Studies of the aetiopathogenesis of pouchitis

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Abstract

The ileal pouch offers a unique opportunity to study the inter-relationships between the gut microbiota, barrier function and host immune responses. Intestinal dendritic cells (DC) are pivotal in the maintenance of gut immune homeostasis. Impaired barrier function due to altered cell to cell junctions, enables interactions between the microbiota and host immune responses prior to the onset of inflammation and epithelial damage. The role of innate immune factors in pouchitis remains unclear.

We performed cross sectional and longitudinal studies of patients following restorative proctocolectomy and assessed DC and tight junction protein (TJP) characteristics in the ileal pouch. Increased expression of the "pore-forming" claudin 2 was an early event in the development of pouch inflammation and aberrant DC expression of gut homing markers was characterised in the ileum and ileal pouch of ulcerative colitis patients without inflammation. DC phenotype in pouchitis suggested an activated innate immune response to microbial signals.

Intestinal immune responses may be manipulated by modification of the gut microbiota. An emerging approach is transplantation of the entire "organ" of the gut microbiota. Effects of faecal microbiota transplantation (FMT) on recipient microbiota and immune responses in inflammatory bowel diseases are unknown.

A single nasogastrically delivered FMT from a healthy donor to patients with chronic pouchitis, resulted in some shift in the composition of the microbiota, with specific changes in the abundance of species suggestive of a “healthier” pouch microbiota. However, microbiota engraftment success varied greatly between recipients and regardless of engraftment success, FMT did not result in immunological response or clinical efficacy.

In conclusion, aberrant DC and TJP characteristics are associated with inflammation of the ileal pouch. Manipulation of the microbiota by FMT may be one means of modifying DC and TJP expression in the ileal pouch. However, these factors were not influenced by a single nasogastrically delivered FMT.

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List of Abbreviations

ACCA	Anti-chitobioside carbohydrate antibodies
AJC	Adherens junctional complex
ANCA	Anti-neutrophil cytoplasm antibody
ASA	Aminosalicylic acid
ASCA	Anti-Saccharomyces cervisiae antibodies
CARD	Caspase recruitment domain containing protein
CCL	Chemokine motif ligand
CCR	CC Chemokine receptor
CD	Cluster of differentiation
CGQoL	Cleveland global quality of life score
CpG	C-phosphate-G
DAPI	4' 6-diamidino-2-phenylindole
DC	Dendritic cell
DC-SIGN	Dendritic cell specific ICAM-3 grabbing non-integrin
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
F. prausnitzii	*Faecalibacterium prausnitzii*
FACS	Fluorescence assisted cell sorter
FAP	Familial adenomatous polyposis
FCS	Foetal calf serum
FISH	Fluorescent in situ hybridisation
FMT	Faecal microbiota transplant
IBD	Inflammatory bowel disease
ICAM	Intercellular adhesion molecule
Ig	Immunoglobulin
IL-	Interleukin
ILC	Innate lymphoid cell
IPAA	Ileal pouch-anal anastomosis
IR	Intensity ratio
JAM	Junctional adhesion molecule
LH	Length heterogeneity
LPMC	Lamina propria mononuclear cells
mDC	Myeloid dendritic cell
MHC	Major Histocompatibility complex
MLCK	Myosin light chain kinase
MUC	Mucin

MyD88	Myeloid differentiation primary-response gene 88
NK	Natural killer
NOD	Nucleotide binding oligomerisation domain
NSAID	Non-steroidal anti-inflammatory drug
OmpC	Outer membrane porin C
OTU	Operational taxonomic unit
PBMC	Peripheral blood monocytic cells
PCR	Polymerase chain reaction
PDAI	Pouch disease activity index
pDC	Plasmacytoid dendritic cell
RA	Retinoic Acid
RNA	Ribonucleic acid
RPC	Restorative proctocolectomy
TECK	Thymus expressed chemokine
Th	T helper
TJ	Tight junction
TLR	Toll like receptor
TNF	Tumour necrosis factor
TRFL-P	Terminal restriction fragment length polymorphism
UC	Ulcerative colitis
vs.	Versus (compared with)
ZO	Zonulin occludens
β7	Beta 7 integrin

Chapter 1. General Introduction

The human gut has evolved to interface with a diverse and dynamic community of microorganisms. Bacteria predominate, but eukaryotes, viruses and archaea are also present. Intestinal bacterial communities comprise up to 1000 different species constituting an incredibly diverse ecosystem (Duerkop et al., 2009). It is estimated that the human microbiota contains up to 10^{14} bacterial cells, an order of magnitude greater than the number of human cells in our body (Sekirov et al., 2010). This has given rise to the view of the microbiota as another organ and a perception of ourselves as ‘‘supraorganisms’’ whose genome is the sum of human and microbial genes (Peterson et al., 2008a).

The co-evolution of the mammalian intestinal microbiota has primarily been driven by the metabolic advantages in enhancing host digestive capabilities. The complex community of bacteria which is rapidly adaptable and evolving, harbours a diversity of saccharolytic enzymes far beyond the limited repertoire encoded by the human genome alone. This association consequently enables flexible dietary changes and optimises efficiency of energy harvesting from our diet (Duerkop et al., 2009).

Millions of years of co-evolution have led to an integral intertwining of host-microbial physiology. Much of our knowledge of gut microbiota function is derived from studies of germ free animals. These demonstrate the commensal microbiota to modulate nutrient absorption, mucosal barrier function, angiogenesis and intestinal maturation (Hooper et al., 2001). The intestinal microbiota plays a critical role in the development and maturation of the gut and systemic immune system. The mucosal immune system needs to be tolerant of the overlying

microbiota, and at the same time to control the gut microbiota to prevent its overgrowth and translocation to systemic sites and respond appropriately to pathogens. This has led to the development of a finely tuned homeostasis between the huge microbial load of the intestine and the host immune system.

The intestinal microbiota is protective from invasion of pathogens. Gut microbiota provides a physical barrier to incoming pathogens by competitive exclusion, competitive consumption of nutrients, and stimulation of host production of antimicrobial substances (Lawley and Walker, 2013). The commensal microbiota signals via TLR-MyD88 dependent pathways enhancing epithelial repair and cross-talk between components of the innate immune responses (Hooper et al., 2012). Commensal species have been shown to influence the make-up of T lymphocyte subsets with distinct effector functions both locally and systemically (Hooper et al., 2012).

At the same time the intestinal immune system has developed strategies to preserve ignorance as well as tolerance of the commensal microbiota. The inner layer of mucus secreted by goblet cells is resistant to bacterial penetration whereas the outer layer is colonised with bacteria (Swidsinski et al., 2005). The epithelial layer bound together by tight junction proteins enables nutrient flux into tissues whilst preventing bacterial penetration. Epithelial cells also produce a number of antimicrobial peptides (Hooper et al., 2012). IgA producing B cells secrete bacteria specific IgA which is transcytosed to the apical layer of the epithelium, confining bacteria to the mucus layer (Peterson et al., 2008a). Symbiotic bacteria that do breach the mucosa are rapidly phagocytosed and killed by macrophages in contrast to pathogens that actively interfere with

macrophage function (Hooper et al., 2012). Dendritic cells sample penetrating and apical bacteria and interact with B and T cells to direct appropriate immune responses (Hooper et al., 2012). Innate lymphoid cells that produce interleukin (IL)–22 are also essential for containment of lymphoid resident bacteria to the intestine, preventing their systemic translocation (Hooper et al., 2012).

Despite huge advances in our understanding of the microbiota and host-microbiota relationships, much of the details of the structure and function of the microbiota are as yet unknown. Inflammatory bowel diseases (IBD) (Crohn's disease, ulcerative colitis (UC) and pouchitis) are considered to be due to an inappropriate inter-relationship between the immune responses and intestinal microbiota (Sartor, 2009). Studies of the aetiology of these diseases are likely to enhance our knowledge of host-microbiota relationships. Genome wide association studies have identified genes contributing to susceptibility to Crohn's disease and UC. The elucidation of these susceptibility loci highlight the role of the immune responses to microbial signalling and processing (Xavier and Podolsky, 2007) as well as epithelial barrier integrity (Anderson et al., 2011, Barrett et al., 2009).

Animal studies of colitis demonstrate an immune driven dysbiosis (i.e. an alteration in the composition of the microbiota to one that is deleterious to host health) highlighting the control the immune system exerts on the structure of the intestinal microbiota (Garrett et al., 2007, Powell et al., 2012). Animal models of colitis also indicate the microbiota drives inflammation in genetically susceptible hosts (Sartor, 2008). Knockout mouse models of colitis have also shown

a transferrable colitogenic microbiota to wild type mice (Garrett et al., 2007). Clinical evidence also points towards the microbiota driving inflammation. Faecal diversion is curative for Crohn's disease whilst reintroduction of the ileal contents to the diverted bowel induces inflammation (D'Haens et al., 1998). However, the study of IBD in the clinical setting with human subjects is limited by the lack of a method that predicts and detects the exact onset of the disease during a first episode of inflammation or relapse. Consequently only clinical investigations in chronic stages of inflammation are possible in UC and Crohn's disease.

The ileal pouch and pouchitis however, offer a unique opportunity to study the inter-relationships between the gut microbiota, epithelial barrier and host immune responses from before the onset of disease. The ileal pouch provides a unique human model for studying IBD pathogenesis for the following reasons: a) the patients have a genetic predisposition to the intestinal inflammation, b) they are most likely to develop inflammation in their pouch in the first year after surgery, so following patients longitudinally from the time of pouch surgery enables access to samples from patients who develop inflammation and those who do not develop inflammation, c) the pouch is accessible with an endoscope and d) there is a human control group (FAP) who have a pouch without a predisposition to intestinal inflammation. For this reason the study of pouchitis could serve as a human model that significantly enhances our understanding of inflammatory bowel diseases in general.

1.1 Restorative proctocolectomy and ileal pouch-anal anastomosis

Between 10 and 35% (Hendriksen et al., 1985, Langholz et al., 1992, Cottone et al., 2008, Hoie et al., 2007) of patients with ulcerative colitis (UC) eventually require surgery. For the majority of patients, restorative proctocolectomy with ileal pouch-anal anastomosis (RPC) is the operation of choice (Becker, 1999). It is also used for patients with Familial Adenomatous Polyposis (FAP). The procedure involves the removal of the colon and rectum and the construction of a reservoir created from 30-40cm of ileum, followed by ileo-anal anastomosis (Parks and Nicholls, 1978). This procedure avoids the need for a permanent stoma and allows good long term function and quality of life (Carmon et al., 2003, Fazio et al., 2013). Over 6000 patients in the UK have undergone restorative proctocolectomy (Burns et al., 2011).

1.1i Pouchitis

Pouchitis is an idiopathic inflammatory condition that may occur in up to 60% of patients after RPC for ulcerative colitis. It is rarely seen in FAP patients after RPC. The cumulative risk of pouchitis increases with longer follow up, but is most frequently seen within the first two years of the pouch functioning. Up to 60% of patients may suffer a recurrence of pouchitis, but the prevalence of chronic pouchitis is only 5-19% (Simchuk and Thirlby, 2000, Stahlberg et al., 1996, Lohmuller et al., 1990, Salemans et al., 1992).

The aetiology of pouchitis remains unclear. There are significant clinical (Gionchetti et al., 1999, Gionchetti et al., 2003, Mimura et al., 2004) and microbiological (Tannock et al., 2012, McLaughlin et al., 2010b) data implicating bacteria in the pathogenesis of pouchitis. Studies

suggest the microbiota stimulate mucosal immune responses in the ileal pouch (Bell, 2004, Pronio et al., 2008). However, the mechanisms by which the pouch microbiota interacts with the mucosal immune system are unclear and data regarding innate immune characteristics and epithelial barrier properties in pouchitis are sparse.

1.1ii Clinical Characteristics of Pouchitis

Clarity of the definition and diagnosis of pouchitis are critical in clinical and aetiological studies of pouchitis. The diagnosis of pouchitis requires the combination of clinical symptoms as well as endoscopic and histological findings. Clinical symptoms include increased stool frequency and liquidity, abdominal cramping, urgency and tenesmus and occasionally bleeding and fever. Endoscopic assessment and biopsy with flexible pouchoscopy (McLaughlin et al., 2009c) should be undertaken. Symptoms do not always correlate with endoscopic and histological findings. A number of pouchitis scoring systems are in use (Sandborn et al., 1994, Shen et al., 2003, Heuschen et al., 2002). The Pouch Disease Activity Index (PDAI) (Table 1.1) is most commonly used. This system attributes numbers to clinical, endoscopic (Figure 1.1) and histological features, which are combined to give a total score. A score ≥7 is diagnostic of pouchitis. However, a flaw of the PDAI is that the presence of acute histological inflammation is not required for a diagnosis of pouchitis.

Pouchitis can be classified according to disease activity, duration, pattern and response to antibiotics. Acute pouchitis can be considered as less than 4 weeks duration, responding to a single antibiotic and occurring less than three times per year. Chronic pouchitis may be

considered to be chronic relapsing if there are three or more episodes per year responding to antibiotics or chronic antibiotic dependent if symptoms are only controlled while maintained on antibiotics. Chronic antibiotic refractory pouchitis is generally defined as pouchitis that no longer responds to a single antibiotic (McLaughlin et al., 2008b, Mahadevan and Sandborn, 2003).

1.1iii Differential Diagnoses

Inflammation of the pouch can also occur for a number of other reasons that should be considered and excluded in the differential diagnosis of pouchitis. Pre-pouch ileitis is inflammation developing in the ileum immediately proximal to the pouch (Figure 1.2). It is usually concurrent with pouchitis, but can occur in isolation. Symptoms include those of pouchitis, but may also include those of intestinal obstruction (McLaughlin et al., 2008a, McLaughlin et al., 2009a). A number of treatments for pre-pouch ileitis have been described including steroids, antibiotics and 5-ASA medications (McLaughlin et al., 2008a).

Table 1.1 Pouch disease activity index

Criteria	Score
Clinical	
Stool Frequency	
Usual postoperative stool frequency	0
1-2 stools/day > postoperative usual	1
≥3 stools/day > postoperative usual	2
Rectal Bleeding	
None or rare	0
Present daily	1
Faecal Urgency or abdominal cramps	
None	0
Occasional	1
Usual	2
Fever (temperature >37.8° C)	
Absent	0
Present	1
Endoscopic Inflammation	
Oedema	1
Granularity	1
Friability	1
Loss of vascular pattern	1
Mucous exudate	1
Ulceration	1
Acute histological inflammation	
Polymorphonuclear leucocyte	
Mild	1
Moderate +crypt abscess	2
Severe +crypt abscess	3
Ulceration per lower power field	
<25%	1
25-50%	2
>50%	3

Figure 1.1 Endoscopic image of active chronic pouchitis

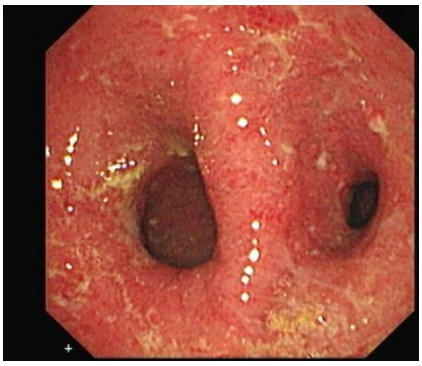
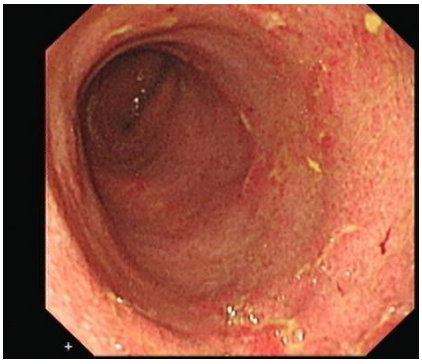


Figure 1.2 Endoscopic image of pre-pouch ileitis



Inflammation of the residual anorectal mucosa (cuffitis) can also be a cause of symptoms similar to pouchitis with endoscopic inflammation. There are a number of different surgical methods used to create the pouch-anal anastomosis. This can be hand-sewn or stapled. The stapled pouch-anal anastomosis leaves a variable length of anorectal mucosa in situ. Persisting proctitis of the residual anorectal mucosa may lead to bleeding, urgency, and frequency of small volume stool. This is less likely following hand-sewn anastomosis with mucosectomy. A high anastomosis can be distinguished clinically by digital examination as well as by contrast

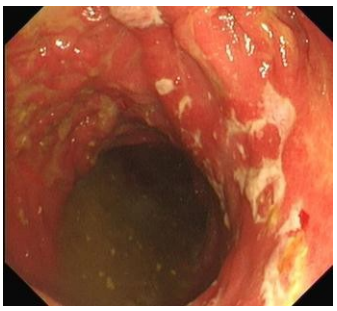
enema and pouchoscopy. The presence of inflamed rectal mucosa can be confirmed on biopsy. Treatment is with 5-ASA or steroid suppositories or in severe persistent cases revision of the anastomosis may be undertaken (Shen et al., 2004, Tekkis et al., 2006, Tulchinsky et al., 2001, Wu et al., 2013).

Two to three percent of patients diagnosed with ulcerative colitis preoperatively are subsequently found to have Crohn's Disease (13). Crohn's disease should be considered where there is fistulation or chronic unremitting pouchitis. Fistulas arising from the anastomosis suggest leak, whilst those arising from the pouch or the pre-pouch ileum usually indicate Crohn's disease. Crohn's disease is generally considered a contraindication to undertaking restorative proctocolectomy. However, a number of studies have assessed the outcomes of pouches performed for patients with Crohn's disease. The majority of patients with Crohn's disease who have undergone RPC were originally considered to have UC. In these patients, failure rates and poor pouch function are significantly greater compared with UC patients following RPC (Reese et al., 2007, Hahnloser et al., 2007). However, a recent study of patients where RPC was used intentionally for well-defined Crohn's disease demonstrated no increase in pouch failure rates although there was an increased incidence of postoperative disease (Le et al., 2012).

It is estimated that approximately 20-30% of patients diagnosed with chronic refractory pouchitis may have identifiable secondary causes of pouch inflammation (Navaneethan and Shen, 2010). Early or late pelvic sepsis are the most frequent cause of pouch failure (Tulchinsky

et al., 2003). Delayed pelvic sepsis may present with systemic illness and pouch dysfunction as well as focal inflammation of pouch mucosa. This should be considered particularly where early complications following ileostomy closure occurred. *Cytomegalovirus* and *Clostridium difficile* may cause pouch inflammation. Both are uncommon, but should be excluded in recurrent or refractory pouchitis (Munoz-Juarez et al., 1999, Moonka et al., 1998, McLaughlin et al., 2010a, Shen et al., 2008a). Pouch ischaemia can occur due to anatomical factors subsequent to the surgical procedure during restorative proctocolectomy where there may be vessel disruption or tension of the pouch-anal anastomosis. Non-steroidal medications can also contribute to inflammation and ulceration of the pouch and these should be withdrawn in patients with pouch inflammation or poor function. Furthermore, stasis due to poor pouch evacuatory function can lead to significant ulceration (Figure 1.3) and appropriate history and investigations should be undertaken to exclude this where suspected.

Figure 1.3 Endoscopic image of pouch ulceration secondary to stasis



1.2 Risk factors for pouchitis

Several risk factors for pouchitis have been reported, although they have not been demonstrated consistently. Extensive or severe UC (Fazio et al., 1995), backwash ileitis (Schmidt et al., 1998), extra-intestinal manifestations of UC (Lohmuller et al., 1990, Hata et al., 2003), pre-colectomy thrombocytosis (Okon et al., 2005, Lian et al., 2009), pANCA positivity (Fleshner et al., 2001, Fleshner et al., 2008), non-smoking status and NSAID use (Fleshner et al., 2007, Achkar et al., 2005, Shen et al., 2005b) have all been reported as risk factors for the development of pouchitis. Primary sclerosing cholangitis is associated with a two fold increase in risk of acute pouchitis (Zins et al., 1995, Penna et al., 1996) and is also associated with pre-pouch ileitis (Shen et al., 2011). Elevated serum IgG4 levels may be associated with a subset of chronic pouchitis patients (Navaneethan et al., 2011). Different risk factors may be associated with acute or chronic pouchitis, suggesting different pathogenic pathways in what may be two distinct diseases or different ends of a spectrum of inflammatory diseases (Fleshner et al., 2007, Achkar et al., 2005, Abdelrazeq et al., 2008).

1.3 Pouch bacteriology and pouchitis

The role of the gut microbiota is increasingly considered to be an important factor in the aetiology of inflammatory bowel diseases (Sartor, 2008, Tamboli et al., 2004). Clinical data implicate bacteria in the pathogenesis of pouchitis. Pouchitis only occurs following closure of the ileostomy when the pouch is exposed to significantly higher concentrations of bacteria. Antibiotics are the mainstay of treatment for acute and chronic pouchitis (Holubar et al., 2010, Gionchetti et al., 1999). Probiotics reduce the risk of disease onset (Gionchetti et al., 2003),

maintain disease remission (Gionchetti et al., 2000b, Mimura et al., 2004) and can induce disease remission (Gionchetti et al., 2007).

The microbiota of the terminal ileum differs significantly from that of the colon with regard to bacterial cell concentration (Sekirov et al., 2010, Frank et al., 2007). In addition, there are significant differences in bacterial composition between the luminal and mucosal compartments of the gut (Swidsinski et al., 2005). Following closure of the ileostomy, the ileal mucosa in the pouch is exposed to faeces containing bacterial concentrations a million or more times greater than in the normal terminal ileum (Nicholls et al., 1981, Santavirta et al., 1991).

In the normal ileum there are approximately 10^7 cells g^{-1} compared with 10^{12} cells g^{-1} in the distal colon (Sekirov et al., 2010). Differences in the diversity of bacterial groups present are also noted between ileal and colonic biopsies. Sequence analysis indicates that the wall of the distal small bowel is colonised by microbial populations that are not radically different from those of the large bowel. Both are dominated by bacteria of the phyla *Bacteroidetes* and *Firmicutes,* but do differ in the relative proportions in which subgroups of these phyla are present (Frank et al., 2007). Small intestine samples show increases in *Streptococcaceae*, *Actinomycinaeae* and *Corynebacteriaceae* compared with the colon, whilst fewer sequences of *Bacteroidetes* and *Lachnospiraceae* are found in small intestine samples compared with colonic samples (Frank et al., 2007). In addition, there are significant differences in bacterial composition between the luminal and mucosal compartments of the gut. Many bacterial

species present in the intestinal lumen do not access the mucus layer and epithelial crypts (Swidsinski et al., 2005).

Many of the studies of the microbiota in the ileal pouch and pouchitis suffer from inconsistencies in the definition and diagnosis of pouchitis, prior to the widespread use of the pouch disease activity index (PDAI) in 1994 (Sandborn et al., 1994) as well as distinctions between acute and chronic pouchitis. Variability in the use of faecal or mucosal samples may also contribute to the inconsistencies in the findings between many of these studies.

The majority of studies of pouch microbiota used culture methods. It is now known that approximately 60-80% of gut bacterial species cannot be cultured (Hayashi et al., 2002, Eckburg, 2005). Molecular techniques have developed to enable microbiota analysis of the entire microbiota without the limitations of culture methods. Various techniques are available, but all are based around the use of the 16S ribosomal RNA (rRNA) gene. The 16SrRNA gene is appropriately small (1.5 kb) and strikes a balance between high conservation and enough variability to distinguish between different species and strains, yet also enough similarity to identify members belonging to the same larger phylogenetic group (Sekirov et al., 2010). The 16SrRNA genes are amplified by polymerase chain reaction (PCR). 16S rRNA sequences are "binned" into operational taxonomic units (OTUs) based on their percent sequence identity (%ID). Specific percentages of identifications are widely accepted as indicators of various tiers of taxonomic resolution. OTUs containing sequences with 99% pairwise sequence similarity

indicate “strain-level” taxa, while 97% designates “species,” 95% “genus,” and 90% “family” (Sekirov et al., 2010).

1.3i Changes in the pouch microbiota following closure of the ileostomy

Santavirta *et al.* (Santavirta et al., 1991) studied faecal samples from 30 UC RPC patients and ten with a conventional ileostomy. Nine patients had a history of pouchitis, although only three patients were symptomatic at the time of the study. The total bacterial count, the numbers of anaerobes and the anaerobe to aerobe ratios were all increased in samples from pouch patients compared with those of patients with a conventional ileostomy. Nasmyth et al. (Nasmyth et al., 1989) also compared faecal samples of pouch patients with those with a conventional ileostomy for UC. They again found an increased anaerobe to aerobe ratio and an increase in *Bacteroides* and *Bifidobacteria* in the samples of pouch patients compared to those with a conventional ileostomy. Two studies have assessed the temporal changes in the pouch microbiota using molecular techniques following ileostomy closure. Over the first year following ileostomy closure, the overall composition of the pouch microbiota showed similarities with the colonic microbiota (Falk et al., 2007, Kohyama et al., 2009).

1.3ii Differences in bacterial cultures between UC and FAP pouches

A number of studies have assessed differences between the microbiota in UC patients and FAP patients with healthy pouches and conventional ileostomies. Using culture of faecal samples Duffy *et al.* (Duffy et al., 2002) studied 25 patients (10 UC RPC, 7 FAP RPC and 8 UC ileostomy). None had a previous history of pouchitis. Eighty percent of the UC RPC samples contained

sulphate reducing bacteria whereas none were found in the samples from the FAP RPC or UC ileostomy patients. There were no significant differences between counts for *Lactobacilli*, *Clostridium Perfringens, Bacteroides, Bifidobacterium* or *Enterococci* and *Coliform* in UC and FAP pouches. Smith *et al.* (Smith et al., 2005) cultured faecal samples from nine UC RPC, five FAP RPC and seven UC ileostomy patients. Sulphate reducing bacteria were exclusively found in samples from the UC RPC patients.

1.3iii Differences in bacterial cultures between the healthy pouch and pouchitis

Several studies have assessed the microbiota of the inflamed and non-inflamed UC and FAP pouch. O'Connell *et al.* (O'Connell et al., 1986) studied 20 UC RPC patients, eight with good function (<6 stools/day) and no pouchitis, six with poor function (>6 stools/day) and no pouchitis and six with pouchitis. Stool culture showed an increased bacterial count in all pouches with no differences between pouchitis or non-pouchitis patients with good or poor function. Onderdonk *et al. (Onderdonk et al., 1992)* cultured mucosal samples from RPC patients with and without pouchitis and samples from conventional ileostomies. Differences between obligate and facultative anaerobe counts were shown between pouchitis and non pouchitis patients with facultative anaerobe counts 50 to 500 times higher than those in non-pouchitis patients. Kmiot *et al.* (Kmiot et al., 1993) studied bacterial counts of stool samples from six non-pouchitis and six pouchitis patients before and following metronidazole. Higher aerobic counts and lower anaerobic counts were associated with pouchitis although these were not significant. Ruseler-van Embden *et al. (Ruseler-van Embden et al., 1994)* studied faecal samples of fourteen pouch patients (12 UC; 2FAP), five with pouchitis and nine classified as

non-pouchitis. There were no differences in the total numbers of bacteria between pouchitis and non-pouchitis patients, but patients with pouchitis had an increased number of aerobes, a decreased ratio anaerobes to aerobes, less *Bifidobacteria* and anaerobic *Lactobacilli*, more *Clostridium perfringens*, and several species such as fungi, that were not found in non-pouchitis patients.

Mcleod *et al.* (McLeod et al., 1994) studied mucosal biopsies from 23 patients with normal pouches, twelve patients with pouchitis, fourteen patients who had either endoscopic or histologic evidence of inflammation but not both, twenty patients with conventional ileostomies, and nine patients without ileostomies from whom biopsy samples of normal ileum were obtained. The total aerobic facultative counts in the biopsy samples from the pouchitis patients were significantly higher when compared with biopsy samples from ileostomy or normal ileum. Intramural bacteria were observed on electron microscopy in biopsy specimens of 47% of patients with pouches compared with fourteen percent of patients with conventional ileostomies or normal ileum.

Sandborn *et al.(Sandborn et al., 1995)* cultured stool from ten UC RPC non-pouchitis, ten UC RPC pouchitis, five FAP RPC non-pouchitis and five ileostomy patients. RPC patients demonstrated a higher ratio of anaerobic gram negative rods compared to ileostomy but no differences between pouchitis and non-pouchitis samples were found. Ohge *et al.* (Ohge et al., 2005) studied faecal samples of 45 UC pouch patients separated into active pouchitis, ongoing pouchitis with antibiotic treatment, previous pouchitis (with no active disease for over a year or

within the last year but inactive within six weeks), eight UC non-pouchitis and five FAP pouch patients. FAP pouch patients demonstrated significantly lower release of hydrogen sulphide gas compared with all UC patients except those on antibiotic therapy. Sulphate reducing bacteria were higher in samples from active pouchitis patients than from all other groups (Table 1.2).

1.3iv Studies of the pouch microbiota using molecular techniques

Several studies to date have used molecular biological methods to analyse differences between the microbiota in pouchitis and non-pouchitis patients (Komanduri et al., 2007, Johnson et al., 2009, Lim et al., 2009, Zella et al., 2010, McLaughlin et al., 2010b). However, differences between the techniques used, the definition of pouchitis, antibiotic washout periods and levels of analysis, complicate comparisons between these studies. Studies of the pouch microbiota using molecular techniques have consequently reached conflicting conclusions regarding differences in the abundance of particular species and phylotypes present in pouchitis or whether there is an increased or reduced diversity of the pouch microbiota (Table 1.3).

Komanduri *et al.* (Komanduri et al., 2007) used length heterogeneity-polymerase chain reaction analysis (LH-PCR) to investigate mucosal biopsies from twenty patients who had undergone restorative proctocolectomy within the previous five years. Five patients had active pouchitis and fifteen patients had healthy pouches. LH-PCR products from three patients in the pouchitis group and five patients in the non-pouchitis group as well as three non-IBD control patients were then pooled. Only phylotypes representing more than five percent of the total clone library were analyzed. There was an increase in bacterial diversity in pouchitis samples

compared with non-IBD controls and an increase in the proportion of *Enterobacter* and *Fusobacter*, a reduction in *Streptococci* and differences in the *Ruminococcus* species comparing pouchitis and non-pouchitis samples. However, pooling of samples (considering the known significant inter-individual variability of the gut microbiota (Eckburg, 2005)) and analysis of only up to 73% of clones may be drawbacks to this study.

Johnson *et al.* (Johnson et al., 2009) assessed mucosal biopsies from seven UC pouchitis, fifteen UC non-pouchitis, one FAP pouchitis and nine FAP non pouchitis patients. Two of the seven UC pouchitis patients met criteria for chronic pouchitis and in the UC non-pouchitis group two patients had either recurrent pouchitis or antibiotic dependent pouchitis. Mucosal biopsies were first cultured and the bacterial DNA was then extracted and amplified and profiles generated using Terminal Restriction Fragment Length- Polymorphisms (TRFL-P). No difference between pouchitis and non-pouchitis groups was demonstrated. However, the use of enrichment culture may have favored identification of bacteria supported by the culture conditions.

Lim *et al.* (Lim et al., 2009) studied faecal samples of fifteen healthy and five inflamed (PDAI >7) UC pouches using a TRFL-P approach. Patients with chronic pouchitis were excluded. Bacterial diversity in healthy pouches was similar to that in inflamed pouches. Bacterial counts of seven dominant organisms were similar in patients with pouchitis and those with a healthy pouch. Seventeen non-dominant organisms were seen exclusively in patients with pouchitis.

Table 1.2. Studies of the pouch microbiota using culture methods

Author	Patients Studied	Faecal/Mucosal Microbiota	Pouch Findings
Santavirta et al. (Santavirta et al., 1991)	30 UC RPC (9 pouchitis), 10 UC ileostomy	Faecal	↑ total bacterial count, ↑ anaerobes, ↑ anaerobe: aerobe ratio
Nasmyth *et al.* (Nasmyth et al., 1989)	15 UC RPC, 14 UC ileostomy	Faecal	↑ anaerobe:aerobe ratio, ↑ *Bacteroides* and *Bifidobacteria*
Duffy *et al.* (Duffy et al., 2002)	10 UC RPC, FAP RPC, 8 UC ileostomy	Faecal	↑ total bacterial count (UC), ↑ sulphate reducing bacteria (UC)
Smith *et al. (Smith et al., 2005)*	9 UC RPC, 5 FAP RPC, 7 UC ileostomy	Faecal	↑ sulphate reducing bacteria (UC), ↑ strict anaerobes (UC)
O'Connell *et al.* (O'Connell et al., 1986)	20 UC RPC (8 good function, 6 poor function-no pouchitis, 6 pouchitis)	Faecal	No differences seen
Onderdonk *et al.* (Onderdonk et al., 1992)	78 biopsies from healthy and inflamed RPC and ileostomy	Mucosal	↑ facultative anaerobes in inflamed pouch
Ruseler van Embden *et al.* (Ruseler-van Embden et al., 1994)	9 non-pouchitis, 5 pouchitis, (12 UC RPC, 2 FAP RPC)	Faecal	↑ aerobes (pouchitis), ↓ anaerobe:aerobe ratio (pouchitis), ↓ *bifidobacteria, lactobacilli* (pouchitis)
McLeod *et al.* (McLeod et al., 1994)	23 non-pouchitis, 12 pouchitis, 14 inflamed non pouchitis, 20 ileostomy, 9 normal ileum	Mucosal	↑ facultative aerobes (pouchitis)
Sandborn *et al.(Sandborn et al., 1995)*	10 UC non-pouchitis, 10 UC pouchitis, 5 FAP non-pouchitis, 5 ileostomy	Faecal	No differences seen between pouchitis and non-pouchitis
Ohge *et al.(Ohge et al., 2005)*	45 UC RPC, 5 FAP RPC	Faecal	↑ sulphate reducing bacteria (UC pouchitis)

Zella *et al.* (Zella et al., 2010) compared the faecal and mucosal microbiota of nine UC pouchitis, three UC non-pouchitis and seven FAP pouchitis patients using a TRFL-P approach. This study again pooled faecal DNA from UC pouchitis and FAP pouches for further species analysis. In this study pouchitis patients had recurrent or persistent pouchitis. TRFL-P data revealed significant

differences in the mucosal and faecal microbiota between the groups. UC pouchitis samples showed more peaks matching *Clostridium* and *Eubacterium* compared with non-pouchitis and FAP pouches and fewer peaks matching *Lactobacillus* and *Streptococcus* compared to FAP. Further sequencing analysis revealed UC pouchitis samples to have more *Firmicutes* and *Verrucomicrobia* and fewer *Bacteroidetes* and *Proteobacteria* compared with FAP.

McLaughlin *et al.* performed a study of the mucosal microbiota in eight UC pouchitis, eight UC non-pouchitis, five FAP non-pouchitis and three FAP pouchitis patients (McLaughlin et al., 2010b). Pouchitis patients included were classified as having chronic pouchitis. PCR amplification of bacterial 16S rRNA genes was performed and the samples were individually cloned and sequenced. There was a significant increase in *Proteobacteria* and a significant reduction in *Bacteroidetes* and *Faecalibacterium prausnitzii* in the UC group compared with the FAP group, but only small differences between the UC pouchitis and non-pouchitis samples and between the FAP pouchitis and non-pouchitis samples. No individual species or phylotype was specifically associated with either UC or FAP pouchitis. However, analysis of bacterial diversity (using the Shannon Diversity Index) revealed significantly greater diversity in the FAP non-pouchitis group compared with the UC non-pouchitis group and lower diversity in the UC pouchitis group compared with the UC non-pouchitis group.

Table 1.3. Studies of pouch microbiota using molecular methods

Author	Patients Studied	Method	Faecal/Mucosal	Findings
Falk *et al.(Falk et al., 2007)*	2 UC, over 1st year from RPC	TRFL-P	Mucosal	More "colonic-like" microbiota, Unstable over 1st year
Kohyama *et al.(Kohyama et al., 2009)*	26 UC <2 years RPC, 11 UC >2 years RPC, 13 UC ileostomy, 31 Healthy controls	TRFL-P	Faecal	Time dependent shift to more "colonic-like" microbiota in UC RPC
Komanduri *et al.(Komanduri et al., 2007)*	5 UC RPC pouchitis, 15 UC RPC non-pouchitis, 13 Healthy control ileum	LH-PCR	Mucosal	↑ bacterial diversity pouchitis v healthy control, ↑ *Enterobacter* and *Fusobacter* in pouchitis v non-pouchitis, ↓ *Streptococci* and *Ruminococci* in pouchitis v non-pouchitis
Johnson *et al.(Johnson et al., 2009)*	7 UC pouchitis, 15 UC non-pouchitis, 1 FAP pouchitis, 9 FAP non-pouchitis	TRFL-P	Mucosal	No differences between pouchitis and non-pouchitis seen
Lim *et al.*(Lim et al., 2006)	5 UC pouchitis, 15 UC non-pouchitis	TRFL-P	Faecal	Similar bacterial diversity between groups, Distinct non-dominant organisms in pouchitis
Zella *et al.* (Zella et al., 2010)	9 UC pouchitis, 3 UC non-pouchitis, 7 FAP	TRFL-P	Faecal and Mucosal	↑ *Clostridium* and *Eubacterium* in pouchitis, ↓ *Lactobacillus* and *Streptococcus*
McLaughlin *et al.(McLaughlin et al., 2010b)*	8 UC pouchitis, 8 UC non-pouchitis, 5 FAP non pouchitis, 3 FAP pouchitis	Full-length sequencing	Mucosal	No individual species or phylotype associated with pouchitis, ↓ bacterial diversity in UC and ↓↓ bacterial diversity in UC pouchitis
Tannock et al.(Tannock et al., 2012)	17 UC non pouchitis, 17 chronic pouchitis on and off antibiotics, 14 FAP	FISH, qPCR, high throughput sequencing	Faecal	↓ diversity of microbiota in chronic pouchitis but ↑ diversity of *Clostridiacaeae* species in chronic pouchitis
Mizoguchi et al. (Mizoguchi et al., 2013)	19 UC non pouchitis, 15 UC pouchitis, 19 CDL-phenotype, 18 FAP	16SrRNA 454 pyrosequencing and qPCR	Mucosal	↑*Proteobacteria* ↓ *Bacteroidetes* in pouchitis, ↓ *Bacteroides*, *Blautia*, *Dorea* and *Sutterella* in pouchitis

Using a combination of culture and molecular techniques, Scarpa et al. (Scarpa et al., 2011a) identified a direct correlation between *Bacteriodaceae spp.* with granulocyte and monocyte infiltration and *Clostridiaceae spp.* with pouch ulceration. Conversely, *Enterococcaceae spp*., *Enterobacteriaceae spp. and Streptococcaceae spp*., correlated inversely with immune cell infiltration. Recently, Tannock et al. demonstrated a reduced diversity of the microbiota in patients with chronic pouchitis and the microbiota of chronic pouchitis patients was composed of less than 200 uncommon faecal species (Tannock et al., 2012). Another study recently characterised the pouch microbiota associated with outcome of pouch function. In this study, inflammatory outcomes were again associated with an increase in *Proteobacteria* and a reduction of *Bacteroidetes* and genera *Bacteroides*, *Blautia*, *Dorea* and *Sutterella* were found less frequently in patients with inflammatory pouch outcomes (Mizoguchi et al., 2013) (Table 1.3).

1.3v Studies of the pouch microbiota following therapeutic intervention

Several studies have assessed changes in the microbiota following interventions of either antibiotic or probiotic therapy (Gosselink et al., 2004a, Kuisma et al., 2003). Analysis of faecal samples before and after treatment showed a significant decrease in total anaerobes and aerobes, *Enterococci, Lactobacilli, Bifidobacteria* and *Bacteroides*, while a non-significant reduction in the number of coliforms and *Clostridium perfringens* was also seen. Gosselink *et al.* (Gosselink et al., 2004b) treated patients with acute pouchitis with either metronidazole or ciprofloxacin and demonstrated different changes in the faecal bacteria depending on the antibiotic used. During pouchitis episodes, a significant decrease of anaerobes, an increase of

aerobic bacteria, more pathogens, such as *Clostridium perfringens* and haemolytic strains of *Escherichia coli* were seen. Treatment with metronidazole resulted in a complete eradication of the anaerobic flora, including *C. perfringens*. However, no changes in the numbers of *E. coli* were found. In contrast, when the patient was treated with ciprofloxacin, not only *C. perfringens*, but also all coliforms including hemolytic strains of *E. coli* were not cultured. The larger part of the anaerobic flora was left undisturbed during the administration of ciprofloxacin.

Two studies assessed the effects of the probiotic *Lactobacillus rhamnosus GG* as prophylaxis for pouchitis following RPC (Gosselink et al., 2004a, Kuisma et al., 2003). *Lactobacillus GG* supplementation changed the pouch intestinal flora by increasing the ratio of total faecal *Lactobacilli* to total faecal anaerobes and enhancing the frequency of *Lactobacilli*-positive cultures in the pouch and afferent limb mucosal biopsy samples. However, only 40% of patients were colonized with *Lactobacillus GG* (Kuisma et al., 2003).

The probiotic VSL#3 (containing 5×10^{11} per gram of viable lyophilised bacteria of four strains of *Lactobacilli*, three strains of *Bifidobacteria*, and one strain of *Streptococcus salivarius* subsp. *Thermophilus)* is effective in maintenance of remission of pouchitis. In the study by Gionchetti *et al.(Gionchetti et al., 2000a),* following induction of remission with open label Ciprofloxacin and Rifaximin, 40 patients received probiotic bacteria (VSL#3 6g/day; 1800 billion lyophilised bacteria) and 20 placebo. Faecal samples were taken before and after antibiotic treatment and monthly during maintenance therapy with VSL#3. Faecal concentration of *Lactobacilli,*

Bifidobacteria and *Streptococcus salivarius* increased significantly in the VSL#3 treated patients. However, no significant changes were noted for *Bacteroides, Coliforms, Clostridia, Enterococci* or in the total aerobe and anaerobe counts compared with baseline levels (Gionchetti et al., 2000a).

In a study of VSL#3 for the prevention of pouchitis onset following RPC similar microbiological findings were noted in the VSL#3 treated patients (Gionchetti et al., 2003). However, in a later study of VSL#3 for maintenance of remission of chronic pouchitis, analysis of mucosal biopsies using denaturing gel gradient electrophoresis (DGGE) analysis demonstrated an increased bacterial diversity and bacterial richness and a reduction in fungal diversity in the VSL#3 treated patients compared with placebo following antibiotic induced remission. The increase of diversity was independent of the bacterial strains included in the VSL#3 treatment (Kuhbacher, 2006).

A recently published study of elemental diet for chronic pouchitis analysed faecal samples of patients before and after treatment using Fluorescent in situ hybridisation (FISH). This did not demonstrate significant clinical or microbiological change, although there was a trend towards an increase in the concentration of Clostridium coccoides-Eubacterium rectale following exclusive elemental diet (McLaughlin et al., 2012) (Table 1.4).

The results from early culture based studies of the pouch microbiota are limited by the more recently acknowledged inadequacies of culture techniques for this analysis as well as

heterogeneity between the classifications of pouchitis and the control groups used. More recent molecular studies also suffer from technical difficulties and heterogeneity of the types and definitions of pouchitis included. Acute and chronic pouchitis may well be distinct conditions or distinct ends of a spectrum of inflammatory disorders. Despite the discrepancies from the numerous studies of the pouch microbiota, a significant body of clinical and microbiological evidence has accumulated to suggest a dysbiosis of the pouch microbiota is likely to be important in the aetiology of this inflammatory disease process. It remains unclear whether the mucosal or faecal microbiota is predominantly responsible for the development of inflammation within the pouch or whether the dysbiosis is primary or secondary. Furthermore, it is unclear whether particular species of pathogenic or commensal bacteria are relevant to pathogenesis or whether the stability and diversity of the microbiota are essential. A dysbiosis of the gut microbiota has also been demonstrated in both Crohn's disease and UC (Sartor, 2008). Dysbiosis of the gut microbiota may be a pathogenic pre-requisite for the idiopathic development of gut inflammation, explaining the comparative frequency of pouchitis in UC RPC patients compared with FAP RPC patients. It is uncertain whether this is an epiphenomenon or predisposes to inflammation, but it is unlikely to singularly be the cause of pouch inflammation.

Table 1.4. Studies of pouch microbiota following therapeutic intervention

Author	Patients Studied	Intervention	Method	Faecal/Mucosal	Findings
Gionchetti *et al.(Gionchetti et al., 1999)*	Chronic Pouchitis	Rifaximin and Ciprofloxacin	Culture	Faecal	↓ anaerobes and aerobes, ↓ *Enterococci, Lactobacilli, Bifidobacteria* and *Bacteroides*
Gosselink *et al.(Gosselink et al., 2004b)*	Acute Pouchitis	Metronidazole or Ciprofloxacin	Culture	Faecal	Metronidazole:↓ anaerobic bacteria, ↔ *E. coli* Ciprofloxacin: ↓ *Clostridium perfringens* and all *E.coli*
Kuisma *et al.(Kuisma et al., 2003)*	Previous pouchitis in remission	*Lactobacillus rhamnosus* GG	Culture	Faecal	↑ Lactobacilli: total anaerobe ratio, ↔ total anaerobe /aerobe/*Lactobacilli.* 40% *Lactobacilli* culture +ve
Gionchetti *et al.(Gionchetti et al., 2000b)*	Maintenance of antibiotic induced remission of pouchitis	VSL#3	Culture	Faecal	↑ *Lactobacilli, Bifidobacteria, Streptococcus salivarius.* ↔ *Bacteroides, Coliforms, Clostridia, Enterococci* or total aerobe and anaerobes
Gionchetti *et al.(Gionchetti et al., 2003)*	Post ileostomy closure prophylaxis of pouchitis	VSL#3	Culture	Faecal	↑ *Lactobacilli, Bifidobacteria, Streptococcus salivarius.* ↔ *Bacteroides, Coliforms, Clostridia, Enterococci*
Kuhbacher *et al.(Kuhbacher, 2006)*	Maintenance of antibiotic induced remission of pouchitis	VSL#3	DGGE	Mucosal	↑ bacterial and ↓ fungal diversity in remission with VSL#3
Mclaughlin et al (McLaughlin et al., 2012)	Chronic pouchitis	Pre and post exclusive elemental diet	FISH	Faecal	Trend to increased Clostridium coccoides-Eubacterium rectale

1.4 The pouch microbiota and host immunity

There is a wealth of literature on the topic of the immune responses in UC and Crohn's disease. Data regarding the immune responses to the alterations in the pouch microbiota are comparatively sparse.

1.4i Genetics and pouchitis

Genome wide association studies have successfully identified genes contributing to susceptibility to Crohn's disease and UC. The elucidation of these susceptibility loci has also furthered our understanding of the underlying pathogenesis, in particular highlighting the role of the immune responses to microbial signalling and processing (Xavier and Podolsky, 2007) as well as epithelial barrier integrity (Anderson et al., 2011, Barrett et al., 2009). A few studies have analysed genetic susceptibility in pouchitis. All of these studies are limited by the small numbers of patients, but they point towards a genetic susceptibility in the immune responses to the gut microbiota.

The interleukin-1 receptor antagonist gene allele 2 (Brett et al., 1996, Carter et al., 2001), tumour necrosis factor (TNF) allele 2 and NOD2/CARD15 polymorphisms (Meier et al., 2005, Sehgal et al., 2010, Tyler et al., 2011) have all been associated with pouchitis. Carriership of the TLR 9-1237C and CD14-260T alleles were significantly more frequent with chronic relapsing pouchitis (Lammers et al., 2005a). A TLR 1 polymorphism was associated with pouchitis, supporting the hypothesis that bacterial recognition is important in the pathogenesis of

pouchitis (Ferrante et al., 2008). Recently a larger study of North American Caucasian patients who had undergone RPC found a NOD2insC gene polymorphism to be associated with worse outcome with chronic pouchitis and a Crohn's like phenotype (Tyler et al., 2012).

1.4ii Serological markers for inflammation of the pouch

The presence of serological markers in IBD has also signaled towards the underlying importance of the host immune responses to the microbiota in the pathogenesis of these diseases. Serological markers such as antibodies against OmpC, an outer membrane porin from *E.coli*, Anti-*Saccharomyces cervisiae* antibodies (ASCA) and antibodies against CBir1, an antigen closely related to flagellin have been studied in UC, indeterminate colitis and Crohn's disease suggesting pathogenic immune responses to bacterial antigens (Mow et al., 2004, Lodes et al., 2004, Targan et al., 2005, Dendrinos et al., 2006).

In a small study, the presence of ASCA antibodies was associated with post-operative fistula development after RPC (Dendrinos et al., 2006). In another small study the presence of ASCA, OmpC and I2 (antigen to *Pseudomonas* fluorescens), were predictive of post-operative continuous inflammation of the pouch (Hui et al., 2005). Both ANCA and CBir1 have been associated with the development of pouchitis in some studies (Aisenberg et al., 2004). Anti-chitobioside carbohydrate (ACCA) antibodies were associated with pouchitis (Ferrante et al., 2008). The presence of these serological markers may be predicting Crohn's disease of the pouch rather than pouchitis in UC patients, but could also represent a subset of the spectrum of pouch inflammatory disorders suggesting pathogenic immune responses to bacterial antigens.

1.4iii Pouch immune responses to bacteria

The hypothesis of immune responses to bacteria or their products as the underlying cause of pouchitis is supported by an *ex vivo* study by Bell *et al.* (Bell, 2004). Bacterial sonicates cultured from mucosal biopsies of UC pouch patients with healthy pouches and pouchitis were cultured with autologous and heterologous peripheral blood (PBMC) and lamina propria monocytes. Sonicates from pouchitis produced intense proliferation of the mononuclear cells (from PBMC and lamina propria) compared with minimal proliferation in sonicates from healthy pouches (Bell, 2004). Lammers *et al.* (Lammers et al., 2005b) demonstrated that patients treated with VSL#3 had significantly lower mucosal mRNA expression levels of interleukin-1 beta (Il1-β), interleukin-8, and interferon-gamma compared with placebo-treated patients. In addition, a lower number of lamina propria monocytes were found in the tissue of patients within the probiotic group compared with the placebo group and patients with pouchitis. Pronio *et al.* (Pronio et al., 2008) demonstrated a significant increase in the percentage of mucosal "regulatory" CD4+ cells in UC pouch patients treated with VSL#3. mRNA expression of Il1-β was reduced in the VSL#3 group and Foxp3 expression increased.

These studies provide strong evidence for the role of bacteria in the stimulation of mucosal innate and adaptive immune responses in the ileal pouch. It is not clear how the pouch microbiota might interact with the mucosal immune system.

1.4iv Cytokine expression in pouchitis

A number of studies have assessed the tissue cytokine profiles in pouchitis. These often show significant overlap with those seen in UC and it is postulated that the mucosal inflammation in pouchitis may be a result of an imbalance of inflammatory and immunoregulatory cytokines that regulate mucosal immune homeostasis. As outlined above a number of studies have suggested genetic polymorphisms relating to interleukin-1 (Brett et al., 1996, Carter et al., 2001) and TNFα (Facklis et al., 1999) are more frequently associated with pouchitis. Elevated levels of IL-1, Il-6, IL-8 and TNFα have been found in tissue from pouchitis patients and also from UC RPC compared with FAP RPC patients (Gionchetti et al., 1994, Patel et al., 1995a, Bulois et al., 2000, Goldberg et al., 1996, Leal et al., 2008).

The prominent neutrophilic inflammatory cell infiltrate seen in pouchitis also suggests an important role for chemo-attractants and adhesion molecules. Few studies have evaluated chemokines or adhesion molecules in pouchitis. A study of pouchitis and non-pouchitis patients demonstrated increased expression of IL-8 as well as chemo-attractants for monocytes and activated T-cells (Helwig et al., 2004). Two studies have assessed expression of adhesion molecules in pouchitis. ICAM-1 and soluble E-selectin were found to be elevated in plasma from pouchitis patients compared to non-pouchitis patients (Patel et al., 1995b). An open label uncontrolled study of twelve patients with chronic unremitting pouchitis treated with an antisense inhibitor of intercellular adhesion molecule-1 for six weeks showed a 58% remission rate (Miner et al., 2006).

As well as an important role in leukocyte migration, ICAM-1 is also an important co-stimulatory signal for T-cells. The dysregulation of serum and mucosal cytokine profiles may be relevant in the persistence of inflammation in the ileal pouch and may define the outcome of immune responses to a dysbiotic microbiota. However, they do not fully inform us of the underlying aberrant cellular mechanisms in the host innate and adaptive immune systems that are responsible for the sensing, processing and directing of appropriate effector responses to the microbiota that may predispose to the development of pouch inflammation.

1.4v Innate immune responses in pouchitis

Appropriate immune responses to the intestinal microbiota are mediated and carefully regulated by epithelial cells, macrophages, dendritic cells (DC), T-lymphocytes and B cells. Defects in these regulatory systems may lead to abnormal inflammatory responses.

Recognition of bacterial antigens is dependent on pattern recognition receptors, such as Toll-like receptors (TLRs) and NOD-like receptors. In humans, TLRs comprise ten transmembrane glycoproteins containing multiple leucine-rich repeat motifs in the divergent ectodomain and a highly conserved region in the intracellular tail. TLRs recognise alarm signals from microbiota/viral-associated and damage-associated molecular patterns (Cario, 2010). Different microorganisms may include different molecular signatures interacting with different members of the TLR family e.g. lipopeptides: TLR2; lipopolysacharride: TLR4; flagellin: TLR5 and CpG DNA motifs: TLR9. Binding of molecular ligands elicits receptor activation. All TLRs may signal through the adaptor protein MyD88, whilst TLR4 uses both MyD88 dependent and independent

pathways. Engagement of MyD88 activates a series of signalling modules i.e. IRAK, TRAF6 and TAK1, ultimately leading to activation of transcription factors (Cario, 2010).

In the normal intestine, TLR2 and 4 are present in low levels on intraepithelial and lamina propria mononuclear cells, minimising recognition of the commensal gut environment and maintaining a basal state of activation. Once host threats are encountered TLR signalling elicits immune responses to eliminate the danger. However, sustained TLR hyper-activation may provoke chronic inflammation as seen in IBD. Furthermore, stimulation of TLR 2 alters epithelial barrier integrity via an apical redistribution of ZO-1 in intraepithelial cells (Cario et al., 2004, Cario, 2008).

Combined carriership of the TLR 9-1237C and CD14-260T alleles were significantly more frequent in chronic relapsing pouchitis (Lammers et al., 2005a). Expression of TLR2 and TLR4 on dendritic cells from colonic samples of UC patients is elevated compared with healthy controls (Hart et al., 2005a). Differences in the expression of TLRs have also been found in pouchitis and between UC RPC and FAP RPC patients (Heuschen et al., 2007, Toiyama et al., 2006, Leal RF, 2010). mRNA expression of TLR 2,3,4 and 5 in UC RPC patients correlated with pouchitis score and compared with normal ileal mucosa, TLR 3 was decreased, whereas TLR 5 was increased in pouch mucosa. TLR 5 expression was increased in pouchitis and no differences were seen in TLR2 or TLR 4 expression (Heuschen et al., 2007). However, another study found TLR 2 and 4 were barely detectable in normal ileal mucosa, but were strongly upregulated in active pouchitis and TLR 4 was also upregulated in the non-inflamed pouch (Toiyama et al., 2006).

Another study showed UC pouch patients (in the absence of clinical, endoscopic and histological inflammation) had significantly higher expression of TLR 4 than FAP pouch patients, whilst TLR 2 expression was similar between the groups (Leal RF, 2010). Scarpa et al. demonstrated the interplay of the mucosal immune response and the microbiota with increased TLR 2 and 4 expression in chronic pouchitis patients correlating with increased *Clostridiaceae spp*. (Scarpa et al., 2011b). These findings may explain an inherent tendency in UC pouch patients towards an increased immune sensitivity to bacterial signals.

De Silva *et al.* demonstrated intense epithelial expression of HLA-DR in inflamed pouch mucosa compared with pouches without inflammation and normal ileum (de Silva et al., 1990). HLA-DR is a MHC class II cell surface receptor that presents peptide antigens to elicit immune system responses. These are typically found on antigen presenting cells such as macrophages and dendritic cells. In the inflamed pouch mucosa, an increase in the number of RFD9+ and CD16+ macrophages were noted (de Silva et al., 1991). CD40, a maturation and co-stimulatory marker on antigen presenting cells necessary for stimulation of naive T cells was significantly elevated on immunohistochemical analysis of biopsy samples from pouchitis compared with non-pouchitis patients (Polese et al., 2005). However, there are no previous studies of dendritic cells in pouchitis.

1.5 Dendritic cells

Mucosal dendritic cells (DC) are at the intersection between the innate and adaptive immune systems and are central to the maintenance of immune homeostasis in the gut. DC continually survey the gut luminal contents, enabling tolerance of commensals and recognising pathogens (Stagg et al., 2003). DC contribute to the expansion and differentiation of most classes of lymphocytes (Ueno 2007). They play a role in the differentiation of B cells, NK cells and NKT cells although relatively little is known about the mechanisms involved. Important interactions have recently been shown between DC and innate lymphoid cells, shaping the innate responses to the microbiota (Powell et al., 2012). DC are critical in determining whether T-cell responses generated are immunogenic or tolerogenic and maintain the delicate balance in the gut between immunogenicity against invading pathogens and tolerance of the commensal microbiota (Hart et al., 2005a).

DC are bone marrow derived antigen presenting cells that are present in most tissues, but comprise only a small proportion of the total circulating leucocyte population. The two major functions of DC are to acquire antigen and to stimulate lymphocytes. DC undergo maturation and migrate to draining lymph nodes in response to signals such as microbial antigen or cytokines. During maturation DC upregulate expression of MHC class II and costimulatory molecules and acquire the ability to stimulate naive T cells. DC can determine whether non-responsiveness or active immune response occurs to a particular antigen and also the type of inflammatory response (Th1, Th2, Th17) that occurs (Stagg et al., 2003).

Functionally distinct subsets of DC have been defined in both mice and humans based on cell surface phenotpye and functional properties. In humans these subsets include myeloid DC (mDC) (CD11c+, CD123low, BDCA2 and 4-) and plasmacytoid DC (pDC) (CD11c-, CD123high, BDCA2 and 4+). The CD11c+ subset follows a myeloid differentiation pathway and is therefore termed myeloid DC (mDC). Recently, mDC have been further subdivided into two groups. Type 1 mDC are identified by BDCA1+ (also known as CD1a+) and represent the majority of mDC in the human body (>90%). These DC are involved in presenting MHC class II antigens, leading to activation of a CD4+ T-cell response. Type 2 mDC (<10% total mDC) are BDCA3+ and carry out MHC class I antigen presentation to CD8+ T-cells with antigen-cross presenting capabilities. pDC come from an independent, possibly lymphoid-related, differentiation pathway. pDC combine features of both classical DC and lymphocytes and display unique molecular sensing of nucleic acids and type 1 interferon production. However, pDC are also capable of generating Th2 responses (Ng et al., 2009c, Ng et al., 2010a, Shortman and Liu, 2002).

The state of maturation and the type of DC can influence the subsequent T cell response. Immature DC, trafficking from peripheral to lymphoid tissue presenting self-antigen, inhibit potentially autoreactive T cells. Non-responsiveness can also be induced to foreign antigen. Mature DC can activate and drive clonal T cell expansion and shape the functional differentiation of dividing T cells through cytokine production (Stagg et al., 2003, Ng et al., 2010a). Naive T-cells require two signals from DC to become fully activated. The antigen (bound to MHC-II) provides the first signal and interacts with the T-cell receptor (TCR). The second signal is antigen non-specific and is provided by co-stimulatory molecules on the surface

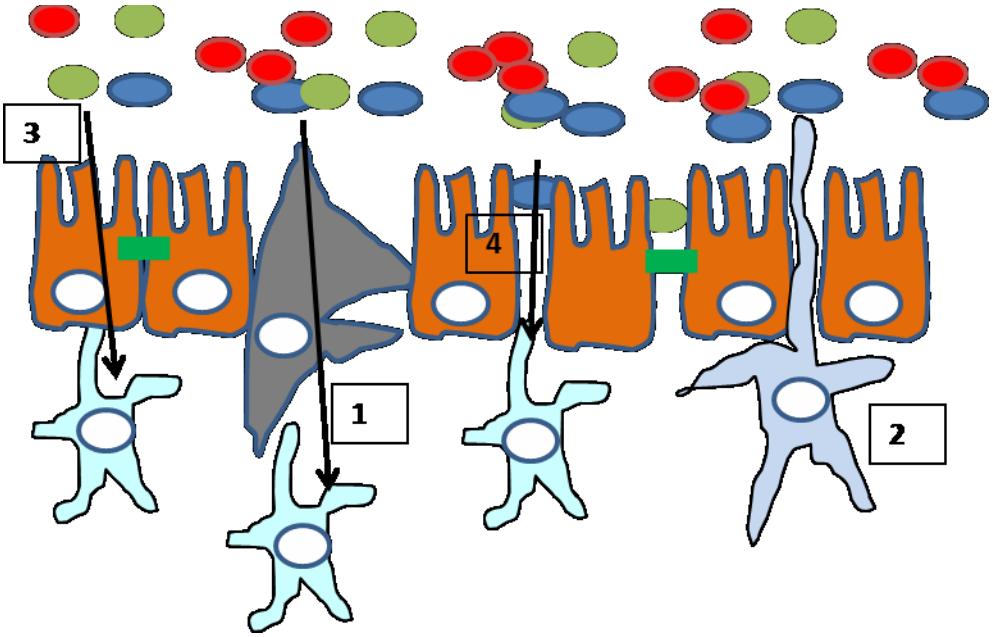
of the DC binding with their corresponding receptors on the naïve T-cell. Examples of such co-stimulatory molecules are CD40, CD80 and CD86. This double signal is necessary for T-cell proliferation, differentiation, survival and cytokine secretion. Activation of T-cells without co-stimulation results in suppression of the immune response, and, in some cases, induces antigen-specific tolerance. Matured or activated DC provide a third signal to the T-cells via secretion of cytokines. The cytokines produced by DC determine the type of effector T-cell response that occurs. The decision between induction of tolerance or active immunity depends on the subpopulation of DC, the surface receptors involved and the tissue environment during DC activation and T-cell priming.

1.5i Dendritic cell interaction with the microbiota

The effects of the microbiota on intestinal DC are pivotal in early bacterial recognition and the shaping of the subsequent innate and adaptive immune responses. DC express pattern recognition receptors including Toll-like receptors (TLRs) and nucleotide binding and leucine rich repeat containing receptors (NLRs NOD1 +2) that recognise common structural elements of microorganisms. DC also express C-type lectin receptors such as DC-SIGN. These receptors interact with ICAM 2 on vascular endothelium promoting extravasation of immature DC and with ICAM 3 on T cells to stabilise the immunological synapse. Within the peripheral tissues and secondary lymphoid organs, immature DC constantly sample foreign and self-antigens (Stagg et al., 2003).

In the gastrointestinal tract a number of routes by which DC can acquire and process specific antigens have been described. The main route for antigen to access the mucosal immune system has traditionally been via M cells. These specialised epithelial cells overlie the dome of Peyer's patches and shuttle luminal antigen to DC in the subepithelial dome regions. M cells may also be found within the villous epithelium to help deliver antigens to lamina propria DC. DC can also acquire antigen via internalisation of apoptotic epithelial cells or uptake of antigen exosomes shed from epithelial cells (Stagg et al., 2003). More recently DC have also been shown to express tight junction proteins and penetrate the epithelium to sample bacteria (Rescigno et al., 2001). Where the epithelial barrier is compromised DC may sample antigen directly (Figure 1.4). DC function can also be shaped indirectly via ligation of pattern recognition receptors and other indirect mechanisms on intestinal epithelial cells (Silva et al., 2008, Iliev et al., 2009).

Figure 1.4 Modes of antigen sampling and uptake by intestinal DC (based on image from Stagg et al. 2003)(Stagg et al., 2003).



Four described pathways of DC sampling of antigen in the intestinal lumen. 1. following transport of antigens by M cells. 2. reaching between epithelial cells to directly sample the lumen. 3. via the epithelial cells, either by pinocytosis of material transported by epithelial cells or following uptake of apoptotic epithelial cells and 4. By direct paracellular transport of antigens as a result of breaks in the integrity of the epithelium.

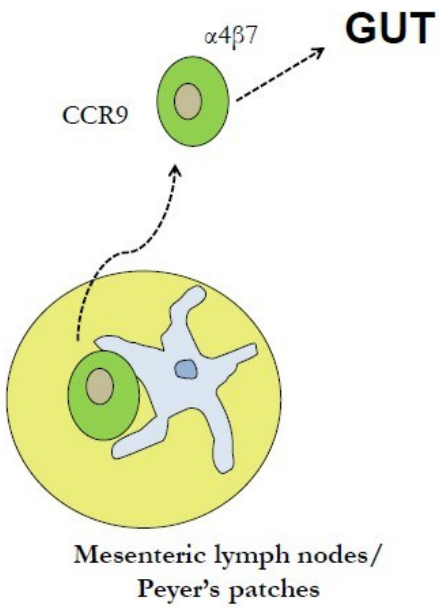
1.5ii Dendritic cells imprint gut-homing properties on lymphocytes

On encountering foreign antigen, DC undergo maturation with upregulation of MHC and co-stimulatory molecules. In addition, expression of CCR7 is upregulated as DC move to the draining lymph node where they present antigens to naïve T cells. DC bearing antigen are consequently critical in "informing" T cells of the tissue and environment from which the DC encountered the antigen and can induce gut homing receptors on T cells enabling them to

home back to the intestine and access the anatomical site most likely to contain their cognate antigen (Ng et al., 2010a, Hart et al., 2010, Mann et al., 2012).

Lymphocytes continuously migrate around the body optimising their probability of encountering an antigen. Lymphocyte subsets express unique patterns of homing molecules enabling guidance to their target tissue via site-specific pathways. Naive T cells express homing receptors allowing migration to lymphoid organs. Once they have become activated by antigen they alter their pattern of homing receptors and can migrate to peripheral sites. The tissue homing phenotype of T cells is established by tissue specific DC during T cell priming and B cell activation (Dudda et al., 2005, Mora et al., 2006). Tissue specific DC can also modulate homing patterns of effector B and T cells during an ongoing inflammatory response (Dudda et al., 2005, Mora et al., 2006). In the intestine, lymphocytes activated in mesenteric lymph nodes have a propensity to home back to the intestine. The molecules enabling homing to the intestine include the integrin α4β7, which is attracted to its ligand mucosal addressin cell adhesion molecule-1 (MadCAM-1). CCR9 and its ligand CCL-25/TECK are involved in homing to the gut, particularly the small intestine (Hart et al., 2010) (Figure 1.5).

Figure 1.5 Induction of intestinal T cell homing by DC (Figure courtesy of Mann ER, APRG)



Intestinal dendritic cells imprint gut homing markers on naive T cells in the mesenteric lymph nodes and peyer's patches in a Retinoic acid dependent manner, leading to gut specific homing of T cells.

Mouse studies suggest that CCR9 expression confers small intestine specific homing properties for DC (Hart et al., 2010). However, a recent study found that the CCR9 ligand, CCL25/TECK, was expressed in the colonic mucosa (Linton et al., 2012). Similar to the gradient in the composition of the microbiota between the small and large bowel, a gradient exists in the small and large intestine with regard to expression of homing molecules and their ligands. In the small intestine both CCR9/CCL25/TECK and α4β7/MadCAM-1 are involved in lymphocyte migration. In contrast, CCR9/CCL25/TECK expression in the colon is limited. There is a gradient of expression of CCL25/TECK from the highest expression in the proximal small bowel to the lower expression in the distal ileum and limited expression is found in the colon. Blockade of CCL25/TECK

prevents T cell migration to the small intestine, but has no effect on lymphocyte homing to the colon (Hart et al., 2010). The relative expression of CCR9 and β7 may therefore determine preferential homing to the small or large bowel.

In mice, CD103+ DC have been shown to possess the ability to generate gut homing T cells in studies involving mesenteric lymph node and small-intestinal DC (Johansson-Lindbom et al., 2005). There is no information regarding the imprinting capacity of colonic CD103+ DC in mice or humans. In mice, it is the CD103+ intestinal DC subset, but not other DC populations, that express the enzyme retinaldehyde dehydrogenase (RALDH) that mediates irreversible conversion of dietary retinal to Retinoic acid (RA) (Mann et al., 2013a). Retinoic acid is essential for the process of DC imprinting gut homing properties on lymphocytes (Dudda et al., 2005, Mora et al., 2006). Retinoic acid may synergise with other factors such as cytokines to determine tissue specific immune responses (Mora et al., 2006, Mann et al., 2013a).

In a recent study, human colonic DC generated gut homing T-cells. The large fraction of CD103− DC identified in the human colon in this study, suggests the existence of CD103− DC subsets with the ability to generate gut homing T-cells (Mann et al., 2012). CD103- DC also conferring a gut homing phenotype to naive T-cells were also recently identified in murine intestinal lymph (Cerovic et al., 2013). CD103− DC may internalise RA from exogenous sources to be released during T-cell activation to generate gut-homing T-cells.

Soluble factors from the tissue microenvironment crucially influence homing receptor polarisation and can over-ride the DC tissue specificity (Dudda et al., 2005). Conditioning human blood DC with supernatants from healthy human colonic biopsies enhances β7 expression on DC and their ability to prime gut-homing T cells (Mann et al., 2012) and is dependent on the presence of RA in the supernatant. MyD88-dependent TLR signalling confers DC ability to imprint gut-specific homing properties on T cells (Wang et al., 2011), directly linking bacterial activation of DC with imprinting capacity. The gut microbiota is likely to be crucial in influencing the gut homing properties of DC and their determination of tissue specific lymphocytes.

1.5iii The role of dendritic cells in inflammatory bowel diseases

The role of DC in IBD is increasingly recognised for their function in regulating intestinal immune responses (Hart et al., 2005a, Stagg et al., 2004). DC are increased and more mature within inflamed IBD tissue. In IBD, DC are activated with upregulated expression of TLRs and co-stimulatory molecules as well as production of pathologically important cytokines (Hart et al., 2005a). Most studies of DC in IBD have focused on myeloid DC.

Recently studies have begun to characterise plasmacytoid DC in IBD. In IBD patients a striking inverse correlation is seen between peripheral pDC and mDC and disease activity (Baumgart et al., 2005). pDC are present in greater numbers in the inflamed colonic mucosa and mesenteric lymph nodes of IBD patients (Baumgart et al., 2011). IBD patients have pDC displaying greater CD40 expression than healthy controls as well as increased secretion of TNF and interleukins 6

and 8 with impaired IFN secretion, suggesting an aberrant distribution and function of pDC in IBD (Baumgart et al., 2011). Lamina propria pDC are increased in inflamed and non-inflamed tissue in UC compared with controls without a concomitant increase of mDC, and these pDC are weakly stimulatory to T cells in mixed leucocyte reactions (Ng et al., 2009c). pDC may be of particular importance in the inappropriate innate immune responses to the microbiota in UC patients.

In IBD, tissue specific homing of lymphocyte populations contribute to the dysregulated immune response. In Crohn's disease, gut-specific adhesion molecules are increased. These are less well defined in UC and in pouchitis no increase in MadCAM-1, ICAM-1 or VCAM-1 was found between UC pouchitis tissue and FAP uninflamed pouch tissue (Thomas et al., 2002). However, activated CD27 and CD30+ T cells were increased in pouchitis mucosa (Thomas et al., 2001). In colonic mucosa, β7+ lymphocytes are increased in active Crohn's disease and UC. Relapse of disease in UC patients is associated with an increase in Th1 and Th2 cytokines from β7+ memory T cells (Hart et al., 2004a). No data currently reports DC or T cell homing properties in pouchitis.

1.5iv Manipulation of DC function with probiotics

Several mechanisms have been identified by which probiotics may exert their therapeutic effects. Mechanisms contributing to altered immune function include modulation of the host microbiota, improved barrier function and direct effects on immune cell types (Ng et al.,

2009a). Attempts to alter the intestinal microbiota may have critical effects on DC function. A number of studies have demonstrated the modulation of DC by probiotic bacteria. In vitro, VSL#3 is a potent inducer of IL-10 by lamina propria DC and reduces co-stimulatory molecule, CD80 expression. Individual strains within VSL#3 display distinct immunomodulatory effects on DC; the most marked anti-inflammatory effects are produced by *Bifidobacteria* (Hart et al., 2004b). In a study of VSL#3 treatment for patients with active UC, a reduction in TLR 2 expression as well as an increase in IL-10 and a reduction in IL-12 production was seen in lamina propria mDC (Ng et al., 2010b). In patients with pouchitis treated with VSL#3 there were enhanced levels of IL-10 in their mucosa and decreased levels of TNF-, IL-1, inducible nitric oxide synthase, and matrix metalloproteinase (Ulisse et al., 2001). *L. reuteri* and *L. casei* bind the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN). Blocking antibodies to DC-SIGN inhibited the induction of "T regs", implying that ligation of DC-SIGN may actively prime DC to induce regulatory T cells (Smits et al., 2005). In an in vivo murine model, administration of VSL#3 skews the proportion of plasmacytoid and myeloid DC in the gut lamina propria, suggesting probiotics may also alter the distribution of intestinal DC subsets (Wang et al., 2009). Heat killed *Lactobacilli caseii shirota* increase DC stimulatory capacity and imprint skin homing markers on T cells (Mann et al., 2013b) and priming of intestinal DC with a secreted peptide of *Lactobacillus plantarum* imprint a skin homing profile on stimulated T cells (Bernardo et al., 2012a).

The immune effects of probiotics are, however, complex. Differential effects are seen between different species and within different strains of the same species. *L. reuteri* and *L. casei*, but not

L. plantarum, primed monocyte-derived DC to drive the development of T cells with regulatory properties (Smits et al., 2005). When tested in vivo, *L. plantarum* and *L rhamnosus GG* exacerbated the development of DSS-induced colitis and caused the death of treated mice, while, conversely *L. paracasei* was protective (Mileti et al., 2009). In addition, secreted peptides from probiotic bacteria (postbiotics) can exert "immunoregulatory" effects on DC (Bernardo et al., 2012a), whilst contact with the bacterium directly may exert stimulatory effects on DC and subsequent T cell responses (Mann ER personal communication). Furthermore, the immune effects of probiotic bacteria may depend on the local microenvironment in which they are acting and the integrity of the epithelial monolayer (Tsilingiri et al., 2012). In an ex-vivo model, probiotic inoculation with IBD tissue exerted destructive effects. This suggests that in the context of a loss of epithelial barrier integrity and increased translocation, otherwise non-pathogenic, non-invasive bacteria may worsen inflammation (Tsilingiri et al., 2012). Currently there are few data regarding the effect of intestinal barrier dysfunction on mucosal DC in human IBD. One limitation is the difficulty in predicting and detecting the onset of the first disease episode or relapse prior to their occurrence. In animal models of colitis, redistribution and maturation of DC are associated with impaired intestinal permeability and are regulated via TLR-2 (Silva et al., 2008, Silva, 2009).

1.6 Epithelial Barrier Function

The mucosal epithelium limits the interactions between microbial antigens and the mucosal immune system at the mucosal surface. The intestinal epithelium is the largest barrier between the body's internal and external environment. Gut epithelial cells prevent bacteria and associated toxins from penetrating the gut lumen. However, the intestinal epithelium is not only a static barrier but has evolved complex mechanisms to control and regulate bacterial interactions with the mucosal surface (Duerkop et al., 2009, Berkes, 2003).

Epithelial goblet cells produce large amounts of mucus glycoprotein regulated by the expression of the MUC2 gene. MUC2 knockout murine models demonstrate the importance of this layer with knockout mice being more susceptible to intestinal inflammation. The outer layer of mucus is populated with bacteria that may be found in the lumen, whilst the inner mucus layer remains relatively sterile. The relative sterility of the inner mucus layer is achieved through a number of mechanisms including the secretion of antibacterial proteins (defensins, cathelicidins and cryptidens) by epithelial cells. IgA sequesters and compartmentalises bacteria away from the epithelial layer and epithelial M cells and intra-epithelial lymphocytes maintain a tight control on local immune responses limiting the passage of pathogens (Salim and Söderholm, 2011).

A number of routes exist for passage across the epithelial barrier between the internal and external environment. Water, small ions and low molecular weight molecules (<600kDa),

typically utilise paracellular routes, whilst macromolecules pass transcellularly via pinocytosis and endocytosis. The intercellular spaces between adjacent cells, linked together by junctional complexes, are critical in regulating the mucosal barrier. The tight junctions form the apical most unit, defining the boundary between the apical and basolateral membranes and are predominantly the rate-limiting factor in paracellular passage (Salim and Söderholm, 2011). Tight junctions (TJ), adherens junctions (AJ) and desmosomes are collectively termed the adherens junctional complex (AJC) (Groschwitz and Hogan, 2009). The tight junction is built up by both transmembrane proteins such as occludin, tricellulin, different claudins and junctional adhesion molecules (JAMs), as well as peripheral membrane proteins such as zona occludens (ZO)-1,-2,-3 and cingulin. The peripheral membrane proteins are linked to the cell cytoskeleton by F-actin and myosin II (Edelblum and Turner, 2009, Groschwitz and Hogan, 2009). These ZO proteins have three PDZ domains that mediate binding to other transmembrane tight junction proteins such as claudins in a dynamic energy dependent manner (Shen et al., 2008b). They are also the direct targets and effectors of different signalling pathways (such as the myosin light chain kinase) thereby altering the assembly, maintenance, and barrier function of the tight junction complex (Schulzke et al., 2009).

The expression of different TJs in the gut varies according to localisation (e.g. villus vs. crypt, small bowel vs. colon) (Rahner et al., 2001, Escaffit et al., 2005), cell membrane localisation (e.g. apical, lateral or basolateral) and the gut's functional properties at the site (Groschwitz and Hogan, 2009, Amasheh et al., 2011, Rahner et al., 2001, Escaffit et al., 2005). For example, claudin 2 is expressed at the apical pole throughout the crypt-villus axis in the jejunum whilst in

the colon expression is restricted to the crypts, whereas claudin 4 expression is throughout the crypt-villus axis in the small and large intestine. The segmental distribution of claudin expression may relate to cell differentiation, carbohydrate metabolism and transcription factors such as HNF1α, Cdx2 and GATA-4 (Escaffit et al., 2005).

In health, the apical TJs construct a dynamic intestinal barrier that regulates the paracellular uptake of water, nutrients and electrolytes (Edelblum and Turner, 2009, Shen et al., 2008b). The majority of TJs are cation selective and prevent contact between the proteins of the two cell poles: the basolateral and apical cell membranes (Hartsock and Nelson, 2008). AJs and desmosomes are mostly involved in communication between neighbouring epithelial cells. (Bruewer et al., 2006, Groschwitz and Hogan, 2009, Hartsock and Nelson, 2008). TJ dysfunction can lead to the disruption of the intestinal barrier integrity. This dysfunction can be initiated by multiple factors, including immunomodulatory cytokine responses, the ingestion of NSAIDs and alcohol, the presence of pathogens with or without the involvement of enterotoxins and other comorbidities such as food allergies, coeliac disease and type 1 diabetes (Groschwitz and Hogan, 2009). Changes in pH, osmotic load or cytoskeleton function all affect the barrier function of TJs (Schneeberger and Lynch, 1992).

There are 24 different claudin isoforms that modulate the paracellular movement of ions based on charge and size (Turksen, 2004). Claudin 1 and claudin 2 are the major components of TJ strands (Morita et al., 1999). Claudin 2 controls the uptake of monovalent cations such as Na^+ to the interstitium and directs the synthesis of aquaporins, thus making the epithelium more

leaky (Amasheh et al., 2002, Rosenthal et al., 2010) in contrast to other claudins (like -1,-3,-4,-5,-8) that tighten the epithelium (Furuse et al., 2002, Milatz et al., 2010, Van Itallie et al., 2001, Amasheh et al., 2005, Angelow et al., 2006, Angelow and Yu, 2007, Angelow et al., 2008). Claudin 2 also directly decreases the barrier function of claudin 1 and claudin 4 strands (Turksen, 2004). Therefore the ratio of different claudins in the TJ determines its functional property as either leaky or tight.

1.6i Tight Junction Proteins in Inflammatory bowel diseases

Patients with clinically active Crohn's disease have increased intestinal permeability (Hollander et al., 1986, Munkholm et al., 1994). Furthermore, increased intestinal permeability in patients with inactive Crohn's disease is predictive of clinical relapse (D'Inca et al., 1999) and has been shown in 10-25% of healthy first degree relatives of patients with Crohn's disease (Peeters et al., 1997, Soderholm et al., 1999) which is associated with CARD15 mutations (Buhner et al., 2006). Increased gut permeability is also characteristic of UC (Munkholm et al., 1994, Schmitz et al., 1999, Gitter et al., 2001) and and recent genetic studies have identified new UC susceptibility loci pertaining to defects of the epithelial barrier (Anderson et al., 2011, Barrett et al., 2009). Barrier properties of pouch mucosa in pouchitis and in UC pouches where backwash ileitis was present prior to restorative proctocolectomy are reduced (Merrett et al., 1996, Kroesen et al., 2006, Amasheh et al., 2009b) and increased bacterial translocation is reported in pouches more than one year following ileostomy closure (Kroesen et al., 2008).

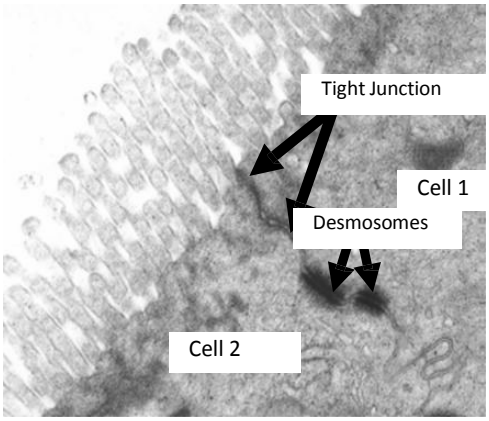
Several studies have focused on expression of claudins in UC patients (Schmitz et al., 1999, Prasad et al., 2005, Oshima et al., 2008). Prasad *et al.* (Prasad et al., 2005) investigated the expression of claudins in the normal colon and the colon from IBD patients, demonstrating a higher expression of claudin 2 in samples from patients with IBD. Additionally, the increase in claudin 2 significantly correlated with disease severity. The changes were more pronounced in UC than in CD samples (Prasad et al., 2005).

Oshima *et al.* (Oshima et al., 2008) found elevated claudin 2 levels in active UC samples, whereas claudin-4 and-7 levels were decreased at both protein and transcriptional level. Claudin 1 and 3 levels remained unchanged. In contrast, Poritz *et al*. (Poritz et al., 2011) found an increase in claudin 1:occludin ratios in colonic samples from UC patients compared with healthy controls and CD samples by Western blot analysis. This change in ratio was the result of both an increase in claudin 1 and a decrease in occludin. Disease severity, measured by the degree of inflammation, was directly proportional to the alterations seen in TJ structure in UC. In another study, claudin 18 was upregulated in the colon of UC patients compared to healthy controls, but did not correlate to disease severity and may represent a primary defect in epithelial barrier integrity (Zwiers et al., 2008).

Barrier properties of ileo-anal pouch mucosa in pouchitis and in non-inflamed pouches are reduced (Kroesen et al., 2008) with increased bacterial translocation (Kroesen et al., 2006). Merrett *et al* (Merrett et al., 1996) assessed permeability through administration and urinary recovery of ^{51}Cr-EDTA in patients prior to ileostomy closure, with samples obtained from a

"healthy" pouch, pouchitis and UC. An increase in pouch permeability in patients with pouchitis compared with those with a normally functioning pouch was found. Similarly, Amasheh *et al*. (Amasheh et al., 2009b) propose that the role of the epithelial barrier is critically important in the pathogenesis of pouchitis. Pouch biopsies were analysed before ileal pouch-anal anastomosis, during acute pouchitis and at a time point over a year after ileostomy closure. Claudin 1, 3, 4, 5 and 7 and occludin showed differential expression in this study, with a decreased level of claudin 1 and an elevation in claudin 2 levels. These observations suggest a mechanism of increased paracellular permeability due to faulty tight junctions as the basis of pouchitis. These changes are similar to the tight junction changes seen in UC.

Figure 1.6 Electron microscopy of pouch epithelium demonstrating tight junctions and gap junctions.



1.6ii Chicken or egg?

It is not clear whether the changes observed in tight junctions lead to abnormal epithelial barrier integrity and the aberrant immune responses, or whether the inflammation itself causes the alterations in tight junction expression and distribution. It is postulated that the primary cause for differences in CD and UC may be their differences in underlying immunological pathways. Inflammatory cytokines associated with gut inflammation alter permeability and tight junction expression (Heller et al., 2005, Zolotarevsky et al., 2002, Arrieta et al., 2008).

Heller *et al*. (Heller et al., 2005) demonstrated a significant upregulation of IL-13 in patients with UC compared to CD patients and non-inflammatory controls. Following the upregulation of IL-13, there was a decrease in trans-epithelial resistance and an increase in epithelial cell apoptosis and conductance. Furthermore, IL-13 caused upregulation of the claudin 2 gene, thus elevating claudin 2 protein production threefold. IL-13 did not have a significant effect on the expression of occludin, claudin 1 and claudin 4. IL-6 is elevated in IBD patients and in CACO-2 cells induces expression of claudin 2 via induction of increased Cdx 2 expression activating the claudin 2 promoter regions (Suzuki et al., 2011).

In CD, interferon gamma (IFN-γ) contributes to impairment of epithelial barrier function through disrupting tight junction complexes by causing decreased expression and increased internalization of occludin and ZO-1 (Scharl et al., 2009). The effects of IFN-γ on barrier function may be mediated through 5' adenosine monophosphate-activated protein kinase (AMPK).

AMPK is key in sensing the cell's energy levels, which decrease during inflammation and subsequently increase the demand for AMPK. Scharl *et al*. (Scharl et al., 2009) showed that inhibiting AMPK not only reverses its disastrous effect on TJs but also its negative effect on trans-epithelial resistance. In T84 cells (model intestinal epithelial cell line) IFN-γ increases intestinal permeability to large molecules such as *E. Coli*-derived lipopolysaccharide; effects were exerted by decreasing expression of occludin and increasing the expression of claudin 1 (Watson et al., 2005).

TNF-α can also affect tight junctions and decrease epithelial barrier function by increasing Myosin Light Chain Kinase (MLCK) phosphorylation. Inhibition of MLCK in TNF-α treated epithelial monolayers can acutely restore barrier function (Zolotarevsky et al., 2002). Furthermore, MLCK-activation promotes IL-13 expression and claudin 2 synthesis (Weber et al., 2010). TNF-α induced MLCK expression may therefore be a critical mechanism for barrier dysfunction in UC and CD. Ileal MLCK expression is increased in ileal biopsies from Crohn's patients compared with controls and increased MLCK correlates with disease activity (Blair et al., 2006).

A recent study of transgenic mice expressing activated MLCK showed increased paracellular permeability without histological inflammation. Further analysis however, found an increase in the absolute numbers of lamina propria CD4+ lymphocytes and a significant redistribution of CD11c+ dendritic cells to the superficial lamina propria as well as polarisation to a Th1 cytokine profile (Su et al., 2009). Other studies also suggest that tight junction abnormalities and

epithelial permeability may precede the increase of inflammatory cytokines. Interleukin-10 (IL-10) blocks IFN-γ induced epithelial permeability and IL-10 knockout mice have increased permeability and spontaneously develop chronic intestinal inflammation (Arrieta et al., 2008). Inhibition of the zonulin receptor (a key receptor in tight junction binding regulation) in IL-10 knockout mice reduced intestinal permeability and attenuated the spontaneous development of colitis (Arrieta et al., 2008). However, increased intestinal permeability in IL-10 knockout mice not only preceded the onset of inflammation but also occurred significantly earlier than any differences in IFN-γ or TNFα. In the cadherin knockout mouse, disruption of the adhesion molecule E-cadherin by tissue specific expression of a dominant negative cadherin in small intestinal epithelial cells throughout the crypt-villus axis resulted in disruption of adherens junctions. When chimeric mice were created, it was evident that a defect in cell migration and proliferation only occurred in epithelial units expressing the dominant negative cadherin. By three months of age, the mice developed typical histological features of inflammatory bowel disease (Hermiston and Gordon, 1995).

1.6 iii Therapeutic manipulation of tight junction proteins in inflammatory bowel diseases

Strategies to manipulate tight junctions and intestinal permeability are likely to have an important role in the future treatment of IBD. Anti-TNF therapy is effective in the treatment of Crohn's disease, UC and chronic pouchitis (Hanauer et al., 2002, Rutgeerts et al., 2005, Barreiro-de Acosta M, 2011). Barrier function is significantly restored following anti-TNF therapy for Crohn's disease (Suenaert et al., 2002, Zeissig et al., 2004). In the study by Zeissig et al, this was

associated with a reduction in epithelial apoptosis but no significant changes in occludin, claudin 1 or claudin 4. However, other claudins including claudin 2 were not assessed (Zeissig et al., 2004). In a study of experimental colitis in mice, both etanercept and infliximab attenuated inflammation induced reductions in ZO-1 and occludin as well as reducing the upregulation of claudin 2 (Fries et al., 2008).

Short chain fatty acids, in particular butyrate are thought to be the principal source of energy for colonocytes and in UC patients colonocytes have demonstrated diminished oxidation of butyrate (Roediger, 1980, Thibault et al., 2010). In vitro culture demonstrated butyrate enhanced claudin 1 transcription and enhanced barrier function (Wang et al., 2012). In colonic epithelial cells treated with butyrate, claudin 2 was downregulated (Daly and Shirazi-Beechey, 2006). However, the clinical efficacy of butyrate for pouchitis has not been demonstrated (Wischmeyer et al., 1993).

Novel therapies that alter epithelial barrier function may be available from nutritional sources. Several plant extracts have been observed to regulate tight junction expression. Quercetin, a common flavanoid, was shown to increase epithelial resistance in CACO-2 cell monolayers by upregulating claudin 4 expression (Amasheh et al., 2008). Berberine, an isoquinolone alkaloid, prevented TNFα induced claudin 1 disassembly and upregulation of claudin 2 in a cell culture model (Amasheh et al., 2010). Polyunsaturated fatty acids can also have beneficial effects on the assembly and morphology of tight junction proteins (Ulluwishewa et al., 2011). Omega-3 and omega 6 polyunsaturated fatty acids up-regulate expression of occludin, reduce

permeability and strengthen the epithelial barrier (Li et al., 2008b) Polyunsaturated fatty acids also reverse the disruptions in tight junction proteins caused by proinflammatory cytokines in CACO-2 epithelial cells (Li et al., 2008a, Amasheh et al., 2009a) and might play a role in preventing the alteration in the epithelial barrier caused by inflammation or proinflammatory cytokines that could be exploited as a therapeutic target in the treatment of gut inflammation.

Zonulin is a key regulator of intestinal permeability through modulation of epithelial tight junctions (Fasano, 2012). A synthetic peptide inhibitor of zonulin known as AT 1001 or Larazotide has undergone clinical studies in the treatment of coeliac disease (Paterson et al., 2007). In the IL-10 knockout mouse, AT 1001 reduced intestinal permeability and attenuated the development of spontaneous colitis (Arrieta et al., 2008). Future studies are necessary to determine the role these proteins may have in modulating tight junctions and epithelial barrier function in inflammatory bowel diseases.

Much attention has focused on the effects probiotic bacteria and their products may have on tight junction expression and epithelial barrier function. In vitro and animal models have shown *Lactobacilli* to attenuate epithelial permeability in experimental colitis and to upregulate tight junction expression of ZO-1, occludin and claudin 3 (Anderson et al., 2010, Liu et al., 2010, Patel et al., 2012). VSL#3 (a mixture of eight probiotic strains) prevented the reduction and redistribution of ZO-1 and claudins 1,3,4 and 5 in a murine model of colitis. Furthermore, bacterial products may be a source of novel therapies affecting epithelial barrier function. In a recent study, uncharacterized extracellular proteins secreted by *B. longum subsp. infantis*,

increased the production of ZO-1 and occludin in epithelial cells (Ewaschuk et al., 2008). Extracellular proteins derived from *Lactobacillus rhamnosus GG* attenuated reduction in epithelial resistance in an in vitro model, preventing the redistribution of tight junction proteins including ZO-1 and occludin in a dose dependent manner (Seth et al., 2008). In a recent study probiotics restored the mucosal barrier to *E. coli* and Horse radish peroxidase in patients with pouchitis and bacterial diversity correlated with barrier function (Persborn et al., 2013).

Dysregulation of tight junction proteins is involved in the pathogenesis of IBD. However, a number of questions remain unanswered regarding the role of tight junctions in the aetiology of IBD. Whether tight junction abnormalities are primary or secondary to the aberrant immune responses present in these diseases remains to be proven. There does not appear to be consensus regarding the up or downregulation of the various claudin isoforms in UC, Crohn's disease or pouchitis. Furthermore, significant differences may exist between animal models and human studies regarding tight junction expression profiles and further human studies are necessary (Alanne et al., 2009).

1.7 Faecal microbiota transplantation

With the application of molecular techniques to the study of gut microbiology, mounting evidence is emerging regarding the relationship between intestinal dysbiosis and aberrant intestinal and also extra-intestinal immune responses (Sartor, 2008, Peterson et al., 2008b, Vrieze et al., 2010, Sekirov et al., 2010). Consequently modification of the microbiota itself is a target for immunotherapeutic manipulation. Studies of immune responses following modification of the microbiota in IBD are likely to further enhance our understanding of these diseases and the relationship between the microbiota and the intestinal immune system.

The possibility of modifying the gut microbiota to replace harmful bacteria with more favourable microbes has been widely explored since Metchnikoff's observations in 1907 of the potential health benefits of the "Bulgarian bacillus" (Metchnikoff, 1908). In vitro studies have demonstrated a positive effect of probiotic bacteria on gut inflammation by modulating gut immune cells (Hart et al., 2004b, Ng et al., 2010b). Probiotics have been extensively investigated in many gastrointestinal disease states where an abnormal microbiota is considered pathogenic (Hart et al., 2003, Preidis and Versalovic, 2009, Shanahan, 2010). The outcomes of these studies have however been variable and modest (Shanahan, 2010). Antibiotics and probiotics are the mainstay of treatment for acute and chronic pouchitis (Holubar et al., 2010, Gionchetti et al., 1999, Gionchetti et al., 2003, Gionchetti et al., 2000b, Mimura et al., 2004, Gionchetti et al., 2007). However, subsequent clinical studies have

reported disappointing results with less than 20% of patients able to maintain remission with VSL#3 following antibiotic induced remission (Shen et al., 2005a, McLaughlin, 2008).

As previously stated, the immune effects of probiotic bacteria may depend on the local microenvironment in which they are acting and the integrity of the epithelial monolayer (Tsilingiri et al., 2012). Another confounding factor of the probiotic approach is the comparatively low number and diversity of bacterial species available in a typical commercial probiotic in comparison with the gut microbiota. Furthermore, probiotic bacterial strains may not be able to compete against the complex interactions of an established and adapted indigenous gut microbial community. An alternative approach is transplantation of the entire "organ" of the gut microbiota.

1.7i Animal studies of faecal microbiota transplantation

There is a considerable body of work involving transplantation into sterile-gut animals. Less work has evaluated the transplantation of microbiota into animals with intestines already populated with bacteria. Studies of knockout mouse models of colitis have shown transfer of colitogenic microbiota to wild type mice by co-housing and cross fostering (Garrett et al., 2007, Garrett et al., 2010, Elinav et al., 2011). Two groups have reported transferring colitis phenotypes from two different knockout mouse models to wild-type mice. Garrett *et al.* found that ulcerative colitis (TRUC) mice, deficient in both innate and adaptive immune responses, transferred their colitis phenotype to wild-type mice by cohousing and cross-

fostering (Garrett et al., 2007). The group showed by pyrosequencing that *Proteus mirabilis* and *Klebsiella pneumonia* were overrepresented and may be responsible for the colitis phenotype (Garrett et al., 2010). In similar experiments, Elinav *et al.* found that *ASC*–/– mice, transfer their colitic phenotype to wild-type mice by cohousing and cross fostering (Elinav et al., 2011). A recent study of NOD2 deficient mice demonstrated transfer of colitogenic microbiota to cohoused and cross-fostered mice and that the colitis susceptibility from NOD2 deficiency was mitigated by faecal microbiota transplantation (FMT) from wild type mice (Couturier-Maillard et al., 2013).

Mouse models of enteric infections have shown prevention of infection following transfer of the microbiota from resistant to susceptible mice (Willing et al., 2011, Ghosh et al., 2011). Manichanh et al. studied the effects of FMT and antibiotic therapy in rats demonstrating three important outcomes: i) there was a marked and a durable increase in the microbial diversity following FMT up to three months following a single gavage of rat caecal content, ii) the increased microbial diversity resulted from both capture of new phylotypes as well as an increase in those already present and iii) antibiotics prior to transplantation are not necessary to engraft the exogenous microbiota and may be deleterious to the intended increase in microbial diversity with faecal transplantation (Manichanh et al., 2010) . When transplantation was performed after antibiotic intake, the resulting state combined the reshaping effects of each treatment. Consequently lowering the bacterial load by antibiotic intake prior to transplantation did not increase establishment of donor phylotypes and in fact reduced the overall diversity of the microbiota following FMT (Manichanh et al., 2010).

A recent study of FMT in a mouse model of *Clostridium difficile* (Gilmore et al., 2012*)* demonstrated that suppression of *Clostridium difficle* following FMT was associated with a shift in the recipients' microbiota to a composition similar to that of the healthy input bacterial community and this was closely linked to a rapid increase in species diversity. Furthermore the critical bacterial mix necessary for the effectiveness of the transplant was phylogenetically diverse, included both obligate and facultative anaerobic species, and represented three of the four predominant intestinal microbiota phyla. Importantly, these species appear to be common inhabitants of the mouse intestine in health and they are phylogenetically distinct from the dominant members of the diseased mice microbiota (Gilmore et al., 2012).

1.7ii Human studies of faecal microbiota transplantation

Faecal microbiota transplantation has been described in ruminants for hundreds of years (Rager et al., 2004). Its use as therapy in humans was first reported by Eisemen *et al*. in 1958 in the treatment of fulminant pseudomembranous enterocolitis (Eiseman et al., 1958). Over the subsequent decades, there have been a small number of case reports and case series of FMT for *Clostridium difficile* (Bowden et al., 1981, Schwan et al., 1983, Tvede and Rask-Madsen, 1989, Borody et al., 1989, Paterson et al., 1994, Gustafsson et al., 1998, Persky and Brandt, 2000, Aas et al., 2003, You et al., 2008, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010, Silverman et al., 2010, Rohlke et al., 2010, Yoon and Brandt, 2010, Khoruts et al., 2010, Garborg et al., 2010) with the recent publication of the first randomised controlled trial of FMT for this indication (van Nood et al., 2013). Faecal microbiota transplantation has also been

performed for constipation (Borody et al., 1989, Grehan et al., 2010, Andrews and Borody, 1993) irritable bowel syndrome and inflammatory bowel diseases (Borody et al., 2003, Grehan et al., 2010, Borody et al., 1989, Bennet and Brinkman, 1989) as well as diseases beyond the gut such as metabolic syndrome (Vrieze et al., 2012). In recent years there has been a resurgence of interest in this procedure and its potential to modify the gut microbiota.

Patient Details

The majority of patients undergoing FMT were treated for *Clostridium difficile* after standard treatments had failed. Borody *et al.* in 1989 (Borody et al., 1989) reported 55 patients treated for constipation, diarrhoea, abdominal pain, ulcerative colitis or Crohn's disease. Andrews *et al.* (Andrews and Borody, 1993) described faecal enema treatment for two patients with constipation and in the recent paper from Grehan *et al*. (Grehan et al., 2010), nine patients had a diagnosis of constipation or diarrhoea predominant IBS and one patient had Crohn's disease. One patient in the series from Aas *et al.* (Aas et al., 2003) had *Clostridium difficile* diarrhoea on a background of Crohn's colitis. Seven other patients with UC are reported to have undergone FMT (Bennet and Brinkman, 1989, Borody et al., 2003). A recent study of ten patients with mild to moderate UC has been reported in the paediatric population (Kunde et al., 2013) as well as studies in adults with UC reported this year including five and six patients respectively (Angelberger et al., 2013, Kump et al., 2013).

Faecal microbiota transplantation has been described in patients as young as two years (Russell et al., 2010) to patients over 90 years of age (Rubin et al., 2009). Several reports include

patients with serious co-morbidities. Three of the four patients reported by Eiseman *et al. (Eiseman et al., 1958)* were in a critical condition requiring the use of vasopressors. In the patients reported by Bowden *et al.* (Bowden et al., 1981), eight had a previously treated carcinoma, two chronic renal failure and two an aortic aneurysm. In the study by Aas *et al.* (Aas et al., 2003) five patients undergoing FMT were hospitalised and of those treated as outpatients, three were nursing home residents. MacConnachie *et al.* (MacConnachie et al., 2009) described FMT in eighteen patients, eleven of whom were hospitalised with significant co-morbidity and a high proportion having hypoalbuminaemia, leucocytosis and renal dysfunction before FMT. The patient in the report of You *et al.*(You et al., 2008) was treated in an intensive care unit with vasopressors and continuous veno-venous haemofiltration.

Donor Screening

The potential risk of transmission of viral, bacterial or parasitic infection during the course of FMT is a concern. No guidelines currently exist regarding faecal transplantation. A number of studies have proposed screening procedures (Aas et al., 2003, Russell et al., 2010). In a recent review of FMT for recurrent *Clostridium difficile* (Bakken, 2009) Bakken suggests a screening process based on previous studies. However, without established guidelines or data from randomised controlled trials, ethical approval for the procedure has to date depended on physician discretion with patient and donor consent, local hospitals' or authorities' approval or occurred within the framework of ethically approved research studies. Screening methods of stool donors are not always detailed. In the majority of reports a spouse or partner, close relative, or household member of the patient is preferred as the stool donor. However, in a

number of reports, donors who are unrelated healthy individuals have been used (Bowden et al., 1981, Gustafsson et al., 1998, Borody et al., 2003, van Nood et al., 2013). Earlier cases did not employ rigorous screening protocols, whereas more recently increased screening of donors' medical histories, blood and stool tests have been implemented.

Donors have been screened for a history of gastrointestinal illness, cancer or polyps, hospitalisation within the three previous months (Silverman et al., 2010) and between 6 weeks (Borody et al., 2003) to 6 months (Aas et al., 2003) without the use of antibiotics. Screening blood tests have included full blood count and liver function tests (Grehan et al., 2010) as well as screening of viral pathogens including HIV 1+2 ,(Paterson et al., 1994, Gustafsson et al., 1998, Persky and Brandt, 2000, Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010, Silverman et al., 2010, Rohlke et al., 2010, Yoon and Brandt, 2010), HTLV I/II (Silverman et al., 2010) hepatitis A, B and C (Gustafsson et al., 1998, Persky and Brandt, 2000, Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010, Silverman et al., 2010, Garborg et al., 2010, Borody et al., 2003), CMV, EBV (Gustafsson et al., 1998, Borody et al., 2003)and also for *Treponema pallidum* (Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010, Grehan et al., 2010)and *Helicobacter pylori* antibody (Silverman et al., 2010).

Donor faecal specimens have been screened for *Salmonella*, *Shigella, Campylobacter spp, Staphylococcus aureus, Aeromonas hydrophila, Yersinia, Vibrio parahaemolyticus, Vibrio cholerae, Candida albicans, Escherichia-coli O157* and *Clostridium difficile* toxins A and B

(Paterson et al., 1994, Gustafsson et al., 1998, Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Garborg et al., 2010, Russell et al., 2010, Silverman et al., 2010, Rohlke et al., 2010, Yoon and Brandt, 2010, Khoruts et al., 2010). Stool microscopy has been screened for protozoa (trophozoites and cysts), helminths and ova including *Entamoeba histolytica, Giardia lamblia, Microspora* (Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010, Silverman et al., 2010, Yoon and Brandt, 2010, Borody et al., 2003*)* , *Cryptosporidium (Silverman et al., 2010)*, *Dientamoeba fragilis, Blastocystis hominis, Ascaris lumbricoides*, trematodes and tape worms (Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010, Silverman et al., 2010, Yoon and Brandt, 2010, Borody et al., 2003) (Table 1.5).

Table 1.5. Suggested screening investigations for faecal transplant donors

Sample	**Investigation**
Blood	Full Blood Count, Liver Function Tests
	Hepatitis A,B,C
	HIV 1+2, HTLV I/II
	CMV, EBV
	Treponema pallidum
Stool	Selective stool culture
	Clostridium difficile toxin A and B
	Microscopy for ova, cysts and parasites

Route of Administration

The initial report of Eisemen *et al.* described administration of faecal enemas (Eiseman et al., 1958), which has been replicated in other studies (Bowden et al., 1981, Schwan et al., 1983, Tvede and Rask-Madsen, 1989, Paterson et al., 1994, Gustafsson et al., 1998, You et al., 2008, Silverman et al., 2010, Grehan et al., 2010, Andrews and Borody, 1993, Borody et al., 2003, Kunde et al., 2013). Others subsequently have used instillation via a colonoscope to the right colon (Persky and Brandt, 2000, Rohlke et al., 2010, Yoon and Brandt, 2010, Khoruts et al., 2010, Garborg et al., 2010, Grehan et al., 2010) or instillation of donor faeces via nasogastric tube (Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010) or duodenal (Garborg et al., 2010, van Nood et al., 2013) or nasojejunal intubation (Bowden et al., 1981, Grehan et al., 2010). The study of Grehan *et al.* employed a combination of colonoscopic instillation followed by enemas or nasojejunal tube (Grehan et al., 2010). The majority of studies entailed a single administration of donor faeces. Some studies used repeated infusions over 2 to 15 days (Eiseman et al., 1958, Bowden et al., 1981, Schwan et al., 1983, Tvede and Rask-Madsen, 1989, Paterson et al., 1994, Borody et al., 2003). In the study by Garborg *et al.* (Garborg et al., 2010), six patients underwent a second infusion of donor faeces having not responded to the initial transplantation.

Patient preparation

Preparation of the patient prior to FMT has varied depending on the method of administration of the donor stool. Studies in which donor stool is instilled at colonoscopy or via rectal enemas include patient preparation with bowel lavage treatments. Bennet and Brinkmann describe a

bowel sterilisation procedure (Bennet and Brinkman, 1989) prior to transplantation of donor stool. Persky and Brandt described the use of prior bowel lavage with polyethylene glycol (Persky and Brandt, 2000) The series of Borody *et al.* in six patients with refractory ulcerative colitis, gave seven to ten days of treatment with vancomycin, metronidazole and rifampicin prior to bowel lavage (Borody et al., 2003). This protocol was repeated in the study by Grehan *et al.* (Grehan et al., 2010). Two recent studies stopped treatment with metronidazole or vancomycin 24-48 hours prior to faecal transplantation (Yoon and Brandt, 2010, Garborg et al., 2010). The study by Silverman *et al.*, included prior treatment with *Saccharomyces boulardii* which was continued up to 60 days after the procedure (Silverman et al., 2010). Patients treated at one centre in the study by Rholke *et al.* (Rohlke et al., 2010) were treated with loperamide immediately following the procedure and again 6 hours later in order maximise contact time of the donor faeces with the colonic mucosa.

Studies of FMT administered into the upper gastrointestinal tract, do not report the use of prior bowel lavage. The method described by Aas *et al.* in 2003 and followed by those of MacConnachie, Rubin and Russell *et al.*, includes pre-treatment with more than four days of vancomycin and 20mg of omeprazole the evening before and the morning of the procedure (Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010). However, a recently published randomised controlled trial of faecal transplantation for recurrent *Clostridium difficile* infection implemented pre-treatment with four days of vancomycin as well as bowel lavage prior to duodenal infusion (van Nood et al., 2013).

Preparation of donor stool

The interval between obtaining donor stool and its administration to the patient has varied between studies, from 24 hours before, 6 hours before (Aas et al., 2003, MacConnachie et al., 2009, Rubin et al., 2009, Russell et al., 2010) or immediately. One study homogenised donor stool in pasteurised cow's milk and filtered the solution which was then stored at -20°C and thawed in water at 37°C 30-60 minutes prior to administration as an enema (Gustafsson et al., 1998). Some studies have described the homogenisation of the stool and filtering to remove debris. The use of between 10 to 200g of stool, diluted in sterile saline 20-500ml has been reported depending on the method of administration. Studies using an upper gastrointestinal protocol for FMT instilled between 30 and 50g of stool homogenised with 50-500ml sterile saline.

Outcomes

In many reports of FMT response is not clearly defined. Resolution of symptoms is most commonly stated. Some papers include absence of *Clostridium difficile* toxin. For *Clostridium difficile,* FMT effective in 87% of patients. Time to response is often not stated, although "immediate", "prompt" or "rapid" response is often reported. Where time to response is stated, this has been recorded to occur within 24 hours to twelve days (Bowden et al., 1981, Gustafsson et al., 1998, Russell et al., 2010, Garborg et al., 2010, Borody et al., 2003). Response appears durable with follow up of patients up to 8 years (Yoon and Brandt, 2010). In the recently published RCT remission rates in the FMT group were 94% at ten weeks (van Nood et al., 2013). In the initial report of Eiseman *et al*. three of the four patients were described as

terminally or critically ill. All of these had cessation of diarrhoea and were completely asymptomatic between 24 hours and ten days following faecal transplantation. The report of Bowden *et al.* describe response as a reduction in frequency of bowel motions, absence of fever, normalisation of leucocyte counts and increased general well-being. Tvede and Rask-Madsen describe normalisation of bowel function as well as reduction in inflammatory markers and increased albumin levels as response to faecal transplantation. In the report of You *et al.* the patient rapidly displayed normalisation of leucocytosis, stabilisation of blood pressure enabling cessation of vasopressors and improvement in renal function allowing cessation of continuous veno-venous haemofiltration as well as normalisation of bowel function. In the reports of Schwann *et al.*, Gustaffson *et al.* Persky and Brandt, Aas *et al.,* MacConnachie *et al.,* Khoruts *et al,* Rholke *et al and* Russell *et al.* cessation of diarrhoea is defined as response. Five of these studies also document a change from a positive to a negative *Clostridium difficile* stool test. The only published RCT of FMT defined cure as an absence of diarrhoea or persistent diarrhea that could be explained by other causes with three consecutive negative stool tests for *C. difficile* toxin (van Nood et al., 2013).

Until recently the majority of UC patients reported responded to FMT and remained in remission from 1 months to 13 years (Borody et al., 2003, Bennet and Brinkman, 1989, Kunde et al., 2013). Patients with UC in the series of Borody *et al*. responded within one to six weeks and were considered in remission by four months following FMT (Borody et al., 2003). Five out of the six patients reported in this series had moderate to severe disease with moderate to severe endoscopic findings. All of the patients were asymptomatic with no endoscopic evidence

of active inflammation following FMT. However, no response was noted in three patients reported by Kunde et al. and only 1 patient showed response to FMT in the two recently published studies (Kunde et al., 2013, Kump et al., 2013, Angelberger et al., 2013)(Table 1.6).

Adverse events

No studies of FMT report any serious adverse events related to the procedure. Some studies report patient deaths due to the underlying disease where the patient has not responded to FMT. In one study in which donor faeces were instilled via a nasogastric tube, the patient died of peritonitis. Although considered unlikely, the nasogastric tube insertion could not be discounted to have been contributory (Paterson et al., 1994). One patient in the study by Silverman *et al.* developed irritable bowel symptoms following FMT (Silverman et al., 2010). In the recent study in paediatric UC patients cramping, bloating and fever were noted (Kunde et al., 2013). Van Nood et al. reported belching, nausea, vomiting and abdominal cramps in the RCT of FMT for recurrent *C. difficile* (van Nood et al., 2013).

Analysis of effects of FMT on stool composition and faecal microbiota

Studies have attempted to analyse stool before and after FMT. Using culture, Tvede and Rask-Madsen observed an absence of *Bacteroides* before bacteriotherapy and during vancomycin therapy whilst patients were symptomatic. During follow up after bacteriotherapy (including faecal enemas in two patients), *Bacteroides* were regularly cultured (Tvede and Rask-Madsen, 1989). Gustafsson *et al.* studied stool short chain fatty acid concentrations before and after faecal transplantation in nine patients. All short chain fatty acids were found to be reduced in

the patient group compared with healthy adults and following faecal enema therapy the relative distribution and absolute amounts of short chain fatty acids returned to patterns similar to those in healthy adults (Gustafsson et al., 1998).

More recently using modern molecular 16S rRNA gene sequencing techniques, two studies have shown a significant change in the microbiota following FMT. Khoruts *et al*. demonstrated a reduction in *Bacteroidetes* and *Firmicutes* in a patient with *Clostridium difficile* diarrhoea. Following faecal transplantation there was a rapid change in the patient's microbiota to a composition that was highly similar to that of the healthy donor for at least four weeks (the duration of follow-up stool analysis) in this study (Khoruts et al., 2010). Grehan *et al.* undertook analysis on the stool of 10 patients who underwent FMT. A dramatic change was shown in the recipients' microbiota to a composition similar to their donors' microbiota. This study analysed stool from patients up to 24 weeks following FMT demonstrating a durable change in the recipients' microbiota up to 24 weeks (Grehan et al., 2010). Following FMT in the randomised trial by van Nood et al., patients showed increased faecal bacterial diversity, similar to that in healthy donors, with an increase in *Bacteroidetes* species and *Clostridium clusters IV and XIVa* and a decrease in *Proteobacteria* species (van Nood et al., 2013).

Two recent studies have assessed the microbiota following FMT in IBD. Faecal microbiota transplantation reduced the relative abundance of *Proteobacteria* and increased *Bacteroidetes* and at family level the most prominent relative changes were a decrease in *Enterobacteriaceae* and *Enterococcaceae* and a relative increase in *Bacteroidaceae*. However, abundant bacteria

from donors established in some of the recipients, but the efficiency and stability of donor microbiota colonization varied greatly (Angelberger et al., 2013, Kump et al., 2013). To date, no studies have attempted to assess the immune responses to modification of the microbiota with faecal transplantation.

Evidence regarding the use of FMT as a means of modifying the gut microbiota and effecting cure of gastrointestinal illness is accumulating. To date the majority of studies of FMT have been in fulminant or refractory *Clostridium difficile*. However, studies to date are heterogeneous regarding the patients treated, donors used, optimal screening protocols, methods and frequency of administration, and definition of response. Furthermore, reports to date may suffer from reporting bias of positive outcomes and under-reporting of adverse effects.

Faecal microbiota transplantation, a therapy used for more than half a century, could hold great promise as a future treatment where a dysbiosis of the gut microbiota is responsible for disease. This therapy is inexpensive as well as being effective in some cases. A second randomised controlled study of FMT in *Clostridium difficile* is on-going in North America and results are eagerly awaited. Standardised controlled studies are necessary to ascertain the most effective regimen as well as the most acceptable method of treatment. Studies of FMT for other gastrointestinal diseases, such as inflammatory bowel diseases, where a dysbiosis of the gut microbiota is evident are necessary. Rigorous screening of potential donors is essential as is the use of partners or close relatives as donors to minimise the potential for transmitting

disease. Close monitoring and long term follow up are necessary. Combining clinical studies with molecular analysis of the microbiota and the effects on the immune response may significantly enhance our understanding of the gut microbiota and its relationship with health and disease.

Table 1.6. Summary of studies of faecal transplantation

Author	Year	Indication	Number of Patients	Route of faecal instillation	Response	Stated Time to Response	Duration of Follow-up
Eiseman	1958	PMC	4	Rectal	4/4	2 days	
Bowden	1981	PMC	16	Rectal/Jejunal	14/16	1-12 days	5 days- 3years
Schwan	1984	Relapsing CDAD	1	Enema	1/1		9 months
Tvede	1989	Relapsing CDAD	2	Enema	1/2		6 months
Bennet	1989	UC	1	Enema	1/1		6 months
Borody	1989	IBS, IBD, CDAD	55	Enema	26 cure 20 response 9 no response		1-12 months
Andrews	1992	Constipation	1	Enema	1/1		18 months
Paterson	1994	Chronic CDAD	7	Enema	7/7		2 years
Gustaffson	1998	AAD/CDAD	9	Enema	9/9	6-10 days	18 months
Persky	2000	Recurrent CDAD	1	Colonic	1/1		5 years
Aas	2003	Recurrent CDAD	18	Nasogastric	15/18		90 days
Borody	2003	UC	6	Enema	6/6	1-6 weeks	1-13 years
You	2008	Fulminant CDAD	1	Enema	1/1	36 hours	
MacConnachie	2009	Recurrent CDAD	15	Nasogastric	11/15		4-24 weeks
Rubin	2009	CDAD	12	Nasogastric	10/12		90 days
Khoruts	2010	Chronic CDAD	1	Colonic	1/1	2 days	6 months
Rholke	2010	Relapsing CDAD	19	Colonic	19/19		6 months- 5 years
Russell	2010	Relapsing CDAD	1	Nasogastric	1/1	36 hours	6 months
Yoon	2010	Refractory/ Recurrent CDAD	12	Colonic	12/12		3 weeks- 8 years
Garborg	2010	Recurrent CDAD	40	Duodenal/Colonic	33/40	24 hours	
Silverman	2010	Chronic CDAD	7	Enema	7/7		
Van Nood	2013	Recurrent CDAD		Duodenal	15/16		10 weeks
Kunde	2013	UC	10	Enema	7/10	1 week	4 weeks

PMC- Pseudomembranous colitis; CDAD- *Clostridium difficile* associated diarrhoea; AAD- antibiotic associated diarrhoea; IBS- Irritable bowel syndrome; IBD- Inflammatory bowel disease; UC- ulcerative colitis.

1.8 Hypothesis

Aberrant host immune characteristics and impaired epithelial barrier properties in the presence of a dysbiosis of the pouch microbiota are responsible for inflammation of the ileal pouch in ulcerative colitis patients.

Manipulation of the pouch microbiota with faecal microbiota transplantation will alter the aberrant immune characteristics and impaired epithelial barrier properties.

1.9 Aims

1. To identify and characterise lamina propria dendritic cells in the ileum, non-inflamed and inflamed ileal pouch of patients with ulcerative colitis
2. To characterise epithelial tight junction expression in the ileal pouch of patients with ulcerative colitis
3. To assess longitudinally the alterations of tight junction protein expression and immune function in ulcerative colitis patients following restorative proctocolectomy
4. To determine the clinical, microbiological and immunological effects of faecal transplantation therapy for patients with chronic refractory pouchitis

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Human Intestinal Tissue

Human intestinal biopsies were obtained at flexible pouchoscopy or surgery from ulcerative colitis patients and FAP patients following restorative proctocolectomy. All patients undergoing flexible pouchoscopy received preparation with a phosphate enema prior to pouchoscopy. Ten mucosal biopsies were taken per patient. Tissues were stored in complete medium on ice and transported immediately for processing. Ethical approval and written informed consents were obtained from all patients [EC nos. 11/LO/0170, 12/LO/0913, 08/H0717/24].

2.1.2 CACO 2 cells

Seeding and culture performed by Drs E Shaw and R Rigby Lancaster University

This is a human caucasian colon adenocarcinoma epithelial cell line isolated from a primary colonic tumour in a 72-year-old Caucasian male using the explant culture technique. These cells form moderately well differentiated adenocarcinomas in nude mice. Cells were seeded in twelve well plates at a density of 2-4x10^4cells/cm^2 and cultured in EMEM (EBSS) + 2mM Glutamine + 1% Non-Essential Amino Acids (NEAA) + 10% Foetal Bovine Serum (FBS) at 37°C. Experiments were performed at day 3 and day 7 post confluence.

2.1.3 Buffers and Media

Complete medium RPMI 1640 Dutch modification (Sigma Aldrich Co. Ltd, Irvine, UK) supplemented with 2mM L-glutamine, 100ug·ml^{-1} streptomycin, 100units·ml^{-1} penicillin and gentamicin (50ug·ml^{-1}). To complete the medium RPMI was supplemented with 10% foetal calf serum (FCS). Dutch modified RPMI 1640 medium incorporates both HEPES buffer for partial pH control and bicarbonate for gas exchange.

FACS buffer

Cells prepared for flow cytometry were washed and re-suspended in FACS buffer. This buffer was prepared from Phosphate buffered saline (PBS) with added FCS (2%), sodium azide (0.02%)(Sigma, UK) and EDTA (1mM)(Sigma, UK). Phosphate buffered saline (PBS) calcium and magnesium free balanced salt solution- maintains the integrity of mammalian cells in vitro (Sigma, Poole).

T0.1E Buffer (Invitrogen 1162066)

Lyophilised primers were diluted in low EDTA concentration buffer. PCR pellets were resuspended prior to storage in T0.1E Buffer.

Sterile Saline Solution

NaCl (0.9%) Baxter was used for preparation of donor faecal transplant material.

1M Saline Solution (Sigma Aldrich)

1M Saline Solution was used for ethanol precipitation of PCR products. 0.3 volume of 1MNACl and 2 volumes of cold 100% ethanol were mixed by inversion and left overnight at -20C. They were then centrifuged at 750g for 20min and 600 microliters of cold 70% ethanol was added prior to recentrifugation at 750g for 15 min before being left to air dry for 30-60 minutes. Pellets were then resuspended in 20-30 microliters of T0.1E buffer and left overnight at 4C.

Trisborate EDTA buffer

Borate- BDH 1005845, EDTA- Sigma E5134 and Tris- Fisus T/3712/56 were used to make Trisborate EDTA buffer used for gel electrophoresis.

RNA later (Ambion)

Used for storage of bio-samples prior to DNA extraction.

2.1.4 Reagents

Foetal calf serum (FCS)

FCS (TCS cellworks, Buckingham, UK) was added to cell culture media to provide growth factors for cells. FCS also blocks non-specific binding during monoclonal antibody labelling.

Normal mouse serum (NMS)

NMS was used to block non-specific binding during monoclonal antibody labelling.

Sodium azide

Sodium azide (Sigma, MO, USA) is an inorganic compound that prevents capping and shedding of monoclonal antibodies used for flow cytometric analysis of cells. Sodium azide (0.02%) was one of the constituents of FACS buffer.

Hanks' balanced salt solution (HBSS)

Calcium- and magnesium-free HBSS (Gibco BRL, Paisley, Scotland) was used to make up EDTA solution and DTT.

Dithiothreitol (DTT)

DTT (Sigma-Aldrich) is a strong reducing agent and was added to calcium- and magnesium- free HBSS to make a solution of 1mmol·L^{-1} concentration in order to remove mucus, faeces and debris from human intestinal biopsies.

Ethylenediaminetetraacetic acid (EDTA)

EDTA (Sigma, Dorset) is a chelating agent; it chelates metal ions to prevent cell clustering. It was added to calcium- and magnesium-free HBSS to remove epithelial cells from human intestinal biopsies to make a solution of 1mmol·L^{-1} concentration.

Collagenase Digestion Medium

Collagenase D (1mg·ml^{-1}) (Roche Diagnostics Ltd, Lewes, UK), in RPMI 1640 Dutch modification was used to digest whole biopsy samples to free cell suspensions.

Leucoperm A and B

Leucoperm A (Serotec, UK) was used as a fixative and contains paraformaldehyde. Leucoperm B (Serotec, UK) was used to permeabilise cells and is detergent based.

Paraformaldehyde

Paraformaldehyde (BDH chemicals, UK) was dissolved in Saline (0.85%) through slow heating at 60°C at pH 7.0-7.4 to make a 1% stock solution which was added directly to antibody labelled cells as a fixative.

Ficoll-Paque

Ficoll- paque (Amersham Pharmacia, Sweden) was used to isolate PBMCs from freshly isolated whole blood.

Monensin

Inhibits intracellular protein transport and causes newly synthesised cytokines to be trapped within the Golgi apparatus of cells. (Sigma, MO, USA).

Mounting medium with 4',6-diamidino-2-phenylindole (DAPI)

DAPI (Vector Laboratories, USA) is a counterstain for DNA and RNA that produces a blue fluorescence. Fluorescence is maintained by storage at 4°C.

2.1.5 Antibodies

Antibodies with the following specificities and fluorochrome labels were used and isotype matched controls were obtained from the same manufacturers.

Antibody	**Clone**	**Channel**	**Isotype**	**Company**
TLR4	HTA125	FITC	mIgG2a	AbdSerotec
TLR2	TLR2.3	FITC	mIgG2a	AbdSerotec
TLR 5	85B152.5	FITC	mIgG2a	Abcam
CD40	LOB7/6	FITC	mIgG2a	AbdSerotec
B7	FIB504	PE	rIgG2a	BD Pharmingen
CCR9	112509	PE	mIgG2a	R&D systems
CD3	UCHT1	PE-CY5	mIgG1	BD Pharmingen
CD16	3G8	PE-CY5	mIgG1	BD Pharmingen
CD14	61D3	PE-CY5	mIgG1	AbdSerotec
CD19	H1B19	PE-CY5	mIgG1	AbdSerotec
CD34	581	PE-CY5	mIgG1	BD Pharmingen
HLA-DR	L243 (G46-6)	APC		BD Pharmingen
CD11c	KB90	FITC	mIgG1	DAKO
CD123	9F5	PE	mIgG1	BD
ZO-1	ZO1-1A12	FITC	mIgG1	Invitrogen
PCK (c-11)		PE	mIgG1	abcam
Claudin-1	421403	Unconjugated	rIgG2a	R&D systems
Claudin-2	12H12	Unconjugated	mIgG2b	Invitrogen
Goat anti-rat IgG Fab2		PE-CY7		Santa Cruz
Goat anti-mouse IgG Fab2		APC-CY7		Santa Cruz
IL4	8D4.8	PE-CY7		BD Pharmingen
IL10	JES3-9D7	PE		AbdSerotec
IL12	C11.5	FITC		BD Pharmingen
IL17	BL168	APC-CY7		Biolegend

Antibodies used for immunofluorescence

Primary Antibodies

ZO-1	Goat anti-ZO-1 (dilution 1:200)	SantaCruzBiotechnology
Claudin 1	Rabbit anti-Claudin 1 (dilution 1:200)	Invitrogen
Claudin 2	Rabbit anti-Claudin 2 (dilution 1:200)	Invitrogen

Secondary Antibodies

ZO-1	Anti-goat FITC (dilution 1:70)	SantaCruz Biotechnology
Claudin 1	Anti-rabbit TRITC (dilution 1:500)	SigmaAldrich
Claudin 2	Anti-rabbit TRITC (dilution 1:500)	Sigma-Aldrich

Blocking Agent

ZO-1	Normal goat serum (1:200)	Vector laboratories
Claudin 1 and 2	Normal rabbit serum (1:200)	Vector laboratories

Anitmouse BD Compensation beads

The BD™ CompBeads Set Anti-Mouse Ig, κ are polystyrene microparticles which are used to optimize fluorescence compensation settings for multicolor flow cytometric analyses. The set provides two populations of microparticles, the BD™ CompBeads Anti-Mouse Ig, κ particles, which bind any mouse κ light chain-bearing immunoglobulin, and the BD™ CompBeads Negative Control (FBS), which has no binding capacity. When mixed together with a fluorochrome-conjugated mouse antibody, the BD™ CompBeads provide distinct positive and negative (background fluorescence) stained populations which can be used to set compensation levels.

2.1.6 Materials for DNA extraction and PCR

Agarose

Type 1 Sigma 083K001 agarose was used for gels for DNA extraction and PCR checks.

SYBR green (Biotium 1161122)

Gelgreen nucleic acid stain 10,000x in water was used to stain nucleic acid for confirmation of DNA or PCR products under UV light examination.

Loading buffer (Promega)

Used for loading of genetic material onto gels for electrophoresis

1Kb Plus DNA ladder (Invitrogen)

Used for sizing DNA fragments

dNTPs

GeneAmp® deoxynucleotide triphosphates (dNTPs) dissolved in glass-distilled water and titrated to pH 7.0 with NaOH were used for the PCR mastermix (dATP, dCTP, dGTP, dTTP). dNTPs were made up to a concentration of 200µM prior to use.

FastDNA® SPIN kit for soil and FastPrep® (MP Biomedical)

Used for DNA extraction as per manufacturer's instructions

Q5™ polymerase (New England Biolabs 0041207)

High fidelity DNA polymerase used for PCR of bacterial DNA.

2.2 Methods

2.2.1 Ethics Committee Approval

The study protocols and associated laboratory investigations were approved by the Brent Research and Ethics Committee EC No 08\H0717\24 and the North London REC 2 EC no. 11/LO/0170. Approval for investigations relating to patients with FAP was given by the South East London NRES committee EC no. 12/LO/0913. Written informed consent was obtained from all participants, patients and healthy volunteers.

Local NHS Research and Development approval and sponsorship to conduct the studies were secured from Imperial College London and North West London Hospital NHS Trust.

2.2.2 Patients and Controls

Patients were recruited from an adult tertiary pouch clinic at St Mark's Hospital. Diagnosis for ulcerative colitis patients with an ileal pouch was made by previous clinical, endoscopic and histological criteria. FAP patients had been previously diagnosed based on family history, endoscopic phenotype and genetic analysis. Diagnosis of pouchitis was based on clinical, endoscopic and histological criteria meeting a PDAI of ≥7 (Table 1). Patients with pouchitis were characterised as acute or chronic as per the definition described above (Section 1.3i). Five patients with UC were followed longitudinally over the first year following ileostomy closure (Table 5.1). In total 15 UC patients with pouchitis, 18 UC patients without pouchitis and 8 patients with FAP were recruited for a cross-sectional study (Table 2.2). Healthy controls (n=9) consisted of patients with macroscopically and histologically normal intestine that had been

referred with symptoms of rectal bleeding, change in bowel habit or a family history of colorectal cancer.

Table 2.2 Patient characteristics of UC pouchitis, UC non-pouchitis and FAP patients in cross-sectional study

Diagnosis	Age (range)	Sex	Median interval since RPC (years)	PDAI	Median pouch frequency in 24 hours (range)
UC Pouchitis (n=15)	51 (24-66)	8 male	7 (1-35 years)	12 (7-14)	12 (6-22)
UC Non pouchitis (n=18)	50 (32-72)	9 male	8.5 (1-33 years)	0 (0-3)	6 (3-20)
FAP (n=8)	42 (25-54)	3 male	10.5 (1-16 years)	0 (0-2)	6 (3-15)

2.2.3 Grading of histological inflammation

The severity of histological inflammation in each pouch biopsy specimen was reported by an expert pathologist. The degree of inflammation was graded according to acute histological criteria as per PDAI (Table 1).

2.2.4 Isolation of peripheral blood mononuclear cells

PBMC were obtained by centrifugation of human peripheral blood over Ficoll-Paque Plus at 800g, for 30 minutes at room temperature (RT) and harvesting the buffy coat layer from the interface. The PBMC were washed twice in complete medium by centrifugation at 650g for five minutes.

2.2.5 Isolation of epithelial cells and lamina propria mononuclear cells

The method used was as described in previous work from the Antigen Presentation Research Group (Bell et al., 2001). Biopsy specimens were collected on cold ice in Complete medium (see above). Tissues were incubated for ten minutes with DTT solution (see above) and then washed with EDTA solution (see above) before being incubated with gentle agitation in 25ml EDTA solution at 37°C for 60 minutes. After 60 minutes the supernatant containing released epithelial cells was removed and passed through a cell strainer. This was then washed with complete medium and then washed again with FACS buffer.

After the 60 minute wash with EDTA solution the remaining biopsy tissues were then digested in 25ml of collagenase digestion medium for 90-120 minutes at 37°C. LPMC released from the tissue samples were passed through a cell strainer and washed in complete medium and then washed again with FACS buffer.

2.2.6 Cell surface and intracellular labelling

Cells were labelled in FACS buffer. Antibodies were added at predetermined optimal concentrations. For surface staining involving only directly conjugated reagents, all antibodies were added simultaneously prior to washing twice with FACS buffer. Where unconjugated antibodies were used, cells were initially labelled with the unconjugated antibody, washed and then labelled with secondary antibody before the cells were washed again. To prevent non-specific binding, unoccupied binding sites were blocked with foetal calf serum prior to the

addition of primary or directly conjugated antibodies. Labelling was performed for 20 minutes in the dark on ice.

For intracellular staining, cells were fixed with 50µl of Leucoperm A for 15 minutes, washed and then 100µl of Leucoperm B, normal mouse serum and the directly conjugated or primary antibodies were added for 20 minutes in the dark on ice. The cells were then washed prior to addition of secondary antibody. Cells were washed twice and finally resuspended in 1% paraformaldehyde and stored at 4°C until acquisition within 24 hours.

2.2.7 Cytokine labelling for ongoing intracellular production of dendritic cell cytokine

Production of cytokines was determined by intracellular staining and flow cytometry. The experimental approach and analysis of data has been described in detail by Panoskaltsis *et al*. (Panoskaltsis et al., 2003). Paired cultures of LPMC, one incubated with monensin to maintain cytokine within the Golgi apparatus of cells and the other incubated without monensin were cultured for four hours at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were fixed with 50µl of Leucoperm A and permeabilised with 100µl of Leucoperm B. To prevent non-specific binding, unoccupied binding sites were blocked with foetal calf serum prior to the addition of 5µl of anti-cytokine antibody. The cells were labelled in the dark for 20 minutes before the cells were washed twice in FACS buffer and then fixed in 1% paraformaldehyde. Samples were then stored at 4°C until acquisition within 24 hours.

2.2.8 Flow Cytometry

Multi-colour flow cytometry characterises and measures the physical properties of individual cells in suspension as they pass through one or more focused laser beams. As the cells pass through the laser beam(s), they disrupt and scatter light in two different planes. This degree of scatter correlates with the cell morphology with forward scatter (FSC) giving a representation of the cell size, and side scatter (SSC) correlating with granularity, allowing cell populations to be identified (Figure 2.1).

Monoclonal antibodies conjugated to fluorescent dyes, which are conjugated to cells, can be detected by colour-specific detectors (channels) of the flow cytometer. Each fluorochrome emits light at a different wavelength (Table 2.1). The FACSCanto flow cytometer (BD Biosciences) was used. The cytometer is capable of detecting the fluorochrome dyes Fluorescein (FITC), Phycoerythrin (PE), PE-Cy5, Allophycocyanin (APC)Pe-Cy7 and APC-Cy7, corresponding to wavelengths FL1, FL2, FL3 and FL4, FL5 and FL6 respectively (Table 2.1).

Table 2.1 Fluorochrome dyes and corresponding wavelengths

Channel	**Name**	**Emission wavelength (nm)**
FL1	FITC	519
FL2	PE	578
FL3	Pe-CY5	670
FL4	Pe-CY7	767
FL5	APC	660
FL6	APC-CY7	780

During acquisition, 20µl of flow-count fluorospheres at a known concentration was added to each tube. Samples were acquired for approximately 3 minutes. All data were then saved as list-mode files and transferred to a computer for analysis.

2.2.9 FACS Analysis

DCs only constitute a small proportion of total leucocytes and cells from different samples emit different levels of innate/background autofluorescence. Therefore, compensation was carried out separately for each individual experiment. To perform partial online compensation, 6 compensation tubes are made, each containing 60µl BD™ CompBeads Anti-Mouse Ig, κ particles, which bind any mouse κ light chain-bearing immunoglobulin, 60µl of the BD™ CompBeads Negative Control (FBS), and 10µl of a mouse κ light chain-bearing antibody for each fluorochrome channel (FL1-6). The tubes were vortexed and incubated in the dark for 30 minutes prior to acquisition. DIVA software (BD Biosciences) was used for partial online compensation prior to cell acquisition, and generation of list mode data files. Completion of compensation and analysis of list mode data was carried out using WinList software (Verity Software House, Maine). Compensation was completed offline using the compensation toolbox on the WinList^{TM} software program, using positive and negative populations of the fluorochrome stained compensation beads. Each individual experiment has its own unique compensation settings, saved as compensation files.

Dead cells and debris were excluded on the basis of light scatter (Figure 2.1)

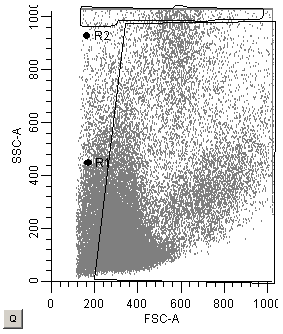


Figure 2.1 Histogram of cell distribution according to forward scatter and side scatter.Live cells were gated within region 1 (R1).

Live cells were analysed on a plot of HLA-DR versus lineage cocktail staining. Lineage cocktail comprised specific monoclonal antibody labels for T cells (CD3), monocytes (CD14, CD16), B cells (CD19) and stem cells (CD34). There is no single specific marker in humans that identifies all DC, but they can be recognised by excluding other cell types. In humans, these cells are characterised by their high expression of HLA-DR and they lack the expression of lineage markers (Bell et al., 2001). Using multicolour flow analysis, DCs were identified as an HLA DR+ lineage – (CD3-, CD14-, CD16-, CD19-, CD34-) population and are defined as such throughout this thesis. (Figure 2.2).

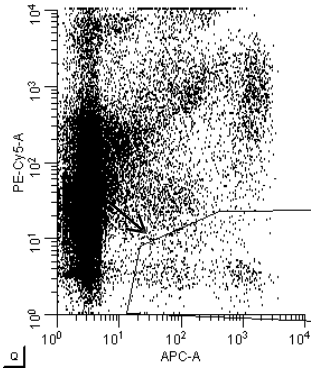


Figure 2.2 Histogram of cell distribution staining positive for DR (x axis) and Lineage (y axis). Dendritic cell population is defined as DR+ ($>10^1$) and Lineage negative ($<10^1$): gated region R3.

Epithelial cells were identified as staining positive for pancytokeratin (C-11) and a positive region was drawn in reference to the isotype antibody e.g. Figure 4.7 Chapter 4.

Enhanced Normalised Subtraction (Winlist software) was used to measure the percentage of positive staining cells and the level of staining for cell surface and intracellular markers as well as the percentage of cytokine-positive cells. The level of staining expressed as an intensity ratio (IR) represents the ratio of the median value of positive events in the test histogram to the median value of staining with the isotype-matched control antibody. The percentage of positive staining cells was determined by superenhanced D_{max} (SED) normalised subtraction. Single parameter histograms were created for both test antibody and isotype control and the isotype control was subtracted from the test antibody using the super-enhanced D_{max} (SED) normalised subtraction facility in WinList. This results in accurate values for percentage expression of that antibody and the intensity ratio (IR) of positive cell staining, i.e. the level of staining of a particular marker. This subtraction technique allows positive cells to be identified in situations where the distribution histograms overlap. The D_{max} is given as a percentage following subtraction of the test and control histograms. If the two histograms are identical and overlap perfectly, a D_{max} of 0% is the result (Figure 2.3).

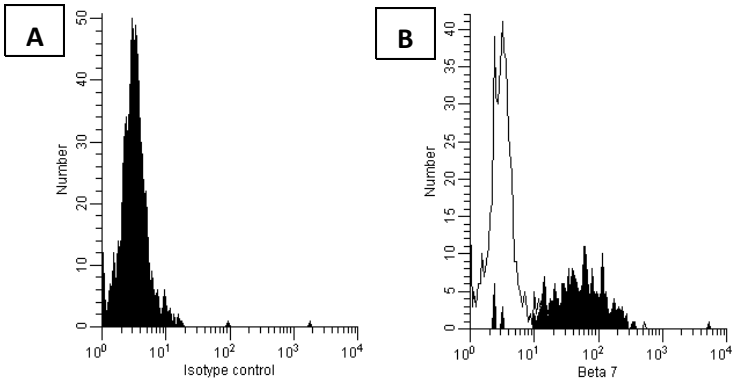


Figure 2.3. Super-enhanced normalised subtraction example histograms A is the isotype control which represents non-specific staining**. B** is the positive staining (shaded area) of the relevant marker (here β7). This represents specific labelling for β7 combined with an element of non-specific staining. Using Enhanced Normalised Subtraction WinList software generates both the percentage of positive staining cells and the IR after subtracting the non-specific binding component from the phenotype labelling histogram. The median channel of fluorescence and distribution of each histogram is compared and the proportion of positive events calculated (B- shaded area).

Normalised cumulative histograms of isotype controls were subtracted from histograms of positive staining. Where cells were cultured with or without monensin, the control samples were used as a reference allowing for gain or loss of cytokine to be measured (Holden et al., 2008). In some cases secreted protein binds back to the surface of cell in the no monensin sample together with the loss of pre-existing cytokine from cells in the monensin sample, which contribute to the overall loss of cytokine. In such a situation the subtraction process can be reversed to generate negative results. In this system, the control sample does not represent zero but a reference point on which to quantify the build-up or loss of cytokine in the test sample.

2.2.10 Multiplex

From each patient, one biopsy sample was blotted and weighed prior to being cultured overnight in complete medium at 37°C in a humidified atmosphere of 5% CO_2 in air. Following removal of the biopsy tissue, the supernatant was then centrifuged to remove residual cell debris and the supernatant was snap frozen in liquid nitrogen and stored at -80°C.
BD Cytometric Bead Array (CBA). Human Th1/Th2/Th17 Cytokine Kit. Catalogue no 560484 Includes: IL-2, IL-4, IL-6, IL-10, TNF, IFN-gamma, IL-17A. The BD CBA Human Th1/Th2/Th17 Cytokine Kit uses bead array technology to simultaneously detect multiple cytokine proteins in research samples. Seven bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for their specific cytokine (IL-2, IL-4, IL-6, IL-10, TNF, IFN-gamma and IL-17A). The seven bead populations are then mixed together to form a bead array, which is analysed in the FL2 (PE) channel of the flow cytometer.

During the assay procedure, the cytokine capture beads were mixed with the recombinant standards and research samples and incubated with FL2 (PE)-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex revealed the concentration of that cytokine. Standard curves were plotted to calculate the detection limit for the concentration of each cytokine. Values falling below the level of the detection limit are reported as being equal to that level.

2.2.11 Electron Microscopy

Performed by Nicholas English of the Antigen Presentation Group

Tissue biopsy (2 mm^3) samples were fixed in 3% glutaraldehyde in 0.1M sodium phosphate buffer ph 7.4 for 2 hours at room temperature. After glutaraldehyde fixation, the samples were kept at 4^oC until convenient for further processing. The samples were given three washes with 0.1M sodium phosphate buffer pH 7.4 and post fixed in 1% osmium tetroxide in 0.1M ph7.4 sodium phosphate buffer for 1 hour. They were given three washes with water to remove the phosphate and kept in water at 4^oC overnight. The samples were given two further washes with water and en bloc stained with 2% aqueous uranyl acetate for 2-4 hours in the dark.

After three washes with water, the tissue samples were gradually dehydrated with changes of increasing concentration of acetone. Acetone concentrations of 25%, 50%, 75% and 95% were used, the samples being kept for 20 minutes in each acetone concentration. The samples were then given four changes of 100 % dry acetone over 1 hour. The small bottles containing the samples were placed on a rotating mixer. The tissue samples were gradually infiltrated with araldite resin in a 1:1 solution of araldite resin in acetone overnight, later with the caps of the bottles loosened or removed to allow the gradual evaporation of acetone. After at least two changes of 100 % araldite over 4- 8 hours, the tissue samples were embedded in the araldite resin and cured for 18 hours at 65 $^{\circ}C$. The araldite blocks of tissue were sectioned using a Reichert –Jung Ultracut E microtome. The ultrathin sections (100 nm thick) were collected on 200 mesh copper grids. They were stained with Reynolds lead citrate and carbon coated.

The grids of sections were viewed using a Jeol JEM- 1200 EX electron microscope. Electron micrographs were taken, the negatives developed and the images printed.

2.2.12 Immunofluorescent microscopy
Assisted by Dr HO Al-Hassi

The fresh human intestinal biopsy samples were snap-frozen and kept in -80°C until embedded in a drop of water. ImmEDge pen was used to draw a hydrophobic barrier around the cells. Sections were (fixed in cold methanol: acetone (1:1) for 1 minute and afterwards washed with 0,1% Tween 20 in PBS detergent. After washing and blocking with with normal goat serum and normal rabbit serum, double labelling with primary antibodies was performed and the sections were left to incubate overnight. After washing, sections were then incubated with secondary fluorescent antibodies (FITC- and TRITC-conjugated) for at least 4 hours (Sigma). Sections were then washed and mounted under cover slip with mounting medium (DAPI) and left overnight to harden. Control experiments were performed by omitting the primary antibodies.

2.2.13 DNA extraction

Biopsy samples were washed in PBS prior to being snap frozen in liquid nitrogen and stored at -80°C. Faecal samples were homogenised by hand before mixing with sterile PBS and vigorous vortexing for five minutes and centrifugation at 250g for 1 min to settle large particulate matter. Aliquots were stored in RNA later (1:10) snap frozen and stored at -80°C.
Samples were thawed slowly on ice and the FastDNA® SPIN kit for soil and FastPrep® (MP Biomedical) instrument were used for DNA extraction as per manufacturer's instructions. DNA was left overnight at 4°C before being frozen at -20°C.

2.2.14 Gel electrophoresis

For confirmation of the DNA test samples of DNA extraction were mixed with loading buffer and run on 2.5% agarose gels mixed with SYBR green at 90V for 1 hour. For confirmation of the PCR products test samples were mixed with loading buffer and run on 1% agarose gels at 130V for 1.5 hours. Moulds were visualised under UV light. Bands were checked against a 1kb DNA ladder.

2.2.15 PCR of bacterial DNA

DNA samples were thawed on ice. 16SrRNA-gene PCR amplicons were generated for Lib-L 454 Titanium sequencing with the use of barcoded primers targeting the V3–V5 regions of the 16S rRNA gene. 16SrRNA genes were amplified using lyophilised Golay barcoded primers (Eurofins MWG Operon). Bacterial primers 454-338F (5'- CCTATCCCCTGTGTGCCTTGGCAGTCT CAGACTCCTACGGGAGGCAGCAG-3') and 926R (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG GAGTCT GAGTCTCCGTCAATTCMTTTRAGT-3') were used to amplify 16Sr RNA genes.

The Golay barcodes that were used are as follows:

Sample	Barcode
Donor1_Faeces	GAGTGGTAGAGA
Patient1_Faeces_Pre	GCATAGTAGCCG
Patient1_Faeces_Post	GCATATAGTCTC
Patient1_Biopsy_Pre	GATCTCATAGGC
Patient1_Biopsy_Post	GATCTTCAGTAC
Donor2_Faeces	GATACGTCCTGA
Patient2_Faeces_Pre	GCATCGTCAACA
Patient2_Faeces_Post	GCATGTGCATGT
Patient2_Biopsy_Pre	GATGATCGCCGA
Patient2_Biopsy_Post	GATGCATGACGC
Donor3_Faeces	GATAGCTGTCTT
Patient3_Faeces_Pre	GCATTGCGTGAG

Patient3_Faeces_Post	GCCACTGATAGT
Patient3_Biopsy_Pre	GATGTCGTGTCA
Patient3_Biopsy_Post	GATGTGAGCGCT
Donor4_Faeces	GATAGTGCCACT
Patient4_Faeces_Pre	GCCAGAGTCGTA
Patient4_Faeces_Post	GCCTATACTACA
Patient4_Biopsy_Pre	GATTAGCACTCT
Patient4_Biopsy_Post	GCAATAGCTGCT
Donor5_Faeces	GATATGCGGCTG
Patient5_Faeces_Pre	GCGACTTGTGTA
Patient5_Faeces_Post	GCGAGATCCAGT
Patient5_Biopsy_Pre	GCACATCGAGCA
Patient5_Biopsy_Post	GCACGACAACAC
Donor6_Faeces	GATCAGAAGATG
Patient6_Faeces_Pre	GCGATATATCGC
Patient6_Faeces_Post	GCGGATGTGACT
Patient6_Biopsy_Pre	GCACTCGTTAGA
Patient6_Biopsy_Post	GCACTGAGACGT
Donor7_Faeces	GATCCGACACTA
Patient7_Faeces_Pre	GCGTACAACTGT
Patient7_Faeces_Post	GCGTATCTTGAT
Patient7_Biopsy_Pre	GCAGCACGTTGA
Patient7_Biopsy_Post	GCAGCCGAGTAT
Donor8_Faeces	GATCGCAGGTGT
Patient8_Faeces_Pre	GCGTTACACACA
Patient8_Faeces_Post	GCTAAGAGAGTA
Patient8_Biopsy_Pre	GCAGGATAGATA
Patient8_Biopsy_Post	GCAGGCAGTACT
Control1_Neg	GATCTATCCGAG
Control1_Pos	GATCGTCCAGAT
Control2_Neg	GCAGTTCATATC
Control3_Neg	GCTAGTCTGAAC
Control4_Neg	GGTGCGTGTATG
Control5_Neg	GTAGAGCTGTTC

Q5™ taq polymerase (New England Biolabs) was used for PCR reactions according to product protocol. Reaction components were assembled quickly on ice and mixed prior to thermocycling. Mastermix was made up as follows for a 25µl reaction: 5µl Q5 reaction buffer,

2.5µl dNTPs, 1µl forward primer, 1µl reverse primer, 0.25µl Q5 high fidelity DNA polymerase, 0.5µl template DNA, 14.75µl nuclease free water. The following PCR cycling conditions were used: 98 °C for 2mins, followed by 25 cycles of 98 °C for 30 seconds, 52 °C for 30 seconds and 72 °C for 2 minutes, followed by a final extension of 72 °C for 5 minutes.

2.2.16 16SrRNA gene sequencing and analysis
Performed by Dr Alan Walker of the Wellcome Trust Sanger Institute

To characterize the microbiota, bacterial 16S rRNA gene sequences were analysed using 454-based analysis tools such as QIIME and mothur. 16SrRNA gene amplicons from each sample were then pooled in equimolar amounts into a mastermix for sequencing using the Lib-L kit on the 454 GS FLX Titanium platform. The resulting sequence data was processed using the mothur software package (Schloss et al., 2009) as described previously (Cooper et al., 2013), except that sequences with less than a minimum length of 320 base pairs rather than 350 base pairs were discarded.

Any contaminant OTUs that were detected in the sequenced negative control samples were removed from the final dataset. Diversity comparisons (Chao, Shannon and inverse Simpson) were carried out using mothur after first sub-sampling the data down to 391 reads per sample to ensure equal sampling depth across all samples. Good's coverage (an estimate of completeness of species sampling) at 391 reads per sample was on average greater than 92% for all sample groups (overall median 95.9%, range 87.9 to 99.7%). Similarity indices were assessed using Bray-Curtis and Theta Yue & Clayton calculators using mothur. OTUs/phylotypes were classified, set at a 97% minimum pair-wise similarity definition against public databases to monitor changes in bacterial composition (The 454 pyrosequence data has been deposited at

the European Nucleotide Archive under Study Accession Number ERP005254/ Sample Accession Number ERS421606). In order to monitor changes in overall bacterial community structure after administration of FMT cluster dendrograms/principal co-ordinates analysis plots were calculated using QIIME/mothur. OTUs that were differentially abundant between cohorts were assessed by Metastats (White et al., 2009), as applied in mothur.

2.2.17 Statistical Analysis (advised by Paul Basset)

Pooled data are expressed as mean and mean standard error for experimental data and median and range for clinical data. Pooled data from separate experiments were compared with the Mann Whitney U test. Where multiple groups (≥3) were analysed Kruskal-Wallis test with Dunn's post test for multiple comparisons was performed. Where appropriate data were paired using Wilcoxon matched-pairs signed rank test. Friedman's test was used to assess repeated measures (over time). A p-value of ≤0.05 was considered significant. GraphPad Prism® Version 5 (GraphPad Software Inc., La Jolla, California, USA) was used for statistical analysis.

Chapter 3. Characterisation of lamina propria dendritic cells in ulcerative colitis patients following restorative proctocolectomy

3.1 Abstract

Introduction: Immune responses to the ileal pouch microbiota instigate pouch inflammation. Lamina propria dendritic cells (DC) are pivotal in the interactions between the host immune system and the gut microbiota. We hypothesised that DC may be involved in the development and perpetuation of pouch inflammation and aimed to characterise DC in the ileum and ileal pouch.

Methods: HLA DR+ lineage negative cells were identified in freshly isolated lamina propria mononuclear cells (LPMC) by multicolour flow cytometry. Bacterial pattern recognition receptors TLR 4 and 2; homing markers β7 and CCR 9 and the co-stimulatory marker CD40 were determined on DC extracted from ileal tissue of ulcerative colitis (UC) patients (9) and controls (9); from pouch tissue of UC patients with (15) and without pouchitis (14) and FAP patients (8).

Results: DC expressing integrin β7 and CD40 were reduced in ileal tissue from UC patients compared with ileal tissue from controls. In UC patients without pouchitis, DC expressing integrin β7 were increased compared with ileal tissue of UC patients and DC expressing gut homing marker CCR9 were increased compared with FAP patients, but no other differences were noted. In UC patients with pouchitis, DC expressing CD40 and TLR 4 and β7 were increased and DC expressing CCR9 were decreased.

Conclusions: DC phenotype in the ileum and in the non-inflamed pouch of UC patients suggests an aberrant expression of homing markers, which could promote colitogenic susceptibility in the pouch of patients with UC. DC phenotype in pouchitis suggests an activated innate immune response to microbial signals in inflammation of the pouch of UC patients.

3.2 Background

Data regarding the immune responses to the alterations in the pouch microbiota are sparse. Previous studies provide evidence for the role of bacteria in the stimulation of mucosal innate and adaptive immune responses in the ileal pouch (Bell, 2004, Lammers et al., 2005b, Pronio et al., 2008). It is not clear how the pouch microbiota might interact with the mucosal immune system. The intestinal immune system maintains a delicate balance between immunogenicity against invading pathogens and tolerance of the commensal microbiota and food antigens. Intestinal dendritic cells (DC) are pivotal in the maintenance of this balance (Chapter 1.5) lying at the intersection between the innate and adaptive immune systems maintaining homeostasis in the gut.

In inflammatory bowel diseases (IBD), DC are increased and more mature within inflamed IBD tissue. In IBD, DC are activated with upregulated expression of TLRs and co-stimulatory molecules as well as production of pathologically important cytokines (Hart et al., 2005a). Most studies of DC in IBD demonstrating these findings have focused on myeloid DC (mDC). Recently studies have begun to characterise plasmacytoid DC (pDC) in inflammatory bowel diseases finding pDC to be present in greater numbers in the inflamed colonic mucosa and MLN of IBD patients (Baumgart et al., 2011). In ulcerative colitis (UC), lamina propria pDC are increased significantly in inflamed and non-inflamed tissue compared with controls without a concomitant increase of mDC (Ng et al., 2009c). pDC may be of particular importance in the inappropriate innate immune responses to the microbiota in UC patients. There are no previous reports of the role of DC in pouchitis.

3.3 Aims

- To identify and characterise lamina propria dendritic cells in the ileum, non-inflamed and inflamed ileal pouch of patients with UC

3.4 Identification of human intestinal lamina propria dendritic cells

Patients were recruited and characterised as described (Chapter 2.2.2). Lamina propria mononuclear cells (LPMC) were obtained from ileal or pouch biopsies by immediate collagenase digestion (Chapter 2.2.5). DC were identified as HLA DR+ lineage- populations on six colour flow cytometry (Chapter 2.2.8, Figure 2.2).

3.5 Aberrant immune characteristics in the ileum of ulcerative colitis patients

The phenotype of HLA DR+ lineage negative cells was determined in ileal tissue from both UC patients and healthy controls. Specifically, antibody staining for the bacterial pattern recognition receptors TLR 4 and 2, homing markers β7 and CCR9 and the co-stimulatory marker CD40 was performed (Chapter 2.2.5) and dendritic cells were acquired and analysed by flow cytometry as described (Chapter 2.2.7-2.2.8).

There were no significant differences between UC patients and healthy controls with regard to the percentage expression of TLR 4 (39 +-5.8% vs. 31 +-6.6%; p=0.4) or TLR 2 (31 +-11.7% vs. 28 +- 8.1%; p=0.8) on ileal dendritic cells (Figure 3.1).

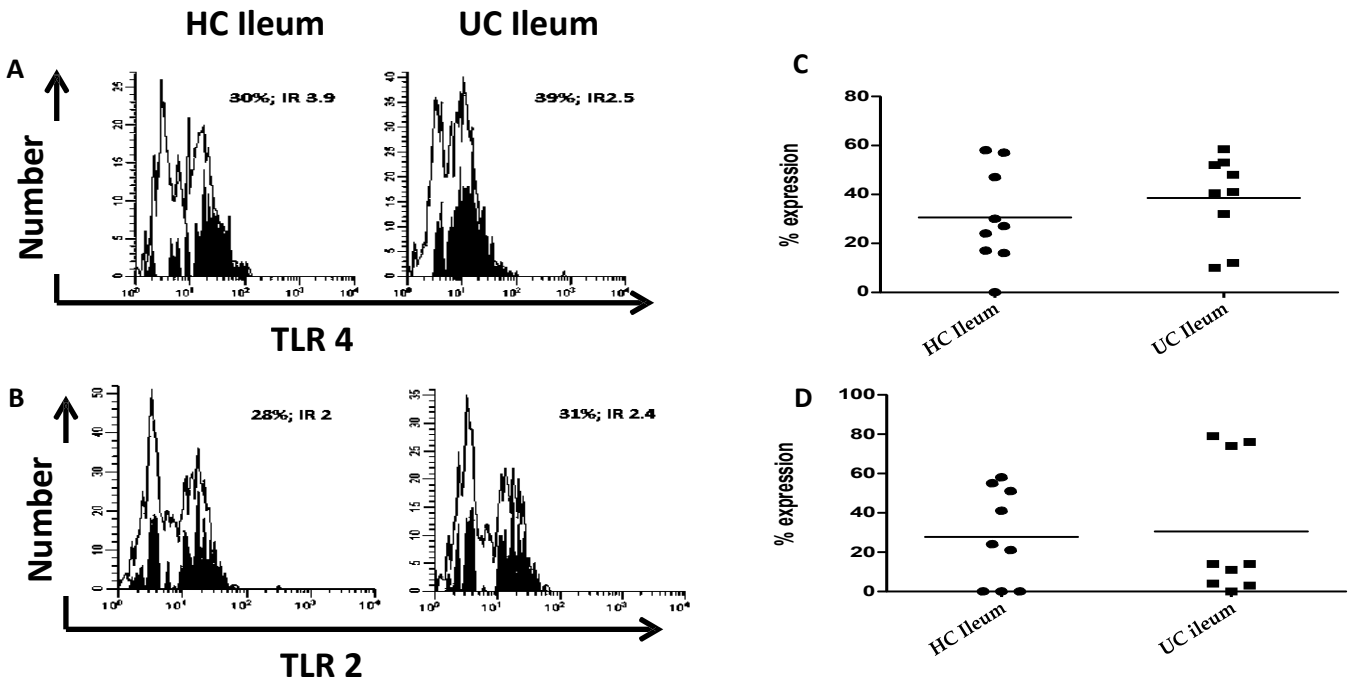


Figure 3.1 TLR 4 and TLR 2 expression on HLA DR+ lineage negative cells in ileal tissue from healthy controls and ulcerative colitis patients. A and B. One parameter histograms representative of TLR 4 and TLR 2 staining in healthy controls (n=9) and ulcerative colitis patients (n=9). C and D. Percentage expression of TLR 4 and TLR 2 in healthy controls and ulcerative colitis patients.

There was significantly lower percentage expression of β7 on DC from the ileum of patients with UC compared with healthy controls (6.4 +- 3.2% vs. 32 +-6.6%; p=0.001). There was no significant difference in CCR9 expression in UC patients (42 +-5.3% vs. 30 +-5.9%, p=0.1) (Figure 3.2).

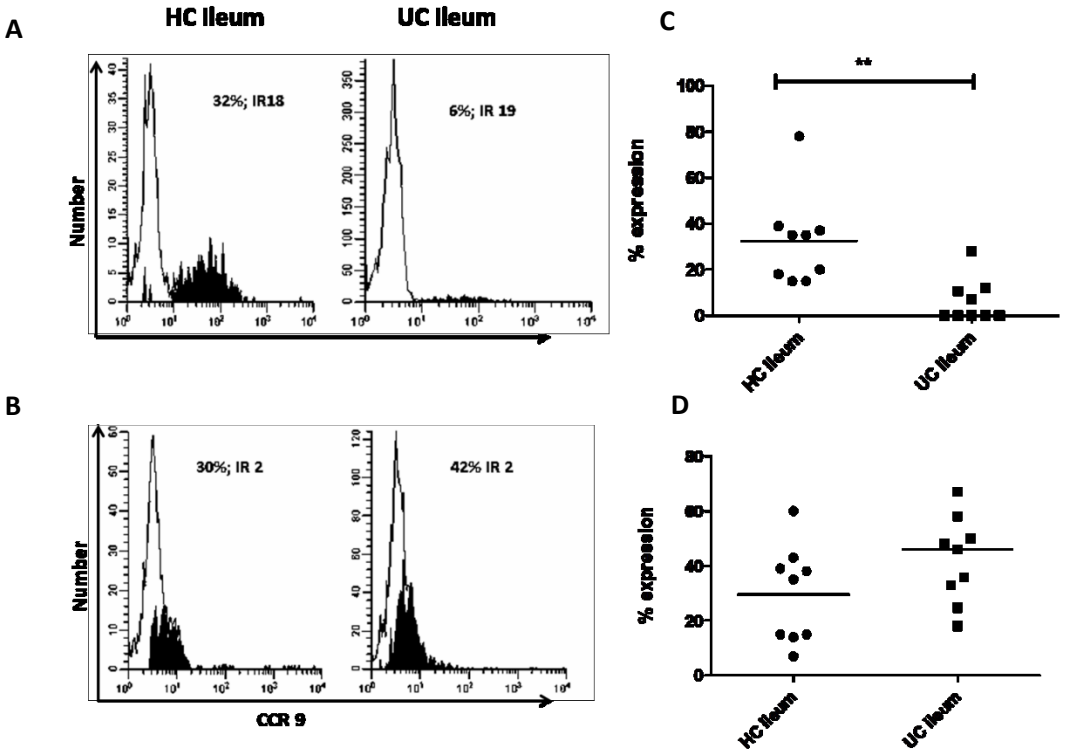


Figure 3.2 β7 and CCR9 expression on HLA DR+ lineage negative cells in ileal tissue from healthy controls and ulcerative colitis patients. A and B. One parameter histograms representative of β7 and CCR9 staining in healthy controls (n=9) and ulcerative colitis patients (n=9). C and D. Percentage expression of β7 and CCR9 in healthy controls and ulcerative colitis patients. **denote p values ≤0.01.

There was a significantly lower percentage expression of the activation marker CD40 on dendritic cells from the ileum of ulcerative colitis patients compared with controls (49 +-5.7% vs. 76 +-3.6%; p=0.004) (Figure 3.3).

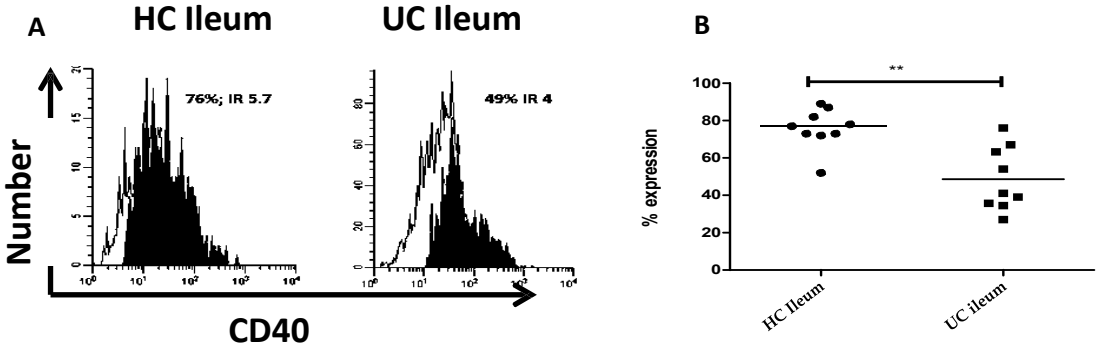


Figure 3.3 CD40 expression on HLA DR+ lineage negative cells in ileal tissue from healthy controls and ulcerative colitis patients. A. One parameter histograms representative of CD40 staining in healthy controls (n=9) and ulcerative colitis patients (n=9). B. Percentage expression of CD40 in healthy controls and ulcerative colitis patients. ** denotes p values ≤ 0.01.

Overall, these data indicate a pre-existing altered dendritic cell phenotype in the ileum of patients with UC. To determine whether the abnormal expression of β7 and CD40 on dendritic cells in the ileum of UC patients was due to differences in DC subsets, further characterisation of myeloid and plasmacytoid dendritic cells was performed.

In three independent experiments LPMC were obtained by collagenase digestion as described and expression of CD123 and CD11c was analysed by six colour flow cytometry within the region of HLA DR+ lineage negative cells. A region was drawn on CD123+ve cells and CD11c+ cells respectively in reference to their isotype antibodies and this positive staining region was back gated to show the regions of CD123+CD11c- and CD11c+CD123- dendritic cells respectively (Figure 3.4).

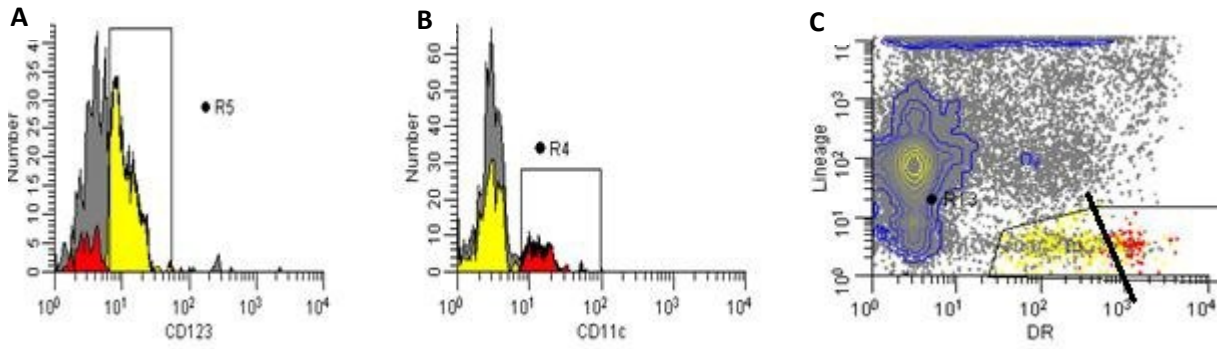


Figure 3.4 Backgating of CD123 and CD11c positive cells on HLA DR versus lineage plot. A and B. Histograms showing region of positive staining (R5) for CD123 and CD11c (R4) in reference to their isotype control (n=3). C. CD11c+ (red) and CD123+ (yellow) DC were backgated to the DC region on the HLA-DR versus lineage cocktail dot plot. All plots were compared to isotype-matched controls (n=3).

The regions denoting CD123+CD11c- and CD11c+CD123- cells that were HLA DR+ lineage negative were drawn for each of the HLA DR versus lineage plots for each experiment to identify putative plasmacytoid and myeloid DC. In addition, plasmacytoid and myeloid DC were also differentiated on the level of staining for HLA DR as previously described (Bell et al., 2001). There was a significantly lower level of expression of HLA DR on putative pDC compared with putative mDC in healthy controls ($p=0.03$), but in UC patients this trend did not reach significance ($p=0.06$) (Figure 3.5).

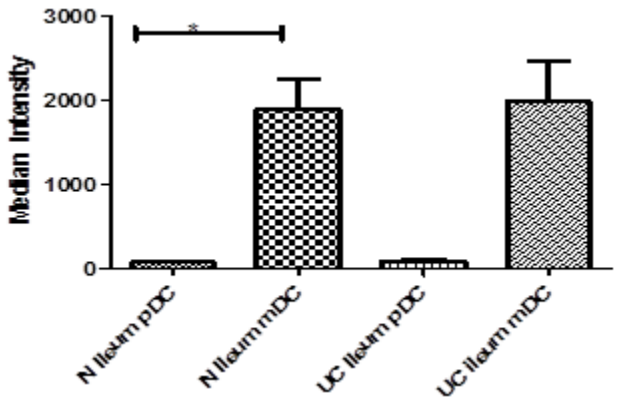


Figure 3.5. Median intensity of HLA DR expression on putative pDC and putative mDC from ileal tissue of healthy controls and ulcerative colitis patients. * denotes p values ≤0.05.

The overall percentage of HLA DR+ lineage negative cells represented approximately 6% of LPMC in the ileum of UC patients compared with 2.5% in the ileum of healthy controls (p=0.008). The increase in DC in UC patients was accounted for only by a significant increase in putative plasmacytoid DC (pDC) (5%+-0.8 vs. 1.7%+-0.4; p=0.01) as there was no difference in putative myeloid DC (mDC) between UC ileum and healthy controls (0.9%+-0.1 vs. 1.1%+-0.3; p=0.7) (Figure 3.6).

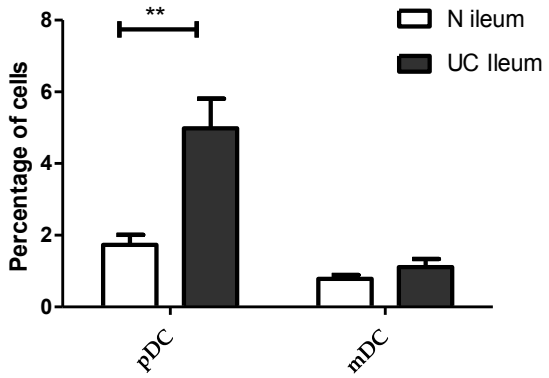


Figure 3.6. Mean percentage of LPMC in the ileum of UC patients and healthy controls. Percentage of cells identified as pDC and mDC in ileum from healthy controls (n=9) and UC patients (n=9). ** denote p values ≤0.01.

Further analysis was performed to assess whether the differences in β7 and CD40 expression were as a result of this discrepancy, or due to genuine differences in the proportion of cells expressing β7 and CD40. A significantly higher percentage of putative mDC expressed β7 compared with putative pDC in both healthy controls (12+-4.8% vs. 67+-9.1%; p=0.004) and UC patients (1.2+-0.8 vs. 49 +-2.8; p=0.01). There was a significantly lower percentage expression of β7 on putative mDC in UC patients compared with healthy controls (p=0.01), but not putative pDC in UC patients compared with healthy controls (p=0.1) (Figure 3.7 A). The percentage expression of CD40 was significantly higher on putative mDC than on putative pDC in healthy controls (87.4 +-3.4% vs. 48.2+-7.8%; p=0.004) and trended towards a higher percentage expression on putative mDC compared with putative pDC in UC patients (91 +-9.8% vs. 55 +-2.0; p=0.06), but there were no significant differences between UC patients and healthy controls (p=0.5). This suggests that the lower proportion of DC expressing CD40 in all DC from UC ileal tissue is due to the increased ratio of pDC to mDC. (Figure 3.7 B).

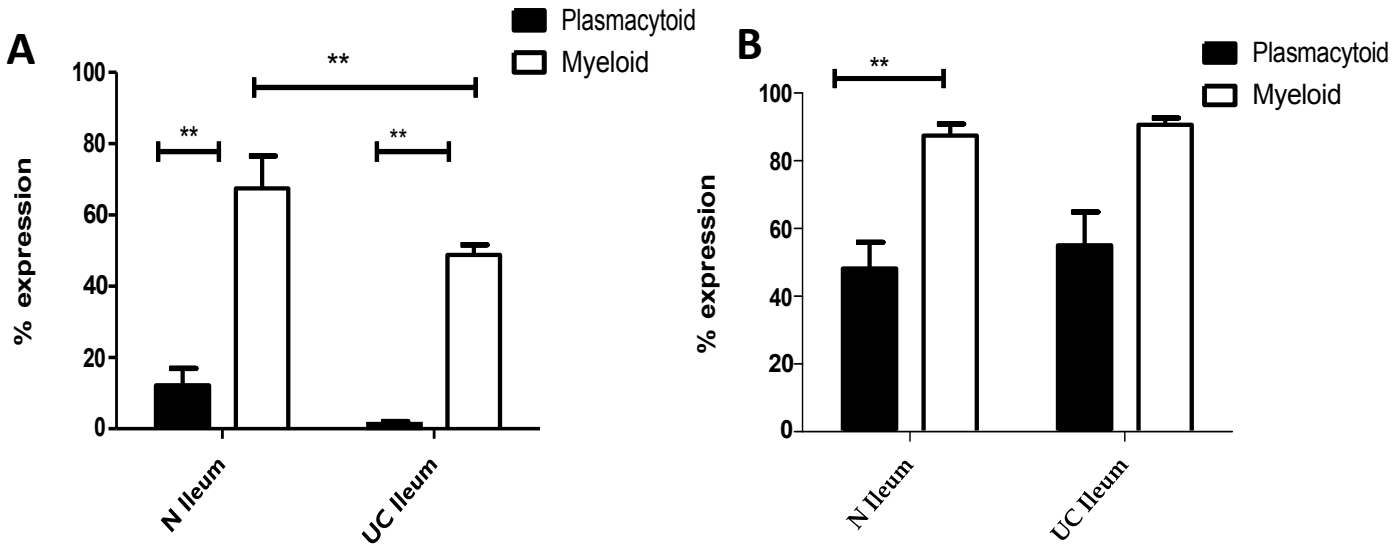


Figure 3.7 Mean percentage expression of β7 and CD40 on DC subsets in the ileum of UC patients and healthy controls. A. Percentage expression of β7 on putative pDC and mDC from the ileum of healthy controls and ulcerative colitis patients. B Percentage expression of CD40 on putative pDC and mDC from the ileum of healthy controls and ulcerative colitis patients. ** denotes p values ≤ 0.01.

3.6 Dendritic cell phenotype differs between the ileum and colon (For these studies ileal tissue experiments were performed by J Landy and colonic tissue experiments by D Bernardo. This is the only work where dual operators were used).

Following ileostomy closure the pouch microbiota has a more colon-like phenotype (Kohyama et al., 2009, Falk et al., 2007). To assess the differences in dendritic cell phenotype that could be attributed to adaptive changes to the more colonic microenvironment rather than the underlying disease, TLR, homing marker and CD40 expression on DC were compared between that found in ileal and colonic tissues in healthy controls. The percentage expression of TLR 4 on colonic DC was significantly higher compared to the ileum of healthy controls (57 +-5.6% vs. 31 +-6.6%; p=0.02) (Figure 3.8 A). There was no significant difference in DC expression of TLR 2 (39 +-5.0% vs. 28 +-8.1%; p=0.3) (Figure 3.8 B).

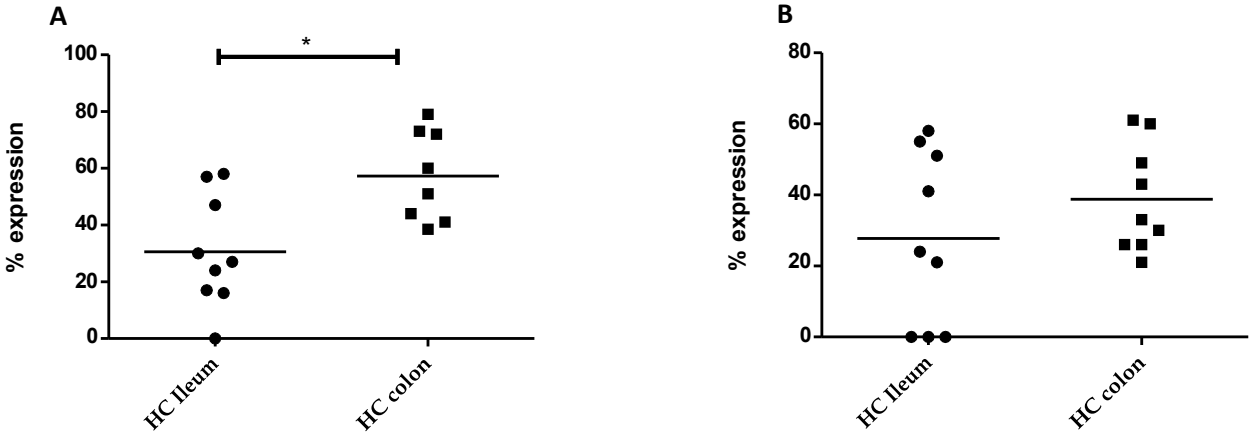


Figure 3.8 TLR 4 and TLR 2 expression on HLA DR+ lineage negative cells in ileal and colonic tissue from healthy controls and ulcerative colitis patients. Percentage expression of A TLR 4 and B TLR 2 in the ileum (n=9) and colon (n=8) of healthy controls. * denotes p values ≤ 0.05.

The percentage expression of β7 on colonic DC was significantly higher compared with the ileum of healthy controls (54 +-6.3% vs. 32 +- 6.6%; p=0.04) (Figure 3.9 A). The percentage expression of CCR9 on colonic DC was significantly lower compared with the ileum (11 +-3.6% vs. 30 +-5.9%, p=0.02) (Figure 3.9 B). The percentage expression of CD40 on colonic DC was significantly lower compared with the ileum of healthy controls (49 +-4.4% vs. 76 +-3.6%; p=0.002) (Figure 3.10).

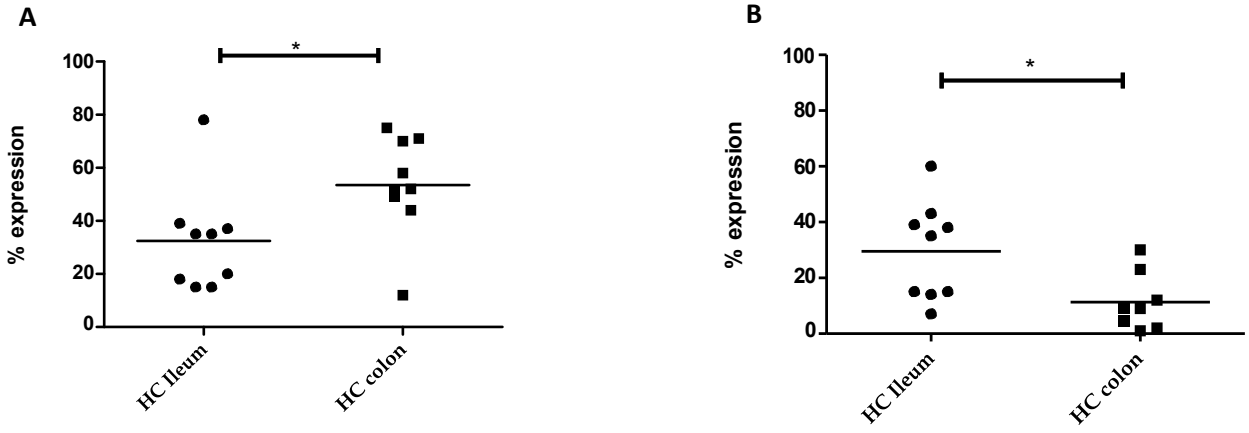


Figure 3.9 β7 and CCR9 expression on HLA DR+ lineage negative cells in ileal and colonic tissue from healthy controls and ulcerative colitis patients. Percentage expression of A β7 and B CCR9 in the ileum (n=9) and colon (n=8) of healthy controls. * denotes p values ≤0.05.

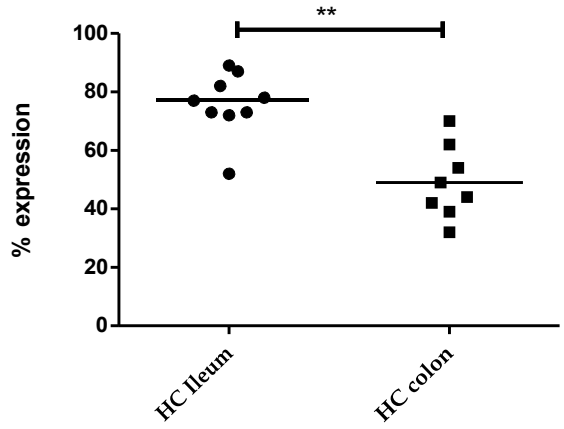


Figure 3.10. CD40 expression on HLA DR+ lineage negative cells from the ileum and colon of healthy controls.

Percentage expression of DC β7 expression in the ileum (n=9) and colon (n=8) of healthy controls. ** denotes p values ≤ 0.01.

3.7 Aberrant immune characteristics in the non-inflamed pouch of ulcerative colitis patients

To assess DC characteristics in the pouch of UC patients LPMC were obtained from UC patients without clinical, endoscopic or histological evidence of pouch inflammation. As a further control group for the non-inflamed UC pouch group, LPMC were obtained from FAP patients with non-inflamed ileal pouches.

There were no significant differences in the percentage expression of TLR 4 on DC from the ileum of UC patients, the pouch of UC patients (39 +-5.8% vs. 29 +- 4.6 %) or the pouch of FAP patients (25 +-6.7%) ($\chi 2=2.53$, $p=0.3$). There were also no significant differences in the percentage expression of TLR 2 on DC from the ileum and pouch of UC patients (31 +-11.6% vs. 27 +- 4.5%) or the pouch of FAP patients (22 +-7.1%) ($\chi 2= 0.15$, $p=0.9$) (Figure 3.11).

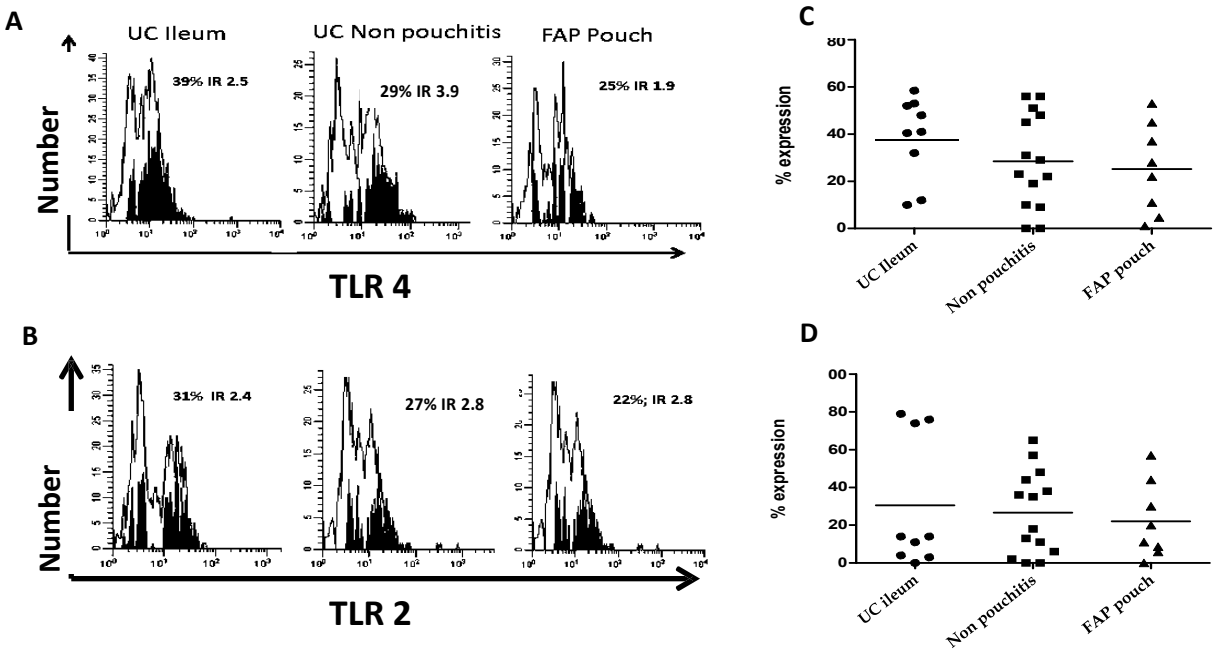


Figure 3.11 TLR 4 and TLR 2 expression on HLA DR+ lineage negative cells in ileal tissue and non-inflamed pouch tissue from ulcerative colitis patients and non-inflamed pouch tissue from FAP patients. A and B. One parameter histograms representative of TLR 4 and TLR 2 staining in the ileum of ulcerative colitis patients (n=9), the non-inflamed pouch of ulcerative colitis patients (n=14) and the non-inflamed pouch of FAP patients (n=8). C and D. Percentage expression of TLR 4 and TLR 2 in the ileum and pouch of UC patients and in the pouch of FAP patients.

There were significant differences in the percentage expression of β7 on DC ($\chi 2=14.9$, $p=0.0006$). The percentage expression of β7 on DC in the non-inflamed pouch of UC patients was higher compared with the ileum of UC patients (27 +-2.5% vs. 6 +-3.2%, $p \leq 0.001$). The percentage expression of β7 on DC from the pouch of FAP patients was elevated compared with the ileum of UC patients (37 +-7.9% vs. 6 +-3.2%, $p=0.002$). The proportion of DC

expressing β7 from the pouch of FAP patients compared with the pouch of UC patients was not significantly different (p=0.2).

There were significant differences in the percentage expression of CCR9 on DC (χ2=7.9, p=0.02). The percentage expression of CCR9 on DC was not significantly different between the pouch of UC or FAP patients compared with the ileum of UC patients (51 +-2.8% vs. 33 +- 3.9% vs. 42 +- 5.3%), but was significantly lower in the pouch of FAP patients compared with the pouch of UC patients (p=0.006) (Figure 3.12).

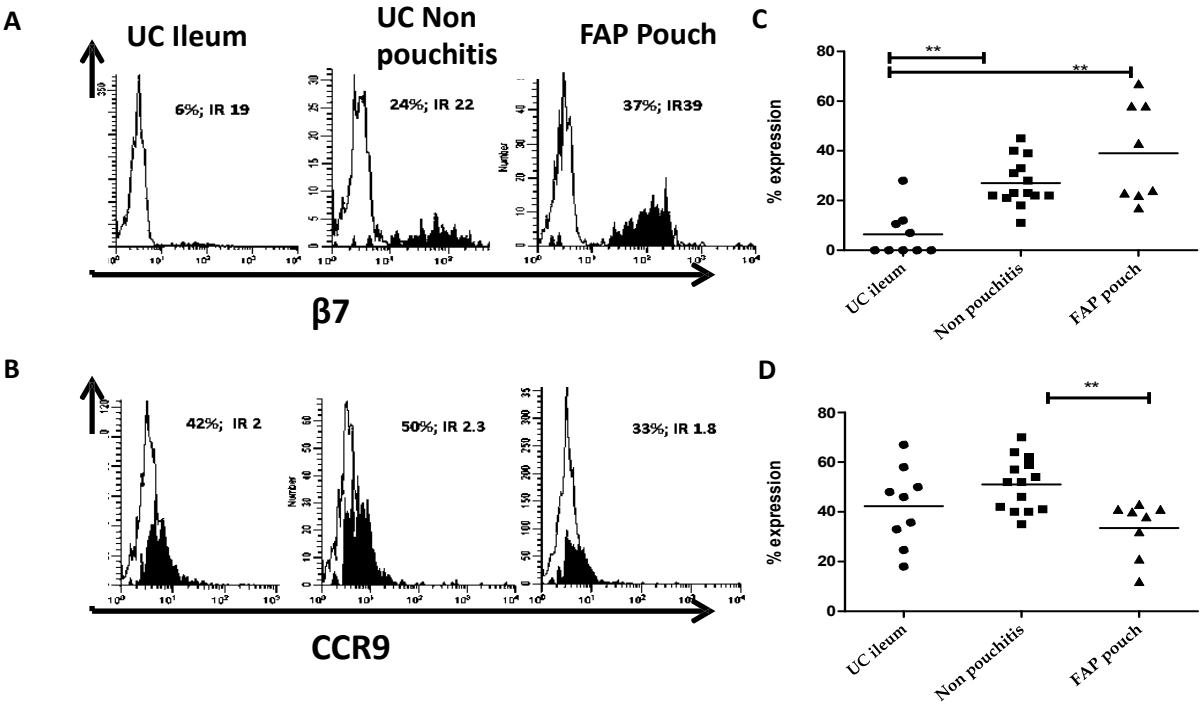


Figure 3.12 β7 and CCR 9 expression on HLA DR+ lineage negative cells in ileal tissue and non-inflamed pouch tissue from ulcerative colitis patients and non-inflamed pouch tissue from FAP patients. A and B. One parameter histograms representative of β7 and CCR9 staining in the ileum of ulcerative colitis patients (n=9), the non-inflamed pouch of ulcerative colitis patients (n=14) and the non-inflamed pouch of FAP patients (n=8). C and D.

Percentage expression of β7 and CCR9 in the ileum and pouch of UC patients and in the pouch of FAP patients. ** denote p values ≤ 0.01.

There were no significant differences in the percentage expression of the co-stimulatory marker CD40 on DC in the pouch of UC (62 +-3.1%) or FAP patients (66 +-5%) compared with the ileum of UC patients (49 +-5.7%) ($\chi 2=4.5$, p=0.09) (Figure 3.13).

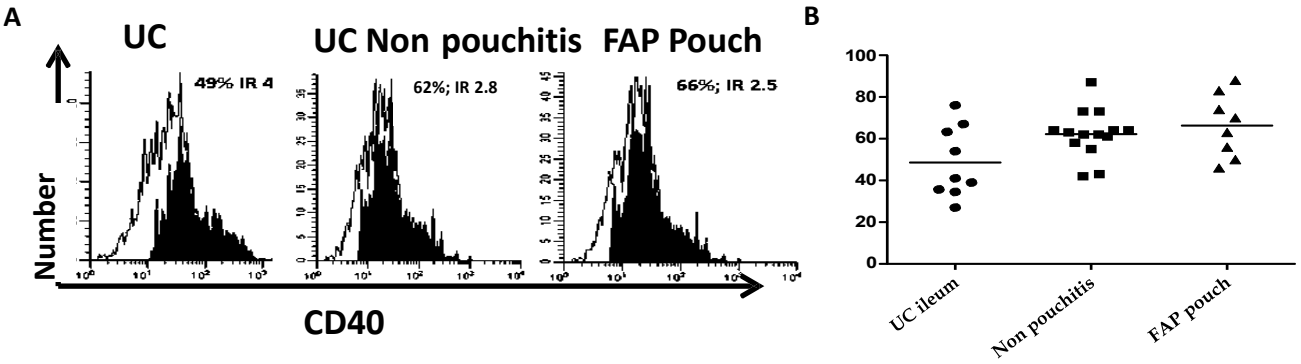


Figure 3.13 CD40 expression on HLA DR+ lineage negative cells in ileal tissue and non-inflamed pouch tissue from ulcerative colitis patients and non-inflamed pouch tissue from FAP patients. A. One parameter histograms representative of CD40 staining in the ileum of ulcerative colitis patients (n=9), the non-inflamed pouch of ulcerative colitis patients (n=14) and the non-inflamed pouch of FAP patients (n=8). B. Percentage expression of CD40 in the ileum and pouch of UC patients and in the pouch of FAP patients.

3.8 Aberrant immune response in ulcerative colitis patients with pouchitis

There are considerable data to suggest that the pouch microbiota drives inflammation of the pouch. Experiments were undertaken to determine whether there were differences in pattern recognition receptor expression on DC in the inflamed pouch of UC patients.

In patients with active pouchitis (diagnosed clinically, endoscopically and histologically) with a PDAI ≥7, there was a significantly higher proportion of DC expressing TLR 4 (44 +-4.8% vs. 29 +-5.3%, p=0.04) but not TLR 2 (42 +-4.2% vs. 27 +-5.9%, p=0.09) compared with UC patients with no pouch inflammation (Figure 3.14).

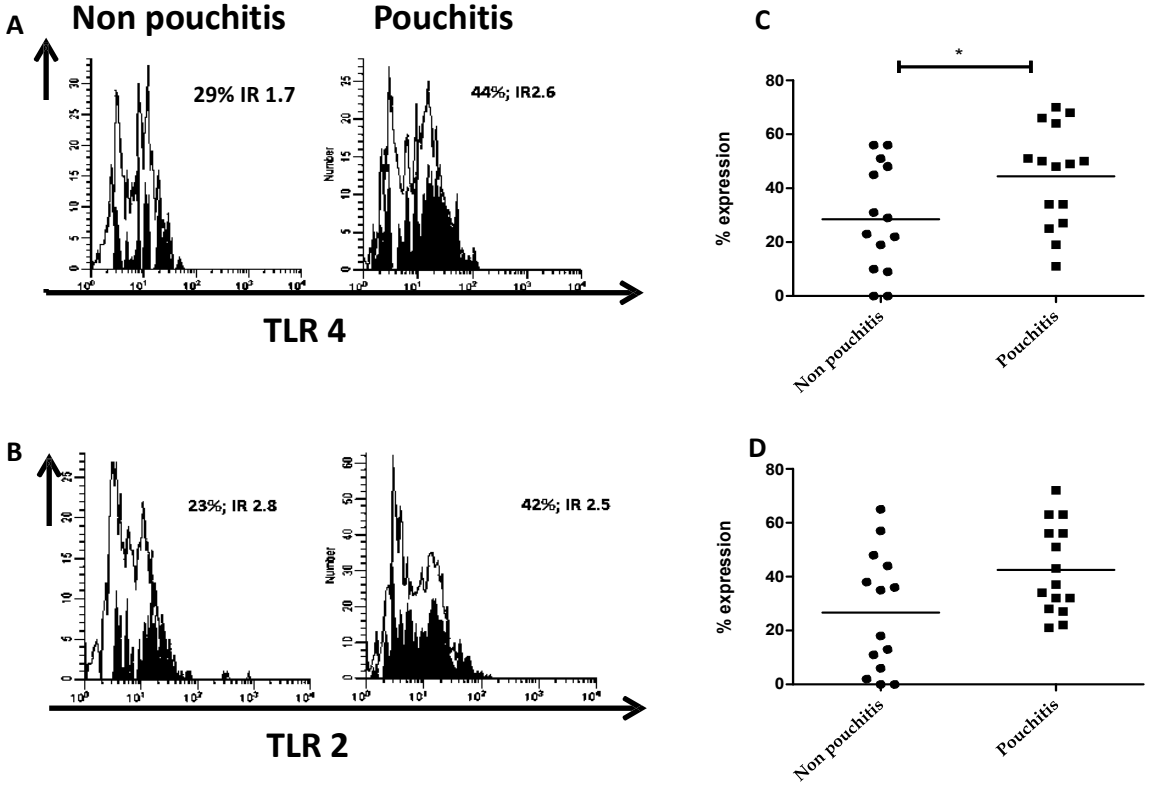


Figure 3.14 TLR 4 and TLR 2 expression on HLA DR+ lineage negative cells in pouch tissue from ulcerative colitis patients without inflammation and with pouchitis. A and B. One parameter histograms representative of TLR 4 and TLR 2 staining in ulcerative colitis patients without pouchitis (n=14) and ulcerative colitis pouchitis patients (n=15). C and D. Percentage expression of TLR 4 and TLR 2 in ulcerative colitis patients without pouchitis and ulcerative colitis pouchitis patients. * denotes p values ≤0.05.

Lamina propria DC in pouch tissue from patients with active pouchitis had significantly altered expression of gut homing markers, with an increase in the percentage expression of β7 (40 +-4.9% vs. 27 +- 2.5%, p=0.04) and a significant reduction in the percentage expression of CCR9 (35 +-4% vs. 51 +- 2.8%, p=0.006) compared with UC patients without pouch inflammation (Figure 3.15). The percentage expression of the co-stimulatory marker CD40 was also significantly elevated on DC in pouch tissue from patients with active pouchitis compared with UC patients without pouch inflammation (80 +-2.6% vs. 62 +-3.1%, p=0.0008) (Figure 3.16).

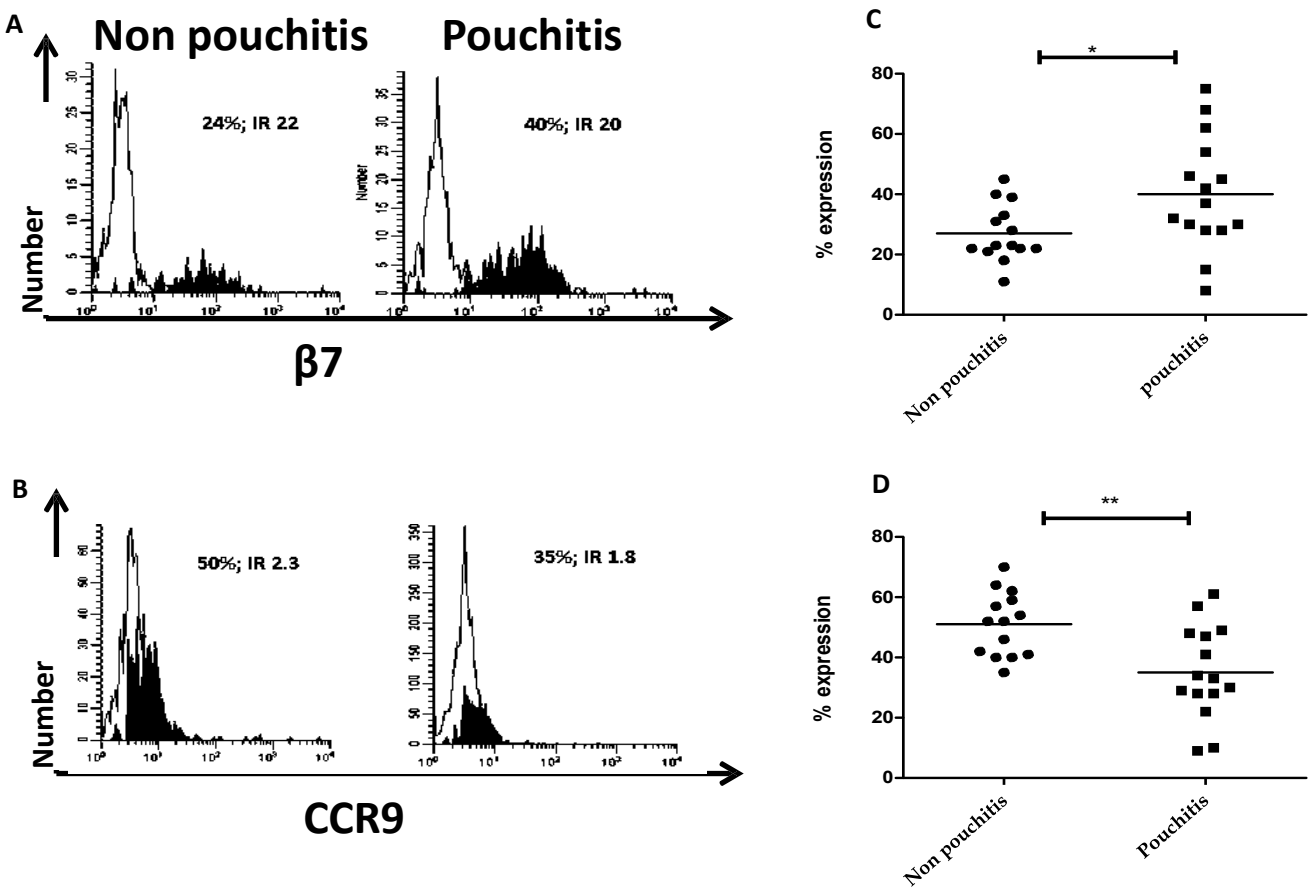


Figure 3.15 β7 and CCR9 expression on HLA DR+ lineage negative cells in pouch tissue from ulcerative colitis patients without inflammation and with pouchitis. A and B. One parameter histograms representative of β7 and CCR9 staining in ulcerative colitis patients without pouchitis (n=14) and ulcerative colitis pouchitis patients (n=15). C and D. Percentage expression of β7 and CCR9 in ulcerative colitis patients without pouchitis and ulcerative colitis pouchitis patients. * and ** denote p values ≤0.05 and 0.01 respectively.

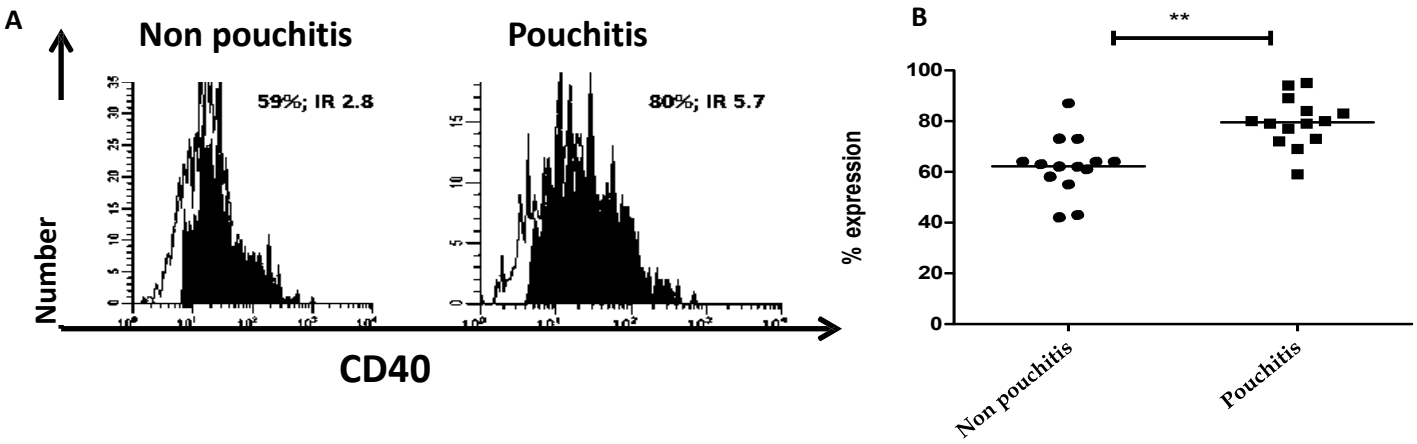


Figure 3.16 CD40 expression on HLA DR+ lineage negative cells in pouch tissue from ulcerative colitis patients without inflammation and with pouchitis. A. One parameter histograms representative of CD40 staining in ulcerative colitis patients without pouchitis (n=14) and ulcerative colitis pouchitis patients (n=14). B. Percentage expression of CD40 in ulcerative colitis patients without pouchitis and ulcerative colitis pouchitis patients. ** denotes p values ≤ 0.01.

3.9 Discussion

This is the first study to identify and characterise DC in the pouch of UC and FAP patients. Here an aberrant DC phenotype was identified to be pre-existent in the non-inflamed ileal tissue of UC patients undergoing RPC. In the ileum of UC patients compared with healthy controls, there was abnormal expression of gut homing markers (reduced β7) and reduced expression of activation marker CD40. Further analysis revealed that the abnormal DC phenotype in UC patients was due to the expansion of the plasmacytoid DC subset in these patients. This has been demonstrated in other studies of colonic mucosa from UC patients (Baumgart et al., 2011, Ng et al., 2009c). Others have also demonstrated CD11c- HLA DR+ cells in UC to express lower levels of activation and co-stimulatory markers including CD40 and to be weak stimulators of allogeneic T cell proliferation (Ng et al., 2009c).

A lower proportion of plasmacytoid DC expressed β7 compared with mDC in both UC patients and healthy controls; but in UC patients, β7 expression was also reduced in mDC compared with controls. Aberrant homing to the intestine in UC patients may therefore not only be as a consequence of the relative expansion of pDC, but a genuine loss of DC expressing β7. β7 pairs with the integrin α4 to designate gut homing. β7 also pairs with the integrin αE (CD103). Our group previously demonstrated reduced expression of CD103 on colonic DC from UC patients. This finding was again present in both the CD11c+ and CD11c- subsets (Ng Sc, 2008).

This may suggest that in UC patients there is an increase in DC homing to the ileum as well as imprinting small bowel homing of lymphocytes (see below). This should be interpreted with caution however. The ileal tissue in UC patients was taken from patients who had previously undergone subtotal colectomy and further work is necessary to evaluate whether our findings are consistently present in the terminal ileum of UC patients with intact anatomy.

In a recent study, DC expressing α4β7 induced Treg and IL-10 producing T cells and lack of α4β7 in the bone marrow innate immune compartment accelerated T cell mediated colitis which correlated with a reduction of intestinal CD103+DC (Villablanca et al., 2013). The expression of CCR9 on DC may also impact on the type of T cell responses. Murine studies demonstrate CCR9+ DC are less activated or mature than CCR9 low DC (Drakes et al., 2009). The expansion of the pDC subset in UC patients and reduced expression of CD40 may lead to weaker stimulation of T cells on presentation of microbial antigens (Steinman et al., 2003) encountered. Recent work from our group also found less mature, less stimulatory DC in the colon of quiescent UC with lower expression of CD40 and CD80 and CD86 and a reduced capacity of colonic and blood DC to stimulate allogeneic T cells (Al-Hassi et al., 2013, Mann et al., 2013b). Further study is necessary to assess the ability of DC in the ileum of UC patients to stimulate T cells, the type of T cell responses they stimulate and the homing markers they imprint. Gut derived DC also effect B lymphocyte intestinal homing and IgA secretion (Mora et al., 2006). This might impact not only on IgA-specific responses to antigen, but also the shaping of the microbiota and control of antigenic exposure at the epithelial surface layer. Further study is necessary to assess the impact of reduced DC β7 expression on B lymphocyte gut homing.

DC characteristics in the ileum and colon of healthy controls were assessed to ascertain the differences that may be accountable to the more colonic microenvironment and those that may be inherent to patients with UC. This is the only part of this work that involved dual operators and this should therefore be taken with caution. However, more recently published work from our group confirms differences between ileal and colonic DC (Mann et al., 2013a) and the differential imprinting of gut homing markers on T-cells by ileal and colonic DC (Mann et al., *in submission*).

In the colon of healthy controls there was increased expression of TLR 4 compared with the ileum. This may reflect the increased requirement for microbial sensing due to the increased number of bacterial antigens present. CD40 expression was reduced in the normal colon compared with the ileum of healthy controls, possibly suggesting an increased tolerance of colonic DC to the increased microbial content.

In the colon, β7 expression was increased and CCR9 expression reduced compared with the ileum. Recent work from our group found ileal, but not colonic DC were able to generate CCR9+ T-cells (Mann et al., *in submission*). The relative expression of CCR9 and β7 may therefore determine a gradient of preferential homing to the small or large bowel. Changes in DC homing markers in the pouch of UC patients might represent changes in the microbial microenvironment following RPC. In UC patients without pouchitis DC β7 expression was increased compared with the ileum of UC patients. However, there was not an expected reduction in the proportion of CCR9+ DC. β7 expression was not significantly increased in FAP

pouch DC compared with UC patients. However, CCR9 expression was significantly reduced in FAP patients compared with UC patients with non-inflamed pouches. The combination of the proportion of DC expressing β7 and CCR 9 in FAP and UC pouches suggest a trend towards less "colon-like" patterns of DC homing marker expression in UC patients that may be aberrant and not solely accounted for by the change in the microenvironment to a more colon like phenotype. The number of FAP patients was small and further samples from patients with FAP are necessary for more robust statistical analysis.

FAP patients are the only pouch control group available and as a group where the development of pouchitis is rare, offer a comparison to the UC patients where pouchitis is common. However, the inclusion of the FAP pouch group as another control may also add complexity as little is known regarding DC characteristics in FAP. FAP may not represent a "normal" pouch response to the altered microbiota following ileostomy closure. Increasing data demonstrate inflammatory signatures in non-colitis associated colorectal cancer (Grivennikov et al., 2012).

In the inflamed pouch of UC patients the proportion of DC expressing TLR 4 was significantly increased. This has been previously demonstrated in colonic DC in IBD (Hart et al., 2005b). Increased expression of TLRs by DCs and other cells interacting with the microbiota may lead to increased recognition of bacterial products and enhanced responses to them. The presence of elevated DC expression of TLRs only in inflammation suggests a number of factors found in inflammation may be responsible. Cytokines, including IL-6, TNF-α, and interferon, that are present in the inflamed mucosa of patients with IBD can cause increased expression of TLR2

and/or TLR4 (Martins et al., 2003). Bacterial products may act directly via TLRs to upregulate further TLR expression on DCs. In a study of TLR 4 and 2 expression in UC patients, positively stained cells accumulated subepithelially and were preferentially detectable close to the crypts (Hausmann et al., 2002). Increased intestinal permeability may therefore lead to greater exposure to microbial products and bacterially driven increases in TLR expression.

In pouchitis, DC expression of β7 was significantly increased and CCR9 expression significantly reduced. This may represent an increased specificity of DC homing and potentially T-cell homing to the pouch in the presence of intestinal inflammation. Increased activation is also supported by the significant increase in DC expressing CD40 in pouchitis patients.

Overall, the DC phenotype in pouchitis suggests a critical role in the maintenance of inflammation in the pouch of UC patients, in response to microbial signals. The findings regarding DC phenotype in the ileum and the non- inflamed pouch of UC patients also suggest that aberrant DC characteristics, in particular aberrant homing marker expression in UC patients may also play a critical role in the colitogenic susceptibility following RPC for UC. Further work assessing DC function and B and T cell responses is necessary to understand the impact these aberrant DC characteristics may hold for UC patients undergoing and following RPC. Understanding the molecular defects enabling interaction between the pouch microbiota and lamina propria DC is likely to further elucidate the aetiology of pouch inflammation.

Chapter 4. Epithelial tight junction protein expression in pouchitis

4.1 Abstract

Introduction: Impaired barrier function is a feature of active IBD, but is also present in quiescent disease. Underlying molecular defects of cell to cell junctions that might selectively enhance the interactions between luminal antigens and lamina propria antigen presenting cells are likely to be critical primary factors in the pathogenesis of pouchitis. We hypothesised that epithelial cell tight junction protein expression would be altered in the non-inflamed as well as inflamed pouch tissue of UC patients and may differ between early and late phases of pouch inflammation. We aimed to characterise epithelial tight junction expression in the non-inflamed pouch of patients with UC and in acute and chronic pouchitis.

Methods: Freshly isolated epithelial cells were identified from pouch mucosal samples of UC patients with pouchitis (13) (chronic pouchitis n=9, acute pouchitis n=4) and without pouchitis (n=18) and FAP patients (8). Epithelial cell expression of ZO-1, claudin 1 and claudin 2 were measured by multicolour flow cytometry.

Results: The proportion of pouch epithelial cells expressing claudin 2 was increased in the non-inflamed pouch of UC patients compared with FAP patients. In acute pouchitis the proportion of epithelial cells expressing claudin 2 was increased whereas in chronic pouchitis there was a reduction in the level of expression of both ZO-1 and claudin 1 expression.

Conclusions: The increased proportion of epithelial cells expressing claudin 2 in the non-inflamed pouch of UC patients and in acute but not in chronic pouchitis suggests that increased expression of claudin 2 is an early event in the development of pouch inflammation in UC patients.

4.2 Background

The intestinal mucosal epithelium limits interactions between luminal microbial antigens and the immune cells in the intestinal lamina propria. Bacterial translocation is defined as the passage of viable bacteria from the gastrointestinal tract to extra-intestinal sites. Three primary mechanisms promoting bacterial translocation are: (a) disruption of the microbiota, (b) increased permeability of the intestinal mucosal barrier, and (c) deficiencies in host immune defenses. These mechanisms act synergistically to promote inflammatory responses in the gut (Berg, 1999, Bowden et al., 1981).

Disruption of the epithelial barrier is a feature of gut inflammation and contributes to the pathogenesis of inflammatory bowel diseases (IBD) (Edelblum and Turner, 2009). Both Crohn's disease and ulcerative colitis (UC) share common features of epithelial barrier disruption, such as epithelial breaks, a reduction in tight junction strands, and glandular atrophy (Bruewer et al., 2006, Weber and Turner, 2007, Clayburgh et al., 2004). Barrier dysfunction is likely to be caused by epithelial damage including apoptosis, erosion and ulceration that are characteristic of gut inflammation. Inflammatory cytokines associated with gut inflammation alter epithelial permeability through their effects on the junctional complexes (Heller et al., 2005, Zolotarevsky et al., 2002, Arrieta et al., 2008).

However impaired barrier function is also evident in quiescent IBD and even in first degree relatives of Crohn's disease patients (D'Inca et al., 1999, Peeters et al., 1997). Dysregulation of

the epithelial barrier with changes in paracellular permeability due to altered cell to cell junctions is likely to be significantly more selective and may be a critical primary factor in the pathogenesis of IBD.

Genome wide association studies (GWAS) in UC have identified susceptibility defects pertaining to barrier dysfunction (Barrett et al., 2009). The most significant association noted is for a single nucleotide polymorphism (SNP) of the HNF4α gene. This gene encodes the transcription factor hepatocyte nuclear factor 4 α that regulates control of multiple elements of cell to cell junction including desmosomes, adherens junctions and tight junctions including claudins (Battle, 2006). Numerous studies demonstrate increased expression of claudin 2 and reduced expression of claudins 1, 4, 5 and 8 in active UC and Crohn's disease (Prasad et al., 2005, Zeissig et al., 2007, Oshima et al., 2008, Poritz et al., 2011, Das et al., 2012).

Barrier properties of ileal pouch mucosa in pouchitis and in non-inflamed pouches are also reduced (Kroesen et al., 2008, Merrett et al., 1996, Kroesen et al., 2006, Amasheh et al., 2009b) and increased bacterial translocation has been reported in pouches functioning for more than twelve months (Kroesen et al., 2008). Amasheh et al. reported increased claudin 2 and reduced claudin 1 expression in acute pouchitis. No previous studies have assessed differences in tight junction expression in acute and chronic pouchitis or in non-inflamed pouches of ulcerative colitis patients compared with FAP patients.

4.3 Aims

- To assess tight junction protein expression in the non-inflamed pouch of ulcerative colitis patients compared with FAP patients with ileal pouches
- To assess tight junction protein expression in the non-inflamed and inflamed pouch of ulcerative colitis patients.
- To assess differences in tight junction protein expression between UC patients with acute and chronic pouchitis.

4.4 Electron micrographs of non-inflamed and inflamed pouch epithelium

(performed by Mr N English APRG)

To gain insight into the differences between the inflamed and non-inflamed pouch epithelium, electron microscopy was performed on mucosal biopsies from UC patients with and without pouchitis. In pouchitis the epithelial villi appeared atrophied with irregularity of the epithelial cell luminal surface. The membranes between cells were more loosely arranged with increased intercellular distance, and condensation of the peri-junctional cytoskeleton (Figure 4.1). In addition, dendritic cells penetrated the epithelial cell layer more frequently (Figure 4.2) whereas in the non-inflamed pouch the dendritic cells appeared to be confined to the lamina propria.

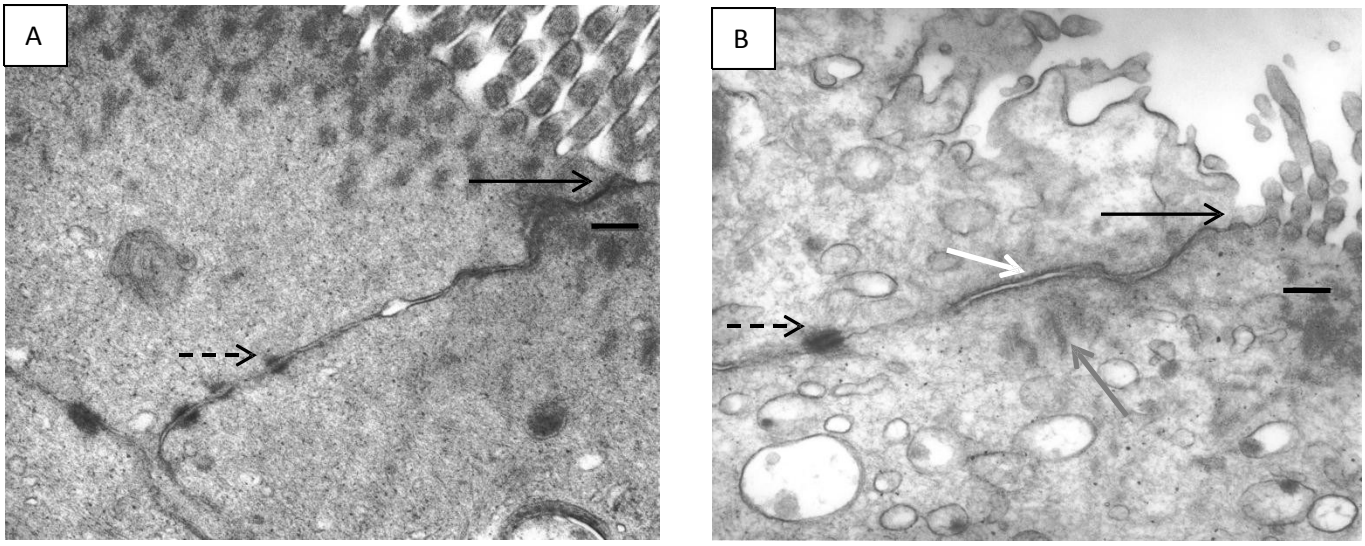


Figure 4.1. Electron micrograph of mucosal epithelium from A. non- inflamed pouch and B. pouchitis. Black bar = 0.2 micron. Solid line black arrow = Epithelial tight junction complex. Dashed black arrow = Desmosomes. White arrow showing increased intercellular distance, grey arrow peri-junctional cytoskeleton condensation.

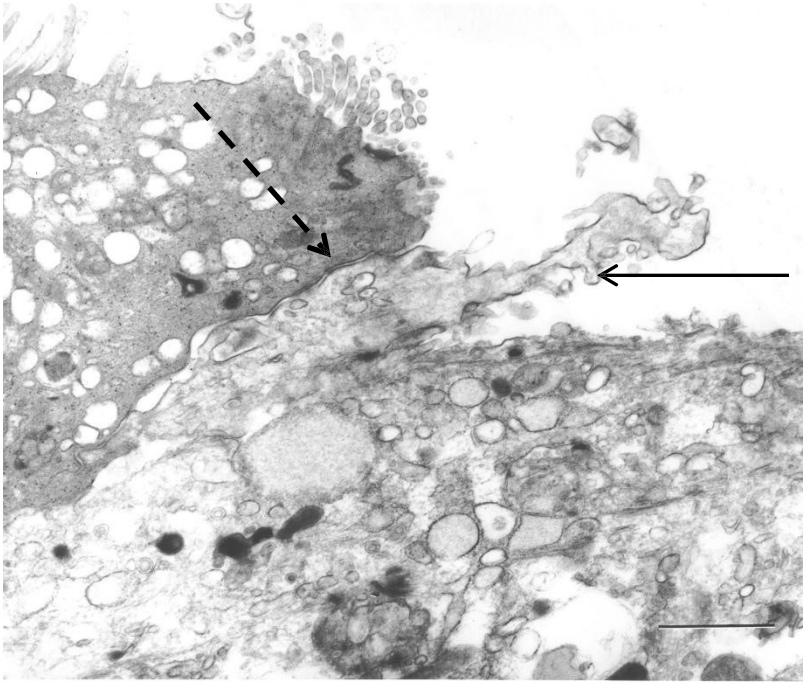


Figure 4.2 Electron micrograph demonstrating dendritic cell penetrating between two epithelial cells in pouchitis. Black bar = 0.1 micron. Solid black arrow = Dendritic cell. Dashed black arrow = Tight junction complex between epithelial cell and dendritic cell.

4.5 Immunofluorescence microscopy of tight junction proteins

To confirm the expression of tight junction proteins ZO-1, claudin 1 and claudin 2, staining of frozen sections of pouch tissue was undertaken (as described in chapter 2.2.12). This demonstrated the expression of these tight junction proteins in pouch tissue (Figure 4.3). It was intended to demonstrate comparisons of the distribution and co-localisation of tight junction proteins by immunofluorescence microscopy between pouch tissue from clinically different groups of patients. However, sections of tissue from pouchitis samples were of poor quality with significant destruction of the epithelial architecture leading to difficulties in adequate staining to assess comparisons between samples and differences between different patient groups.

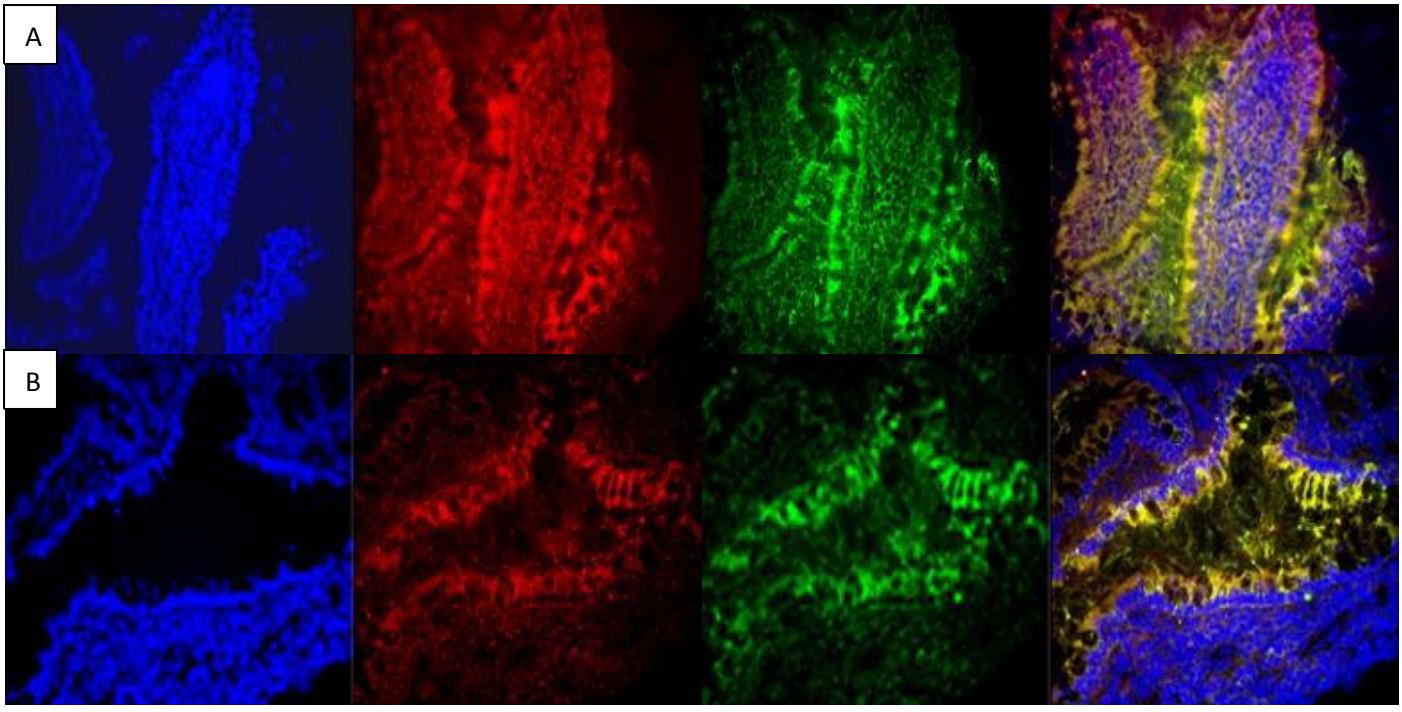


Figure 4.3. Immunofluorescence microscopy of tight junction protein expression in pouch and pouchitis samples. **Panel A**. ZO-1 and claudin 1 staining on epithelium from an ulcerative colitis patient with a non-inflamed pouch. From left to right panel shows staining with DAPI only, claudin 1, ZO-1 and all three stains combined. 20x magnification. **Panel B**. ZO-1 and claudin 2 staining on epithelium from an ulcerative colitis patient with pouchitis. From left to right panel shows staining with DAPI only, claudin 2, ZO-1 and all three combined. 20x magnification.

4.6. Flow cytometry for the assessment of epithelial tight junction protein expression- optimisation of methodology

The vast majority of studies assessing quantitative differences in tight junction expression employ either western blot analysis or RT-PCR. Neither of these methods enables analysis of expression on specific cell types, assuming the vast majority of tight junctions expressed will be derived from the epithelium. Furthermore, mRNA expression does not necessarily correlate with protein expression (Ogasawara et al., 2009a). In order to assess quantitative differences in the epithelial expression of ZO-1, claudin 1 and claudin 2 between non-pouchitis and pouchitis samples in this study, flow cytometry was undertaken. This has previously been described (Drudy et al., 2001, Russ et al., 1998).

Removal of the epithelium from biopsy tissue requires incubation with EDTA. This methodology has been described previously (Mennigen et al., 2009). Western blotting and RT-PCR, to quantify tight junction expression also require the use of EDTA (Amasheh et al., 2012). However, calcium chelation by EDTA is used as a method of disrupting tight junctions and dissociating confluent monolayers of epithelial cells. EDTA in monolayers of epithelial cell lines affects the distribution of ZO-1 (Siliciano and Goodenough, 1988). To demonstrate expression

of tight junction proteins by flow cytometry and to assess the effect of EDTA incubation on tight junction protein expression, a group of cells previously well defined using flow cytometric analysis and from a readily accessible source was initially assessed.

Dendritic cells express tight junction proteins in order to penetrate the epithelium whilst still preserving barrier function (Rescigno et al., 2001). Studies have also demonstrated expression of JAM-A and mRNA expression of other tight junction proteins in circulating dendritic cells and other peripheral blood cells, which may be necessary for trafficking of immune cells across the endothelium (Ogasawara et al., 2009a, Ostermann et al., 2002, Liu et al., 2000). The expression of other tight junction proteins has not previously been demonstrated in human peripheral blood cells. ZO-1, claudin 1 and claudin 2 expression were therefore assessed on HLA DR + lineage negative cells from PBMCs (Figure 4.4). ZO-1 (96 +-2.8%; IR 8.8 +-1) and to a lesser extent claudin 1 (33 +-7.3%; IR 5 +-2.7), but not claudin 2 were expressed on dendritic cells from peripheral blood of healthy controls.

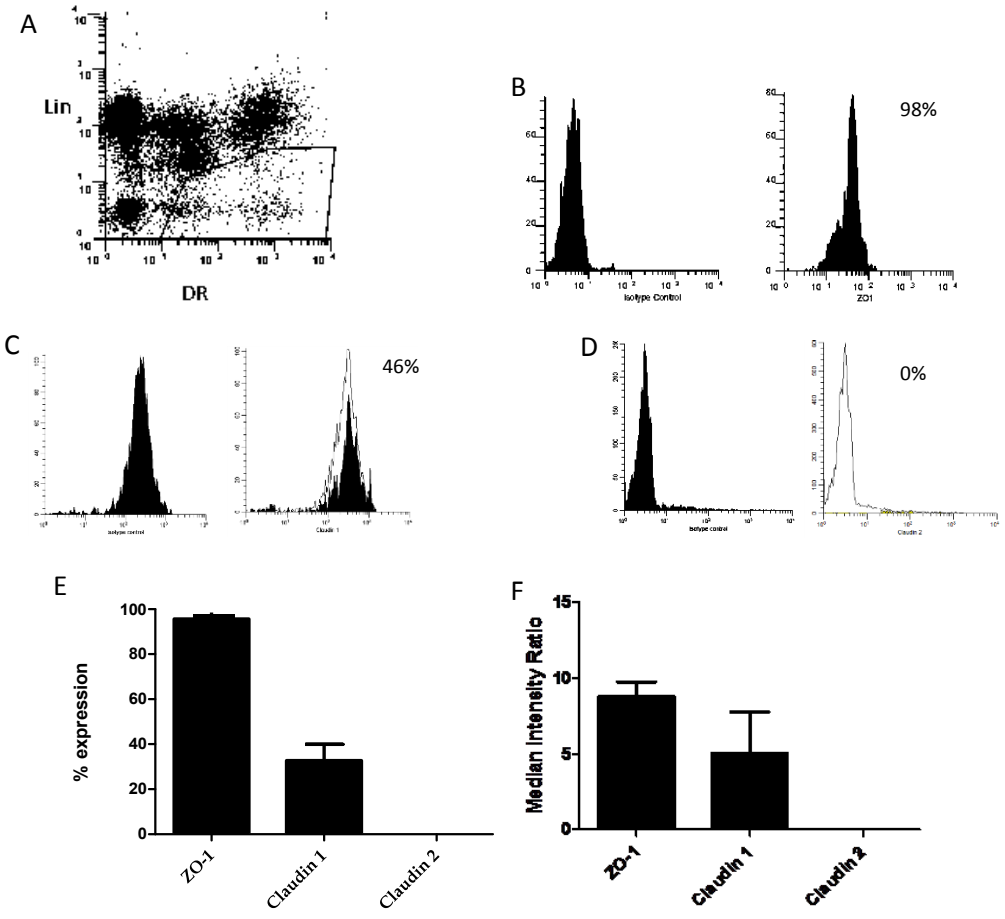


Figure 4.4. ZO-1 and claudin 1 expression on HLA DR+ lineage negative PBMCs. A. Histogram of cell distribution staining positive for DR (x axis) and Lineage (y axis). Dendritic cell population is defined as DR+ (>$10^{1)}$ and Lineage negative (<10^{1}). B. Single parameter histograms representative of isotype control and ZO-1 expression, C. claudin 1 expression and D. claudin 2 expression on peripheral blood DCs. E. Percentage expression and F. Median intensity ratio of ZO-1, claudin 1 and claudin 2 representing the levels of tight junction expression on healthy control peripheral blood dendritic cells (n=3).

To assess whether EDTA incubation may reduce the level of expression in addition to redistributing tight junctions, PBMCs were incubated without EDTA and with 1mM EDTA for 30 and 60 minutes (Figure 4.5). Contrary to the expected reduction in expression of ZO-1 following EDTA incubation, there was a slight increase in the level of expression, although there appeared to be no effect of a longer incubation at 60 minutes compared with 30 minutes incubation with 1mM EDTA (IR 17 +-1.4 vs. 23.5 +-2.1 vs. 21 +-2.8).

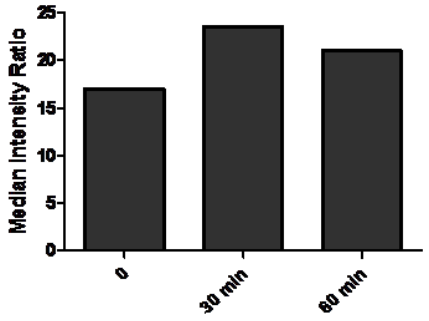


Figure 4.5 ZO-1 expression on HLA DR+ lineage negative PBMCs with and without EDTA incubation. Median intensity ratio of ZO-1 expression after 0, 30 and 60 minutes incubation with 1mM EDTA (One experiment performed in duplicate).

Experiments on CACO-2 cells were next performed to provide a positive control and to further assess the effect of EDTA incubation on the level of tight junction expression in an intestinal epithelial cell line. CACO-2 cells are of an epithelial cell line originally obtained from a human colonic adenocarcinoma. However, differentiation of these cells on culture leads to a heterogeneous population of cells ranging between colonocytes and foetal and mature ileal enterocytes (Sambuy et al., 2005). CACO-2 cells have been extensively used as a model of the intestinal epithelial barrier and barrier properties and tight junction expression on these cells is well established (Sambuy et al., 2005, Sun et al., 2008).

CACO-2 cells were cultured as described (Chapter 2.1.2). Cells were removed from wells using a sterile cell scraper and treated with 1mM EDTA for 30 or 60 minutes. Staining of CACO-2 cells with antibodies for ZO-1, claudin 1 and claudin 2 was then undertaken. ZO-1 (80 +-4%, IR 13.7 +-2.8) and claudin 1 (30 +-17% IR 10.8 +-9.6), but not claudin 2 were detected on CACO-2 cells by flow cytometry after 1mM EDTA incubation for 30 minutes. There were no significant changes in ZO-1 (80 +-4%, IR 12 +- 3; p=1, 0.3) or claudin 1 (29 +-17%, IR 9.4 +-8; p=0.4, 0.5) expression following incubation with 1mM EDTA for 60 minutes (Figure 4.6).

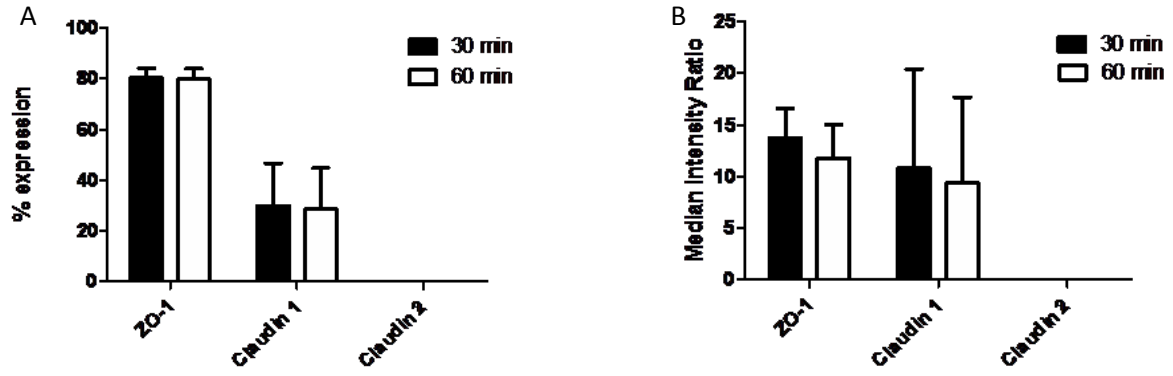


Figure 4.6. ZO-1 and claudin 1 expression on CACO-2 cells. A. Percentage expression of CACO-2 cells expressing ZO-1, claudin 1 or claudin 2 after 30 and 60 minute incubations with 1mM EDTA. B. Median intensity ratio of ZO-1, claudin1 or claudin 2 expression after 30 and 60 minute incubations with 1mM EDTA. Results representative of three independent experiments.

Mucosal biopsies of pouch tissue were next taken from patients undergoing follow-up pouchoscopy. The epithelium was removed following incubation with EDTA. To assess the distribution of the mucosal epithelial cells on the basis of light scatter, the epithelium removed

following EDTA incubation was combined with the lamina propria cells following separate collagenase digestion and staining for pancytokeratin (PCK) was undertaken. A region was drawn to exclude the lymphocytes and include only the PCK positive staining region when back gated to show the distribution of PCK positive cells on the light scatter histogram. This gating strategy was then used to define the distribution of epithelial cells on the light scatter histogram when the epithelium was analysed without the addition of the lamina propria cells. Epithelial cells were identified as staining positive for pancytokeratin (C-11) and a positive region was drawn in reference to the isotype antibody and ZO-1, claudin 1 and claudin 2 were analysed gating on the PCK positive region (Figure 4.7).

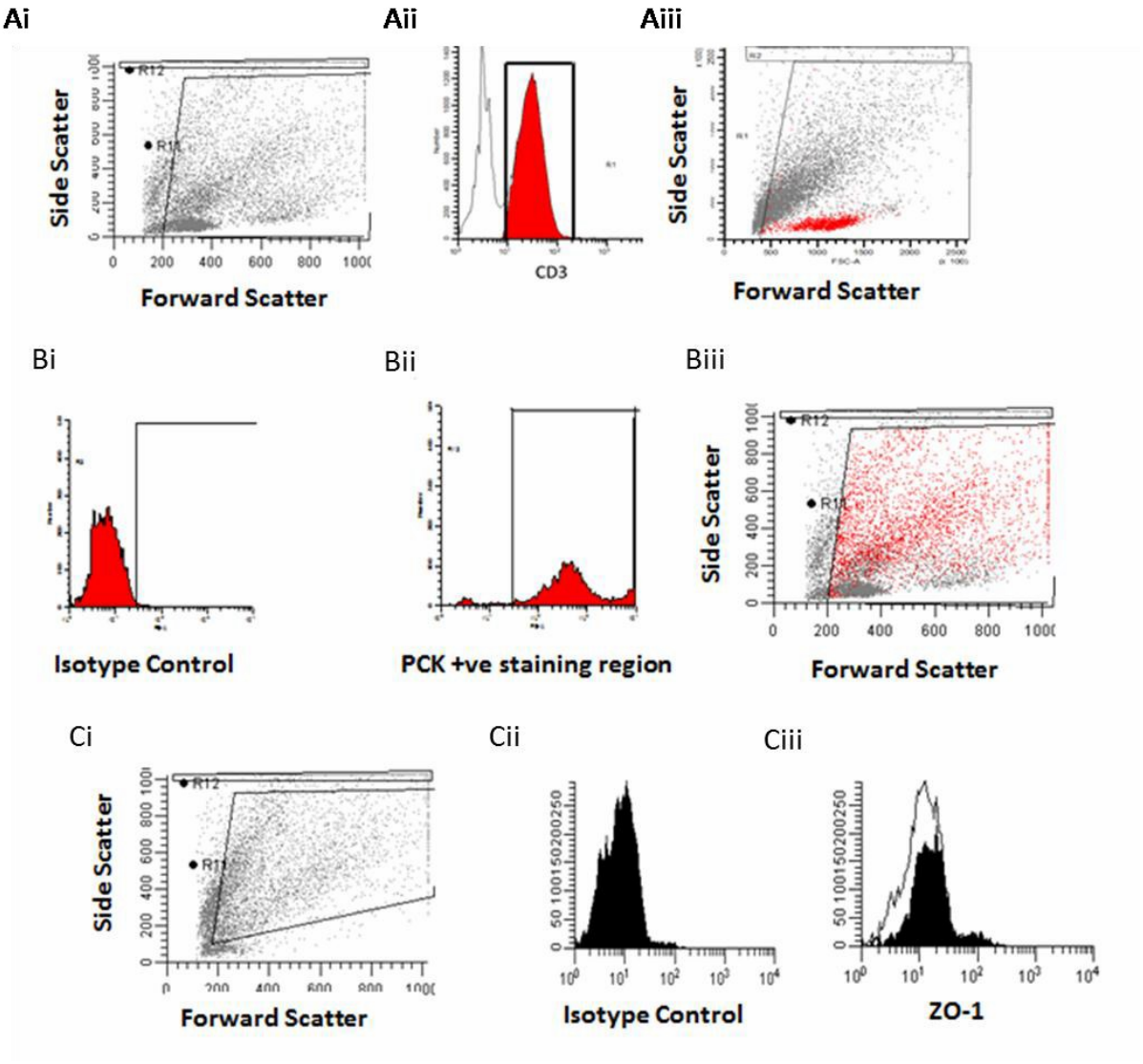


Figure 4.7. Identification of epithelial cells and tight junction protein expression. Ai. Forward scatter versus side scatter plot of mixed epithelial and lamina propria cells within live cell gate. Aii. Single parameter histogram demonstrating region of CD3+ staining cells. Aiii. CD3+ staining back gated on light scatter histogram. Bi and Bii. Histograms demonstrating positive region of PCK positive staining in reference to isotype control antibody. A region was drawn that excludes irrelevant isotype-matched control antibody and nonspecific staining and all events exceeding isotype control staining intensity were regarded as positively labeled epithelial cells. Biii. PCK+ staining back gated on light scatter histogram. Ci. Forward scatter versus light scatter plot of isolated epithelial cells with region drawn to exclude CD3+ contamination (percentage expression of CD3+ cells= 0.4%; n=3). Cii and

Ciii. Single parameter histograms representative of isotype control and epithelial positive staining of tight junction protein antibody (here ZO-1) using super-enhanced D_{max} (SED) normalised subtraction. The shaded areas of histograms represent the proportion of DC expressing each marker after subtraction of staining with an isotype-matched control.

4.7 Tight junction protein expression in the non-inflamed pouch of ulcerative colitis patients and FAP patients

Employing the methods described above, flow cytometric analysis of tight junction expression was next undertaken in non-inflamed pouch tissue from patients with UC and with FAP. There was a significantly greater proportion of epithelial cells expressing claudin 2 in UC patients compared with FAP patients (28 +-7% vs. 6 +-3.9%; p=0.05). There were no significant differences between UC patients without pouchitis and FAP pouch patients, in the proportion of epithelial cells expressing ZO-1 (81 +-2.5% vs. 78 +-2.9%; p=0.4) or claudin 1 (28 +-5.7% vs. 19 +-6.8%; p=0.4). There were no significant differences between UC patients without pouchitis and FAP pouch patients in the median intensity ratio of epithelial cell expression of ZO-1 (5.6 +-0.6 vs. 5.9 +-0.4; p=0.9), claudin 1 (4.2 +-1 vs. 2.6 +-0.9; p=0.4) or claudin 2 (0.8 +-0.2 vs. 0.5 +-0.5; p=0.2) (Figure 4.8).

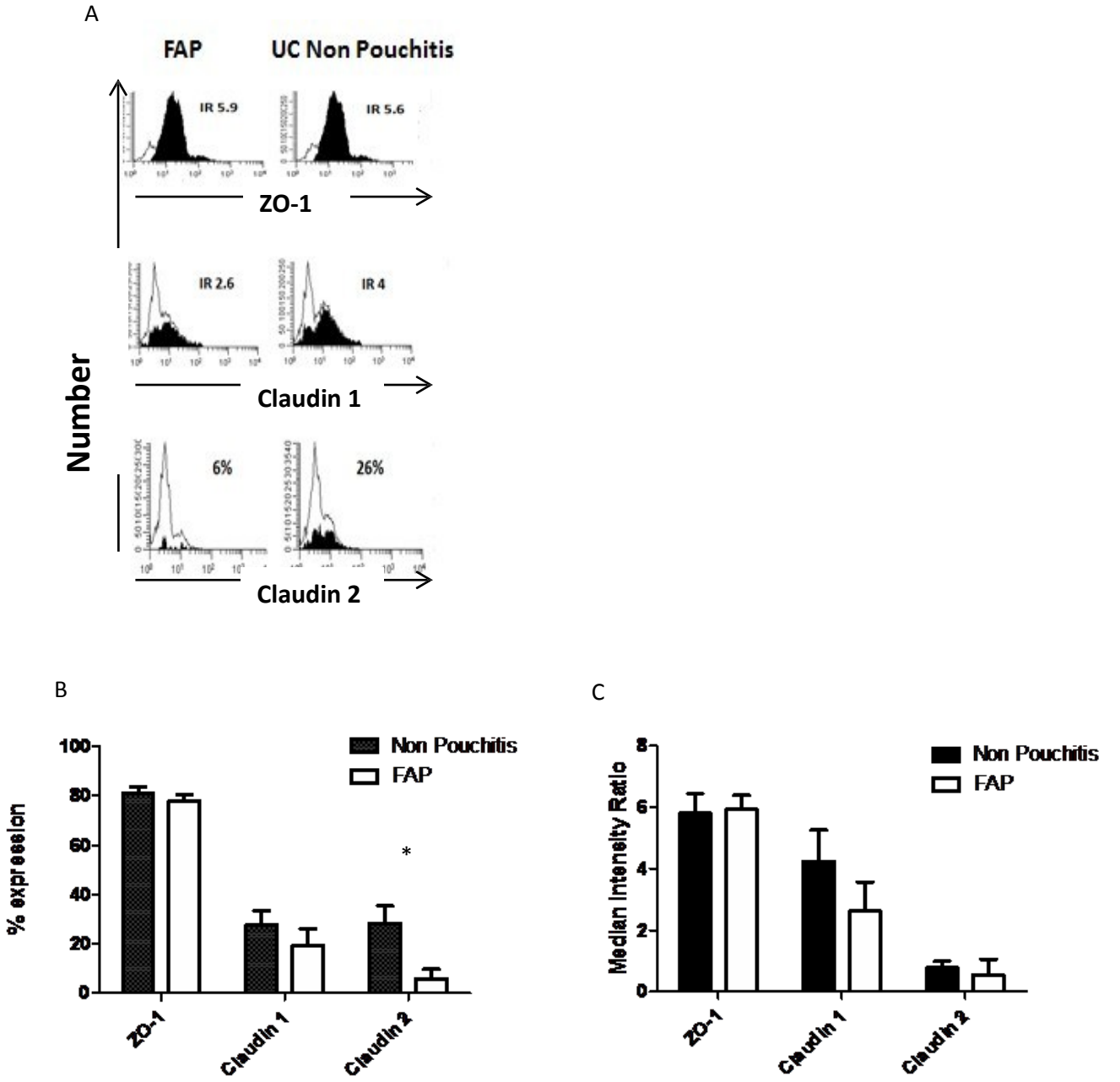


Figure 4.8. Epithelial cell tight junction protein expression in ulcerative colitis patients without pouchitis and FAP patients with ileal pouches. A. Representative histograms of ZO-1, claudin 1 and claudin 2 staining for FAP and non pouchitis. B. Percentage expression of ZO-1, claudin 1 and claudin 2 in epithelial cells of ulcerative colitis patients without pouchitis (n=18) and FAP pouch patients (n=8). C. Median intensity ratio of ZO-1, claudin 1 and claudin 2 in

epithelial cells of ulcerative colitis patients without pouchitis and FAP pouch patients. * denotes p values ≤0.05.

4.8 Tight junction protein expression in the non-inflamed pouch of ulcerative colitis patients and acute and chronic pouchitis patients

The median intensity ratio of ZO-1 (5.8+-0.6 vs. 3.6 +-0.7; p=0.009) and claudin 1 (4.2 +-1 vs. 1.3 +-0.2; p=0.01) (i.e. the level of staining per cell) was significantly lower in pouchitis compared with non-pouchitis patients. However, there were no significant differences in the proportion of epithelial cells expressing ZO-1 (81 +-2.5% vs. 83 +-2.2%; p=0.7) or claudin 1 (28 +-5.7% vs. 42 +-8.9%; p=0.1) between UC patients without pouchitis and those with pouchitis. There were no significant differences in either the proportion of epithelial cells expressing claudin 2 (28 +-7% vs. 31 +-9.5%; p=0.8) or the median intensity ratio of claudin 2 expression between pouchitis and non-pouchitis patients (0.8 +-0.2 vs. 1.5 +-0.5; p=0.2) (Figure 4.9).

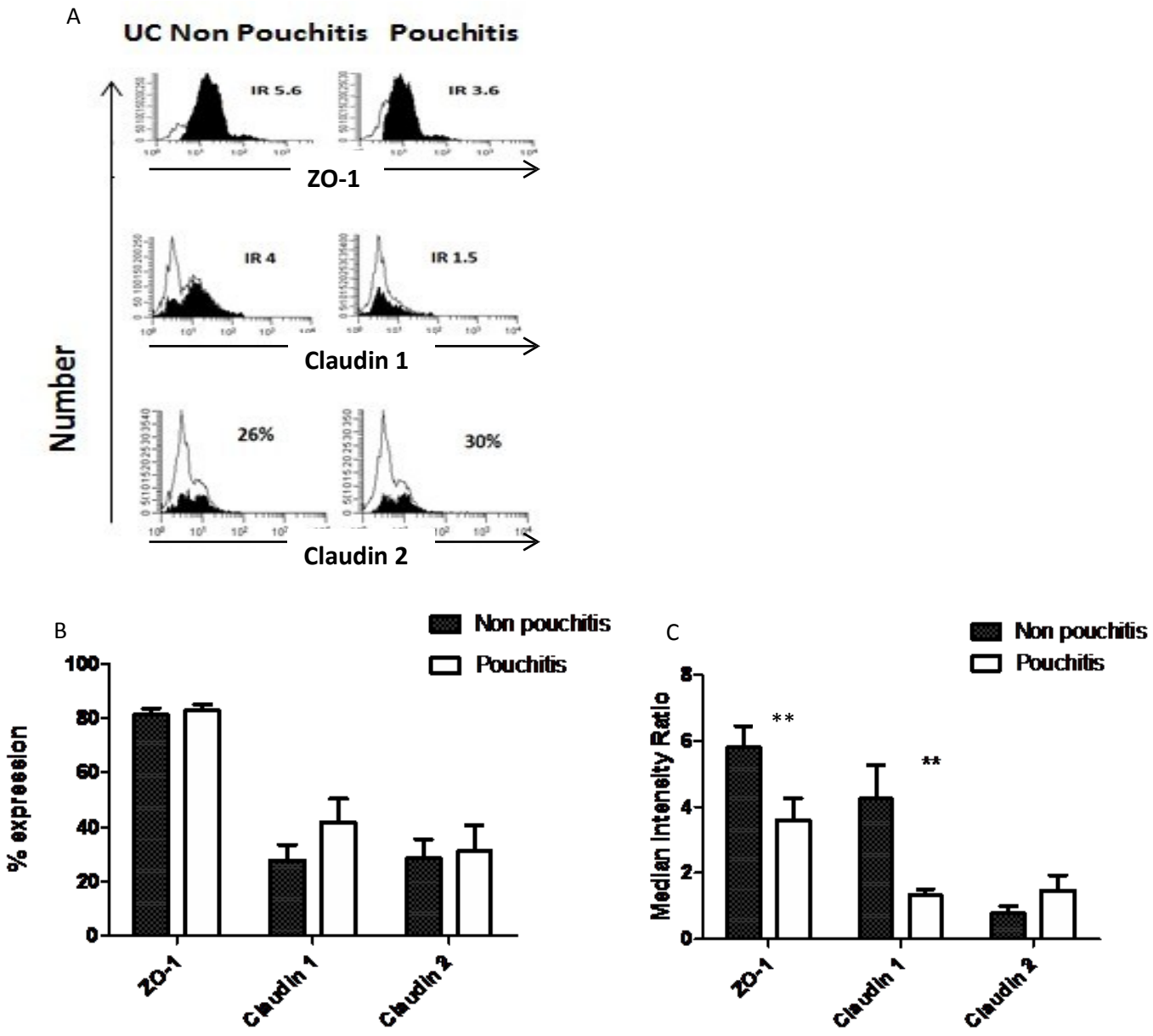


Figure 4.9. Epithelial cell tight junction protein expression in ulcerative colitis patients with and without pouchitis. A. Representative histograms of ZO-1, claudin 1 and claudin 2 staining for non pouchitis and pouchitis. B. Percentage expression of ZO-1, claudin 1 and claudin 2 in epithelial cells of ulcerative colitis patients without pouchitis (n=18) and pouchitis patients (n=13). C. Median intensity ratio of ZO-1, claudin 1 and claudin 2 in epithelial cells of ulcerative colitis patients without pouchitis and pouchitis patients. ** denotes p values ≤0.01

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On further analysis, differences were noted in tight junction expression between UC patients with acute and with chronic pouchitis (as defined in section 1.3i). There were significant differences in the percentage expression of ZO-1 on epithelial cells between patients with acute and chronic pouchitis and those without pouch inflammation ($\chi 2=8.7$, $p=0.01$). The percentage expression of ZO-1 on epithelial cells was significantly higher in acute pouchitis compared with chronic pouchitis (92 +-0.9% vs. 79 +-2%, $p=0.01$) and compared with non-pouchitis patients (81 +-2.5%, $p=0.01$). There were also significant differences in the percentage expression of claudin 2 ($\chi 2=8.95$, $p=0.01$). In acute pouchitis the proportion of epithelial cells expressing claudin 2 (74 +-3%) was significantly greater compared to both patients without pouchitis (28 +-7%, $p=0.006$) and those with chronic pouchitis (12 +-7%, $p=0.02$) (Figure 4.10 A).

There were significant differences in the median intensity ratio of ZO-1 between patients with acute and chronic pouchitis and those without pouch inflammation ($\chi 2=12.5$, $p=0.002$). The median intensity ratio of ZO-1 was significantly lower in chronic pouchitis patients (2.5+-0.3) compared with non-pouchitis patients (5.6 +-2.6, $p=0.007$), greater in acute pouchitis patients compared with chronic pouchitis patients (6 +-1.6 vs. 2.5 +-0.3; $p=0.03$), but not significantly different between acute pouchitis and non-pouchitis patients ($p=0.8$). There were significant differences in the median intensity ratio of claudin 1 expression ($\chi=6.4$, $p=0.04$), but there were no significant differences with Dunn's post-test comparison.

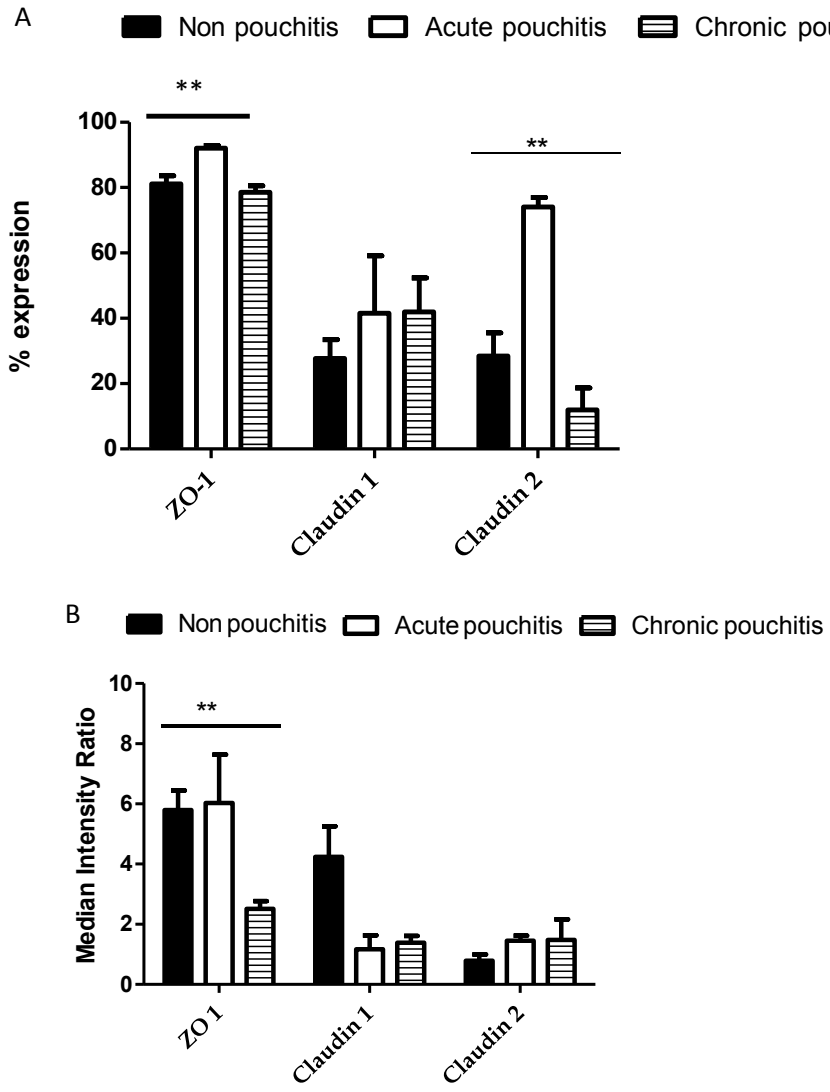


Figure 4.10. Epithelial cell tight junction protein expression in ulcerative colitis patients without pouchitis and with acute and chronic pouchitis A. Percentage expression of ZO-1, claudin 1 and claudin 2 in epithelial cells of UC patients without pouchitis (n=18) and acute (n=4) and chronic pouchitis (n=9) patients. B. Median intensity ratio of ZO-1, claudin 1 and claudin 2 in epithelial cells of UC patients without pouchitis and acute and chronic pouchitis patients. ** denotes p values ≤ 0.01.

4.9 Cytokine expression in the inflamed and non-inflamed pouch of ulcerative colitis patients

It is not clear whether the alterations in tight junction protein expression in IBD are primary or secondary to inflammation. A number of studies show pro-inflammatory cytokines regulate tight junction proteins (Heller et al., 2005, Suzuki et al., 2011, Weber et al., 2010). To assess the cytokine levels in supernatants of mucosal samples from ulcerative colitis patients without pouch inflammation and those with pouchitis, cytokine bead array (multiplex analysis) was undertaken (Chapter 2.2.10).

IL-6 levels in the supernatants of mucosal biopsies from patients with pouchitis (2371+-538pg·ml^{-1}) were significantly higher compared with UC patients without pouchitis (637 +-102pg·ml^{-1}, p=0.02) and this difference was most significant in patients with acute pouchitis (2404 +- 599pg·ml^{-1}) compared to patients without pouchitis (637 +-102pg·ml^{-1}, p=0.009), but showed only a trend towards significance for patients with chronic pouchitis (2360 +-716 pg·ml^{-1}, p=0.08; $\chi 2$=8.8, p=0.03). TNF levels in the supernatants of mucosal biopsies from pouchitis patients compared with UC patients without pouchitis were significantly higher (20 +-5.5pg·ml^{-1} v 10 +-0.5pg·ml^{-1}, p=0.005). The level of TNF was significantly greater in the supernatants of mucosal biopsies from patients with chronic pouchitis compared with ulcerative colitis patients without pouchitis (20 +-7.8 pg·ml^{-1}, p=0.008), but did not reach significance in patients with acute pouchitis (18 +-7.9 pg·ml^{-1}, p=0.06; $\chi 2$=7.9, p=0.02) (Figure 4.11).

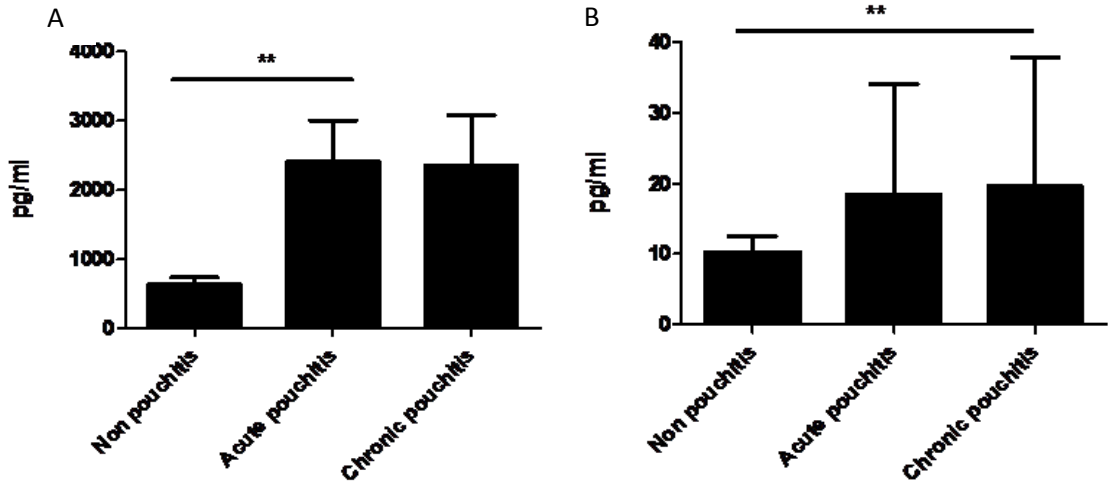


Figure 4.11. IL6 and TNF levels from pouch biopsy supernatants in ulcerative colitis patients without pouchitis and with acute and chronic pouchitis. A. IL6 levels in supernatants of ulcerative colitis patients without pouchitis (n=16) and those with acute (n=4) and chronic pouchitis (n=9). B. TNF levels in supernatants of ulcerative colitis patients without pouchitis (n=16) and those with acute (n=4) and chronic pouchitis (n=9). ** denotes p values ≤0.01.

There were no differences in levels of IL-2 (11.9 +-0.8 vs. 12+-0.5 $pg \cdot ml^{-1}$ vs. 12+-0.6 $pg \cdot ml^{-1}$, $\chi 2$ =1.53 p=0.46), IL-4 (13 +-0.5 $pg \cdot ml^{-1}$ vs. 13.5 +-0.7 vs. 13.7 +-0.9 $pg \cdot ml^{-1}$, $\chi 2$=1.6, p=0.46) or IL-10 (10.6 +-0.7 $pg \cdot ml^{-1}$ vs. 12.3 +-2.7 $pg \cdot ml^{-1}$ vs. 10.7 +-0.7 $pg \cdot ml^{-1}$, $\chi 2$=0.9, p=0.6) in the supernatants of mucosal samples between UC patients without pouch inflammation and those with acute or chronic pouchitis (Figure 4.12).

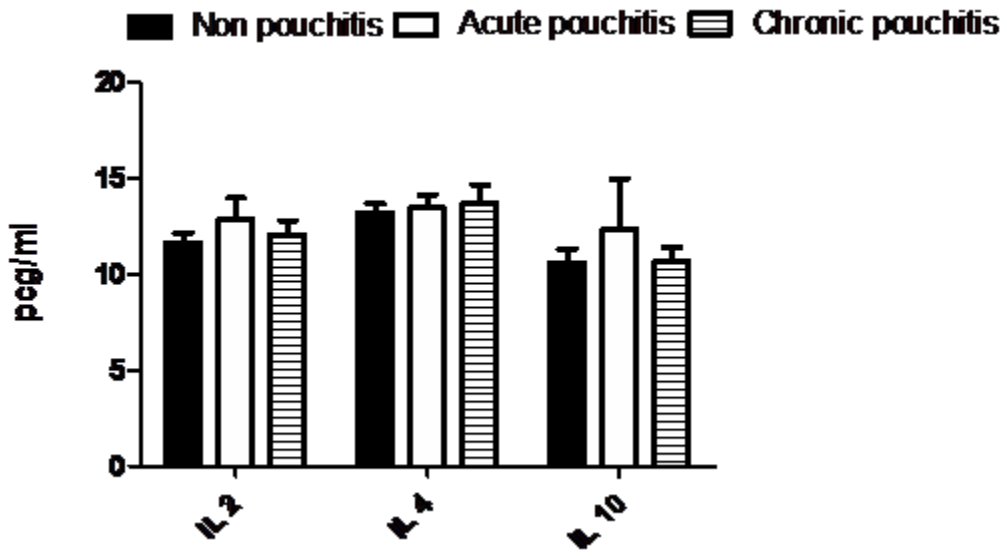


Figure 4.12. Mucosal cytokine levels from pouch biopsy supernatants in ulcerative colitis patients with and without pouchitis. IL-2, IL-4 and IL-10 levels in supernatants of ulcerative colitis patients without pouchitis (n=16) and those with acute (n=4) and chronic pouchitis (n=9).

4.10 Discussion

Significant differences in tight junction protein expression were found between UC patients and FAP patients, and between UC patients without pouch inflammation, those with pouch inflammation and those with acute or chronic pouchitis. There are no previous studies comparing tight junction protein expression between UC and FAP pouch patients. Similarly there are no previous studies assessing differences between acute and chronic pouch inflammation.

These results suggest an increase in claudin 2, the "pore-forming" tight junction protein, in the non-inflamed pouch of UC patients compared with FAP patients. In acute pouchitis the proportion of epithelial cells expressing claudin 2 and ZO-1 was increased whereas in chronic pouchitis there was a reduction in the level of expression of both ZO-1 and claudin 1 expression. Claudin 2 is a "pore-forming" tight junction that reduces trans epithelial resistance and can also directly decrease the "tightening" barrier function of claudin 1 (Turksen, 2004). In the pouch, the distribution of claudin 2 along the crypt-villus axis is not known. However, in the distal ileum and colon, expression is restricted to the crypts (Rahner et al., 2001). An increase in the proportion of epithelial cells expressing claudin 2 could present a mechanism by which UC patients are more susceptible to microbial signalling compared with FAP patients.

The elevation of claudin 2 expression in acute but not in chronic pouchitis compared with the non inflamed pouch of ulcerative colitis patients may suggest that increased expression of the

"pore forming" tight junction claudin 2 is an early event in the development of inflammation. In chronic pouchitis, there was lower expression of ZO-1 and claudin 1 that represents a loss of integral tight junction function and a reduction in the "tightening" effect of claudin 1 expression. Electron micrographs of the epithelium from pouchitis suggested a destruction of the villi and a loosening of cell to cell contacts in the inflamed pouch. This could represent an ongoing defect in epithelial barrier function leading to the persistence of microbial stimulation of the immune response as suggested in chapter 3 by increased TLR 4 and CD40 expression on DC in pouchitis.

Tight junction expression may be secondary to increases in the levels of pro-inflammatory cytokines. The levels of both IL-6 and TNF were elevated in acute pouchitis compared with non pouchitis samples. IL-6 has been demonstrated to be elevated in UC, Crohn's disease and in the inflamed and non-inflamed pouch of UC patients when compared with FAP patients (Hart et al., 2005a, Bernardo et al., 2012b, Leal et al., 2008, Gionchetti et al., 1994). In CACO-2 cells IL-6 increases expression of claudin 2 via trans-signalling by activating the MEK/ERK and PI3K pathways and increased expression of Cdx2 acting at the promoter region of claudin 2 (Suzuki et al., 2011). TNF alters epithelial barrier function by reorganisation of tight junction proteins including ZO-1 and claudin 1 (Bruewer et al., 2003). TNF-α increases Myosin Light Chain Kinase (MLCK) phosphorylation and inhibition of MLCK in TNF-α treated epithelial monolayers can acutely restore barrier function (Zolotarevsky et al., 2002). Furthermore, MLCK-activation promotes IL-13 expression and claudin 2 synthesis (Weber et al., 2010).

The distribution and co-localisation of tight junction proteins are likely to be critical with regard to their functional effects on barrier function in addition to the relative amounts of the different tight junction proteins expressed. Tight junction proteins are found both localised to the tight junction complex as well as within an intracellular pool, with dynamic shifts between these two pools enabling rapid structural and functional responses to stimuli (Shen et al., 2008b). It had been intended to study the localisation of tight junction proteins in inflamed and non inflamed pouches of patients with ulcerative colitis by immunofluorescence microscopy. However, using frozen sections, the epithelial architecture, particularly from patients with severe inflammation was too distorted to allow for any relevant comparisons, although staining for ZO-1, claudin 1 and claudin 2 was demonstrated on inflamed and non-inflamed pouch samples. The disruption of the epithelial architecture in severe pouchitis itself demonstrates the disturbance of the epithelial barrier in pouchitis.

Flow cytometry with labelling of epithelial cells enables a quantitative analysis of tight junction protein expression specifically on epithelial cells. The use of EDTA, a chelating agent that is used to dissociate epithelial cells in confluent monolayers may have altered the level of expression of tight junction proteins. However, the results from both the experiments of PBMC and CACO-2 cells did not demonstrate any significant alteration in the level of tight junction protein expression following incubation with EDTA.

Expression of ZO-1 and Claudin-1 has not been previously demonstrated in human peripheral dendritic cells. JAM-A has been identified on human peripheral blood neutrophils, monocytes,

lymphocytes and erythrocytes (Liu et al., 2000), but only ZO-1 mRNA expression has been previously identified in peripheral blood dendritic cells (Ogasawara et al., 2009b). The finding of ZO-1 and claudin 1 expression on peripheral blood DC may have implications regarding the trafficking of these cells across the endothelium. Further investigation regarding the expression of tight junction proteins on PBMCs and their functional importance in endothelial transmigration in health and disease is necessary.

CACO-2 cells are an established model of the intestinal barrier and were studied as a positive control for the expression of tight junction proteins. ZO-1 and claudin 1, but not claudin 2 were identified on CACO-2 cells. CACO-2 cells in culture undergo a process of differentiation leading to the formation of a monolayer of cells, which express several morphological and functional characteristics of mature enterocytes. Depending on the degree of differentiation and other factors including the number of passages, plating and culture conditions, CACO-2 cells will be characterised by subpopulations with differing morphologies of both enterocytic and colonocytic characteristics (Engle et al., 1998). Subsequently, endogenous expression of claudin 2 on these cells is controversial. Previous studies have suggested that CACO-2 cells do not endogenously express claudin 2 (Amasheh, 2008). Other studies suggest claudin 2 expression in CACO-2 cells may depend on the degree of differentiation of these cells with loss of expression of claudin 2 following differentiation and an absence of claudin 2 expression within between one and five days post confluence (Escaffit et al., 2005).

Altered expression of the tight junction proteins ZO-1, claudin 1 and claudin 2 are likely to be critical to the mechanism of impaired epithelial barrier function in pouch inflammation. Increased claudin 2 expression could be an early event in the development of pouch inflammation and may be key in the aetiology of pouchitis. However, the findings of altered tight junction expression may be secondary to the elevation in inflammatory cytokines that were demonstrated in both acute and chronic pouchitis. In this study analysis of cytokines from FAP patients was not available. Previous studies demonstrate elevated IL-6 levels in non-inflamed pouches of ulcerative colitis patients when compared with FAP patients (Leal et al., 2008). The interaction of altered epithelial barrier function through altered tight junction expression and aberrant immune activation may occur concomitantly creating an amplifying cycle leading to the development of histologically and clinically apparent mucosal inflammation (Clayburgh et al., 2004). To determine the aetiological role of these factors, longitudinal studies of patients prior to disease onset are needed. These are not feasible in Crohn's disease or ulcerative colitis, but the pouch offers a unique opportunity for such investigation.

Chapter 5. Longitudinal assessment of epithelial and immune cell changes following ileostomy closure in patients with ulcerative colitis

5.1 Abstract

Introduction: The temporal inter-relationships between defective barrier function, abnormal immune responses and the microbiota in the development of gut inflammation are unknown. The ileal pouch offers a unique opportunity to study these factors before the onset of disease and map temporal changes between these factors to inform differences between cause and effect. We aimed to assess changes in epithelial tight junction expression, dendritic cell phenotype and mucosal cytokine production over the first year following restorative proctocolectomy (RPC) for UC.

Methods: Mucosal biopsy samples were taken from the same UC patients (5) undergoing RPC, from the ileostomy afferent loop, the pouch pre-ileostomy closure (P0) and the pouch 6 and 12 months post-ileostomy closure. Epithelial cells and lamina propria DC were isolated from biopsy tissue and epithelial cell expression of ZO-1, claudin 1, claudin 2 and DC expression of TLR 2 and 4, CCR9, β7 and CD40 were measured by multicolour flow cytometry. Cytokines were assessed by multiplex analysis of biopsy supernatants.

Results: Epithelial expression of claudin 2 was increased at 6 months. No changes were seen in ZO-1 or claudin 1 expression. DC expressing integrin β7 and CCR9 were increased after ileostomy closure, but no differences in TLR or CD40 expression were seen. There was an increase in IL-6 levels at 12 months after ileostomy closure.

Conclusions: In patients with UC, altered tight junction expression with increased epithelial expression of the "pore-forming" tight junction claudin 2 was an early event after ileostomy closure that was concurrent with alterations in DC homing marker expression and preceded

increased IL-6 levels, as well as increased TLR4 and CD40 activation marker expression in patients with mucosal inflammation of the pouch at twelve months following ileostomy closure.

5.2 Background

The temporal inter- relationships between defective barrier function, abnormal immune responses and the microbiota in the development of gut inflammation are uncertain. Animal models are inconclusive, but suggest a primary defect in epithelial barrier function as an early event in the development of gut inflammation.

In the SAMP murine model, impaired epithelial barrier function most likely secondary to altered tight junction protein expression preceded the development of pathogenic lymphocytic properties and inflammation. The commensal microbiota was not necessary for the development of the epithelial defect in this model (Olson et al., 2006). Increased intestinal permeability in IL-10 knockout mice not only precedes the onset of inflammation but also occurs prior to increased levels of pro-inflammatory cytokines (Arrieta et al., 2008).

Other models demonstrate epithelial barrier dysfunction to occur in parallel with redistribution of DC, elevations in pro-inflammatory cytokines and recruitment of lymphocytes that predispose to the early development of inflammation and amplify immune-mediated inflammation (Silva et al., 2008, Su et al., 2009). Transgenic mice expressing activated MLCK show increased paracellular permeability without histological inflammation with an increase in the absolute numbers of lamina propria CD4+ lymphocytes and a significant redistribution of CD11c+ DC to the superficial lamina propria as well as polarisation to a Th1 cytokine profile (Su et al., 2009). A recent study of a MLCK and RAG2 knockout model demonstrated that barrier

loss in immune-mediated colitis occurs by two temporally and morphologically distinct mechanisms. MLCK dependent tight junction permeability increases occur early and promote disease initiation. Later, as disease progresses, apoptosis and gross ulceration of the epithelium causes tight junction-independent barrier loss (Su et al., 2013).

The onset of the first episode of pouchitis is most common within twelve months of ileostomy closure (Abdelrazeq et al., 2008). Following closure of the ileostomy, the ileal mucosa in the pouch is exposed to faeces containing bacterial concentrations a million or more times greater than in the normal terminal ileum (Nicholls et al., 1981, Santavirta et al., 1991) and the overall composition of the pouch microbiota shows similarities with the colonic microbiota (Falk et al., 2007, Kohyama et al., 2009). Mucosal morphological changes occur within six weeks of ileostomy closure and remain stable at six months post ileostomy closure (Apel et al., 1994). Groups of patients without pouchitis, or with acute or chronic pouchitis, may be differentiated on the basis of mucosal histological changes at six months following ileostomy closure (Stallmach et al., 1999, Setti Carraro et al., 1994). Early responses of epithelial barrier function and immune responses to the changes in the microbiota are therefore likely to be critical in determining the predisposition to and development of pouch inflammation.

The two preceding chapters have demonstrated aberrant DC phenotype and tight junction expression in UC patients with and without pouchitis. It is not clear whether these are primary factors in the aetiology of pouch inflammation or secondary to inflammation. There are no data regarding the early changes in the innate immune system or tight junction protein expression

that may predispose to pouchitis. The ileal pouch offers a unique opportunity to study the development of inflammation in the gut before disease onset and this opportunity may also afford new insights into the pathogenesis of gut inflammation in other inflammatory bowel diseases.

5.3 Aims

- To assess changes in tight junction expression from the ileum and pouch of ulcerative colitis patients before and twelve months after closure of ileostomy
- To assess changes in dendritic cell phenotype from the ileum and pouch of ulcerative colitis patients before and twelve months after ileostomy closure
- To assess cytokine profiles in the ileum and pouch of ulcerative colitis patients before and twelve months after ileostomy closure

5.4 Patient characteristics

Ten patients were initially recruited to participate in the study, but only five completed twelve months follow up. All patients had a previous history of UC diagnosed clinically, endoscopically and histologically. The median age of the five patients was 37 years (range 29-40 years). All of the patients had a J pouch and underwent a three stage procedure. None of the patients had extra-intestinal manifestations or primary sclerosing cholangitis (PSC). One patient developed pouchitis at twelve months. Two further patients developed mild endoscopic and histological inflammation but were asymptomatic (Table 5.1)

Table 5.1 Patient characteristics

Patient No.	Duration UC (years)	Indication for colectomy	EIM/PSC	Backwash Ileitis	PDAI at 6 months	PDAI histology score at 6 months	PDAI at 12 months	PDAI histology score at 12 months
1	6	ASUC	N	N	4	2	12	4
2	10	CRSUC	N	N	2	2	2	2
3	5	ASUC	N	N/K	3	2	7*	4
4	14	ASUC	N	N	3	2	6*	3
5	3	ASUC	N	N	2	2	2	2

ASUC- acute severe UC. CRSUC- chronic refractory severe UC. N/K- not known, original histology specimen not available. *No clinical symptoms of pouchitis.

For all subsequent figures patient 1 at twelve months follow up (PDAI 12) will be marked with the colour red. Patients 3 and 4 at twelve months with histological and endoscopic feature of pouchitis but no clinical symptoms will be marked with the colour orange.

5.5 Altered epithelial tight junction expression in the pouch of ulcerative colitis patients following restorative proctocolectomy

Mucosal biopsies were taken from the ileum and diverted ileal pouch prior to ileostomy closure and during flexible pouchoscopy at six and twelve months following. Biopsies were processed and analysed as described (Chapter 2.2.5- 2.2.10 and Chapter 4.6).

ZO-1, claudin 1 and claudin 2 expression were assessed over the twelve months following ileostomy closure. There were no significant differences in the proportion of epithelial cells expressing ZO-1 at six months (89 +-2.4%, $\chi 2=1.2$, $p=0.7$) or at twelve months (90 +-1.9%, $\chi 2=0.4$, $p=0.95$) following ileostomy closure compared with the ileum (88 +-3.5%) or diverted pouch (90 +-3.6%) prior to closure. There were no significant differences in the median intensity ratio of ZO-1 at six months (8.4 +-1.3, $\chi 2=2.1$, $p=0.4$) or at twelve months (4.2 +-0.6, $\chi 2=0.4$, $p=0.8$) following ileostomy closure compared with the ileum (6.8 +-2.5) or diverted pouch (5.1 +-1.3) prior to ileostomy closure (Figure 5.1).

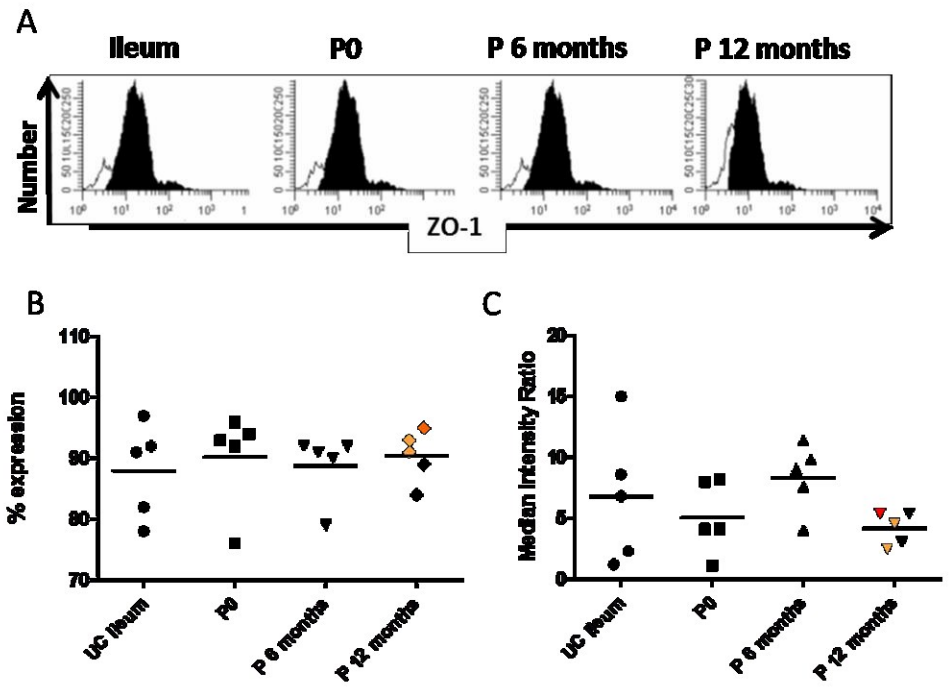


Figure 5.1 Epithelial cell expression of ZO-1 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure. A. Representative single parameter histograms of epithelial cell ZO-1 staining. B. Percentage expression and C. Median intensity ratio of epithelial ZO-1 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure (n=5).

There were no significant differences in the proportion of epithelial cells expressing claudin 1 at six months (38 +-11%, $\chi 2=0.3$, p=0.9) or at twelve months (58 +-10%, $\chi 2=3.6$, p=0.2) following ileostomy closure compared with the ileum (25 +-14%) or diverted pouch (35 +-15%) prior to ileostomy closure. There were no significant differences in the median intensity ratio of claudin 1 at six months (3.2 +-1.0, $\chi 2=2.4$, p=0.3) or at twelve months (2.3 +-0.6, $\chi 2=1.2$, p=0.6)

following ileostomy closure compared with the ileum (1.3 +-0.6) or diverted pouch (1.7 +-0.8) prior to closure (Figure 5.2).

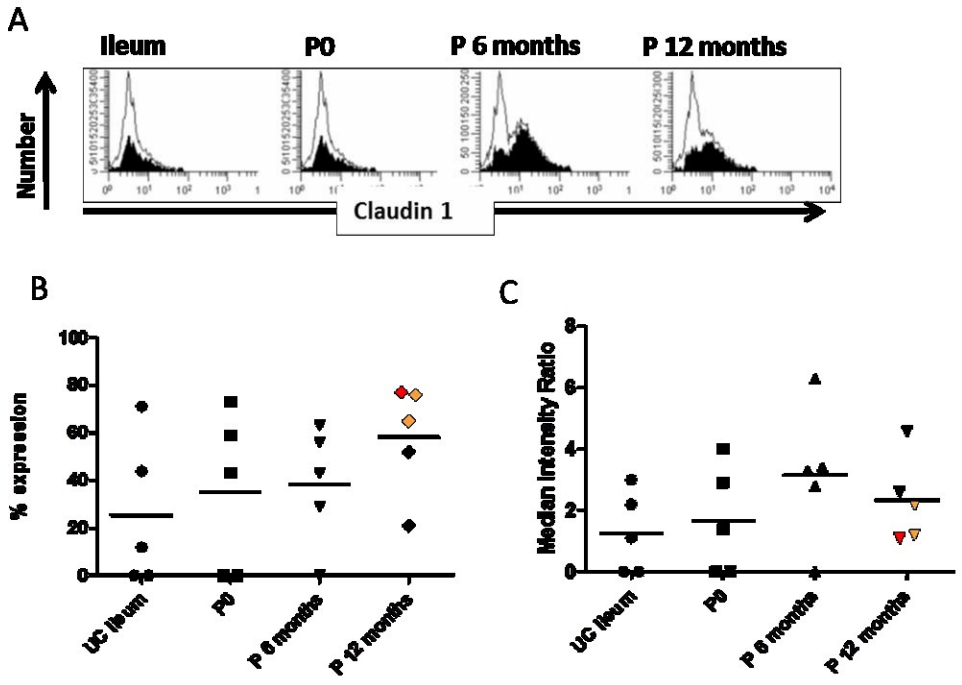


Figure 5.2 Epithelial cell expression of claudin 1 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure. A. Representative single parameter histograms of epithelial cell claudin 1 staining. B. Percentage expression and C. Median intensity ratio of epithelial claudin 1 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure (n=5).

There was a significant increase in the proportion of epithelial cells expressing claudin 2 at six months (48+-15%, $\chi 2=6.5$, $p=0.02$) but this was not significant at twelve months (46 +-19%, $\chi 2=1.7$, $p=0.4$) following ileostomy closure compared with the ileum (8.6 +-5%) or diverted pouch (15 +-10%) prior to ileostomy closure. There were no significant differences in the median intensity ratio of claudin 2 at six months (1.6 +-0.5, $\chi 2=2.8$, $p=0.12$) or at twelve months

(0.9 +-0.4, χ2=1.7, p=0.4) following ileostomy closure compared with the ileum (0.6 +-0.4) or diverted pouch (1.4 +-0.7) prior to ileostomy closure.

The proportion of epithelial cells expressing claudin 2 was elevated in four of the five patients at six months following ileostomy closure. Three of these patients had endoscopic and histological inflammation of the pouch at twelve months. One patient had an increased proportion of epithelial cells expressing claudin 2 at six months and did not develop pouch inflammation, but had a low proportion of epithelial cells expressing claudin 2 at 12 months (Figure 5.3).

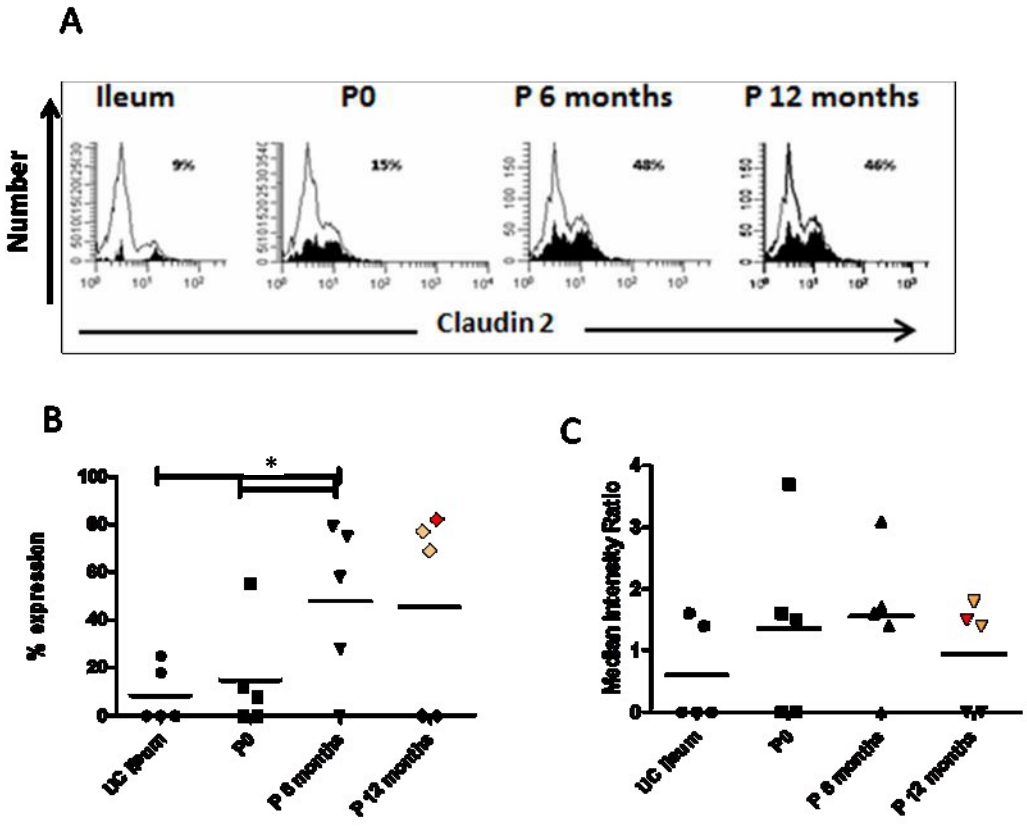


Figure 5.3 Epithelial cell expression of claudin 2 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure. A. Representative single parameter histograms of epithelial cell claudin 2 staining. B. Percentage expression and C. Median intensity ratio of epithelial claudin 2 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure (n=5). * denotes p values ≤0.05.

5.6 Altered dendritic cell phenotype in the pouch of ulcerative colitis following restorative proctocolectomy

There were no significant differences in the proportion of DC expressing TLR 4 at six months (42 +-3.2%, $\chi 2=5.2$, p=0.09) or at twelve months (47 +-7.0%, $\chi 2=3.6$, p=0.2) following ileostomy closure compared with the ileum (34 +-9.6%) or diverted pouch (18 +-6.4%) prior to ileostomy closure. There was no significant change in the proportion of DC expressing TLR 2 at 6 months (28 +-5.0%, $\chi 2=1.2$, p=0.7) or at twelve months (33 +-11%, $\chi 2=1.4$, p=0.5) following ileostomy closure compared with the ileum (24 +-7.4%) or diverted pouch (21 +-10%) prior to ileostomy closure (Figure 5.4).

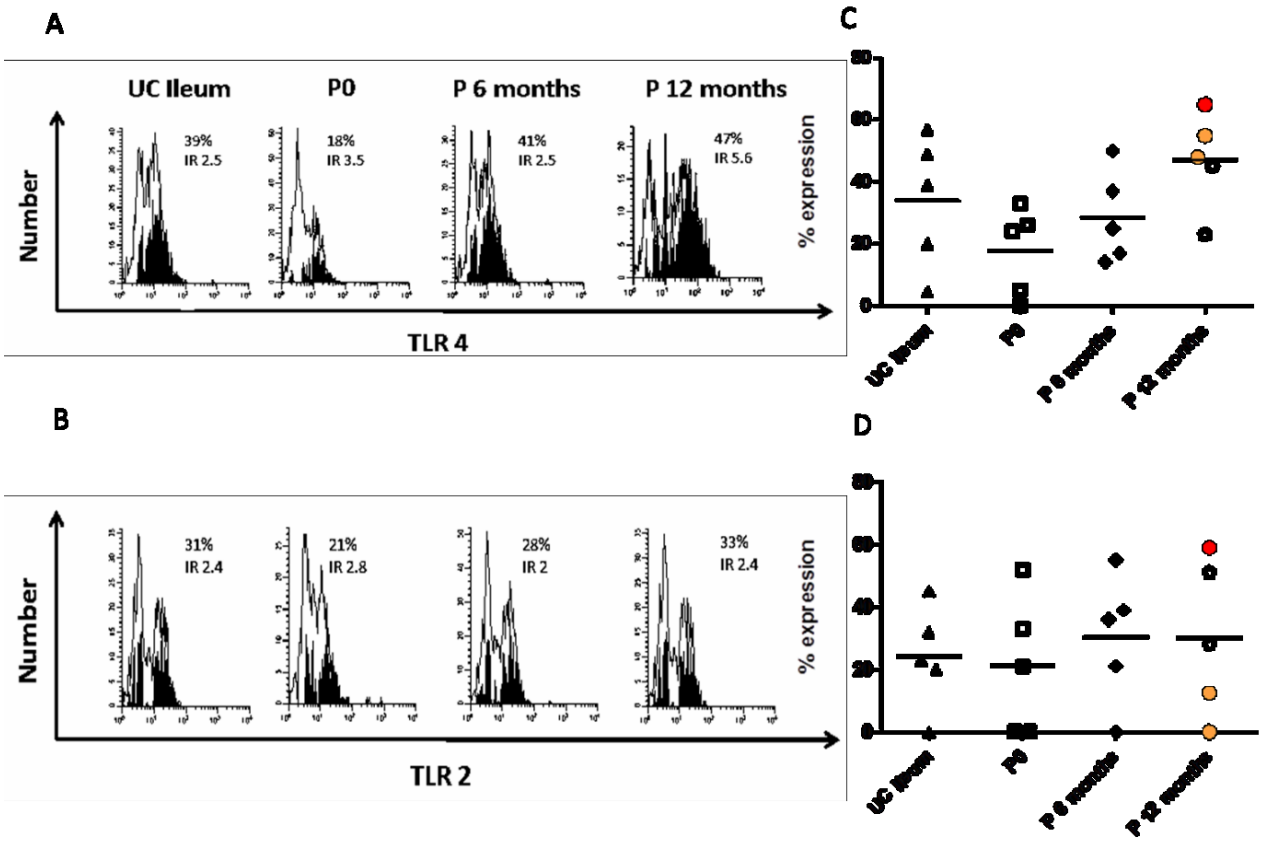


Figure 5.4 TLR 4 and TLR 2 expression on lamia propria dendritic cells in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure. A and B. One parameter histograms representative of TLR 4 and TLR 2 staining cells and C and D. Percentage expression of TLR 4 and TLR 2 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure (n=5).

There was a significant increase in the proportion of DC expressing β7 at six months (17 +-3.1%, $\chi 2=6.6$, $p=0.02$) and at twelve months (37 +-2.2%, $\chi 2=7.6$, $p \leq 0.01$) following ileostomy closure compared with the ileum (8.4 +-4.2%) or diverted pouch (3.4 +-2.0%) prior to ileostomy closure. There was a significant increase in the proportion of DC expressing CCR9 at six months (46 +-

2.3%, χ2=9.6, p≤0.001) but this was not significant at twelve months (50 +-6.5%, χ2=4.5, p=0.1) following ileostomy closure compared with the ileum (41 +-4.1%) or diverted pouch (26+-6.5%) prior to ileostomy closure (Figure 5.5).

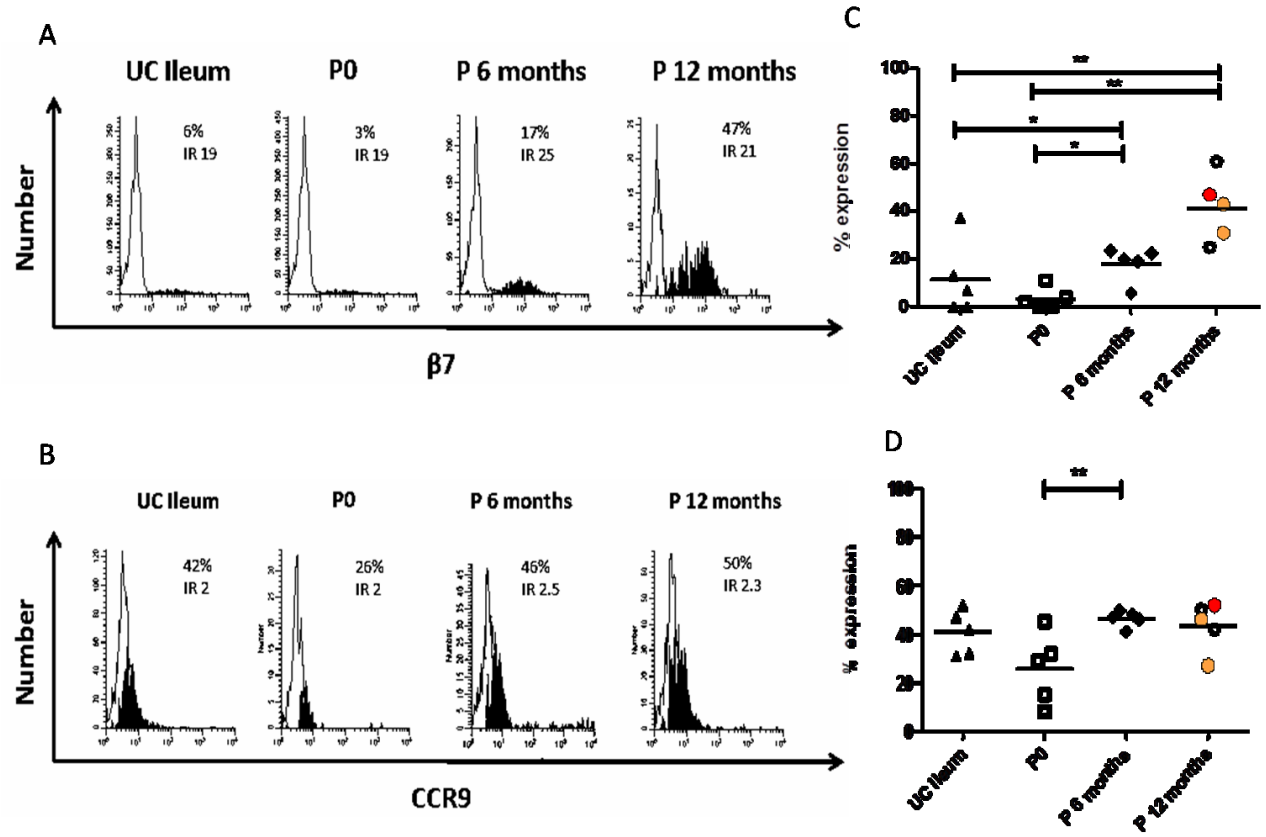


Figure 5.5 β7 and CCR9 expression on lamina propria dendritic cells in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure. A and B. One parameter histograms representative of β7 and CCR9 staining cells and C and D. Percentage expression of β7 and CCR9 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure (n=5). * and ** denote p values ≤0.05 and 0.01 respectively.

There were no significant differences in the proportion of DC expressing CD40 at six months (59 +-3.3%, $\chi 2=2.8$, $p=0.4$) or at twelve months (75 +-6.6%, $\chi 2=2.8$, $p=0.4$) following ileostomy closure compared with the ileum (49 +-7.6%) or diverted pouch (54 +-3.3%) prior to ileostomy closure (Figure 5.6).

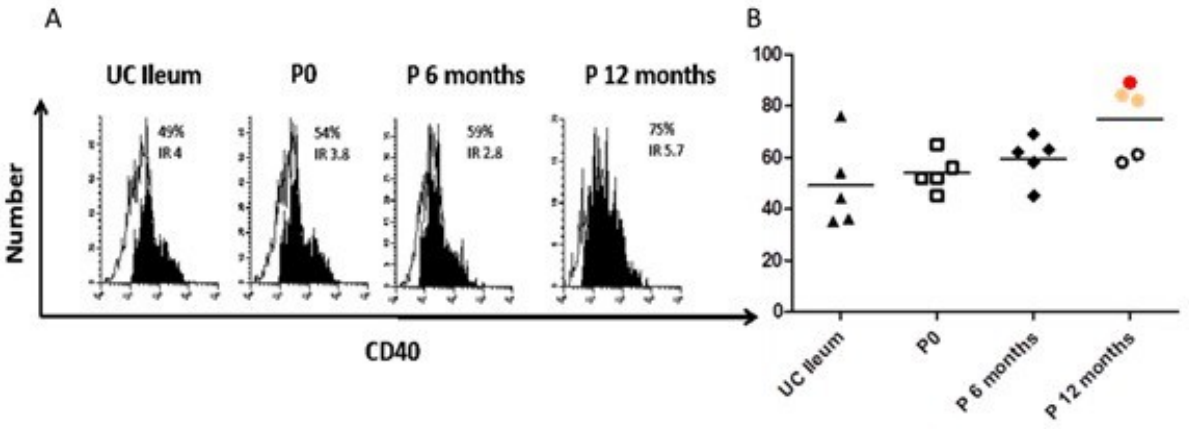


Figure 5.6 CD40 expression on lamina propria dendritic cells in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure. A. One parameter histograms representative of CD40 staining cells and B. Percentage expression of CD40 in the ileum and diverted pouch of ulcerative colitis patients prior to ileostomy closure and at six and twelve months following ileostomy closure (n=5).

5.7 Increased proinflammatory cytokines in the pouch of ulcerative colitis patients following restorative proctocolectomy

There were no significant changes in the levels of IL-2 ($\chi 2=1$, $p=0.7$), IL-4 ($\chi 2=2$, $p=0.4$), IL-10 ($\chi 2=2$ $p=0.4$) or TNF ($\chi 2=4$, $p=0.1$) in the supernatants of mucosal biopsy samples over the twelve months following ileostomy closure. There was a significant increase in IL-6 levels at twelve months following ileostomy closure ($\chi 2=6.6$, $p=0.04$) (Figure 5.7).

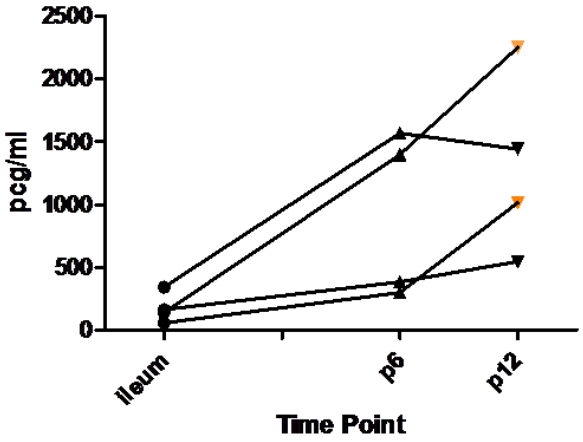


Figure 5.7. IL-6 levels in biopsy supernatant of ulcerative colitis patients before and at six and twelve months following ileostomy closure. IL-6 levels in supernatants of mucosal biopsies from the ileum prior to ileostomy closure and from the pouch at six and twelve months following ileostomy closure (n=4)

5.8 Discussion

This is the first study to longitudinally assess DC characteristics and epithelial cell tight junction protein expression following ileostomy closure in UC patients. The increased expression of claudin 2 in the pouch of UC patients at six months following ileostomy closure suggests this is an early event in the development of inflammation. The proportion of epithelial cells expressing claudin 2 was increased at six months in four of the five patients studied. There was no evidence of histological inflammation in any of these patients at this time point. Three of these patients went on to develop histological inflammation at 12 months post ileostomy closure with one meeting criteria for a diagnosis of pouchitis. This could present a mechanism for

barrier dysfunction by which UC patients are more susceptible to immune activation by the more “colon-like” microbiota following ileostomy closure.

Animal models demonstrate epithelial barrier dysfunction to occur in parallel with redistribution of DC, elevations in pro-inflammatory cytokines and recruitment of lymphocytes that predispose to the early development of inflammation and amplify immune-mediated inflammation (Silva et al., 2008, Su et al., 2009). Similarly here, increased claudin 2 expression was concurrent with alterations in DC homing marker expression and preceded increased IL-6 levels, as well as increased TLR4 and CD40 activation marker expression in the patients with inflammation of the pouch at 12 months.

Changes in DC homing markers in the pouch of UC patients also represent an aberrant response to the more colonic microenvironment following RPC. Following ileostomy closure there was an increase in the proportion of DC expressing β7 that might be expected with the changes towards a more colonic microenvironment. However, CCR9+ DC were also increased with a more “small bowel” profile that may be an aberrant response to the altered microenvironment.

The levels of IL-6 increased in the biopsy supernatants of pouch samples following ileostomy closure. As mentioned previously IL-6 levels are elevated not only in the inflamed, but also the non-inflamed pouch of UC patients compared with FAP patients (Gionchetti et al., 1994, Leal et al., 2008). IL-6 is produced by many cell types, but the main sources in the intestinal lamina propria are dendritic cells and macrophages. IL-6 is a key signal in the transition from the initial

innate immune response to infection to a more sustained, adaptive immune response (Naugler and Karin, 2008). IL-6 also synergises with intestinal DC and retinoic acid to induce gut specific IgA secreting B cells. The increase in IL-6 following ileostomy closure may therefore impact on epithelial expression of tight junction proteins (Chapter 4) and the type of immune responses following DC activation of lymphocytes.

There are several limitations to this study. The number of patients studied is small, reflecting the difficulties of such a longitudinal study in clinical practice. Furthermore, the addition of FAP patients would aid the differentiation of responses following ileostomy closure that are specific to UC patients. Correlation with changes in the microbiota would also be important in determining the response to changes in the microbiota as well as analysis of DC function.

Nonetheless, this is the first truly longitudinal study of immune characteristics and epithelial tight junction protein expression following ileostomy closure and re-introduction of the faecal stream. Increased epithelial cell expression of claudin 2 and increased DC gut homing marker expression are early changes following ileostomy closure and the introduction of the faecal stream. It may not be possible to resolve the “chicken and egg” dilemma as to the primary aetiological factor in the development of intestinal inflammation.

However, this study demonstrates the potential utility of the ileal pouch as a human model that can enhance our understanding of the inter-relationships of key factors in the aetiology of inflammatory bowel diseases. Studies of these immunological factors following modification of

the microbiota in the context of pouch inflammation may further enhance our understanding of the inter-relationships between the microbiota and the gut immune responses.

Chapter 6. A pilot study of faecal microbiota transplantation for chronic pouchitis

6.1 Abstract

Introduction: Faecal microbiota transplantation (FMT) is effective in the treatment of recurrent *Clostridium difficile* infection, where resolution of disease is tightly linked to post-FMT changes in microbiota diversity and composition. Effects of FMT in inflammatory bowel diseases (IBD) remain unclear. We undertook a pilot study to assess the clinical outcome in patients with chronic pouchitis after FMT.

Methods: Eight patients with chronic pouchitis (with a current PDAI ≥7) were treated with FMT via nasogastric administration. Clinical activity and faecal coliform antibiotic sensitivities were assessed before and four weeks following FMT.

Results: None of the patients achieved clinical remission and no patients achieved clinical response with a PDAI ≤7 at four weeks post FMT. There were no significant changes in pouch frequency or Cleveland global quality of life score. Four patients had coliform resistant to ciprofloxacin on faecal analysis prior to FMT. Two of these patients demonstrated coliforms that had regained sensitivity to ciprofloxacin four weeks following faecal transplantation.

Conclusions: FMT appears to be safe and acceptable to patients with chronic pouchitis. However, in this study a single nasogastric administration of healthy donor stool was not clinically effective. Further studies of FMT for chronic pouchitis and other inflammatory bowel diseases will need to consider alternative protocols.

6.2 Background

Antibiotics are the mainstay of treatment for pouchitis. The majority of patients will respond to a course of a single agent or combination of antibiotics and remain in remission (Hurst et al., 1996). Patients who fail to respond to a single antibiotic, or have early relapse, may respond to combination therapy with two antibiotics (Abdelrazeq et al., 2005, Gionchetti et al., 1999, Mimura et al., 2002, Shen et al., 2007). Around 5-19% (Madiba and Bartolo, 2001, Mowschenson et al., 2000) will develop chronic pouchitis.

A proportion of patients will go on to suffer from frequent episodes which remain responsive to antibiotics, others will require continuous antibiotic or probiotic treatment to maintain a remission (Mimura et al., 2004) and a proportion of patients will not respond to antibiotics. These patients are difficult to manage and few evidence based treatments are available (Pardi and Sandborn, 2006). Long term treatment with antibiotics may lead to the development of antibiotic resistance and extended spectrum beta-lactamase-producing (ESBL) bacteria (McLaughlin et al., 2010a) rendering patients resistant to antibiotic therapy (McLaughlin et al., 2009b).

Long-term follow-up studies demonstrate that pouchitis accounts for 10% of pouch failures (Tulchinsky et al., 2003). The only proven treatment for these individuals is conversion to a permanent ileostomy which may hold significant physical and psychological morbidity. New therapies that target the pouch microbiota and alter immune responses are needed. Probiotics

have shown some promise in the treatment of pouchitis (Gionchetti et al., 2000a, Mimura et al., 2004, Gionchetti et al., 2003, Gionchetti et al., 2007) and studies have demonstrated their immunoregulatory effects in UC patients who have undergone restorative proctocolectomy (Lammers et al., 2005b, Pronio et al., 2008). However, results with probiotics in clinical practice are less convincing (Shen et al., 2005a). Other strategies to alter the composition of the pouch microbiota are needed.

An alternative approach is transplantation of the entire "organ" of the gut microbiota from a healthy donor. Faecal microbiota transplantation (FMT) is effective in the treatment of recurrent *Clostridium difficile* infection, where resolution of disease is tightly linked to post-FMT changes in microbiota diversity and composition (van Nood et al., 2013). Case reports and small case series have suggested positive clinical outcomes following FMT in IBD (Borody et al., 2003, Grehan et al., 2010, Borody et al., 1989, Bennet and Brinkman, 1989). However, recent studies of FMT in UC did not demonstrate clinical efficacy and suggest variable efficiency of colonisation by the donors' microbiota (Angelberger et al., 2013, Kump et al., 2013).

6.3 Aims

- To conduct a prospective pilot study of faecal microbiota transplantation (FMT) for chronic refractory pouchitis.

6.4 Primary end point

The number of patients in clinical remission (Clinical PDAI 0 or Total PDAI <4) four weeks after treatment with faecal microbiota transplantation.

Secondary end points

- The number of patients to achieve clinical response (reduction in PDAI score ≥3 points (Abdelrazeq et al., 2005)) four weeks after treatment with faecal microbiota transplantation
- The number of patients demonstrating changes in pouch faecal bacterial sensitivities following faecal microbiota transplantation

6.5 FMT Protocol

To be included in the study UC patients had an ileal pouch with chronic pouchitis (Chapter 1.1ii), diagnosed clinically, endoscopically and histologically) with a current pouch disease activity index (PDAI) ≥7. Patients were excluded if they had used antibiotics, probiotics, non-steroidal medications, steroids or immunosuppressants within 2 weeks of study participation. Stool samples were taken prior to study entry to exclude *Cryptosporidium*, *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Campylobacter jejuni*, *C. difficile* toxin, helminths, ova and parasites.

Healthy donors were nominated by the patients receiving FMT and included relatives, partners or an anonymous previously screened donor. Healthy donors were excluded if they had a history of gastrointestinal illness, inflammatory bowel disease, bowel cancer, antibiotic or probiotic use within the preceding six months, or hospitalisation within three months of study participation. Screening of healthy donors included blood tests for full blood count, renal and liver function, serology for hepatitis A, B, C, E, CMV, EBV, HTLV I/II, HIV and *Treponema Pallidum*. Three stool samples were taken to exclude Cryptosporidium, *Salmonella* spp., *Shigella* spp., *Escherichia coli*, *Campylobacter jejuni*, *C. difficile* toxin, helminths, ova and parasites.

Donor stool was prepared as previously described (27). Stool donors were asked to provide a stool sample less than six hours prior to faecal transplantation. Thirty grams of stool was homogenised in 50ml of 0.9% saline and filtered through a sterile gauze to produce a faecal-saline solution. The night before the procedure the recipient was treated with proton pump inhibitor to provide a favourable gastric pH. A nasogastric tube was inserted and the position confirmed according to local protocols. Thirty millilitres of the faecal-saline solution was administered via the nasogastric tube which was then flushed with 50ml of normal saline solution.

A flexible pouchoscopy was performed and biopsy and stool samples were collected prior to FMT and at four weeks following transplantation. PDAI and Cleveland Global Quality of Life Scores (CGQoL) were recorded at each time point.

6.6 Patient characteristics

Ten patients were recruited to the study. One withdrew prior to faecal transplantation. Another patient was diagnosed with adenocarcinoma of the rectal cuff at the screening pouchoscopy and so did not proceed to faecal transplantation. Therefore eight patients were studied of whom five were female. The median age was 46 years (range 24-63 years). The median number of years since RPC was 10 years (range 4-22 years) (Table 6.1).

Table 6.1. Patient characteristics

Patient	Sex	Time since RPC (years)	Pouch type	Pre-pouch ileitis	Baseline pouch frequency
1	F	6	J	Yes	24
2	F	6	J	Yes	20
3	F	10	J	Yes	11
4	F	22	W	Yes	15
5	M	16	J	Yes	9
6	M	22	W	Yes	13
7	M	4	J	Yes	15
8	F	4	J	Yes	20

6.7 Clinical outcomes following FMT

None of the patients achieved clinical remission following FMT. Two patients (patients 2 and 3) achieved a reduction of total PDAI score >3 points at 4 weeks post FMT. However, both patients had a total PDAI score >7 (PDAI 9 and 10 respectively) at 4 weeks post FMT. There were no significant differences in clinical PDAI (4, range 3-5 vs. 4, range 3-5; p=0.3) (Figure 6.1a), endoscopy (5, range 3-6 vs. 4.5 range, 3-6; p=0.6) (Figure 6.1b) or histology sub-scores (2.5, range 2-4 vs. 2, range 2-4; p=0.4) (Figure 6.1c) before and four weeks after FMT.

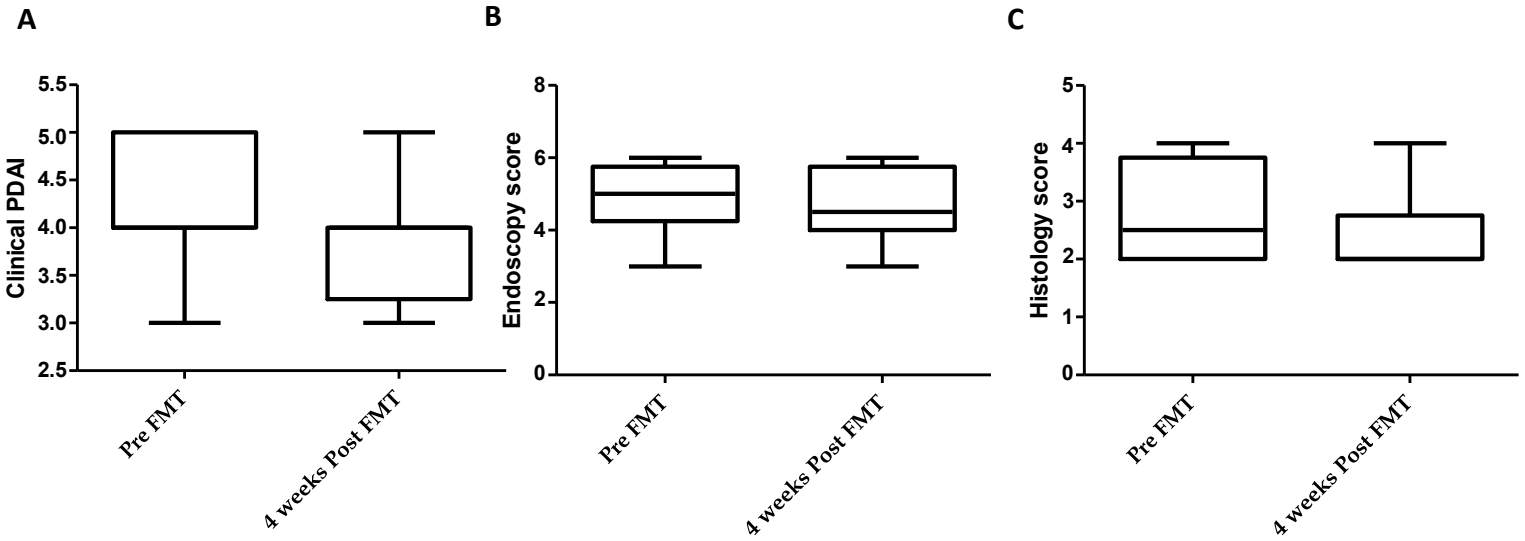


Figure 6.1 A. Median Clinical PDAI, B. Endoscopy subscore, C. Histology subscore before and 4 weeks following FMT (n=8).

There were no significant differences in pouch frequency (15, range 9-24 vs. 15, range 8-25, p=1) (Figure 6.2), Total PDAI (11.5, range 10-14 vs. 10.5 range 9-14; p=0.2) (Figure 6.3a) or Cleveland Global Quality of Health Score (0.41, range 0.2-0.7 vs. 0.47, range 0.2-0.7; p=0.8) (Figure 6.3b) before and four weeks after faecal transplantation.

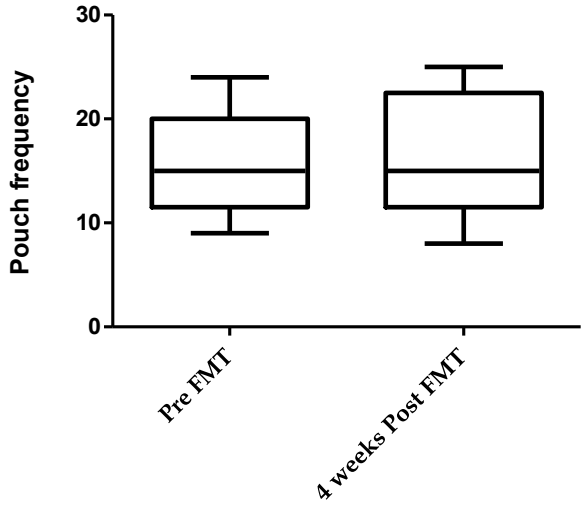


Figure 6.2. Median pouch frequency before and 4 weeks after FMT (n=8). No change in pouch frequency was noted 4 weeks after FMT.

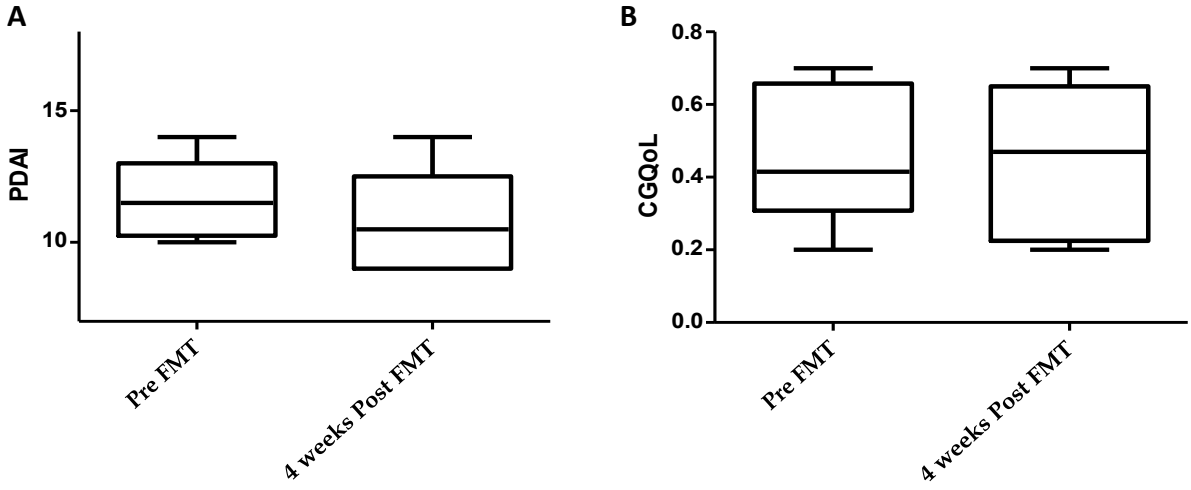


Figure 6.3 A. Median total PDAI and B. Median Cleveland Global Quality of Life (CGQoL) score before and 4 weeks after FMT (n=8).

6.8 Changes in coliform sensitivities before and after FMT

Four patients had faecal sensitivities which demonstrated coliforms that were resistant to ciprofloxacin and in three patients stool testing detected ESBL-coliforms. Two of these patients demonstrated coliforms that had regained sensitivity to ciprofloxacin four weeks following faecal transplantation (Table 6.2). For these patients, regaining sensitivity to ciprofloxacin enabled further effective antimicrobial therapy during their ongoing disease management.

Table 6.2. Changes in faecal coliform sensitivities before and four weeks after faecal transplantation.

Patient	Pre-faecal transplant		Post-faecal transplant	
	ESBL coliform	Ciprofloxacin sensitive coliform	ESBL coliform	Ciprofloxacin sensitive coliform
1	No	Yes	No	Yes
2	No	No	No	No
3	**Yes**	**No**	**No**	**Yes**
4	No	Yes	No	Yes
5	**Yes**	**No**	**No**	**Yes**
6	Yes	No	Yes	No
7	No	Yes	No	Yes
8	No	Yes	No	Yes

6.9 Adverse Events

There were no major adverse events following FMT. Three patients reported adverse effects including nausea (n=3); vomiting (n=1); bloating (n=2); fever (n=1). All adverse events were transient, lasting less than 24 hours.

6.10 Discussion

This was the first study of FMT for chronic pouchitis. In this study FMT was not effective in achieving either clinical response or remission for patients with chronic pouchitis. No improvement in any parameter was seen at four weeks following FMT. Two patients (patients 2 and 3) achieved a clinical response with a reduction of total PDAI score ≥3 points at 4 weeks post FMT. However, both patients still had a total PDAI score ≥7 at 4 weeks post FMT. Two of the four patients (patients 3 and 5) demonstrating resistant coliform in stool samples prior to FMT, regained sensitivity to ciprofloxacin at four weeks following FMT, enabling further efficacy of antibiotic therapy. Regaining sensitivity to antimicrobials may enable further effective antimicrobial therapy in this group of unresponsive patients. This study demonstrates that patients with chronic inflammatory bowel disease are willing to undergo the treatment and that FMT is safe and well tolerated although side effects were noted as have been previously documented in other reports of FMT (Silverman et al., 2010, van Nood et al., 2013).

Previous reports of FMT for IBD report the use of more frequent faecal infusions as well as rectal or colonic administration. However, in this study FMT was performed via nasogastric tube in accordance with previously reported efficacious protocols for *Clostridium difficile* (Aas et al., 2003, MacConnachie et al., 2009). This was due to the potential difficulties this group of patients have in retaining enemas as well as the need to standardise the administration of the faecal-saline solution.

Previous case reports of FMT for IBD also include pre-treatment with antibiotics and 5 ASA, steroids or immunosuppressant medication. However, animal studies demonstrate that antibiotics are not necessary to establish engraftment of exogenous microbiota (Manichanh et al., 2010). The use of other medications for IBD concurrently with FMT raise the question of the efficacy of FMT in the treatment of active disease or the maintenance of remission once induced by other medications. The patients included in this study had longstanding severe inflammation and may represent a refractory spectrum of disease.

It remains to be seen whether FMT is effective in induction of remission for chronic active IBD. There is only limited data regarding the efficacy of probiotics for the induction of remission of colitis and pouchitis. The vast majority of data suggesting efficacy of probiotics in UC or pouchitis are in the context of quiescent disease for the prevention of onset or maintenance of remission. The timing of interventions altering the microbiota may be relevant in the efficacy of this strategy in changing the course of IBD.

FMT appears to be safe and acceptable to patients with chronic pouchitis. However, in this study a single nasogastric administration of healthy donor stool was not clinically effective. Further controlled studies of FMT for chronic pouchitis and other inflammatory bowel diseases will need to consider alternative protocols including combined upper and lower gastrointestinal administration as well as repeated infusions. Studies will need to ascertain the role of pre-treatment antibiotics, probiotics, bowel lavage and proton pump inhibition in FMT. Furthermore, studies including selection of patients with less chronic and refractory disease or

patients conventionally induced into remission are necessary. Understanding the effect of FMT on the microbiota in pouchitis will enhance our understanding of the immunotherapeutic potential of FMT in IBD.

Chapter 7. The microbiological effects of faecal microbiota transplantation in chronic pouchitis

7.1 Abstract

Introduction: The efficacy of faecal microbiota transplantation (FMT) in the treatment of *Clostridium difficile* infection is tightly linked to changes in microbiota diversity and composition. Effects of FMT on recipient microbiota in inflammatory bowel diseases (IBD) remain unclear. We undertook a pilot study to assess the effects of FMT on microbiota composition in patients with chronic pouchitis.

Methods: Eight patients with chronic pouchitis (with a current PDAI ≥7) were treated with FMT via nasogastric administration. Changes in pouch faecal and mucosal microbiota were assessed by 16S rRNA gene pyrosequencing before and four weeks after FMT.

Results: There was significant inter-individual variation in microbiota content, in both the donors and pouch patients. Significant taxonomic differences were found between healthy donors and patients with pouchitis. Following FMT, there were no overall changes in bacterial richness or diversity of the faecal or mucosal microbiota. However, post FMT there were variable shifts in faecal and mucosal microbiota composition and changes in proportional abundance of species suggestive of a "healthier" pouch microbiota.

Conclusions: FMT via a single nasogastric administration in patients with chronic pouchitis resulted in variable changes in microbiota composition and did not alter the diversity of the pouch microbiota. Further modifications in the methodology of FMT may be required to impact on the microbiota for patients with IBD.

7.2 Background

A significant body of clinical and microbiological evidence has accumulated to suggest a dysbiosis of the pouch microbiota is likely to be important in the aetiology of this inflammatory disease process. Studies of the pouch microbiota have reached conflicting conclusions regarding differences in the abundance of particular species and phylotypes present in pouchitis or whether there is an increased or reduced diversity of the pouch microbiota. Predominantly studies find a reduced diversity of the microbiota in pouchitis with increased proportion of *Proteobacteria* and reduced *Bacteroidetes* (Mizoguchi et al., 2013, Tannock et al., 2012, McLaughlin et al., 2010b).

Several studies have assessed changes in the pouch microbiota following interventions of either antibiotic or probiotic therapy (Gosselink et al., 2004a, Kuisma et al., 2003, Gionchetti et al., 2000b) with limited success in achieving overall change in the composition of the microbiota (see Chapter 1.3v, Table 1.4).

Faecal microbiota transplantation may be a means of altering the composition of the microbiota as a whole. Animal and human studies have demonstrated the effective and durable transfer of donor microbiota using faecal transplantation for *Clostridium difficile* infection (Manichanh et al., 2010, Lawley et al., 2012, Grehan et al., 2010, van Nood et al., 2013). These studies demonstrate FMT to increase bacterial diversity and particularly replenish *Bacteroidetes* and *Clostridia* and reduce the proportions of *Proteobacteria* (van Nood et al., 2013, Tvede and

Rask-Madsen, 1989). Two recent studies have assessed the microbiota following FMT in IBD. Faecal microbiota transplantation reduced the relative abundance of *Proteobacteria* and increased *Bacteroidetes* and at family level the most prominent relative changes were a decrease in *Enterobacteriaceae* and *Enterococcaceae* and a relative increase in *Bacteroidaceae*. Abundant bacteria from donors established in some of the recipients, but the efficiency and stability of donor microbiota colonization varied greatly (Angelberger et al., 2013, Kump et al., 2013). No previous studies have assessed the pouch microbiota following FMT.

7.3 Aims

- To assess the faecal and mucosal microbiota in patients with active chronic pouchitis before and four weeks after FMT

7.4 Methods

FMT was undertaken as described in Chapter 6.5. Stool and biopsies were collected for microbiological analysis before and four weeks following FMT. Biopsies (n=2) for microbiological assessment were immediately washed in phosphate buffered saline (PBS) and snap frozen in liquid nitrogen and stored at -80°C. 2ml of stool for microbiological and metabolic assessment was mixed with 8ml of PBS, vortexed and centrifuged at 250g for 1 minute to exclude large particulate matter. Aliquots (1:10) were stored in RNA later (Ambion) at -80°C. DNA extraction**,** PCR and 16S rRNA gene sequencing and analysis were performed as described in Chapter 2.2.13-16.

7.5 Results

A total of 156963 sequences were generated with an average of 3982 reads per sample (range 391-12592). Samples with less than 250 reads were excluded from analysis (n=4). Most sequences were related to phyla *Firmicutes* (57%), *Bacteroidetes* (26%), *Proteobacteria* (14.5%) and *Actinobacteria* (1.6%). A total of 105 bacterial families were detected in samples, sixteen of these accounting for more than 90% of operational taxonomic units (OTUs) in the whole dataset. Good's coverage (an estimate of completeness of species sampling) at 391 reads per sample was greater than 92% for all sample groups (overall median for all samples was 95.9%, range 87.9 to 99.7%).

7.6 Donor and patient samples at baseline

There was significant inter-individual variation in microbiota content, in both the donors and pouch patients. Despite this, some general trends were observed. At the phylum level, prior to FMT, pouchitis patient stool and biopsy samples were characterised by a lower proportion of *Bacteroidetes* (stool, p=0.05; biopsies, p=0.0003) and a higher proportion of *Proteobacteria* (stool, p=0.02; biopsies, p=0.04) compared with donor stool samples. Patient mucosal samples also had a higher proportional abundance of *Firmicutes* (p=0.009) compared with donor stool samples (Figure 7.1).

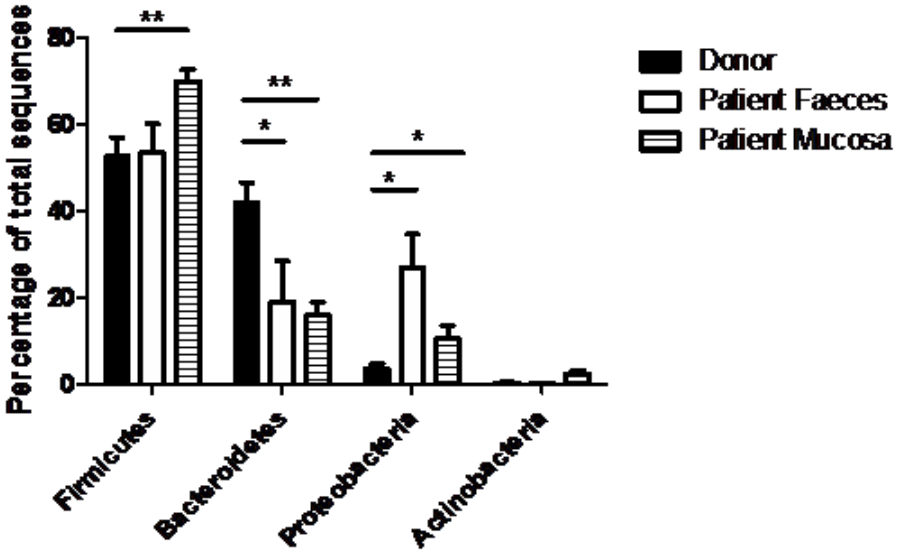


Figure 7.1 Percentage of sequences identified from the four predominant bacterial phyla in donor and patient samples at baseline. Percentage of total sequences from the predominant bacterial phyla in donor stool (n=8), patient stool pre FMT (n=7) and patient mucosal samples pre FMT (n=8). * and ** denote p values ≤0.05 and 0.01 respectively.

At the family level, patient stool and biopsies prior to FMT were characterised by a lower proportion of *Ruminococcaceae* (stool, p=0.0003; biopsies, p=0.01) and higher *Enterobacteriaceae* (stool, p=0.0003; biopsies, p=0.0003). Patient mucosal samples prior to FMT also demonstrated higher proportional abundances of *Lachnospiraceae* (p=0.004), and *Clostridiaceae* (p=0.02) (Figure 7.2).

At genus level analysis both patient stool and mucosal samples prior to FMT demonstrated reduced proportions of *Faecalibacterium* (p=0.003) and increased proportions of *Escherichia-shigella* (p=0.001) compared with donor stool samples (Table 7.1).

Patient stool prior to FMT was characterised by low phylotype richness (observed OTU diversity p=0.001; Chao 1 p=0.0003) and diversity (Shannon diversity index p=0.001; Simpson diversity index p=0.02) compared with donor stool samples. However, patient mucosal samples prior to FMT demonstrated no differences in richness (observed OTU diversity p=0.9; Chao 1 p=0.6) or diversity (Shannon diversity index p=0.9; Simpson diversity index p=0.8) compared with donor stool (Figure 7.3).

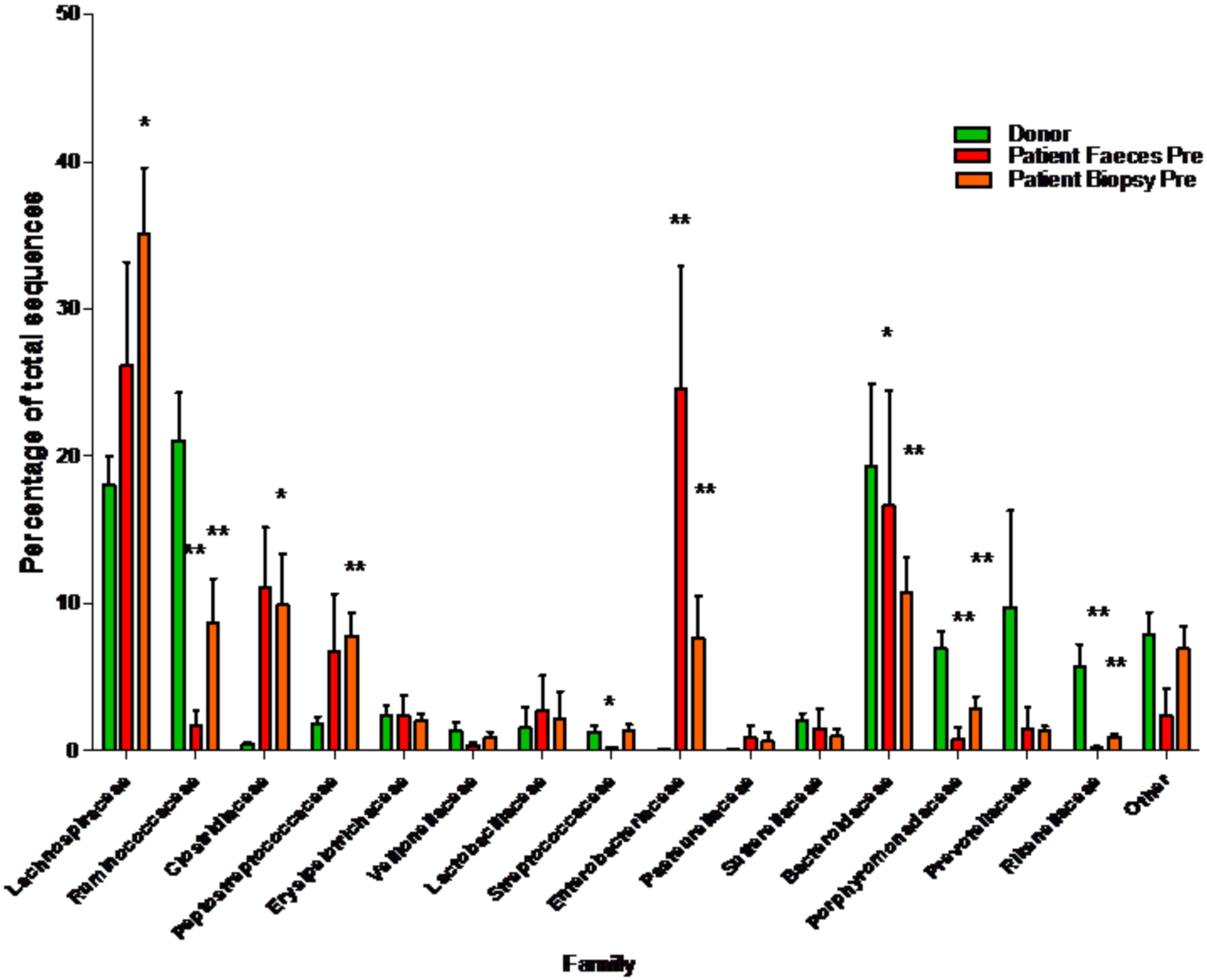


Figure 7.2 Percentage of sequences identified from the bacterial families of >1% total abundance in donor and patient samples at baseline. Percentage of total sequences from the predominant bacterial families in donor stool (n=8), patient stool pre FMT (n=7) and patient mucosal samples pre FMT (n=8). * and ** denote p values ≤0.05

Table 7.1 Analysis of genera from donor and patient samples at baseline

Genus	Donor stool (%)	Patient stool baseline (%)	p-value	Patient mucosa baseline (%)	p-value
Bacteroides	19.3 +- 5.6	16.6 +- 7.8	0.4	10.7 +-2.4	0.2
Escherichia_Shigella	0.06 +- 0.02	22.0 +-8.3	0.001**	7.3 +-2.8	0.001**
Prevotella	9.3 +-6.3	0.07 +-0.03	0.9	0.93 +-0.3	0.3
Lachnospiracea_incertae_sedis	4.6+-0.6	10.6 +- 3.5	0.3	11.7 +-3.6	0.009**
Clostridium_sensu_stricto	0.45 +-0.1	11.1 +-4.1	0.2	9.9 +-3.5	0.02*
Lactobacillus	1.6 +- 1.4	2.7 +- 2.4	0.6	1.9 +- 1.6	0.9
Faecalibacterium	10.9 +-2.1	1.4 +-0.9	0.003**	4.6 +-1.7	0.03*
Blautia	2.5 +-0.4	5.0 +-2.3	0.7	7.2 +- 2.3	0.2
Clostridium_XI	1.8 +-0.5	6.8 +-3.9	0.9	7.8 +-1.6	0.001**
unclassified Lachnospiraceae	4.6 +-0.8	2.8 +-1.0	0.2	6.7 +-0.9	0.1
Sutterella	2.0 +-0.5	0.2 +-0.2	0.004**	1.0 +-0.5	0.1
Anaerostipes	2.6 +-0.3	3.6 +-2.5	0.2	2.7 +-0.7	0.6
Clostridium_XVIII	1.5 +-0.4	2.2 +-1.4	0.2	1.2 +-0.4	0.3
Alistipes	5.7 +-1.5	0.2 +-0.1	0.003**	0.9 +-0.2	0.03*
Streptococcus	1.3 +-0.4	0.2 +-0.1	0.04*	1.3 +-0.4	0.8
Clostridium_XlVa	0.9 +-0.4	1.4 +-0.9	0.3	1.8 +-0.5	0.2
Barnesiella	2.8 +-0.5	0.1 +-0.1	0.001**	1.1 +-0.7	0.02*
Subdoligranulum	3.0 +-0.7	0.04 +-0.0	0.0003**	1.0 +-0.5	0.04*
Roseburia	1.0 +-0.2	2.4+-2.3	0.03*	2.7 +-2.0	0.6

Percentage of sequences identified from genera of >1% total abundance in donor and patient samples at baseline. Percentage of total sequences from the predominant bacterial genera in donor stool (n=8), patient stool pre FMT (n=7) and patient mucosal samples pre FMT (n=8). * and ** denote p values ≤0.05 and 0.01 respectively.

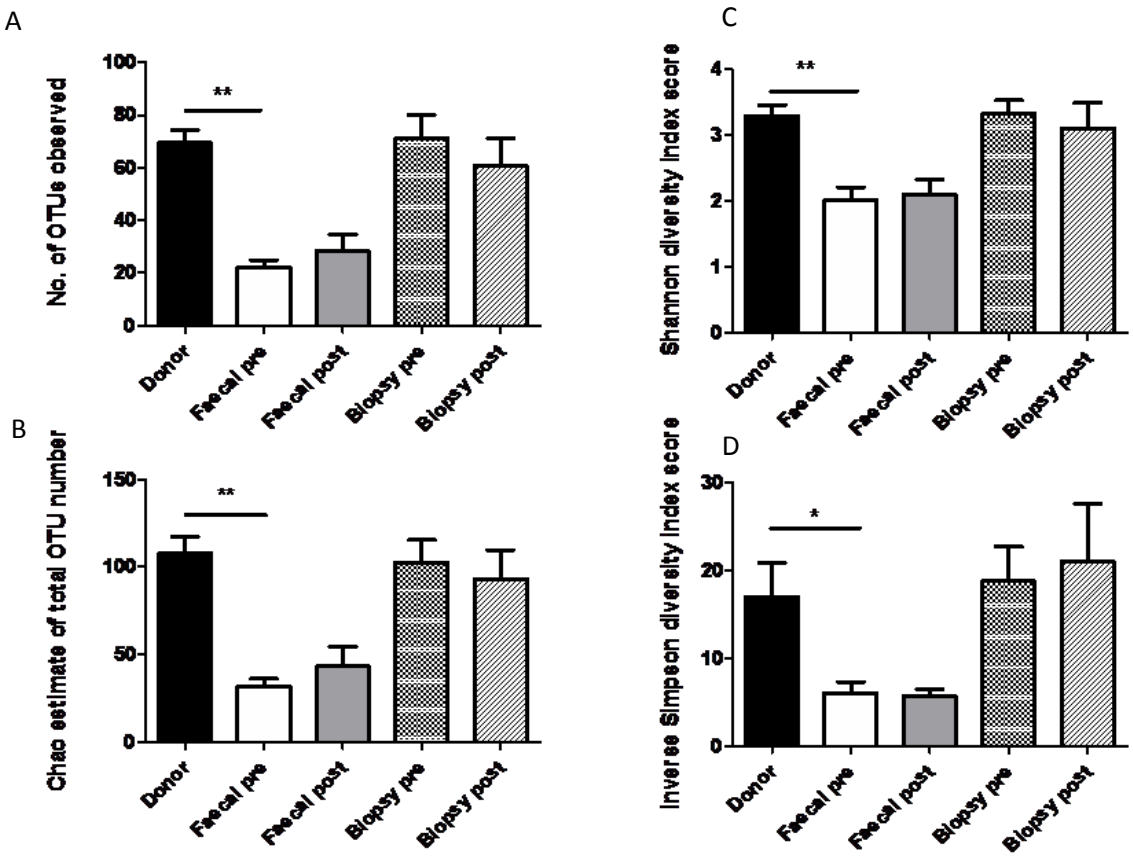


Figure 7.3 Phylotype richness and diversity in donor and patient samples A. Comparison of the number of operational taxonomic units (OTUs) observed. B. Chao estimate of total OTU number. C. Shannon diversity index. D. Simpson Diversity index. donor stool (n=8), patient stool pre FMT (n=7), patient mucosal samples pre FMT (n=8), patient stool post FMT (n=8), patient mucosal samples post FMT (n=5). * and ** denote p values ≤0.05 and 0.01 respectively.

7.7 Changes in microbiota composition post faecal transplantation

There were no overall changes in bacterial richness or diversity of the faecal or mucosal microbiota post FMT (Figure 7.3). However, Non-metric multidimensional scaling (NMDS) analysis using the Bray Curtis calculator, which measures dissimilarity in microbial community structure between samples by comparing overlapping OTU membership and their relative abundances, indicated a shift in both the stool and mucosal microbiota towards a composition with greater similarity to donor stool following FMT (Figure 7.4). Analysis of molecular variance (AMOVA) using the Yue and Clayton calculator, which also takes into account OTU membership and relative abundances when comparing bacterial community structures also suggested less difference between recipient and donor microbiota compositions following FMT (Donor vs. recipient faeces pre FMT p=0.005; Donor vs. recipient faeces post FMT p=0.034; Donor vs. recipient biopsy pre FMT p=0.003; Donor vs. recipient biopsy post FMT p=0.174).

Despite a high degree of inter-individual variation, assessment by metastats revealed an overall significant reduction in proportional abundance of *Escherichia coli/Shigella* spp. (p=0.005) and *Ruminococcus gnavus* (p=0.01) in patients post FMT, and an increase in relative abundance of *Sutterella stercoricanis* (p=0.003) and *Dorea longicatena* (p=0.01) as well as a trend towards increased proportional abundance of *Faecalibacterium prauznitzii* (p=0.09).

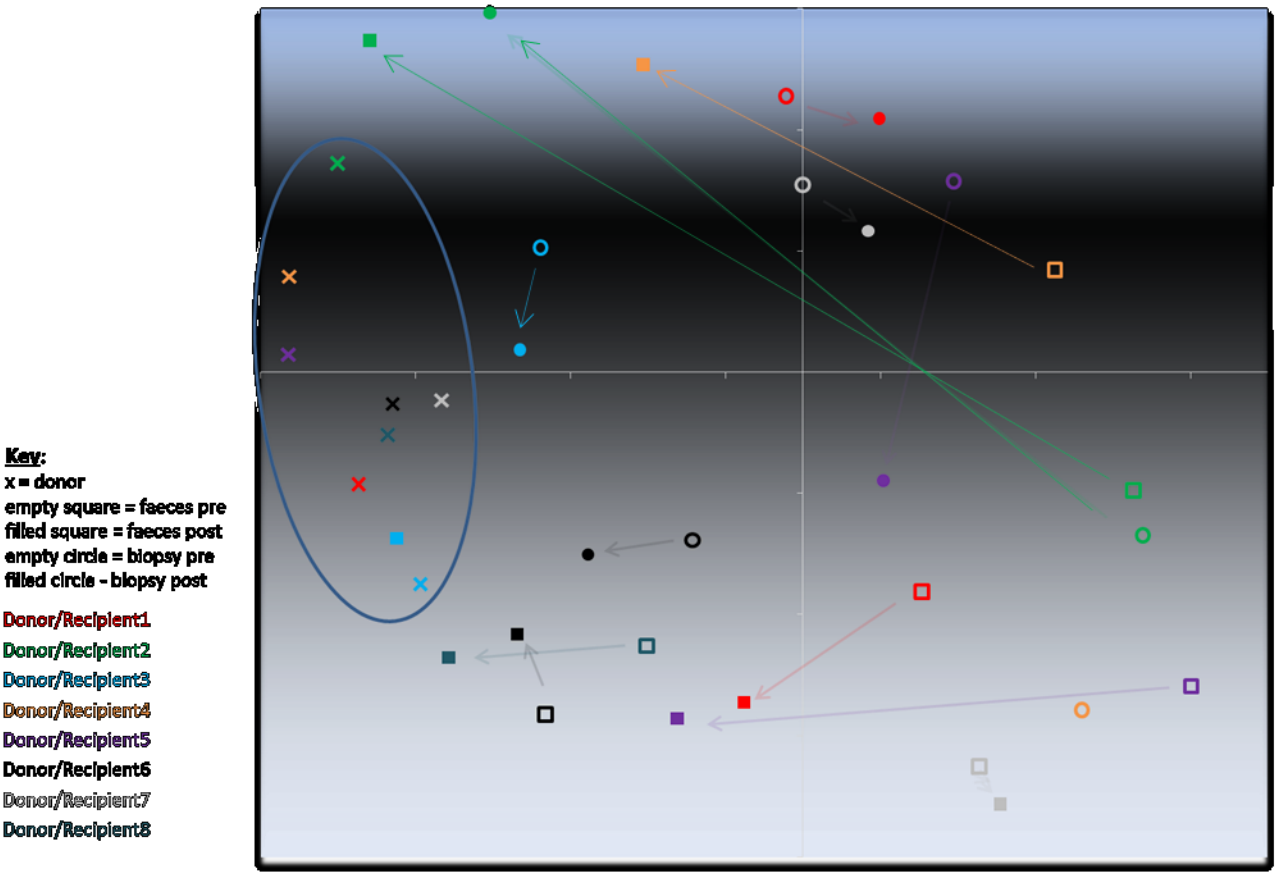


Figure 7.4 (Created by Dr A Walker) **Principal coordinate analysis for donor and patient samples pre and post FMT.** Principle coordinate analysis of donor stool (x) and patient stool pre FMT (open squares) and post FMT (filled squares) and patient mucosal samples pre FMT (open circles) and post FMT (filled circles) for each patient and donor. Oval shows distinct clustering of healthy donor faecal microbiota sample profiles in comparison to the pouchitis patient samples, arrows indicate directional shifts in patient samples post FMT.

There were no significant changes at phylum level in either the stool or mucosal microbiota post FMT (Figure 7.5).

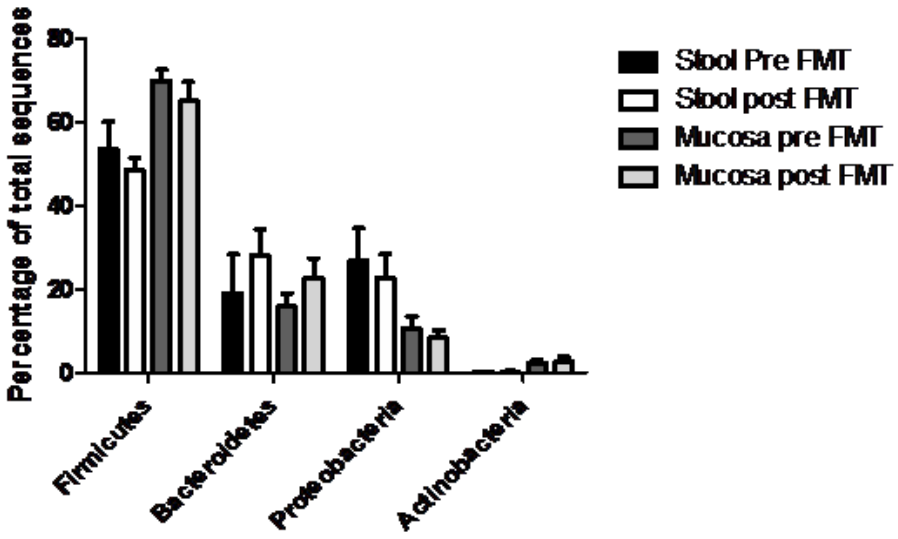


Figure 7.5 Percentage of sequences identified from the four predominant bacterial phyla in patient samples pre and post FMT. Percentage of total sequences from the predominant bacterial phyla in patient stool pre FMT (n=7) and patient stool post FMT (n=8); patient mucosal samples pre FMT (n=8) and patient mucosal samples post FMT (n=5).

At family level analysis there was a significant increase in the proportion of *Suterellaceae* in both stool and mucosal samples post FMT (p=0.02) as well as a significant reduction in the proportion of *Clostridiaceae* (p=0.03) in mucosal samples post FMT (Figure 7.6 and Figure 7.7). At genus level, the proportion of *Suterella* was significantly increased post FMT in both stool and mucosal samples (p=0.02). There was a trend towards increased *Faecalibacterium* in stool (p=0.07), but not in mucosal (p=0.4) samples post FMT. There was a significant reduction in the proportion of *Echerischia-shigella* in stool (p=0.03), but not in mucosal (p=0.6) samples post FMT (Table 7.2).

Table 7.2 Analysis of genera from patient stool and mucosal samples pre and post FMT

Genus	Patient stool baseline (%)	Patient stool post FMT (%)	p-value	Patient mucosa baseline (%)	Patient mucosa post FMT (%)	p-value
Bacteroides	16.6 +- 7.8	20.7 +-6.2	1	10.7 +-2.4	11.9 +-3.2	0.9
Escherichia_Shigella	22.0 +-8.3	4.5 +-2.6	0.03*	7.3 +-2.8	3.2 +-1.1	0.6
Prevotella	0.07 +-0.03	6.2 +-5.7	0.3	0.93 +-0.3	7.5 +_5.4	0.5
Lachnospiracea_incertae_sedis	10.6 +- 3.5	4.0 +-1.4	0.3	11.7 +-3.6	9.2 +-2.5	0.9
Clostridium_sensu_stricto	11.1 +-4.1	6.5 +-5.0	0.3	9.9 +-3.5	2.9 +-0.8	0.2
Lactobacillus	2.7 +- 2.4	6.4 +-6.2	1	1.9 +- 1.6	7.2 +-7.0	0.9
Faecalibacterium	1.4 +-0.9	5.2 +-2.4	0.07†	4.6 +-1.7	6.1 +-1.3	0.4
Blautia	5.0 +-2.3	2.3 +-0.8	0.2	7.2 +- 2.3	5.0 +-1.6	0.3
Clostridium_XI	6.8 +-3.9	5.2 +-2.6	0.9	7.8 +-1.6	5.2 +-1.6	0.3
unclassified Lachnospiraceae	2.8 +-1.0	2.0 +-0.6	0.5	6.7 +-0.9	5.6 +-1.5	0.3
Sutterella	0.2 +-0.2	9.7 +-3.7	0.02*	1.0 +-0.5	2.7 +-0.8	0.02*
Anaerostipes	3.6 +-2.5	1.4 +-0.7	0.7	2.7 +-0.7	3.6 +-1.2	0.5
Clostridium_XVIII	2.2 +-1.4	3.4 +-1.7	0.6	1.2 +-0.4	3.5 +-1.8	0.3
Alistipes	0.2 +-0.1	0.2+-0.2	0.4	0.9 +-0.2	1.3 +-0.5	0.7
Streptococcus	0.2 +-0.1	4.6 +-2.6	0.3	1.3 +-0.4	2.6 +-1.3	0.9
Clostridium_XlVa	1.4 +-0.9	1.4+-0.8	0.6	1.8 +-0.5	2.9+-0.9	0.5
Barnesiella	0.1 +-0.1	0.7+-0.	0.8	1.1 +-0.7	1.2 +-0.6	0.9
Subdoligranulum	0.04 +-0.0	0.5 +-0.3	0.6	1.0 +-0.5	1.2 +-0.4	0.6
Roseburia	2.4+-2.3	0.2 +-0.1	0.4	2.7 +-2.0	0.8 +-0.2	0.9

Percentage of sequences identified from genera of >1% total abundance in patient samples at baseline. Percentage of total sequences from the predominant bacterial genera in patient stool pre FMT (n=7) and post FMT (n-8) and patient mucosal samples pre FMT (n=8) and post FMT (n=5). * denotes p values ≤0.05.

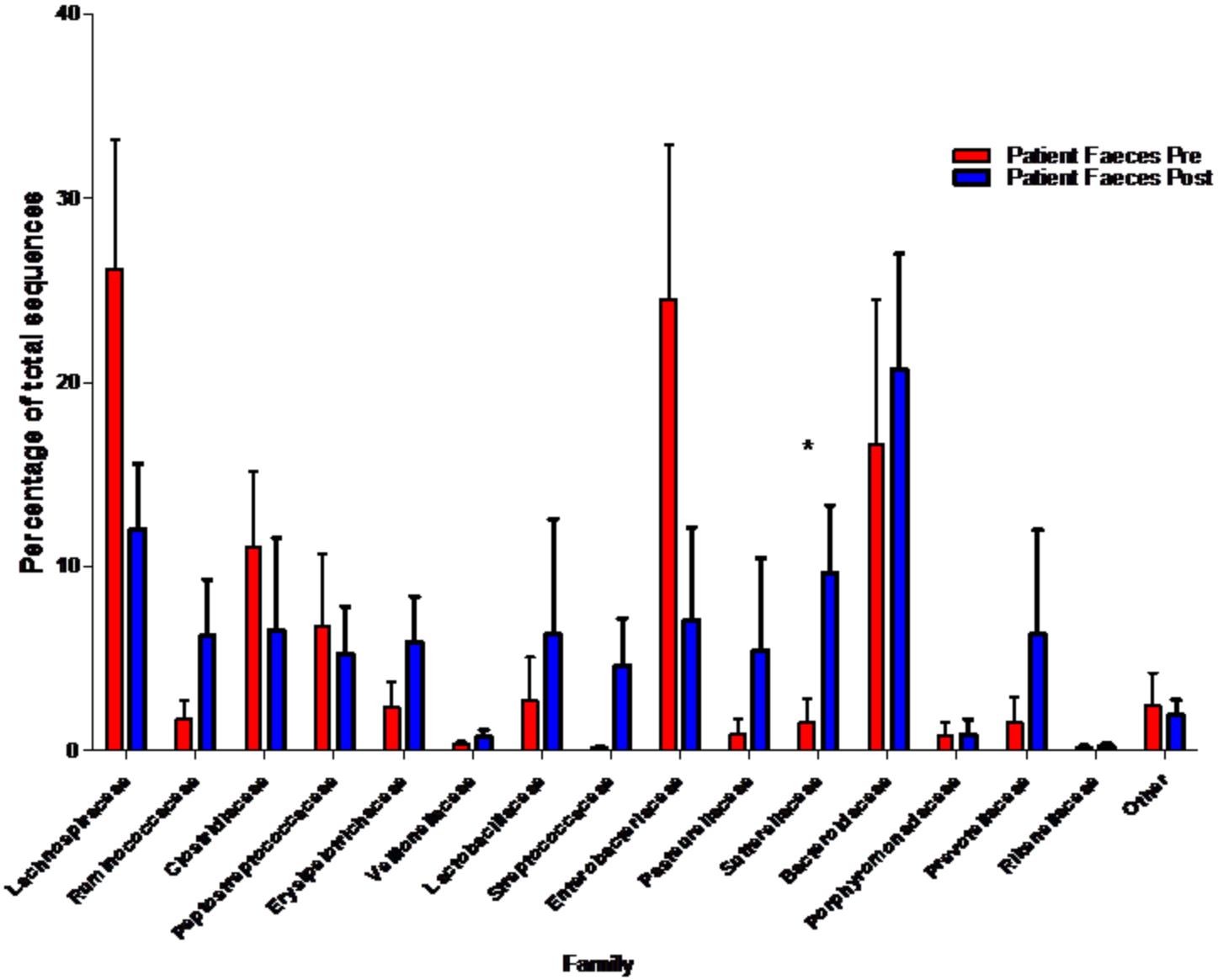


Figure 7.6 Percentage of sequences identified from the four predominant bacterial phyla in patient samples pre and post FMT. Percentage of total sequences from the predominant bacterial families in patient stool pre FMT (n=7) and patient stool post FMT (n=8). * denotes p values ≤0.05.

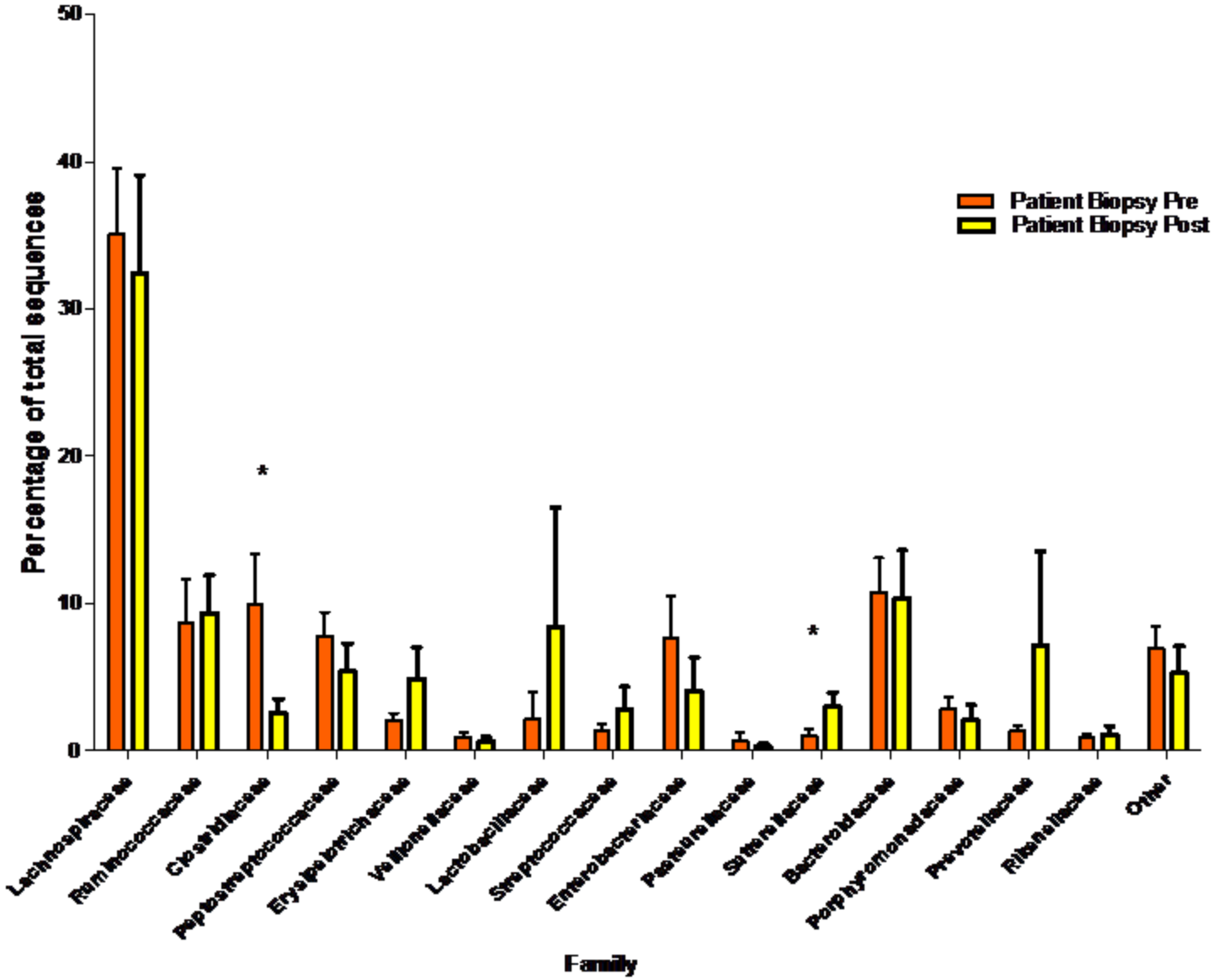


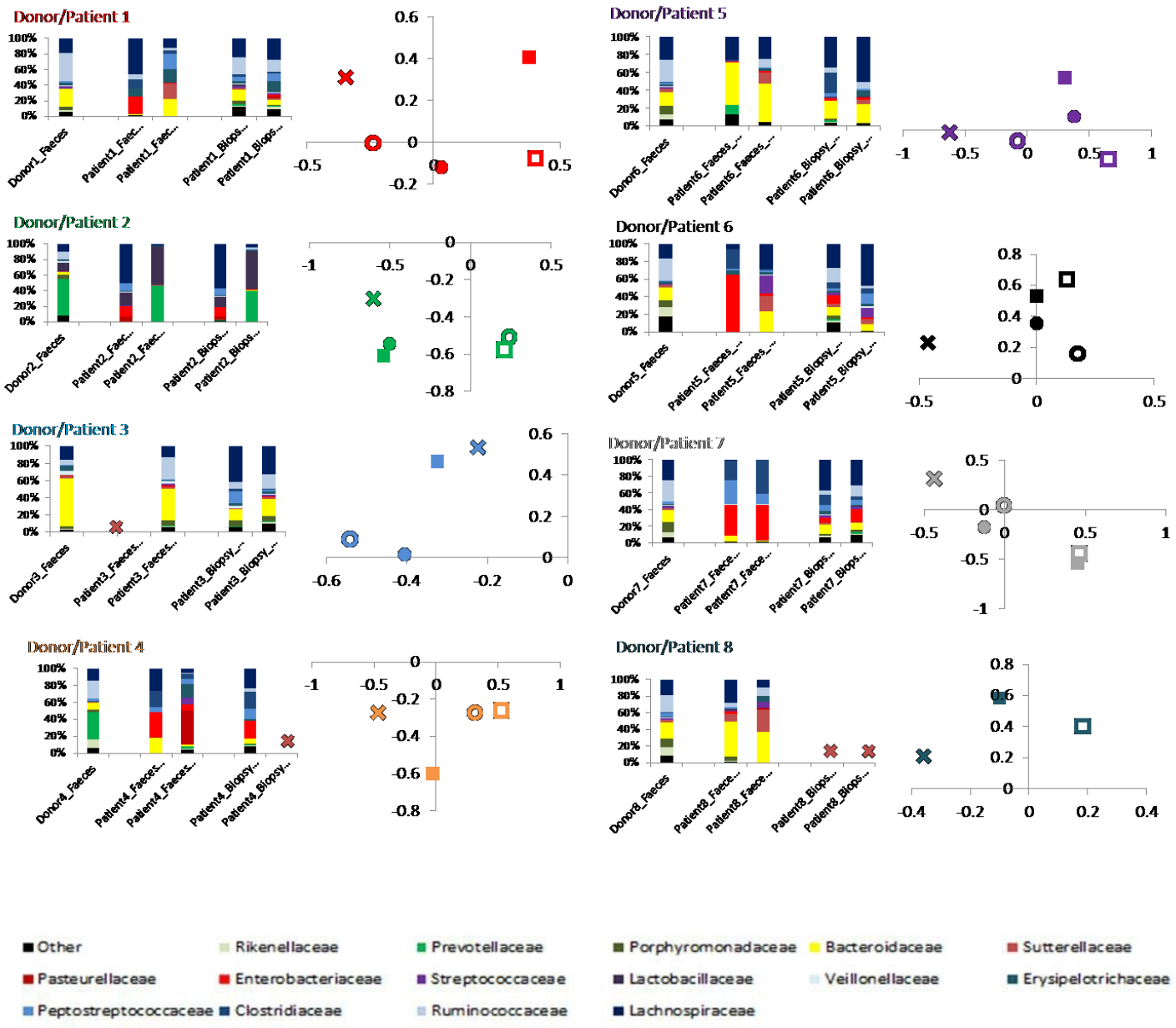
Figure 7.7 Percentage of sequences identified from the bacterial families of >1% total abundance in patient mucosal samples pre and post FMT. Percentage of total sequences from the predominant bacterial families in patient mucosal samples pre FMT (n=8) and patient mucosal sampels post FMT (n=5). * denotes p values ≤0.05.

7.8 Inter-individual variation

The significant inter-individual variation of the baseline faecal and mucosal microbiota was also reflected in the varying responses following FMT by each of the recipient patients (Figures 7.4 and 7.8). Broadly, Patients 2 and 3 showed a shift in their faecal and mucosal microbiota towards that of their respective donor's stool following FMT. Patient 5 showed a shift in the faecal and mucosal microbiota post FMT, although this shift was not towards a similar microbiota as Donor 5. Patients 1 and 4 showed a slight shift in the composition of their faecal microbiota, but not of their mucosal microbiota. Patients 6, 7 and 8 showed no shift in either stool or mucosal microbiota following FMT. There was an increased proportion of *Ruminococcaceae* with a concomitant reduction in the proportion of *Enterobacteriaceae* in the samples post FMT in patients 2, 3, 4 and 5.

Figure 7.8 Individual patient/donor combination analysis pre and post FMT (created by Dr A Walker)

A



B

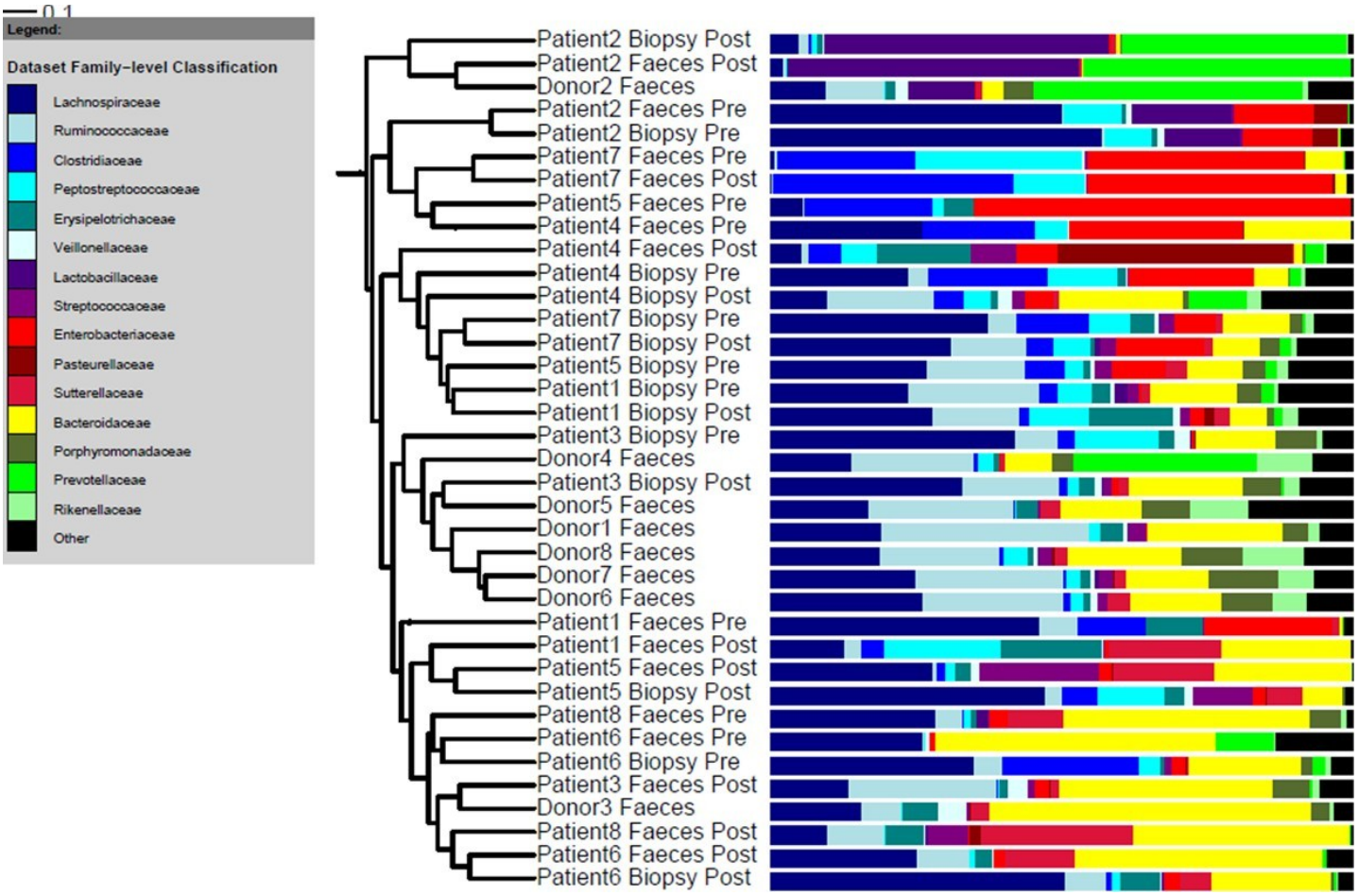


Figure 7.8. A. Family-level compositional analysis, and Bray-Curtis NMDS plots for each patient/donor combination. Key: X=donor; empty square=faeces pre; filled square=faeces post; empty circle=biopsy pre; filled circle=biopsy post. **B**. **B**ray Curtis dendrogram showing overall comparison between samples. This illustrates how some samples (e.g. from Patient 2) come to resemble the donor's microbiota following FMT, while others (e.g. Patient 7) remain distinct from the donor's microbiota following FMT.

7.9 Discussion

Studies of FMT for IBD are rare and restricted to case reports and small case series. Only two studies recently published have assessed the changes in the microbiota following FMT in the context of UC in a total of eleven patients (Kump et al., 2013, Angelberger et al., 2013). This is the first study to assess the microbiological effects of FMT in chronic pouchitis.

In this study there were no major overall shifts in the composition of the patients' microbiota at four weeks following FMT. There were no changes in either faecal or mucosal microbiota diversity at four weeks following FMT. However, there was some shift in the composition of the microbiota, with specific changes in the abundance of some species suggestive of a "healthier" pouch microbiota.

A number of studies have documented the association between increased *Proteobacteria*, specifically *Escherichia*, in IBD (Giaffer et al., 1992, Pilarczyk-Zurek et al., 2013, Gosselink et al., 2004b). It is unclear whether this finding is causative or secondary to inflammation. However, it is of interest that post FMT, *Escherichia* were significantly less proportionally abundant in the patients' stools. Other post FMT microbiota changes also suggested a shift towards a healthier bacterial composition. The mucolytic bacterium *Ruminococcus gnavus*, which was significantly reduced in proportional abundance following FMT, is increased in the epithelium of UC and CD patients (Png et al., 2010). *Clostridiaceae*, also reduced following FMT, are associated with pouch inflammation (37). Furthermore, potentially beneficial bacterial groups were increased in

proportional abundance following FMT. For example, *Faecalibacterium prauznitzii* is reduced in colitis patients (Sokol et al., 2009) and has anti-inflammatory properties , *Dorea* has been associated with non-inflammatory pouch outcomes (Mizoguchi et al., 2013) and a recent study associated increased abundance of *Sutterella* with pouch health (Mizoguchi et al., 2013).

There were also significant inter-individual differences with some patients demonstrating a shift in the microbiota either towards that of their donor or a merged composition of recipient and donor microbiota. However, patients 2 and 3 (who showed some response following FMT, see Chapter 6) showed a shift in their faecal and mucosal microbiota towards that of their respective donor's stool following FMT (although no stool sample was available for analysis for patient 3 prior to FMT and close similarity of the microbiota in this sample cannot be ruled out). However, although patients 2 and 3 demonstrated some changes towards a "healthy" pouch following FMT on family and genus level analysis, these changes were neither consistent, nor entirely restricted to these patients.

As expected, the patients' faecal and mucosal microbiota differed greatly from healthy donors' faecal microbiota. This difference may in part be due to the comparison of pouch and colonic samples. Nonetheless, the baseline pouch samples demonstrated findings in keeping with a dysbiosis as (previously found in IBD and specifically pouchitis) that may be attributable to the inflammatory state and underlying disease (Angelberger et al., 2013, Kump et al., 2013, Mizoguchi et al., 2013, Tannock et al., 2012, McLaughlin et al., 2010b, Scarpa et al., 2011a).

The diversity of the pouch microbiota was reduced in the faecal samples, but not in the mucosal samples at baseline. This is an unexpected finding, but may represent the severity and longevity of the refractory inflammation in the mucosa of the patients included in this study. The chronic refractory exposure to inflammation may have enabled the residual mucosal microbiota to create a niche with reasonable diversity, whilst the high pouch frequency in these patients may have counteracted the stability of the faecal microbiota. Previous studies have suggested a higher richness of the mucosal microbiota than the faecal microbiota. Higher phylotype richness (''biodiversity''), enhances the robustness and stability of an ecosystem and might be an intrinsic safeguard against perturbations (Lawley and Walker, 2013). Consequently, oosmotic diarrhoea also has a greater impact on the diversity of the faecal compared with mucosal microbiota (Gorkiewicz et al., 2013). Patients with milder or quiescent disease may therefore be more amenable to durable alteration of the microbiota with FMT.

There are several possible explanations for the lack of significant engraftment of the donors' microbiota to the recipients. The protocol of FMT used in this study consisted of a single nasogastric administration without pre-treatment with antibiotics or bowel lavage. This protocol is effective for *Clostridium difficile* infection (Aas et al., 2003, MacConnachie et al., 2009). However, more recently evidence has suggested greater efficacy with nasoduodenal administration (van Nood et al., 2013) and repeated infusions have been suggested for the use of FMT in chronic intestinal inflammation (Borody and Khoruts, 2012) although this has been demonstrated only in small case series to date. No human data exist regarding the benefit of prior antibiotics in FMT. One study in rats suggested this was not beneficial for engraftment of

the transplanted microbiota and instead led to a merging of antibiotic effects and transplant microbiota (Manichanh et al., 2010).

A study of FMT in mice showed that successful alteration of the recipient's microbiota depended on the donor microbiota being both phylogenetically diverse and distinct from that of the recipient (Lawley et al., 2012). Donors were not selected for their microbiota compatibility and indeed relatives and household contacts were used in some cases. Relatives of patients with IBD have been shown to have alterations in their microbiota distinguishing them from other "healthy" donors (Joossens et al., 2011). Donors' microbiota were dissimilar to recipients and were significantly more diverse than recipients' faecal microbiota, but not mucosal microbiota. However, donor microbiota may not be applicable to the niche of the UC pouch and perhaps FAP RPC or UC RPC patients without a history of pouchitis would be a more appropriate donor in this context.

The two recent studies of FMT in UC demonstrate a transient shift in the recipients' microbiota following FMT. Sampling at week four in this study may have missed some of the changes in the microbiota that occurred following FMT. This effect may be more exaggerated in pouch patients where frequency and bowel transit are increased. Finally, the patient group included in this study may be responsible for the lack of success of FMT engraftment. The patients included in the study had severe, refractory chronic disease with high pouch frequency. This in itself may have reduced the chances of successful transplantation due to their frequency and the likelihood of a well-established microbiota in the presence of chronic inflammation.

Future studies of the effects of FMT on the microbiota in inflammatory bowel diseases will need to address a number of questions in addition to those raised in Chapter 6:

1. Which donors are most appropriate microbiologically?
2. Does engraftment of particular species or a complex community altering microbiota diversity lead to functional efficacy of FMT?
3. Does alteration of the microbiota with FMT result in significant functional change of the microbiota?
4. What are the immunological consequences of FMT?

Chapter 8. The mucosal immunological effects of faecal microbiota transplantation in chronic pouchitis

8.1 Abstract

Introduction: Studies of the immune responses to modification of the microbiota in inflammatory bowel diseases may enhance our understanding of these diseases. Probiotics alter immune responses and epithelial barrier function in IBD. To date no previous studies in humans with IBD have assessed the immunological effects of faecal microbiota transplantation (FMT). We aimed to assess alterations of mucosal dendritic cell and tight junction protein expression following FMT in patients with chronic pouchitis.

Methods: Eight patients with chronic pouchitis (with a current PDAI ≥7) were treated with FMT via nasogastric administration. Mucosal biopsy samples were taken before and four weeks after FMT. Epithelial cells and lamina propria DC were isolated from biopsy tissue and epithelial cell expression of ZO-1, claudin 1, claudin 2 and DC expression of TLR 2 and 4, CCR9, β7 and CD40 were measured by multicolour flow cytometry. Ongoing production of cytokines by dendritic cells were assessed following incubation with and without monensin and cytokine levels in biopsy supernatants were assessed by multiplex analysis .

Results: There were no significant changes in dendritic cell expression of TLRs, homing markers, activation markers or cytokine production following FMT. There were no significant changes in epithelial cell tight junction protein expression or cytokine levels in whole biopsy supernatants following FMT.

Conclusions: FMT via a single nasogastric administration in patients with chronic pouchitis did not result in immunological response. Further studies that include assessment of the functionality of the microbiota post FMT in conjunction with immunological assessment are required.

8.2 Background

Probiotics alter the innate and adaptive immune responses in IBD (Hart et al., 2004b, Ng et al., 2009b). Probiotics also alter epithelial barrier function and the expression and distribution of epithelial tight junctions (Ng et al., 2009b, Mennigen et al., 2009, Madsen et al., 2001, Karczewski et al., 2010, Persborn et al., 2013). Studies of the immune effects of probiotics in IBD are predominately in vitro, ex-vivo or in animal models. Few studies demonstrate the immune effects of probiotic administration in vivo in UC and pouchitis patients.

Crohn's disease and UC patients treated with a probiotic yoghurt containing *L. rhamnosus* GR-1 (GR-1) and *L. reuteri* RC-14 (RC-14) demonstrated expansion of $CD4+CD25^{high}$ cell population with a decrease in serum IL-12 concentration and a decreased percentage of TNF-α and IL-12-producing monocytes and myeloid DC. Colonic myeloid DC of UC patients treated with VSL#3 down regulated TLR2 expression and increased IL-10 production whilst decreasing IL-12p40 production (Ng et al., 2010b).

Ulcerative colitis patients treated with VSL#3 prophylactically following RPC have lower mucosal mRNA expression levels of interleukin-1beta, interleukin-8, and interferon-gamma compared with placebo treated patients (Lammers et al., 2005b). Patients with active pouchitis where remission was induced with combination antibiotics and maintained with VSL#3 for nine months demonstrated additional immune effects to VSL#3 beyond those seen following antibiotic induced remission with reductions in tissue TNFα and IL-1α and increased IL-10 as

well as reduced matrix metalloproteases (Ulisse et al., 2001). In another study of VSL#3 treatment of non-inflamed ileal pouch patients a significant increase in mucosal CD4+CD25high cells as well as increased Foxp3 mRNA expression was found after treatment compared with baseline (Pronio et al., 2008).

To date no previous studies in humans have assessed the immunological effects of faecal microbiota transplantation (FMT). In a recent study in a mouse model of *Clostridium difficile*, high levels of expression of pro-inflammatory genes were suppressed following FMT (Lawley et al., 2012). Transplantation of the microbiota as an "organ" rather than specific strains or combinations of bacteria may have significant effects on the immune responses in inflammatory bowel diseases.

8.3 Aims

- To assess dendritic cell phenotype and cytokine production in patients with active chronic pouchitis before and after FMT
- To assess cytokine levels in biopsy supernatants of patients with active chronic pouchitis before and after FMT
- To assess epithelial tight junction protein expression in patients with active chronic pouchitis before and after FMT

8.4 Methods

FMT was undertaken as described in Chapter 6.5. Biopsies were taken for immunological assessments before and four weeks after FMT. Biopsies were processed and analysed as described (Chapter 2.2.5- 2.2.10 and Chapter 4.6).

8.5 Dendritic cell phenotype before and after FMT

There were no significant changes after FMT in DC expression of TLR 4 (46 +- 6.5% vs. 34 +- 7%, p=0.2) or TLR 2 (43 +-5.4% vs. 49 +-8.8%, p=0.3) (Figure 8.1). There were no significant changes in DC expression of homing markers β7 (40 +-7.6% vs. 32 +-4.5%, p=0.4) or CCR 9 (39 +-5.2 vs. 40 +-3.5%, p=0.8) before and after FMT (Figure 8.2) or expression of activation marker CD40 (81 +-2.9% vs. 76 +- 2.8%, p=0.3) (Figure 8.3).

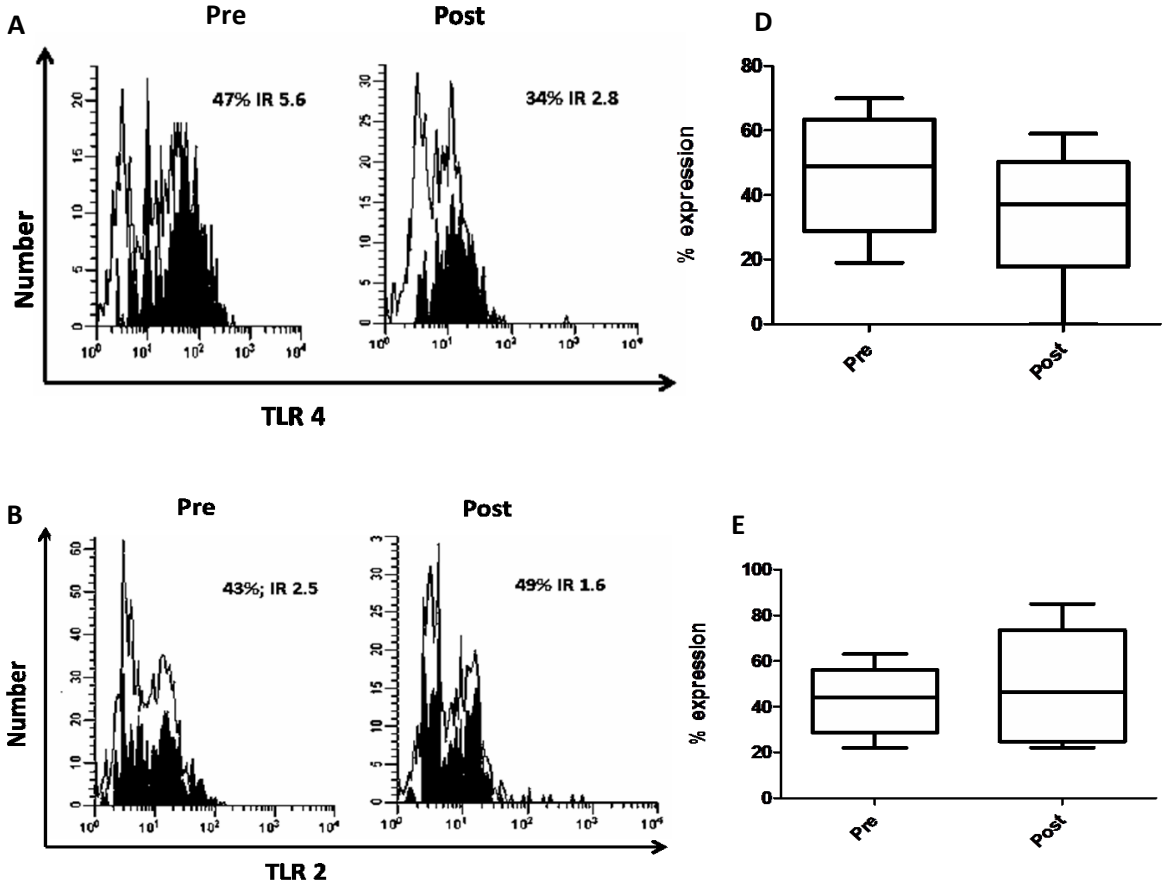


Figure 8.1 TLR 4 and TLR 2 expression on HLA DR+ lineage negative cells in pouch tissue in patients with active chronic pouchitis pre and post FMT. A, B and C. One parameter histograms representative of TLR 4 and TLR 2 (n=8) staining pre and post FMT. D, E and F. Percentage expression of TLR 4 and TLR 2 pre and post FMT.

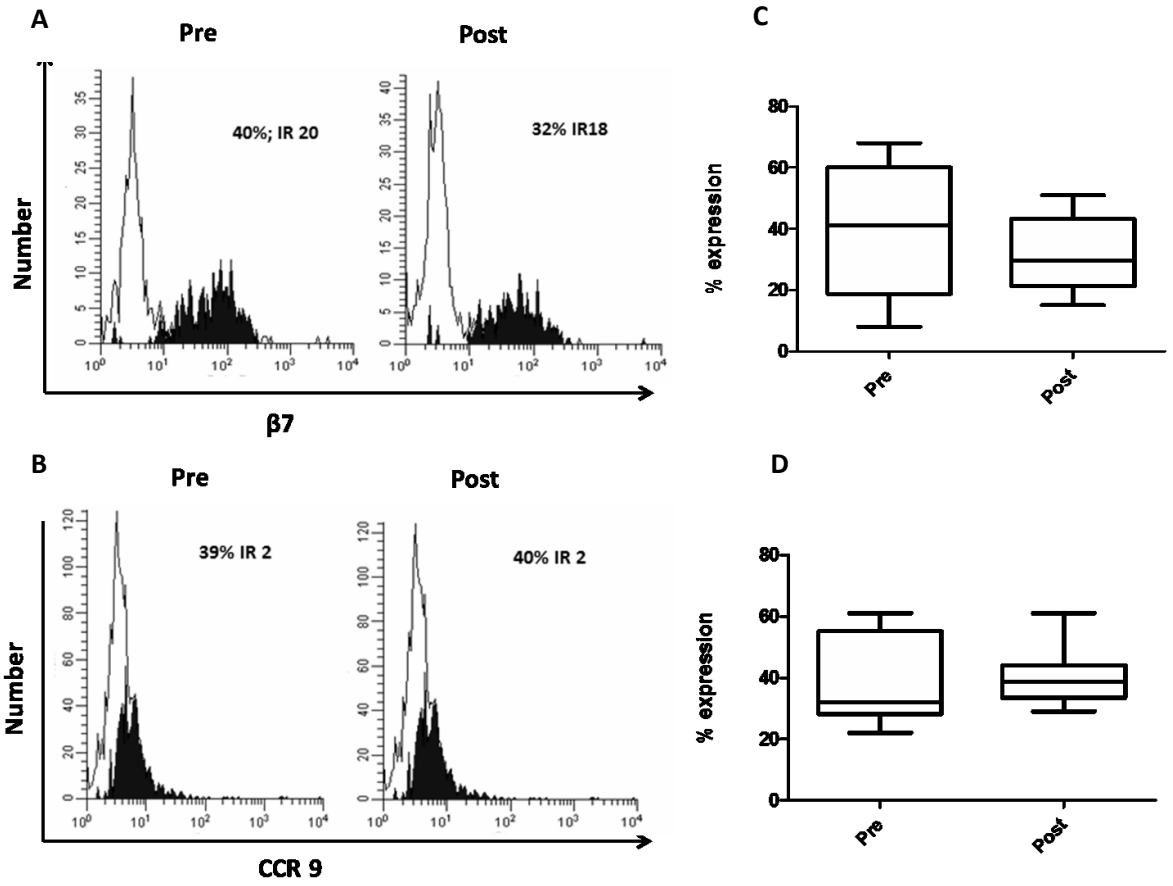


Figure 8.2 β7 and CCR9 expression on HLA DR+ lineage negative cells in pouch tissue in patients with active chronic pouchitis pre and post FMT. A and B. One parameter histograms representative of β7 and CCR9 staining pre (n=8) and post (n=8) FMT. C and D. Percentage expression of β7 and CCR9 pre and post FMT.

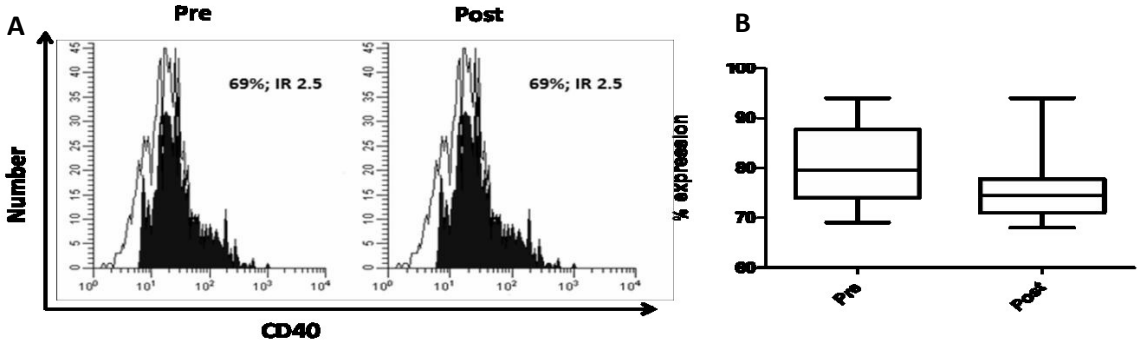


Figure 8.3 CD40 expression on HLA DR+ lineage negative cells in pouch tissue in patients with active chronic pouchitis pre and post FMT. A and B. One parameter histograms representative of CD40 staining pre (n=8) and post (n=8) FMT. C and D. Percentage expression of CD40 pre and post FMT.

8.4 Ongoing production of cytokines in dendritic cells before and after FMT

For a functional assessment of DC before and after FMT, ongoing intracellular production of cytokines in DC was assessed as described previously (Chapter 2.2.6) in the absence of exogenous stimulation. Levels of IL-12, IL-10, IL-17 and IL-4 production were assessed. There were no significant changes in ongoing production of cytokines in DC before and after FMT. There were no changes in IL-12 (-14 +-8.7% vs. 12 +-12.6%, p=0.3), IL-10 (0 +-11% vs. 7 +-5%, p=0.5), IL-17 (27 +-13% vs. 14 +-7%, p=0.5) or IL-4 production (17 +-7% vs. 34 +-17%, p=0.4) (Figure 8.4).

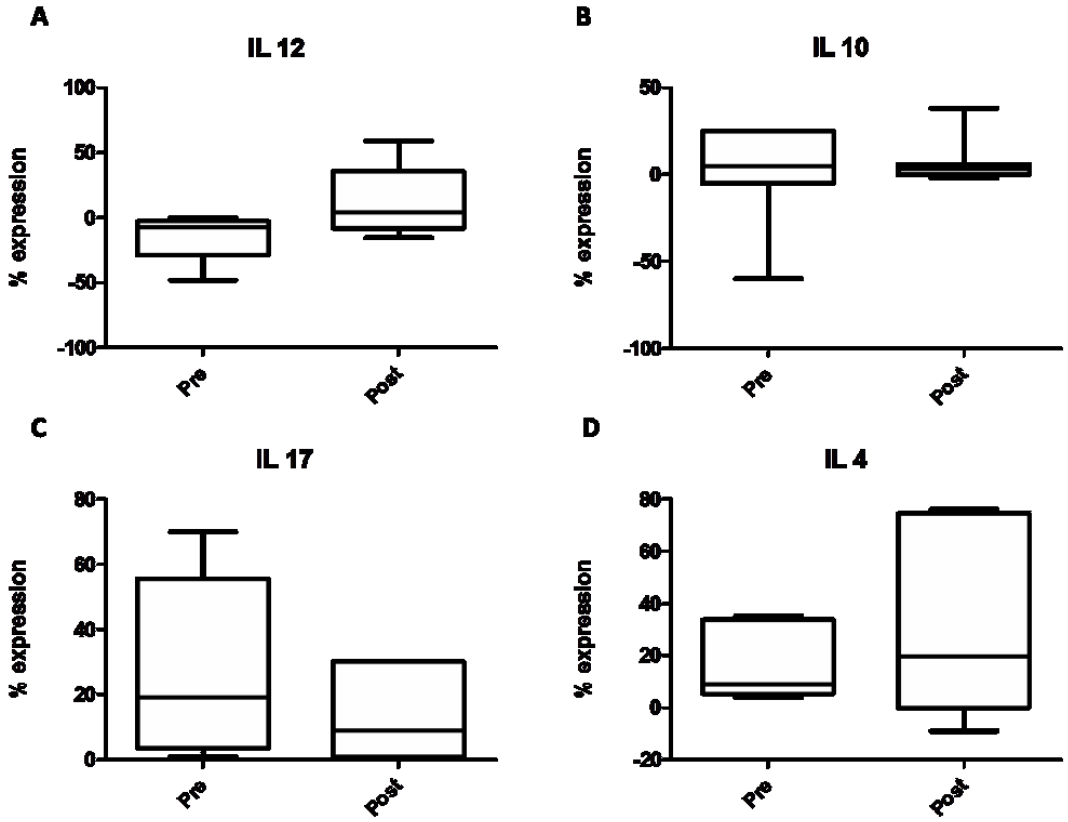


Figure 8.4 Percentage expression of ongoing production of cytokine in dendritic cells from pouch tissue of patients with active chronic pouchitis pre (n=5) and post FMT (n=5) of A. IL-12; B. IL-10; C. IL-17; D. IL-4.

8.5 Cytokine levels in biopsy supernatants of patients with active chronic pouchitis before and after FMT

No changes were demonstrated in either DC phenotype or function following FMT. In order to assess whether other aspects of the mucosal response were altered following FMT, cytokine levels of biopsy supernatants were assessed before and after FMT. There were no significant changes in any of the cytokine levels assessed following FMT: IL-2 (12.2 +-0.98 $pg{\cdot}ml^{-1}$ vs. 11.8 +-0.6 $pg{\cdot}ml^{-1}$, p=0.8); IL-4 (13.5 +-1.1 $pg{\cdot}ml^{-1}$ vs. 12.3 +-0.3 $pg{\cdot}ml^{-1}$, p=0.3); IL-6 (1325+-750 $pg{\cdot}ml^{-1}$ vs. 1666 +-946 $pg{\cdot}ml^{-1}$, p=0.4); IL-10 (10.4+-0.8 $pg{\cdot}ml^{-1}$ vs. 14.1 +-2.43 $pg{\cdot}ml^{-1}$, p=0.1); TNF (12.0 +-1.5 $pg{\cdot}ml^{-1}$ vs. 15.5 +-2.5 $pg{\cdot}ml^{-1}$, p=0.3) (Figure 8.5).

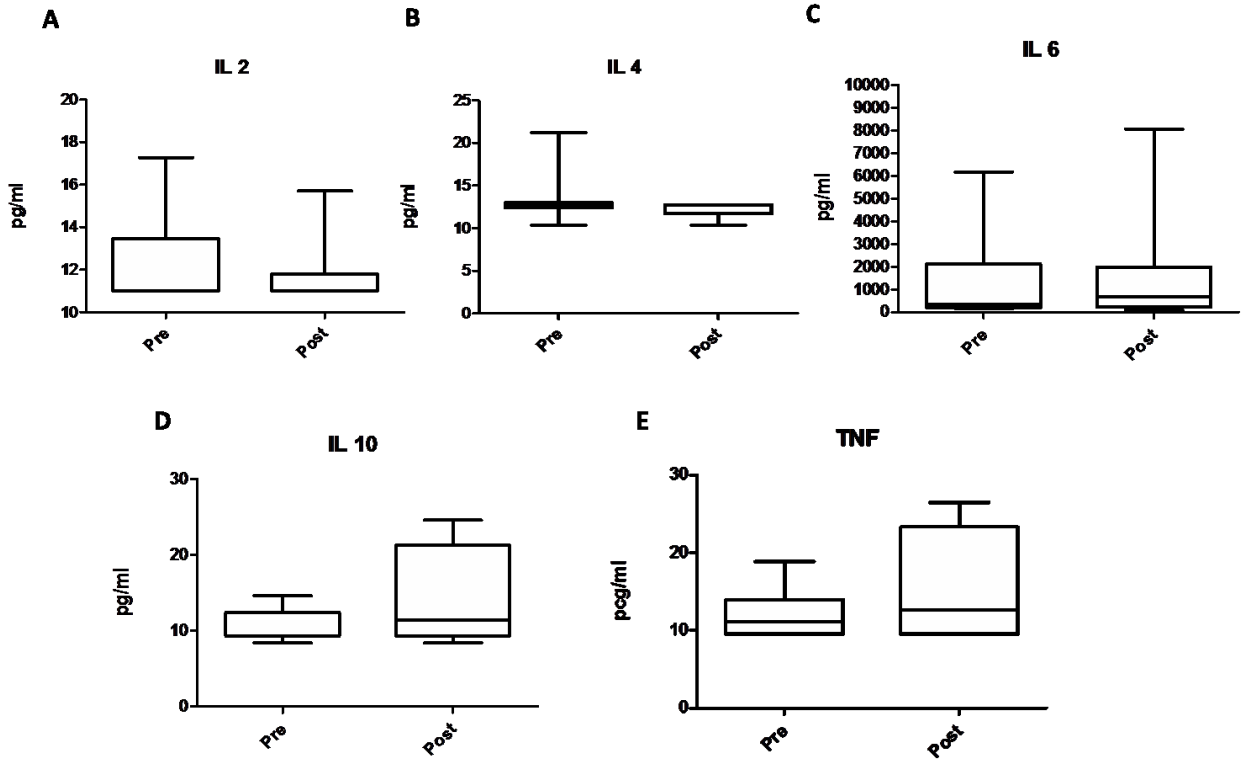


Figure 8.5 Cytokine levels in biopsy supernatants of pouch biopsies of patients with active chronic pouchitis before (n=8) and after (n=8) FMT. A. IL-2; B. IL-4; C. IL-6; D. IL-10; E. TNF.

8.6 Epithelial tight junction protein expression before and after FMT

There were no significant changes in tight junction expression before and after FMT. There were no changes in the median intensity ratio of epithelial expression of ZO-1 (2.9 +-0.4 vs. 3.4 +-0.7, p=0.4) or Claudin 1 (1.3 +-0.3 vs. 1.2 +-0.6, p=0.9) or the percentage epithelial cell expression of Claudin 2 (19 +-11% vs. 40 +-13%, p=0.3) (Figure 8.6).

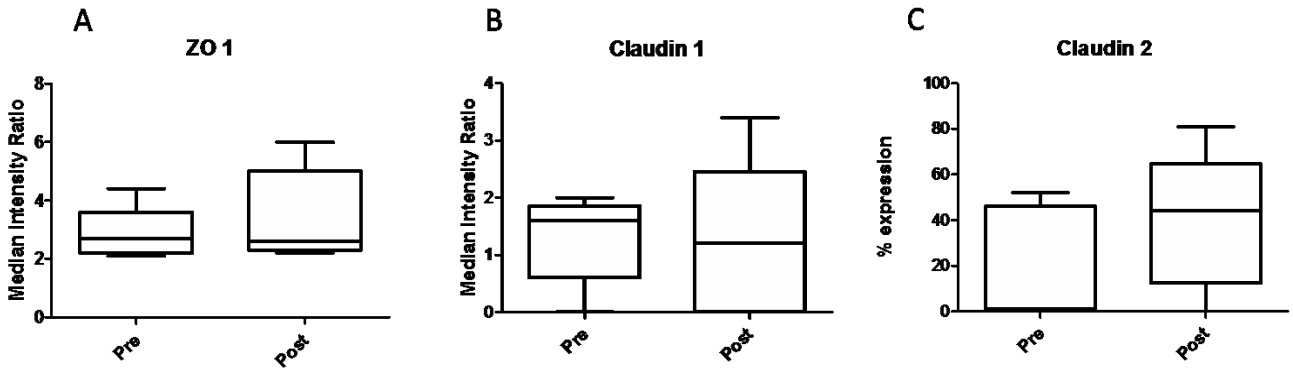


Figure 8.6 Epithelial tight junction expression from pouch tissue of patients with active chronic pouchitis pre (n=5) and post (n=5) FMT. A. Median intensity ratio of epithelial expression of ZO-1 and B. claudin 1. C. Percentage epithelial cell expression of claudin 2.

8.7 Discussion

This is the first study to assess the immunological outcomes following FMT in humans. There were no changes in DC phenotype or cytokine production following FMT. Neither were there any alterations in cytokine levels in the biopsy supernatants or in tight junction protein expression.

Despite the potentially beneficial shifts in the microbiota post FMT as described in Chapter 7 no immunological response was seen. The changes in composition of the microbiota reflect

alterations in relative abundance rather than absolute abundance and may therefore not be reflected by immunological responses. Furthermore, abundant molecular functions are not necessarily provided by abundant species (Arumugam et al., 2011) and core metabolic functions may be shared between bacteria, promoting stability in metabolic function, and maintaining homeostasis. Consequently, the changes in the microbiota that were demonstrated post FMT might not lead to significant metabolic and subsequently immunological changes.

The patients included in the study had severe, refractory chronic disease. The immune effects of bacteria may also depend on the local microenvironment in which they are acting and the integrity of the epithelial monolayer (Tsilingiri et al., 2012). Loss of epithelial barrier integrity with increased translocation of otherwise non-pathogenic, non-invasive bacteria may worsen inflammation (Tsilingiri et al., 2012). FMT in this group of patients may therefore have deleterious rather than positive clinical and immunological outcomes.

Despite a large body of evidence suggesting the microbiota drives gut inflammation, it is uncertain whether dysbiosis itself causes IBD or if it represents an epiphenomenon. In addition, whether the absolute or relative abundance of particular species or the overall complexities of the microbial ecosystem are predominantly responsible for host immune responses to the microbiota is not known. Future studies will need to assess these issues as well as the functional aspects of the microbiota.

Chapter 9. General discussion and future work

9.1 General discussion

The ileal pouch provides a unique human model for studying IBD pathogenesis. The first part of the thesis focuses on characterising lamina propria dendritic cell phenotype and epithelial cell tight junction expression in the UC pouch and pouchitis. This is the first study to identify and characterise DC in the ileal pouch mucosa of UC and FAP patients. The focus of the work here is more descriptive than mechanistic. However, before moving forward into the mechanisms responsible for the development of pouchitis we must first understand its natural evolution.

An aberrant DC phenotype was identified in the non-inflamed ileal tissue of UC patients undergoing RPC. In the ileum of UC patients compared with healthy controls there was abnormal expression of gut homing markers and reduced expression of activation marker CD40. Further analysis revealed expansion of the plasmacytoid DC subset in UC patients. However, the aberrant gut homing marker expression in UC patients was not only a consequence of the relative expansion of pDC, but a genuine reduction of DC expressing β7 on myeloid DC. This suggests abnormalities of the innate immune response in the ileum of UC patients prior to RPC that may lead to a susceptibility to inflammation following restoration of the microbiota with ileostomy closure. Aberrant expression of gut homing markers was also noted in the non-inflamed pouch of UC patients compared with FAP patients. Changes in DC homing markers in the ileal pouch of UC patients are likely to represent an aberrant response to the altered microenvironment following RPC.

The findings regarding DC phenotype in the ileum and the non-inflamed pouch of UC patients suggest that aberrant DC characteristics, in particular aberrant homing marker expression, in UC patients may play a critical role in the colitogenic susceptibility following RPC for UC. We speculate that prior to the onset of inflammation, the aberrant homing profile and expansion of pDC in UC patients may lead to a loss of gut homing marker imprinted lymphocytes and reduced activation of tolerogenic T cell responses to the colonic microenvironment in the functioning pouch. Furthermore inappropriate recruitment of DC to the lamina propria may lead to a mis-shaping of the microbiota in UC patients and increased levels of microbial interaction at the epithelium. We also identified differences in DC expression of gut homing markers as well as TLRs and activation markers between the inflamed and non inflamed ileal pouch in UC patients and FAP patients. These findings suggest DC play a critical role in the maintenance of inflammation with increased homing of activated DC to the inflamed pouch in response to microbial signals.

The aberrant immune responses to the microbiota depend on interactions at the epithelium. We demonstrated the development of a methodology using flow cytometry to quantitatively assess tight junction protein expression on epithelial cells. We identified abnormalities in pouch epithelial TJPs in UC patients with an increase in expression of the “pore forming” TJP, claudin 2. Furthermore, we demonstrated differences in the expression of claudin 2 in acute pouchitis and of ZO-1 and claudin 1 in chronic pouchitis. We suggest that increased epithelial expression of claudin 2 is an early event in the development of inflammation which could present a mechanism by which UC patients are more susceptible to microbial signalling compared with

FAP patients. However, the findings of altered tight junction expression may be secondary to the elevation in inflammatory cytokines that were demonstrated in both acute and chronic pouchitis.

The temporal inter-relationships between defective barrier function, abnormal immune responses and the microbiota are not known. To determine the aetiological role of these factors, longitudinal studies of patients prior to disease onset are needed. These are not feasible in Crohn's disease or UC, but the pouch offers a unique opportunity for such investigation. We undertook the first study to longitudinally assess DC characteristics and epithelial cell tight junction protein expression following ileostomy closure (with reintroduction of the faecal stream) in UC patients.

The increased expression of claudin 2 in in the ileal pouch of UC patients at six months following ileostomy closure suggested this is an early event in the development of inflammation. Changes in DC homing markers in the pouch of UC patients also represent an aberrant response to the more colonic microenvironment following RPC. This could present a mechanism for barrier dysfunction by which UC patients are more susceptible to immune activation by the more "colon-like" microbiota following ileostomy closure.

The second part of the thesis focuses on an in vivo study of faecal microbiota transplantation (FMT) in patients with chronic pouchitis. A significant body of evidence suggests a dysbiosis of the pouch microbiota is likely to be important in the aetiology of pouchitis. No previous studies

have assessed the effects of FMT in pouchitis or the immunological effects of FMT in humans or in the context of IBD.

FMT was safe and acceptable to patients with chronic pouchitis. However, in this study a single nasogastric administration of healthy donor stool was not clinically effective. There were no changes in either faecal or mucosal microbiota diversity at four weeks following FMT. However, there was some shift in the composition of the microbiota, with specific changes in the abundance of some species suggestive of a “healthier” pouch microbiota. There were also significant inter-individual differences between patients. There were no changes in dendritic cell phenotype or cytokine production following FMT. Neither were there any alterations in cytokine levels in the biopsy supernatants or in tight junction protein expression. This most likely is a consequence of the lack of overall changes in the microbiota of the recipients. However, may also be due to the severe refractory nature of the inflammatory response in the patients studied.

This study did not elucidate the key changes in the microbiota necessary for resolution of pouch inflammation. However, these data stimulate a number of questions regarding the role of FMT in IBD and the likelihood of successful engraftment of donor microbiota in the context of chronic mucosal inflammation:

1. What is the most appropriate protocol regarding route of administration and frequency of infusions as well as the need for pre-treatment antibiotics, probiotics, bowel lavage and proton pump inhibition?

2. Which donors are most appropriate both by relationship to the recipient and microbiologically?

3. In which patient group will FMT be most effective- acute or chronic active disease or once remission has been induced by standard therapies

4. Whether engraftment of particular species or a complex community altering microbiota diversity is necessary for functional efficacy?

9.2 Future work

Data presented in the first part of this thesis characterise an aberrant dendritic cell phenotype in the development and maintenance of pouch inflammation. There are small numbers of patients in these studies and larger cohorts of UC and FAP patients following RPC are necessary. Further studies are also necessary to characterise the role of DC subsets, particularly plasmacytoid DC, CCR9+ and CD103+ DC in these patient groups. Furthermore, future studies are needed to assess the functional outcomes of the findings presented in this thesis.

The relative expression of CCR9 and β7 on lamina propria DC may determine a gradient of their preferential homing to the small or large bowel reflecting the gradient in the composition of the microbiota between the small and large bowel. Further studies, designed to assess the relative co-expression of β7 and CCR9 on tissue DC in the ileum, non-inflamed and inflamed pouch of UC patients are necessary. Furthermore, transwell migration studies are required to assess the migratory affinity of β7CCR9+ DC to their respective ligands depending on their relative proportions found in different patient groups as well as the effects of biopsy supernatants from the different patient groups.

In addition, future studies should assess the functional consequences of the DC phenotype in the UC ileum and normal ileum and in UC patients with and without pouchitis and FAP patients. Mixed leucocyte reaction experiments are needed to ascertain differences in T cell responses as

a consequence of the DC phenotypes demonstrated by this data. B cell properties and IgA levels should also be assessed in future studies.

We demonstrated the development of a methodology using flow cytometry to quantitatively assess tight junction protein expression on epithelial cells. In the process of this methodological development novel findings regarding expression of tight junctions were noted on blood dendritic cells and further studies of tight junction protein expression in peripheral blood dendritic cells should be undertaken in health and in IBD.

The distribution and co-localisation of tight junction proteins are likely to be critical with regard to their effects on barrier function and measures of epithelial barrier function were not assessed here. Further studies using immunohistochemistry in addition to quantitative analyses are necessary to assess tight junction protein distribution. Further assessment with electron microscopy to assess structural alterations of the apical junctions and assessment of trans-epithelial electrical resistance would contribute to characterisation of the changes in barrier function resulting from the altered tight junction protein expression demonstrated by our data. Clinical data pairing tight junction protein expression with intestinal permeability using lactulose-rhamnose tests in patients are also required.

The unique opportunity afforded by the pouch to assess the temporal inter-relationships between defective barrier function, abnormal immune responses and the microbiota require further study with functional assessments and correlation with data regarding the microbiota

using molecular techniques. This work also requires comparative study to a cohort of FAP patients undergoing RPC.

Treatment with VSL#3 immediately following ileostomy closure is effective in the prevention of the onset of acute pouchitis (Gionchetti et al., 2003). Further work assessing changes in barrier function and immune responses as a consequence of probiotic therapy post ileostomy closure should be undertaken to assess the critical factors in development of pouchitis. In addition, larger and longer term studies are necessary to establish molecular markers that may be predictive of poor outcome when assessed either prior to ileostomy closure or at six months post ileostomy closure to enable the identification and early management of at risk patients.

The data regarding the study of FMT raise a number of questions that should be addressed in future studies. The optimal protocol of FMT in inflammatory bowel disease requires further definition as well as the group of patients or phase of disease in which this therapy is likely to be most effective. Increasing data suggest that strategies to alter the microbiota in IBD may be most effective in quiescent disease. Future studies should consider the role of FMT in "resetting" the colitogenic potential of patients' microbiota following induction of remission and resolution of the inflammatory responses.

Donor microbiota used in this study may not be applicable to the niche of the UC pouch and perhaps FAP RPC or UC RPC patients without a history of pouchitis would be a more appropriate donor in this context. This option may raise considerable ethical and medical

concerns as the long-term sequelae of FMT are unknown and protocols to date ensure only healthy individuals are recruited as donors.

Therefore as a preliminary study, in vitro work to assess the immunological effects of bacterial and mucos

al supernatants from FAP and UC patients without pouchitis are needed to assess their potential as donors as well as the luminal micro-environmental factors that may differentiate these patients immunologically. Further in vitro and animal work is also necessary to assess the immunological effects of defined mixtures of specific bacteria that are most likely to re-establish a health-associated microbiota as has been suggested for *Clostridium difficile* (Lawley et al., 2012).

Throughout this thesis we have stated the importance of the interactions between the intestinal microbiota and host immune responses in health and disease. Inflammatory bowel diseases are considered to be due to an inappropriate inter-relationship between the immune responses and intestinal microbiota and studies of the aetiology of these diseases enhance our knowledge of host-microbiota relationships. The ileal pouch offers a unique opportunity to study the inter-relationships between the gut microbiota, epithelial barrier and host immune responses from before the onset of disease and serves as a human model that significantly enhances our understanding of inflammatory bowel diseases in general. Further studies of the ileal pouch will most likely lead to a greater understanding of host immune-microbiota interactions and opportunities to manipulate these relationships for the prevention and treatment of pouchitis and other inflammatory bowel diseases.

Publications (related to the thesis)

Landy J et al. Variable alterations of the microbiota without metabolic or immunological change following fecal microbiota transplantation in patients with chronic pouchitis. (*In submission*)

Landy J, Al-Hassi HO, Ronde E, English NR, Mann ER, Bernardo D, Ciclitira PJ, Clark SK, Knight SC, Hart AL. Innate immune factors in the development and maintenance of pouchitis. Inflamm Bowel Dis. 2014 Sep 12. [Epub ahead of print]

Landy J, Hart AL. The microbiome in inflammatory bowel disease and beyond. *Clin Med*. December 2013 13:s29-s31

Mann ER, Bernardo D, Ng SC, Rigby R, Al-Hassi HO, Landy J. Human gut dendritic cells drive aberrant, gut-specific T-cell responses in ulcerative colitis, characterised by increased IL-4 production and loss of IL-22 and IFNγ. Accepted for publication in Inflammatory bowel diseases 2014

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The effects of faecal microbiota transplantation on the innate immune system and epithelial tight junction expression in chronic refractory pouchitis. Oral presentation DDF, Glasgow June 2013.

Alteration in epithelial and dendritic cell tight junction proteins expression in the ileo-anal pouch following ileostomy closure. Oral poster presentation ECCO Congress Barcelona 2012.

Management of Chronic Pouchitis and Current Research. Ileo-anal Pouch Advanced Master class. St Mark's National UK Pouch study day. July 2011 +2012.

Faecal transplantation for chronic pouchitis. St Mark's Association Day 2011.

Pouchitis. Red Lion Group (Patient group). AGM. April 2011.

The aetiopathogenesis of pouchitis- new therapeutic opportunities. St Mark's Grand Round. 2011

Awards and Travel Grants

British Society of Gastroenterology poster of distinction for poster presented at BSG 2013

United European Gastroenterology poster of excellence for poster presented at UEGW 2012

United European Gastroenterology Travel Grant awarded for abstract presented at UEGW 2012

Highly commended abstracts presented at ECCO congress 2012, Barcelona.

Synergy/Shire SpR Innovation Fund Travel Bursary awarded for abstract presented at ECCO 2012

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