, such as butyric acid, produced by intestinal microbiota (Takaishi *et al.*, 2008). Individuals living in rural areas of Japan and adhering to a traditional diet presented with higher levels of *Bifidobacteria*, whereas lower *Clostridium* levels observed in individuals regularly consuming brown rice (Benno *et al.*, 1989; Benno *et al.*, 1989). The elemental diet, which has been used to treat CD with similar efficacy to steroidal therapies, provides nutritional requirements in simple forms such as free amino acids and short chain carbohydrates (Gorard *et al.*, 1993). This diet not only alters the intestinal microbiota but also reduces levels of IL-1β, IL-6 and TNF-α (Yamamoto *et al.*, 2005; Kajiura *et al.*, 2008). In the current study, the increased levels of pro-Th17 cytokines in intestinal biopsies from IBD patients may therefore have represented the stimulation of an immune response driven by an imbalance in commensal bacteria due to diet-related factors.

Antigen and Antigen Presenting Cells

Since no cells of the immune system act in isolation, it is important to consider antigen presentation in the gut. Activation of the immune response first requires the presentation of antigen, by antigen presenting cells such as dendritic cells. Dendritic cells constantly sample food-derived antigen and bacterial antigens from the intestinal lumen, and then migrate to the draining lymph nodes where they initiate an immune response. Dendritic cells are involved in both activating the immune system and maintaining tolerance, via their expression of unique costimulatory molecules and production of cytokines that differentiate naïve T cells into Th1, Th2, Th17 or Treg

phenotypes (Blanco *et al.*, 2008). The differentiation of T cell lineages is not inherent to specific subsets of dendritic cells, but dependent upon environmental triggers. For example, immature dendritic cells require IFN-γ exposure in order to produce IL-12, and subsequently drive the differentiation of naïve T cells into Th1 cells (Vieira *et al.*, 2000).

The production of cytokine by dendritic cells may be dependent upon the microbial stimulus, with different bacterial species promoting the secretion of various cytokines (Bilsborough and Viney, 2004). Exposure of dendritic cells to certain microbial products generates specific T cell responses, with dendritic cells capable of distinguishing between similar microbial structures (Stagg *et al.*, 2003). Different microbial compounds are known to drive human myeloid dendritic cells into Th1 or Th2-promoting dendritic cells. For example, extracts from the helminth *Schistosoma mansoni* induced the development of Th2-promoting dendritic cells, whereas *Bordetella pertussis* generated dendritic cells producing IL-12, thus promoting a Th1 response (de Jong *et al.*, 2002). Exposure of dendritic cells to gram-negative bacteria also induces the production of IL-23 thereby promoting Th17 development (Smits *et al.*, 2004). Dendritic cells recognise microbial structures via the expression of pattern recognition receptors, such as toll-like receptors (TLR). Specific toll-like receptors recognise particular structural components of microbiota. For example, TLR4 is required for the recognition of LPS derived from *E.coli*, TLR5 is required for the identification of flagellin from Gram-negative bacteria and TLR9 recognises bacterial DNA (Stagg *et al.*, 2003). TLR9 polymorphisms are also associated with CD, and interactions between other associated variants, such as NOD2 and IL-23R, increase susceptibility to CD (Torok *et al.*, 2009).

Specific microbiota have also been reported to promote Th17 differentiation within the small intestine, with *Cytophaga-flavobacter-bacteroidetes* increasing Th17 production with a concomitant decrease in Treg cells (Ivanov *et al.*, 2008). ATP derived from commensal bacteria has been reported to act on a specific dendritic cell subset inducing production of IL-6, IL-23 and TGF-β and promoting Th17 differentiation in mice (Atarashi *et al.*, 2008). A dendritic cell subset ($CD11_c^{high}$ $CD11_b^{high}$) that expresses TLR5 has been shown to promote the differentiation of Th1 and Th17 cells with exposure to bacterial flagellin ligand (Uematsu *et al.*, 2008). The DNA of commensal bacteria is also involved in intestinal homeostasis, with TLR-9 specific engagement promoting Th1 and Th2 differentiation thereby reducing Treg development (Hall *et al.*, 2008). An imbalance in commensal bacteria, or specific dendritic cell subsets, may therefore result in induction of pathology through the unnecessary activation of an immune response.

Retinoic Acid

Elevated Th17 cell numbers and decreased Tregs observed in IBD cohorts of the current study may have reflected a deficiency in retinoic acid due to Vitamin A deficiency, or alternatively the enzymes required for retinoic acid metabolism. Retinoic acid has been reported to support TGF-β dependent differentiation of induced Tregs that maintain a stable Treg lineage and promote Th2 differentiation, whilst reducing the differentiation of Th1 and Th17 cells (Iwata *et al.*, 2003; Mucida *et al.*, 2007). Specific lamina propria dendritic cells are capable of converting dietary Vitamin A to retinoic acid, and retinoic acid also enhances expression of the gut homing receptors, α4β7 and CCR9, on T cells (Iwata *et al.*, 2004). Deficiencies in both Vitamin A and retinaldehyde dehydrogenase

have been previously confirmed in IBD (Main *et al.*, 1983; Bousvaros *et al.*, 1998). Indeed, a deficiency in Vitamin A may have a range of immunological consequences, as it is crucial for formation of the epithelial lining of the intestinal system and may play an important role in intestinal barrier function (McCullough *et al.*, 1999). A deficiency in Vitamin A has also been linked to the over-production of IFN-γ and the promotion of Th1 cell differentiation (Cantorna *et al.*, 1994).

Environmental Toxins

Environmental toxins may also be involved in skewing Treg and Th17 homeostasis, thereby supporting the geographical distribution of IBD in developed countries. The aryl hydrocarbon receptor (AHR) is a cytosolic transcription factor with the ability to bind to a range of synthetic and natural ligands, including those found in industrial pollutants, cigarette smoke and in char grilled meat products (Quintana and Weiner, 2009; Stockinger *et al.*, 2009). Upregulation of AHR expression has been documented in Th17 cells, however, AHR activation can promote Treg and Th17 development in a ligand specific manner. Activation of AHR by 2,3,7,8-tetrachlorodibenzo-p-dioxin, found in tryptophan derivates, promoted the development of Tregs that actively suppress experimental autoimmune encephalitis (EAE) in mice, whereas the activation by 6-formylindolo[3,2-b]carbazole promoted Th17 differentiation and exacerbated the symptoms of EAE (Quintana *et al.*, 2008).

7.1.5 Implications

The identification of a Treg/Th17 imbalance in IBD has a number of practical implications for the diagnosis and treatment of IBD. Firstly, the variation in Treg and Th17 cell numbers in the peripheral blood may provide a non-invasive diagnostic tool

for IBD as an initial screen. High serum levels of IL-17, IL-6 and IL-1β may also provide information regarding clinical activity and may be predictive of relapse, with IL-6 already identified as a good predictor of relapse in IBD (Van Kemseke *et al.*, 2000). The inability of current medications to completely control IBD, and the association of these therapies with an increased risk of infection and malignancy, calls for the development of new and improved therapeutics. The findings of the current study suggest the re-establishment of effector and regulatory cell homeostasis as a primary goal in favour of the general suppression of the immune system. Suppression of specific effector cells combined with promotion of regulatory T cells may provide the key in regaining immune homeostasis, whilst minimising negative side-effects.

Regulatory T cell therapy to control inflammation has been explored in the setting of organ transplantation. Low Tregs levels have been documented in patients with chronic rejection to transplanted organs, and high levels in those with a natural tolerance to donor organs who do not require immune suppressor medications for transplant tolerance (Louis *et al.*, 2006). Manipulation of Tregs to promote tolerance to donor organs, however, has shown limited success. The principal challenge in applying Tregs to a clinical setting includes the difficulty in purifying *bona fide* human Tregs due to the inability to use Foxp3 as a biomarker. Not only does detection of Foxp3 require permeabilisation and therefore the death of cells for its labelling, but as previously discussed, it may also be expressed by activated T cells and by cells that produce IL-17 (Allan *et al.*, 2007; Wang *et al.*, 2007; Koenen *et al.*, 2008; Voo *et al.*, 2009). The plasticity of Tregs also presents a significant challenge in their therapeutic use. Exploitation of the reciprocal relationship between Treg and Th17 cells may be a more appropriate avenue for the development of new therapies.

Success of the elemental diet in controlling IBD in a manner analogous to steroidal therapy, and its ability to reduce levels of the proinflammatory cytokines IL-1β and IL-6 suggests an important role for diet in controlling inflammation (Gorard *et al.*, 1993; Yamamoto *et al.*, 2005; Kajiura *et al.*, 2008). Association of IBD with an imbalance in commensal bacteria may be linked to diets high in carbohydrates, refined sugars, dairy products and animal fats stereotypically consumed by the Western World (Asakura *et al.*, 2008; Takaishi *et al.*, 2008). The results of other diet therapies that specifically balance Treg and Th17 cells, such as the synthetic retinoic acid AM80, are greatly anticipated. Most recently, animal models of autoimmune disease have shown the ability of the Chinese medicinal fungus, *Cordyceps sinensis* to increase the ratio of Treg to Th17 cells and successfully reduce the incidence of diabetes in non-obese diabetic mice (Shi *et al.*, 2009). Measurement of the effector and regulatory cell ratio after treatment with various modalities may therefore provide methods for measuring effectiveness of new therapies for controlling diseases such as IBD.

7.1.6 Summary

The current study has demonstrated that IBD is characterised by a decrease in number of Treg and an increase in proinflammatory Th17 cells in the peripheral blood. A state of equilibrium between Treg and Th17 cell numbers within the periphery was described in the control group, in which a near 1:1 ratio of Treg and Th17 cells existed. In IBD however, this symmetry is disrupted with significantly greater Th17 cell numbers, concomitant with fewer $Foxp3^+$ regulatory T cells, hence a lower Treg:Th17 ratio. Investigation into markers of Treg and Th17 cells at the site of disease using real time RT-PCR indicated an increase in surrogate markers for Treg and Th17 cells, namely

Foxp3 and IL-17a. Significantly higher Foxp3 expression was observed in IBD, and an increase in IL-17a expression in IBD and coeliac disease. Examination of the cytokine microenvironment of the intestinal mucosa of IBD patients uncovered a proinflammatory milieu with high levels of IL-1β and IL-6 that encourages Th17 development and suppresses Treg function. We hypothesize that Tregs may be actively recruited from the periphery to the site of inflammation, where their suppressor functions may not only be 'paralysed' by the proinflammatory milieu, but may also culminate into their conversion into IL-17 producing cells (Koenen *et al.*, 2008; Beriou *et al.*, 2009; Voo *et al.*, 2009). The validated techniques for measuring these cell types as described in this study may provide new non-invasive diagnostic tools for detecting IBD. The reciprocal relationship between Treg and Th17 cells, and their skewed development in IBD and in other diseases further encourages the development of new therapeutic modalities that focus on re-establishing this equilibrium to restore health.