UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE

Division of Infection, Inflammation and Immunity

The role of Intestinal Tissue Macrophages in the Pathogenesis of Inflammatory Bowel Disease

By

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PhD Thesis

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ABSTRACT

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THE ROLE OF INTESTINAL TISSUE MACROPHAGES IN THE PATHOGENESIS OF INFLAMMATORY BOWEL DISEASE

Suranga Dharmasiri

The inflammatory bowel diseases, Crohn's disease (CD) and Ulcerative colitis (UC) are chronic relapsing remitting inflammatory diseases of the gastrointestinal tract. Over the last 20 years significant advances in the scientific understanding of these complex diseases has been made and several new pharmacological therapies with new mechanisms of action are now available. It is accepted that the inflammatory bowel diseases result from a complex interaction between environmental factors, host genetics, the immune system responses and the intestinal microbiota. Evidence from genetic, animal and human studies have identified a key role for the innate immune system in the pathogenesis of these diseases. Although several mouse studies have implicated mechanisms by which intestinal macrophages may be involved in inflammatory bowel disease pathogenesis, data from humans with the disease is sparse. In this study we have recruited patients with ulcerative colitis and colonic Crohn's disease as well as a group of health controls. In these patients we have isolated intestinal tissue macrophages and subjected the RNA from these cells to high throughput RNA-Seq. We have demonstrated that the gene expression profiles of the IBD macrophages are profoundly altered. We have found that in IBD patients the macrophages promote inflammation, recruitment of T-cells through the release of chemokines such as CXCL9 & CXCL10 and that cellular metabolic process are down regulated in these cells. We were able to show that although the macrophages from UC and CD have similar gene expression there are important differences such as expression of the M2 phenotype, up-regulation of pathways associated with fibrosis and granuloma formation in CD but not the UC macrophages.

Table of Contents

List of Tables

List of Figures

DECLARATION OF AUTHORSHIP

I, Suranga Dharmasiri .....

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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.....

I confirm that:

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Transcriptomic Profiling of Intestinal Macrophages Isolated from Patients Reveals a Profound Gene Expression Reprogramming Underlying IBD Pathogenesis. S Dharmasiri, E Garrido Martin, F Cummings, T Sanchez-Elsner. Gastroenterology 152(5):S612. Poster Presentation April 2017

Signed: .....

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Definitions and Abbreviations

AEC: Aminoethyl carbazole

ASC: Acute Severe Colitis

5-ASA: 5-aminosalicylic acid

CD: Crohn's disease

CDAI: The Crohn's disease activity index

CT: Computed tomography

DEGs: Differentially Expressed Genes

EDTA: Ethylenediaminetetraacetic acid

ESR: Erythrocyte sedimentation rate

FACS: Fluorescence activated cell sorting

FDR: False discovery rate

FMT: Faecal microbiota transplant

GMA: Glycol Methacrylate

GSEA: Gene Set Enrichment Analysis

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GWAS: Genome-wide association study

HBI: The Harvey-Bradshaw Index

IBD: Inflammatory bowel disease

IBDU: Inflammatory bowel disease unclassified

IHC: Immunohistochemistry

iNOS: Inducible nitric oxide synthase

IPA: Ingenuity Pathway Analysis

LPMC: Lamina Propria Monocytic Cells

LPS: Lipopolysaccharide

M-CSF: Macrophage colony stimulating factor

MRI: Magnetic resonance imaging

mTNF: Membrane bound TNF

NF-κβ: Nuclear factor-kappa B

NO: Nitric oxide

NSAIDS: Non-steroidal anti-inflammatory drugs

PAMPs: Pathogen-associated molecular patterns

PML: Progressive multifocal leukoencephalopathy

PPAR-γ: Peroxisome proliferator-activated receptors

PPRs: pattern-recognition receptors

PSC: Primary sclerosing cholangitis

PCA: Principal Component Analysis

RPKM: reads per kilobase transcript length per million mapped reads

SNP: Single nucleotide polymorphism

sTNF: soluble TNF

TACE: TNF-alpha converting enzyme

TLRs: Toll Like Receptors

TNBS: 2,4,6-trinitrobenzene sulfonic acid

UC: Ulcerative colitis

Chapter 1: Introduction

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) consists of two chronic disorders, Ulcerative colitis (UC) and Crohn's disease (CD). In some instances, CD and UC cannot be differentiated and the diagnosis IBD unclassified (IBDU) is given. These conditions are incurable, can affect individuals at any age and have a significant burden on patients, their families and healthcare resource.(1) Principally IBD affects the intestinal tract, manifesting in symptoms of diarrhoea, rectal bleeding, abdominal pain and weight loss. These are however systemic diseases, which can also affect the skin, joints, eyes and liver, a phenomenon known as extra intestinal manifestations of IBD

There is evidence that IBD results from the complex interplay between host genetics, the microbiome, immune response and environmental triggers.(2) In recent years a great deal of progress has been made in our understanding of IBD aetiology however the exact mechanisms by which individuals develop IBD remains unknown

1.1.1 Epidemiology of IBD

The incidence and prevalence of IBD varies by geographic region. Molodecky et al(3) analysed 238 studies that reported IBD incidence, prevalence or both. This systematic analysis estimated the incidence of UC in Europe to be 0.6 to 24.3 per 100,000 person years, in Asia and Middle East 0.1 to 6.3 per 100,000 person years and in North America 0 to 19.2 per 100,000 person years. For CD the estimates were, Europe 0.3 to 12.7 per 100,000 person years, Asia and Middle East 0.04 to 5 per 100,00 person years and North America 0-20.2 per 100,000 person years. The prevalence estimates from this study follow a similar pattern with the highest prevalence of IBD in Europe and North America.

In North American and Europe, a north-south gradient has been described with the prevalence greatest in the northern regions decreasing moving southwards on these continents. In Europe the mean incidence for UC in the North is 11.8 per 100,000 compared with that of 8.7 per 100,000 in the south and for CD, 6.3 per 100,000 and 3.6 per 100,000 comparing north and south respectively.(3)

IBD is traditionally considered a disease of Western developed populations. Recent epidemiological studies however show that IBD is present in developing world populations and is becoming more common even in those of Non-European ancestry.(4-7) In the early 1990s Probert et al (8, 9) studied a migrant population of South Asians in Leicester, U.K. They found that this

group of migrants had a higher incidence of UC but a lower incidence of CD than the indigenous European population. The UC that they suffered seemed to be less severe but these migrants had a higher incidence of UC than their country of origin. Further research has shown that the increased risk of UC is most marked in second generation migrants whist the first generation migrants tend to retain the lower risk of their country of origin.(10) These findings imply that environmental exposures during childhood are important in the development of IBD. An established pattern of emerging IBD has been observed in developing countries, UC tends to emerge initially followed by a rising trend in the incidence of CD around 10 years later.(11)

As well as variation with geography, race is also important. The risk of IBD is three fold higher in the Jewish population and particularly high in the Ashkenazi Jewish population.(12) These observations are most likely due to the genetic component in the development of IBD.

IBD can develop at any age, the peak age of onset for CD is reported as between 20-30 years and for UC it is 30-40 years.(13) Some studies suggest a bimodal distribution with a further peak in incidence of IBD between the 6^{th} and 7^{th} decade, although this is not currently widely accepted. (13, 14)

In adults, UC is slightly more common in males and CD is more frequent in females. (15-17) In children suffering with IBD this distribution is reversed with CD being more common in boys and UC more frequent in girls. (13)

Epidemiological studies have consistently found the incidence of IBD to be higher in urban areas compared to those living in rural regions.(18, 19) It has been shown that being born and raised on a livestock farm for the first five years of life lowers IBD risk.(20) There is also evidence that the larger the family size of an individual and the greater the number of siblings they have, the lower their risk of IBD. (14) These findings suggest a role for changes in host microbiota in IBD pathogenesis.

1.2 Clinical Aspects of Inflammatory bowel disease

This section provides a clinical overview of both CD and UC, from presentation through to current and emerging therapeutic options.

1.2.1 Ulcerative Colitis

1.2.1.1 Introduction

Ulcerative colitis is a relapsing remitting disease of inflammation limited to the colonic mucosa. UC was the first subtype of IBD to be identified as a distinct disease entity. Although there are earlier descriptions of UC, Sir Samuel Wilks, was the first to use the term "ulcerative colitis".(21) The disease typically affects the rectum and extends proximally in the colon varying degrees. In some patients there is continuous extension of inflammation (either macroscopic, microscopic or both) into the terminal ileum termed backwash ileitis. This phenomenon occurs in up to 20% of patients and is due to reflux of caecal contents in to the ileum.(22)

The Montreal classification (23) describes ulcerative colitis according to the degree to which the disease extends proximally. Disease that affects the rectum only is termed proctitis (E1), disease extending to the splenic flexure is known as left sided colitis (E2) and disease that extends beyond the splenic flexure is termed extensive colitis (E3). If the whole of the colon is affected the terms "total colitis" or "pan-ulcerative colitis" are often used. In some patients who have a less extensive colitis, a small patch of inflammation in the caecum may be present known as a "caecal patch".(24) The extent of disease is an important factor in the choice of drugs and the frequency of surveillance for cancer, as disease extent is a major determinant of cancer risk.(25)

1.2.1.2 Clinical Presentation

The most common presenting features of UC are frequent diarrhoea associated with rectal bleeding and urgency to defecate. The onset is usually insidious rather than sudden. Nocturnal defecation is often reported by patients and systemic symptoms of malaise, anorexia or fever are markers of severe disease.(22) UC may present with intermittent symptoms which progress over several months. Such patients may present with systemic upset of weight loss and fever.(26) Extra-intestinal manifestations which include; axial or peripheral arthropathy, episcleritis, erythema nodosum and primary sclerosing cholangitis occur in up to 31% of patients.(27)

Active tobacco smoking appears to be protective against the development of UC (28), though smoking does not improve the natural history of the disease when compared to non-smokers.(29)

1.2.1.3 Diagnosis of Ulcerative Colitis

A careful and detailed clinical assessment together with utilisation of appropriate specialist investigations is required to make an accurate diagnosis of UC. The first stage of this assessment is a thorough clinical history, to establish the onset, duration and severity of symptoms. The majority of patients describe visible blood in their stools.(22) An enquiry into systemic upset including weight loss, fevers and extra-intestinal manifestations should be made. A clear family history of IBD adds weight to a suspicion of UC given the heritability associated with UC. (30-32) There are a number of alternative pathologies that can cause colitis and therefore present in a similar fashion to ulcerative colitis. These can be divided in to infective and non-infective causes **(table 1)**. The history must therefore address these alternative pathologies.

A physical examination should be performed and includes measurement of pulse rate, body temperature, body weight and height. The abdomen is examined for tenderness, the oral cavity for aphthous ulceration, the eyes, joints and skin for involvement.

Alternative causes of Colitis

Non-Infective	Infective		
Diverticulitis Eosinophilic gastroenteritis Radiation related Sarcoidosis Bechçet's Disease Non-steroidal anti-inflammatory drugs Vascular –Ischaemic colitis	Viral	Bacterial	Parasitic
	Cytomegalovirus Herpes simplex HIV	Salmonella spp Campylobacter spp Shigella spp Escherichia Coli Yersinia app Gonococci Mycobatcerium tuberculosis Atypical mycobacterium	Entamoeba histolytica Cryptospora spp Isospora spp Trichuris trichura Strongyloids

Table 1 The causes of Non-inflammatory bowel disease that need to be considered prior to making the diagnosis.(26)

A stool sample is sent for microscopy and culture to exclude an infective cause, although this is rare in cases where the diarrhoea has persisted beyond 6 weeks.(33) In addition to a stool test, initial investigations include blood tests for CRP, renal function and liver function tests. In

patients who are presenting with severe symptoms an abdominal x-ray is preformed to assess for toxic megacolon. Increasingly centres are now utilising faecal calprotectin, which is reliable in identifying intestinal inflammation though cannot be used to differentiate between the causes of intestinal inflammation.(34) The blood tests may reveal markers of chronic inflammation and iron deficiency anaemia but in mild cases the blood results may be normal. With the exception of proctitis the CRP generally correlates with disease activity.(35) In order to confirm a clinical suspicion of UC, it is common practice to perform an endoscopic examination of the lower gastrointestinal tract. This should ideally be a full colonoscopy with terminal ileal intubation to ensure that more proximal inflammation and the alternative diagnosis of CD is not missed. In clinical practice however this is not always possible or safe and therefore a flexible sigmoidoscopy is performed to examine the left colon. Endoscopic examination provides objective evidence of UC and allows for biopsies to be taken for microscopic examination. The microscopic features of UC included architectural changes of crypt branching and distortion, mucin depletion and paneth cell metaplasia. In inflamed samples there is increased lamina propria cellularity, basal lymphoplasmacytosis and crypt abscesses.(36)

1.2.1.4 Assessment of disease severity in ulcerative colitis

A number of clinical scoring indices are utilised to measure disease severity. Identifying the severity of disease is important as it informs treatment decisions and determines prognosis. The Truelove and Witts classification (37) published in 1955 remains the most widely used severity index in routine clinical practice. A severe episode is defined as greater than 6 bloody stools a day associated with at least one defined systemic feature **(table 2).** In the UK this severity index is utilised to identify patients suffering severe flares who require inpatient treatment with intravenous steroids and close monitoring.

Activity	Mild	Moderate	Severe
Number of bloody stools per day	<4	4-6	>6
Temperature (^{O}C)	Afebrile	Intermediate	>37.8
Heart rate (beats per minute)	Normal	Intermediate	>90
Erythrocyte sedimentation rate	<20	20-30	>30
Haemoglobin (g/dl)	>11	10.5-11	>10.5

Table 2 Truelove and Witt's Classification for UC severity

The Truelove and Witt's criteria although useful in identifying severe cases, is not quantitative and therefore of minimal use in clinical trials to measure response to treatments and therefore changes in disease activity. The Simple Clinical Colitis Activity Index (38) and the Mayo score (39) are regularly used in clinical trials of UC (**tables 3 & 4**).

Simple Clinical Colitis Index

Variable	Scores				
	0	1	2	3	4
Bowel frequency (day)					
Urgency of defecation					
Blood in stool					
General well-being					
Arthritis, pyoderma gangrenosum, uveitis		Score 1 for each manifestation			

Table 3 Simple Clinical Colitis Activity Index

Mayo scoring system

Stool frequency

0 = Normal number of stools for patient

1 = 1-2 stools more than normal

2 = 3-4 stools more than normal

3= 5 or more stools more than normal for patient

Rectal bleeding

0 = No blood

1 = Streaks of blood

2 = Obvious blood with stool most of the time

3= Blood alone passed

Mucosal appearance at endoscopy

0 = Normal or inactive disease

1 = Mild disease (erythema, decreased vascular pattern, mild friability)

2 = Moderate disease (marked erythema, absent vascular pattern, friability, erosions)

3 = Severe disease (spontaneous bleeding, ulceration)

Physician's global assessment

0 = Normal

1 = Mild disease

2 = Moderate disease

3 = severe disease

Table 4 Mayo Score

1.2.1.5 Disease course and natural history of ulcerative colitis

IBD is a rapidly changing field; our understanding of IBD and the treatment options available for UC have changed significantly over the last two decades. It is therefore difficult to truly give an account of how the disease will behave in the next 20 years given these changes. Our current understanding of the outcomes and natural course of UC is based on historical data where clinical practice and available resources were quite different to the current accepted standards. In addition the term natural history is a misnomer, as it is very rare that the disease is allowed to run a "natural course" without some form of therapeutic intervention. It is however true to say that before the first effective treatments of UC were introduced in the form of corticosteroids and safe surgical practice, the risk of death in an individual presenting with acute severe UC was significant, 33% if presenting for the first time with a 12% risk of death from a further relapse if they survived the initial episode.(40)

Large cohort studies have shown that UC is a dynamic disease in which the disease extent may progress over time. In the Norwegian IBSEN cohort 14% of patients with proctitis progressed to extensive colitis over a 10-year period of follow up after their initial diagnosis.(41) This is consistent with the finding by Langholz et al (42) that over 50% of patients with left sided colitis progressed over a 25 year period to have disease that had extended proximally. The extent of disease influences the clinical course and cancer risk. Patients with extensive disease appear to have greater resistance to first line therapies and may have increased risk of colectomy.(43) The risk of developing colorectal cancer is increased in patients with UC with disease extending beyond the rectum. The risk increases with the extent of the disease.(44-46) An associated diagnosis of primary sclerosing cholangitis (PSC) and a family history of colorectal cancer further increase the risk.(47, 48) For this reason national guidelines recommend endoscopic surveillance approximately 10 years after the onset of disease.(49)

Colectomy rates are often utilised as an endpoint for disease progression, severity and medical treatment failure. Previously colectomy was thought of as a "cure" of UC. It is now recognised that for patients undergoing colectomy surgery, normal bowel function and lifestyle are often not restored. A recent systematic review has demonstrated that between 11-44% of patients experience early complications and around a third of patients suffer long term post operative complications following colectomy surgery.(50) Even when the continuity of the bowel is restored by the creation of an ileal pouch anal anastomosis (IPAA), normal bowel function cannot be presumed to return. UC patients with an IPAA often have increased bowel frequency, incontinence and up to 70% developed pouchitis over a 20-year follow up.(51) There are also a

number of long-term extra-intestinal complications that patients suffer with following colectomy including depression, issues with body image and sexual dysfunction.(52)

A number of factors have been investigated as possible factors influencing the course of UC. The existence of a family history of IBD (either CD or UC) is a risk factor for developing UC and those with a family history seem to have an earlier documented onset of disease. The clinical course and outcomes of UC however are not affected by the presence of a family history.(53, 54) There is evidence that appendectomy has a protective role in UC reducing the risk of developing the disease and in those already with the disease the severity and frequency of recurrence may be reduced by appendectomy.(55-57)

Development of acute severe colitis (ASC) is an adverse prognostic marker.(43) Data from a large tertiary centre suggests that around a quarter of all patients with UC develop at least one episode of ASC, 20% have a colectomy on the first admission and for patients admitted with a second episode of ASC the rate of colectomy was 40%.(58) Patients with ASC who do not respond early in their admission to intravenous corticosteroids are at higher risk. Travis et al identified that at day three of treatment with intravenous corticosteroids, patients with a CRP >45 mg/L or passing greater than eight stools a day were at high risk of colectomy.(59) This has led to the development of medical rescue therapeutic strategies in an effort to avoid colectomy in these patients.(60)

The presence of extra-intestinal manifestations of UC is associated with a greater extent of disease and a worse disease course.(61, 62)

1.2.2 Crohn's Disease

1.2.2.1 Introduction

Crohn's disease is a chronic relapsing remitting disease characterised by trans-mural inflammation of the gastrointestinal tract. CD can have a significant negative impact of the quality of life for those affected.(63) Any part of the gastrointestinal tract from the oral cavity to the anus may be involved. Generally the disease affects the gastrointestinal tract in a patchy manner with healthy tissue interspersed between active lesions. The trans-mural (full intestinal wall thickness) nature of the inflammation can lead to complications such as the development of strictures, fistulae and perforations leading to abscess formation. CD is a lifelong systemic disease in which patients may experience extra-intestinal manifestations that affect the joints, eyes, skin and liver.

In CD, the ileum and colon are the most commonly affected areas.(64, 65) The most widely accepted clinical classification system for CD is the Montreal Classification system, in which patients' are classified according to age of onset, disease location and whether the disease has exhibited penetrating or stricturing behaviour **(table 5)**. (23)

Montreal Classification	
Age at diagnosis (A)	
A1	16 Years or younger
A2	17 – 40 years
A3	Over 40 years
Location (L)	
L1	Terminal ileum
L2	Colon
L3	Ileocolon
L4	Upper GI (L4, may be added to L1-3)
Behaviour (B)	
B1	Non-stricturing, non-penetrating
B2	Stricturing
B3	Penetrating Perianal modifier (p) can be added to and behaviour (B)

Table 5 The Montreal classification of Crohn's disease

1.2.2.2 Clinical presentation

The clinical presentation of CD is varied and largely dependent on the site of disease. Patients' with disease limited to the distal colon will present with symptoms of diarrhoea, rectal bleeding and urgency that may be clinically indistinguishable from ulcerative colitis. The most common presenting symptom of CD is chronic diarrhoea.(66) Abdominal pain and weight loss are also common presenting features seen in 70% and 60% of patients respectively.(67) Some patients present acutely with symptoms of acute abdominal pain and/or bowel obstruction due to stricturing or penetrating disease.

In a small number of patients with CD the presenting features are that of isolated perianal disease.(68) These patients present with perianal pain and discharge arising from the development of perianal abscesses and fistulae. The majority of these patients will subsequently go onto develop intestinal CD lesions more proximally in the gastrointestinal tract.(69) In a small proportion of patients extra-intestinal features predate the development of any gastrointestinal symptoms; these symptoms are commonly related to joint pain. (67)

1.2.2.3 Diagnosis of Crohn's disease

There is no gold standard test or single clinical diagnostic feature of CD. The diagnosis therefore is dependent on a careful thorough history, physical examination and specialist investigations to provide evidence to support the clinical suspicion of the diagnosis. This is not a perfect process, a change in diagnosis from UC to CD occurs in approximately 3-14% of patients in the five years after diagnosis.(70)

The clinical history should clarify the onset and frequency of symptoms, recent travel and medications particularly the use of non-steroidal anti-inflammatory drugs (NSAIDs) or antibiotics. Exposure to antibiotics and use of NSAIDs increases the risk of development of CD.(71, 72) The risk of CD is elevated in those with a family history of IBD (73-76) and therefore the presence of family members with IBD supports the clinical diagnosis. An honest smoking history is essential as there is clear evidence that smoking increases the risk of development of CD which is independent of genetic susceptibility.(77) A concerted effort is made to elicit a history of infective diarrhoea, not only as this is an alternative diagnoses and can mimic CD (78), but patients with a history of infective gastroenteritis are at increased risk of developing CD.(79, 80) A full surgical history is important, previous CD lesions may have been resected without the underlying diagnosis being appreciated and patients with a previous history of appendectomy are at increased risk of developing CD.(81)

At the time of initial presentation a general physical examination is performed that includes measurement of the weight, pulse, blood pressure and temperature. The oral cavity is inspected for aphthous ulcers. The abdomen is examined for evidence of tenderness and abdominal masses, particularly in the right iliac fossa where a terminal ileal inflammatory mass may be palpable. The joints, skin, eyes and perianal area are inspected for involvement.

The initial laboratory tests that are sent include; a full blood count, CRP, erythrocyte sedimentation rate (ESR), renal function and liver function tests. These tests can all be normal in CD. The CRP does however generally correlate with disease activity in CD.(82, 83) The full blood count may show evidence of iron deficiency anaemia and a raised platelet count. Faecal calprotectin concentrations can be used to identify the presence of intestinal inflammation in CD (84), although not a diagnostic test, it is useful in identifying patients who merit further investigation and also as a tool for monitoring disease activity in those with an established diagnosis of CD.(85) At the time of the initial presentation, stool samples are also sent for microbiological assessment to exclude an infective cause for the symptoms.

It is routine practice to perform an ileocolonoscopy in patients with suspected CD. This allows direct visualisation and biopsy of lesions for histological assessment. In the majority of individuals most disease can be reached by ileocolonoscopy. The classical endoscopic appearances of CD are discontinuous involvement, deep ulcers and cobble stoning. A variety of microscopic features of CD have been identified, the key features however are, focal or patchy inflammation, crypt distortion and the presence of granulomas.(67) The presence of granulomas (aggregates of macrophages cells) is useful in distinguishing CD from UC, although they are not always present in CD.

In some patients, the disease cannot be accessed by ileocolonoscopy. This may be due to strictures, patient discomfort, technical issues or that the disease is too proximal in the small bowel to be reached by a colonoscope. In these cases or when there is clinical suspicion of proximal small bowel disease radiological imaging can be utilised. Cross sectional imaging in the form of computed tomography (CT) and magnetic resonance imaging (MRI) have largely superseded the previously much utilised dynamic barium fluoroscopic techniques.(86) More recently ultrasound is increasingly being utilised in the assessment of CD.(87) Ultrasonography, CT and MRI all appear to be accurate in the evaluation of suspected CD and disease activity.(88) Ultrasonography and MRI have the advantage of not utilising ionising radiation. This is an important consideration given that CD is a chronic disease and these patents are likely to have several radiological investigations over a lifetime. Ultrasonography is however user dependent and its accuracy lower for proximal disease. MRI is therefore often the modality of choice when

available. In addition to identifying the mucosal lesions of CD, radiological assessment is vital for identification of the complications of CD such as fistulisation, abscess formation and stricturing disease. In cases where the affected segments of bowel are beyond the reach of a standard colonoscope, direct visualisation can still be accomplished with either wireless capsule enteroscopy or push enteroscopy. Upper gastrointestinal disease is assessed by gastroduodenoscopy. In clinical practice, radiological and endoscopic techniques are used as complementary techniques in the assessment of patients with suspected and established CD.

1.2.2.4 Assessment of disease activity in CD

In the treatment of patients with CD a balance must be struck between ensuring that patients are treated aggressively to prevent future complications whist not exposing patients to unnecessary therapy. It is important that patients are not given therapy where the benefits are outweighed by the risks. In order to achieve this balance treatment must be matched to the disease severity.

A number of CD activity indices have been validated and are utilised particularly in pharmacological clinical trials. The Crohn's Disease Activity index (CDAI) is a well established scoring system that combines symptoms over a 7 day period with basic laboratory investigations to score the severity of disease between 0 and 600.(89) Disease is graded depending on this score, a score of 120-220, 220-450 and greater than 450 is described as mild, moderate and severe disease respectively.(67) Changes in score are used to describe relapses and response. The CDAI score does have some important limitations, it is not accurate for patients with fistulising or stricturing disease, irrelevant for patients with stomas and does not include a component for mucosal inflammation.(90) A co-diagnosis of irritable bowel syndrome can therefore artificially elevate the CDAI score. The CDAI score is time consuming and not suitable for everyday clinical practice. The Harvey-Bradshaw Index (HBI) (91) which only requires one day of symptom diary is more suited to clinical practice and correlates well with the more complex CDAI score.(92) The HBI consists of 5 variables which are each individually scored as per **table 6*,*** remission is classed as a score less than 5, mild disease 5-7, moderate disease 8-16 and a score of > 16 is considered severe disease activity. The CDAI and the HBI however poorly correlate with mucosal disease activity.(93, 94) Endoscopy is the gold standard for the assessment of mucosal lesions of CD. Several endoscopic scoring indices have been validated including the Crohn's Disease Endoscopic Index of Severity (95) and the Simple Endoscopic Score for Crohn's Disease.(96) These scores are generally utilised alongside the CDAI or HBI in clinical trials but rarely in routine clinical practice. A pragmatic approach is to simply classify patients according to the presence or absence of ulcers at endoscopy.(97)

In recent times particularly with regard pharmacological IBD clinical trials, patient reported outcomes are gaining prominence and are now a requirement for drug approval.(98) These patient reported outcomes in IBD clinical trials so far have focused on quality life indices such as the Inflammatory Bowel Disease Questionnaire. (99)

There is no single biomarker than can be used to assess disease severity in isolation. The two major biomarkers utilised for monitoring disease activity are CRP and faecal calprotectin. The CRP is a non-specific marker of inflammation, though there is inter-individual variation in CRP levels and the CRP may be normal even during a flare.(83, 97)

The historical behaviour of an individual's disease is an important consideration when assessing disease severity. Patients with penetrating disease, flares requiring admission to hospital for treatment, previous requirement for surgical intervention, with extra-intestinal manifestations involving 2 or more systems or poor response to currently available therapy have been defined as having aggressive disease.(100)

In practice, clinicians take a composite approach, using a combination of clinical and endoscopic scores, patent reported symptoms, biomarkers and disease history to make a clinical judgement of disease severity in each individual patient to guide therapy for that individual.

Harvey-Bradshaw Simple Index	
Variable	**Score**
General well being	0 = Very well 1 = Slightly below par 2 = Poor 3 = Very poor 4 = Terrible
Abdominal Pain	0 = None 1 = Mild 2 = Moderate 3 = Severe
Number of liquid stools daily	Score 1 per occurrence
Abdominal mass	0 = None 1 = Dubious 2 = definite 3 = definite and tender
Complications	Score 1 per item • Arthralgia • Uveitis • Erythema nodosum • Aphthous ulcers • Pyoderma grangrenosum • Ana fissure • New fistula • Abscess

Table 6 Harvey-Bradshaw index

1.2.2.5 Disease course and natural history of Crohn's disease

Crohn's disease like UC is a chronic relapsing remitting disease. CD however is a destructive condition causing structural and anatomical damage. Overtime the disease appears to be progressive though this progression may be altered by medical therapy.(101)

The age at which the disease develops has a significant bearing on the disease course. The earlier the disease onset the more severe the disease, the more complications patients experience and the disease tends to be more extensive in distribution with upper GI involvement being more common in those presenting at a young age.(102-107) A severe disease course is defined as one complicated by one of: stricturing, steroid dependency, the need for surgery, the requirement for steroid treatment at the time of diagnosis, extensive small and/or large bowel disease, perianal disease or the presence of extra-intestinal manifestations.(107, 108)

The majority of patients at initial diagnosis have ileo-colonic disease (40-50%), around 30% will have isolated small bowel disease and in a further 30% only the colon is affected.(109) A change in the anatomic distribution of the disease is rare, only 15.9% patients in a cohort of 125 patients followed up for 10 years experience a change in their disease location.

Population based studies have shown consistently that the majority of patients with CD will require surgery at some point in their lives, the cumulative probability of surgery was 61% after 10 years and 82% after 20 years in the Copenhagen County Cohort.(110) Within 1 year of surgery Rutgeerts et al showed that 73% of patients had recurrent lesions that were visible endoscopically although only 20% of these patients had symptoms at this point.(111)

1.2.3 Summary of the clinical differences between CD and UC

The clinical presentation for CD and UC can be very similar and clinically difficult to differentiate between the two diseases. The clinical features that are utilised to make this differentiation are summarised in **Table 7.**

Key Feature	Crohn's disease	Ulcerative Colitis
Distribution	Any part of the intestinal tract from the oral cavity to the anus	Involves the rectum and colon only
Histological findings	Transmural inflammation, presence of granulomas	Mucosal and sub-mucosal inflammation polyomorphonuclear cells aggregate
Endoscopic findings	Discontinuous lesions, strictures, linear ulcerations	Continuous lesions from the rectum
Fistula formation	Common	Uncommon
Peri-anal involvement	Common	Uncommon

Table 7 A comparison of the clinical features of CD and UC

1.3 Pharmacological Therapy in IBD

There have been significant advances in medical management of patients with IBD over the last 30 years, driven by pharmaceutical advances beginning in the late 1990s with the use of anti-TNF alpha medications (112). Prior to this point the therapeutic options for these patients were limited to surgery, steroids, aminosalicylates and immunomodulators such as azathioprine. This section provides a detailed description of the established and emerging drugs used for treating inflammatory bowel disease. The drug section shows that whist the number of therapeutic options and mechanistic targets have greatly increased in recent years no drug at present directly targets intestinal macrophages.

1.3.1 Aminosalicylates

The parent drug sulphasalazine, a combination of sulfapyridine and 5-aminosalicylic acid linked by an azo bond has been used for over 40 years in the treatment of IBD. The discovery that the active component of sulphasalazine was the 5-aminosalicylic acid (5-ASA or mesalazine) and that the sulfapyridine only acted as a carrier molecule(113) yet was the major cause of the associated side-effects led to the development of numerous topical (rectally administered) and oral 5-ASA based formulations.(114) Although these different preparations all essentially contain the same active ingredient, mesalazine, they have been engineered to have various different release mechanisms. Some of these drugs are time release drugs, such as pentasa whilst others are pH release dependent such as asacol and salofalk.

1.3.1.1 Mechanism of action

Despite these drugs being used for a number of years in IBD the exact mechanism of action is still debated. A number of potential mechanisms of action have previously been purposed for 5-ASA drugs. These mechanisms include disruption of inflammatory pathways through the inhibition of cyclo-oxygenase, lipoxygenase, platelet-activating factor, interleukin-1 and nuclear factor-kappa B (NF-κβ).(115) A direct role on intestinal epithelial cells by inhibition of apoptosis and cell injury by oxidative stress has also previously been proposed and investigated.(116, 117)

The current evidence, from both human and animal studies suggests that the anti-inflammatory effects of 5-ASA medications are mediated through the activation of peroxisome proliferator-activated receptors (PPAR-γ).(118)

1.3.1.2 Role and use in inflammatory bowel disease

The 5-ASA drugs are the first line drugs for the treatment of mild to moderate UC, they are used both for induction and maintenance of remission.(119) In UC the route of delivery should be matched to the disease extent in order to achieve satisfactory drug levels in the affected parts of the colon. In distal disease topical preparations of 5-ASA are used in preference, suppositories for isolated proctitis and enemas for left sided disease. The rectal route has been proven to be effective in the induction of remission in UC.(120) Topical 5-ASAs are more effective than topical steroids and oral 5-ASA alone for proctitis.(121-123) Combination of oral and rectal 5-ASA is more effective than either route alone in the treatment of left sided UC that does not extend beyond 50cm from the anal verge.(124) Therefore, current guidelines recommend combine oral and topical mesalazine as first line therapy for mild to moderately active left sided UC.(119) In clinical practice sulphasalazine is rarely used despite being as efficacious as mesalazine because the newer 5-ASA drugs have a better side effect profile.(118, 125)

Once daily dosing of 5-ASA has been shown to be at least as effective as multiple dosing regimes, patients given once daily 3g of mesalazine achieved a clinical remission rate of 86% Vs 71.8 % ($p=0.0298$) in the group given 1g mesalazine three times a day. (126) It has been proposed that 5-ASA medications have a chemo-preventative role against colorectal cancer (127), the data is conflicting with two meta-analyses providing opposing results, long term 5-ASA use in UC is however recommended for maintenance of remission.(128)

The role of 5-ASAs in the treatment of CD is more controversial. A meta-analysis has demonstrated that 5-ASA medications are no better than placebo in inducing remission in CD.(129) A small benefit of 5-ASA medications in preventing relapse following surgically induced remission has been demonstrated, however as the effect is modest, this treatment strategy is only valid in patients who cannot be treated with immunosuppressants.

1.3.2 Corticosteroids

The anti-inflammatory properties of corticosteroids are utilised in a number of chronic inflammatory disorders including chronic obstructive pulmonary disease, asthma and rheumatoid arthritis. Although fast acting, the systemic use of corticosteroids is limited by both short term and long-term serious side effects.

1.3.2.1 Mechanism of Action

Corticosteroids are potent inhibitors of T-Cell activation and pro-inflammatory cytokines. Corticosteroids interact with the cytosolic glucocorticoid receptor (GR), which belongs to the

nuclear receptor superfamily.(130) This allows the formation of a homodimer of two activated GRs, which is then transferred into the nucleus of the cell. This in turn leads to binding to specific sequences of the DNA, inhibiting the promotor regions of pro-inflammatory genes such as NF-κβ and activator protein-1 which are important transcription factors for several pro-inflammatory cytokines.(131)

1.3.2.2 Role and use of corticosteroids in IBD

Oral, rectal and intravenous steroids are used in the treatment of both CD and UC. Their role in IBD is as inducers of remission. Corticosteroids should not be used in long-term management strategies because of potential serious long-term side-effects associated with chronic use.(132) The efficacy of oral steroids in the form of oral prednisolone has been well established for a number of years for the induction of remission in mild to moderate UC.(133) Oral prednisolone is also effective in the induction of remission of CD(134) though the majority of patients who are treated with oral prednisolone for the first time are not in remission a year later.(49)

Oral Budesonide is an alternative option to oral prednisolone. Budesonide undergoes extensive first pass hepatic metabolism and as a consequence is associated with fewer systemic side effects when compared to prednisolone.(132, 135) Budesonide is better than placebo in the treatment of mild to moderate UC though the effect size is small.(135, 136) Budesonide does however appear more effective in CD though it is still less effective than either oral prednisolone or azathioprine.(137, 138)

Rectally administered corticosteroids in the form of suppositories, foam or liquid enemas are regularly utilised in the treatment of proctitis and left sided colonic inflammation. In UC they have been shown to be effective add on therapy in addition of topical 5-ASA but they are less effective than topical 5-ASA alone.(122, 139) The intravenous route for steroids is primarily utilised for patients presenting with acute severe colitis and those in which the enteral route is not appropriate.(140)

1.3.3 Thiopurines

The thiopurines; azathioprine, 6-mercaptopurine and to a lesser extent thioguanine are used extensively in the treatment of IBD primarily for maintenance of remission and more recently as a method for reducing antibody formation to biologic therapies.

1.3.3.1 Mechanism of action of thiopurines

Azathioprine is a pro-drug that is metabolised in the liver to release mercaptopurine and 6-thioguanine. Mercaptopurine undergoes further metabolism to either the inactive inosine-triphosphate or 6 thioinosine-5' monophosphate which inhibits nucleic acid synthesis and is therefore cytotoxic.(141) A key mechanism for the immunosuppressive effects of thiopurines is the induction of T-cell apoptosis though the inhibition of Rac1 target genes and therefore inhibition of adaptive immune system activation.(142)

1.3.3.2 Role and use of thiopurines in IBD

Thiopurines have proven efficacy in the induction of remission of CD (143, 144) and in UC, azathioprine has been shown to be more effective than 5-ASA in the induction of remission after 6 months of treatment.(144) In clinical practice however thiopurines are not utilised as induction agents as the time for their peak onset of action is long, with response rates increasing up to 17 weeks after initiation. The major role of thiopurines in IBD therapy is the maintenance of remission.(145, 146)

1.3.4 Methotrexate

Methotrexate is an antimetabolite drug that was originally conceived as an anticancer medication. Methotrexate in lower doses has also been used extensively for a number of years in the treatment of autoimmune conditions such as Wegener's disease, psoriasis, and rheumatoid arthritis. Methotrexate is used less extensively in IBD despite evidence of its efficacy and favourable cost comparisons with newer biological agents.(147)

1.3.4.1 Mechanism of action of methotrexate

The principle cytotoxic action of methotrexate arises from the inhibition of the enzyme dihydrofolate reductase.(148) The anti-inflammatory effect of low dose methotrexate seen in IBD is not mediated through cytotoxic effects but the inhibition of other folate dependent enzymes. Long-term low dose methotrexate leads to the accumulation of adenosine, which is lymphotoxic and has anti-inflammatory effects such as inhibition the pro-inflammatory cytokine IL-1 by blocking binding to the receptor, increasing IL-2 production, decreasing production of IL-6, IL-8 and the leukotriene B4.(149, 150)

1.3.4.2 The role and use of methotrexate in IBD

Methotrexate is used in a similar way to thiopurines in IBD. Randomised control trials and meta-analysis have confirmed that methotrexate is effective both in the induction and maintenance of

remission in CD.(151, 152) The evidence for the role in UC is less clear, small studies have shown some benefit(153, 154), however systematic review, which assessed low dose oral methotrexate (12.5mg/week or 15mg/week) suggested no benefit.(155, 156) The studies that have demonstrated efficacy of methotrexate in CD have used intra-muscular or sub-cutaneous methotrexate at a dose of 25mg. It may be that the lack evidence of efficacy for methotrexate in UC is due to lack of studies with appropriate dosing and route of delivery rather than a true lack of efficacy of the drug in UC.

1.3.5 Biological agents

Over the last 20 years major advances in the treatment of IBD has been driven by the development of biological agents. Biologics are medicinal products that have been manufactured or derived from a natural biological living system. They included hormones such as insulin, blood factors, antibodies and vaccines. The biological therapies approved for IBD are antibodies targeting particular molecules that have a role in propagating inflammation. The first biological therapy approved for the treatment of IBD was anti-TNF alpha medication.(112) Although the number of drugs approved targeting TNF alpha increased, this remained the only target of biological therapy in IBD for a number of years. More recently several other targets have been investigated. Anti integrin and anti IL12/23 biological therapies have recently been approved for the treatment of IBD.(157-159)

1.3.5.1 Anti-TNF alpha medication

There are currently four anti-TNF drugs that have been approved for treatment of IBD, infliximab, adalimumab, golimumab and certolizumab although the latter has not been licensed for use in Europe.(160)

1.3.5.1.1 Mechanism of action of anti-TNF alpha medication

TNF alpha is a cytokine that plays an important role in the activation of inflammatory pathways in IBD. It is well established that TNF-alpha levels are increased in the serum, stool and mucosa of patient with active IBD.(161-163) TNF alpha is a protein that is mainly secreted by activated macrophages and T-lymphocytes. It is initially produced as a transmembrane protein. The extracellular domain is subsequently cleaved off by TNF-alpha converting enzyme (TACE) to generate soluble TNF. Therefore two forms of TNF exist; membrane bound TNF (mTNF) and soluble TNF (sTNF). Both forms of TNF alpha can bind to the TNF alpha-receptor 1 or 2.(164) This leads to activation of a number of inflammatory pathways including the release of IL-1, IL-6 and interferon-γ.(165) It was initially believed that anti TNF medications acted by neutralising TNF

alpha and therefore preventing the activation of these pro-inflammatory pathways. Further experimental investigation however has revealed that this is unlikely to be the mechanism by which anti TNF alpha therapy has its therapeutic effects. Animal experiments have demonstrated that TNF alpha plays an important role in wound healing, and could be required to suppress intestinal inflammation.(166) The evidence now indicates that the clinically active anti-TNF alpha medications exert their anti-inflammatory effect through the inhibition of mTNF signalling to induce T-cell death. This is supported by the finding that patients with high levels of mTNF positive cells in the mucosa responded significantly better to anti TNF alpha therapy than patients with low amounts of mTNF positive mucosal cells.(167)

1.3.5.1.2 The role and use of Anti TNF alpha medication in IBD

Anti TNF alpha medications are effective in both the induction and maintenance of remission of CD and UC.

1.3.5.1.2.1 Crohn's disease

The first anti TNF alpha drug proven to have clinical efficacy in IBD was infliximab, a chimeric IgG1 antibody to TNF alpha. Infliximab consists of human constant and murine variable regions.(168) Targan et al performed the first randomised placebo control study of Anti TNF alpha therapy in CD demonstrating that 4 weeks after a single dose of infliximab 41% of the treated patients had a clinical response compared with 12% in the placebo group ($p=0.008$).(169) The ACCENT-1 study subsequently demonstrated infliximab to be more effective than placebo in the maintenance of remission in CD patients who had achieved remission with infliximab therapy, 39% of infliximab treated patients were in remission compared with 21% in the placebo group ($p=0.003$) at week 30.(170) Similarly the CLASSIC 1 trial demonstrated the efficacy of the subcutaneously delivered adalimumab in the induction of remission of CD, 36% of treated patients in remission Vs 12% in the placebo group ($p=0.001$) at week 4.(171) The CHARM study established the role of adalimumab in maintenance of remission in CD, 36% in the adalimumab treated group Vs 16% in the placebo group ($p<0.001$) at 52 weeks.(172).

Certolizumab is a fully humanised monoclonal antibody to TNF alpha that has been approved for use in CD in the America, Canada and Switzerland, though has not gained a licence for use in IBD in Europe.(173) The efficacy of certolizumab in CD induction and maintenance however has been well demonstrated in the PRECISE I and II studies.(174, 175) At the time of writing no study has yet been published testing the efficacy of Golimumab in CD.

The ACCENT-2 study investigated the role of infliximab in fistulising CD, in this study infliximab (5mg/kg) was more effective treatment than placebo for closure of fistulae after 54 weeks of treatment (36% in infliximab group Vs 19% in the placebo group, p=0.009).(176)

The beneficial role for combination therapy of infliximab with thiopurines in CD was demonstrated in the pivotal SONIC study, at week 50 clinical remission was achieved in 74.1% of patients receiving combination therapy compared with 66% of patients in the infliximab monotherapy group and 54.7% in the azathioprine monotherapy group. It is now routine practice to treat patients with CD with combination therapy where possible safely.

1.3.5.1.2.2 Ulcerative Colitis

The ACT I and ACT II studies established the role for infliximab for induction and maintenance therapy in UC. These studies compared the 5mg/kg and 10mg/kg does with placebo. In the ACT I trial, 69% of patients treated with 5mg/kg and 61% of those treated with 10mg/kg showed a clinical response at week 8 compared with 37% in the placebo group ($p<0.001$). The induction rates in the ACT II study were similar. In the ACT I study, patients in the infliximab groups were more likely to have clinical response than the placebo group at the week 54 time point; 45% for 5mg/kg, 44% for 10mg/kg and 20% for placebo ($p<0.001$).(177)

Infliximab is the Anti-TNF agent of choice for rescue therapy in acute severe ulcerative colitis refractory to corticosteroids, two large studies have demonstrated similar efficacy to cyclosporine in this scenario.(178, 179)

As with CD, there appears to be a beneficial role in combination therapy of infliximab with a thiopurine in UC, which was demonstrated in the SUCCESS study.(180)

The ULTRA 1 and 2 studies investigated adalimumab for induction and maintenance of remission in UC respectively. The ULTRA 1 trial compared two induction regimes (160/80mg Vs 80/40mg) with placebo. At week at 8 remission was achieved in 18.5%, 10% and 9.2% of the 160/80mg, 80/40mg and placebo groups respectively. The difference between the placebo group and the 80/40mg group did not reach statistical significance highlighting the 160/80mg induction dose as the most effective regime.(181) The ULTRA 2 study showed a greater superiority of the adalimumab 160/80mg induction therapy over placebo for clinical remission at 8 weeks, 16.5% for adalimumab compared with 9.3% for placebo ($p<0.005$). The ULTRA 2 study also identified the efficacy of adalimumab for maintenance therapy over placebo, at week 52 clinical remission was seen in 17.3% of the adalimumab group Vs 8.5% of those treated with placebo ($p=0.004$).(182) Similar results were achieved in a Japanese cohort of patients.(183)

Golimumab is the most recent anti-TNF drug to be approved for use in UC. The PURSUIT-SC study (184) compared two golimumab induction regimes (200/100mg and 400/200mg) with placebo. The active treatment groups (200/100mg and 400/200mg) had higher rates of clinical response and remission at week 6 compared to placebo. The rates of clinical response were 51.0%, 54.9% and 30.2% ($p<0.001$) in the three groups respectively. The rates of clinical remission were 17.8%, 17.9% and 6.4%, respectively. The PURSUIT-M trial (185), evaluated the efficacy of golimumab as maintenance therapy for UC in those that had responded to induction therapy with golimumab. Two golimumab doses (50mg and 100mg, subcutaneously every 4 weeks) were compared with placebo. The clinical response in the 50mg, 100mg and placebo groups were 47.0%, 49.7% and 31.2% respectively after 54 weeks of treatment (both groups compared to placebo reached statistical significance).

1.3.5.2 Anti-integrin medications

There are currently two anti-integrin medications licenced for use in IBD (Natalizumab and Vedolizumab) with further agents undergoing clinical trials. Natalizumab the humanised antibody against the α_4 integrin is licensed for use North America but not Europe, due to concerns regarding the risk of infection with JC virus leading to progressive multifocal leukoencephalopathy (PML). This increased risk is most likely due to natalizumab blocking both $\alpha_4\beta_1$, which plays an important role in T cell homing to the central nervous system, in addition to the gut specific $\alpha_4\beta_7$. Vedolizumab targets the $\alpha_4\beta_7$ heterodimer only and is therefore a gut specific drug.

1.3.5.2.1 Mechanism of action

It is widely accepted that T lymphocytes play an important role in the development and propagation of intestinal inflammation in IBD.(186) Integrins are secondary adhesion molecules that are important in for the migration of lymphocytes from the blood stream into the gastrointestinal mucosa. Integrins expressed on the cell surface of lymphocytes bind specific ligands called addressins on the endothelium. The $\alpha_4\beta_1$ integrin binds to vascular adhesion molecule 1 (VCAM-1) and the $\alpha_4\beta_7$ found on gut homing lymphocytes (187) binds mucosal addressin cell adhesion molecule 1 (MAdCAM-1). It has been observed that in inflamed mucosa $\alpha_4\beta_7$ expressing cells are increased (188) suggesting that MadCAM-1 is up-regulated.(189) Anti-integrin medications target the interaction between T cell integrins and addressins reducing T-cell recruitment to the intestinal mucosa. As the $\alpha_4\beta_7$ integrin is specific to gut homing T-lymphocytes, blocking this molecule reduces the migration of T-lymphocytes into the intestinal mucosa specifically.(190)

1.3.5.2.2 Role and use of anti-integrin therapy in IBD

Natalizumab was the first anti-integrin medication to be investigated in IBD. The first randomised control trial was reported in 2003 by Ghosh et al (191), 248 patients with moderate to severe CD were allocated to one of four treatment arms: two infusions of placebo (0-0mg/kg), one infusion of 3mg/kg of natalizumab followed by placebo (3-0mg/kg), two infusions of 3mg/kg (3-3mg/kg) or two infusions of 6mg/kg of natalizumab (6-6mg-kg). At week 6 the remission rate was 27%, 29% (not significant), 44% (p=0.03), and 21%(not significant) of patients respectively. The response rates defined as >70 point fall in CDAI at week 6 was 38%, 59% (p=0.022), 71% (p<0.001) and 57% (p= 0.039) of patients respectively. The subsequent ENACT(192) study which further assessed induction treatment of natalizumab in CD found the drug was no better than placebo in inducing a clinical response or remission at week 10. A sub-group analysis however did find that in patients with a raised CRP, natalizumab appeared to be better than placebo for remission and response. This was confirmed in the ENCORE (193) study, which only included CD patients with moderate to severe active disease with an elevated CRP. In this study 51% in the active group responded compared with 37% in the placebo group (p= 0.001) at week 8. The role of natalizumab in the maintenance of remission of CD was assessed in the ENACT II (192) study, which took responders from the ENACT I study at week 10 and re-randomised them to receive either placebo or 300mg of natalizumab every 4 weeks. At week 60 response was maintained in 54% of the active group compared with 20% in the placebo group (p<0.01). One patient in this trial died of progressive multifocal leukoencephalopathy (PML). Despite the trial data suggesting that natalizumab is effective in induction and maintenance therapy in CD its use is restricted by the rare but fatal complication of PML. There is no clinical trial evidence for the efficacy of natalizumab in UC.

The GEMINI II (194) study has demonstrated efficacy of Vedolizumab for both induction and maintenance therapy in CD. In this placebo controlled randomised study, 14% of those in the active treatment arm achieved clinical remission compared with 6.8 % in the placebo group (p=0.02). In the maintenance phase patients who had responded to vedolizumab were re-randomised to treatment with either 300mg of vedolizumab every 8 or 4 weeks or placebo. At week 52 the remission rates were 39%, 36% and 21.6% in the placebo group respectively (Both dose groups p value significant compared with placebo).

Vedolizumab has also been studied in UC. The GEMINI I study found Vedolizumab to be effective in moderate to severe UC for both induction and maintenance therapy. Patients entering this study were randomised to either Vedolizumab at weeks 0 and 2 or a matched placebo in cohort 1. In cohort 2 of the study, patients received open label Vedolizumab. At week 6 in cohort 1 the remission and response rates were superior in the Vedolizumab group. For response, 47%

(Vedolizumab) compared with 25.5% (placebo, $p < 0.001$) and for remission 16.9% (Vedolizumab) compared with 5.4% (placebo, $p=0.001$). At week 6, patients from both cohorts who had a clinical response to Vedolizumab were randomised to receive Vedolizumab 8 weekly, Vedolizumab 4 weekly or placebo for up to 52 weeks. At 52 weeks patients allocated to the Vedolizumab groups were more likely to be in remission, 41% (8 weekly), 44.8% (4 weekly) compared with 15.9% of the placebo group ($p<0.001$ for both).

Etrolizumab, a humanised antibody that binds selectively to the β_7 subunit of the heterodimeric intergrins $\alpha_4\beta_7$ and $\alpha_E\beta_7$ has shown efficacy in IBD in phase 2 studies and is undergoing further evaluation in phase 3 clinical trials.(195)

1.3.6 JAK inhibitors

Janus Kinase inhibitors are small molecules that target the Janus Kinase family of which there are 4 members, JAK1-4. The JAKs to varying degrees are involved in intracellular signalling involved in in the release of a number of pro-inflammatory cytokines. A number of JAK inhibitors are currently under development and undergoing clinical trials for both UC and CD. At present only Tofacitinib has been licenced for use in UC only. The OCTAVE 1 and OCTAVE 2 studies demonstrated the efficacy of Tofacitinib in moderate to severe UC compared to placebo both for induction and maintenance of remission in UC.(196)

1.3.7 Summary of pharmacological treatments in IBD

The detailed account of the pharmacological therapeutic options in this section demonstrate that over the last 20 years the pharmacological options to treat IBD have greatly expanded and generally these drugs are used for both UC and CD with the exception of Aminosalicylates which have limited evidence of efficacy in CD (197) are utilised in both diseases.

Ulcerative Colitis	Crohn's disease
Aminosalicylates	Corticosteroids
Corticosteroids	
Methotrexate	Methotrexate
Anti TNF alpha Therapy Infliximab Adalimumab Golimumab	Anti TNF alpha therapy Infliximab Adalimumab
Anti Integrin therapy Vedolizumab	Anti integrin therapy Vedolizumab
Anti IL12/23 therapy Ustekinumab	Anti IL12/23 therapy Ustekinumab
JAK inhibition Tofacitinib	

Table 8 Summary of the current licensed drug treatments for IBD in the U.K

1.4 Aetiology and Pathophysiology

1.4.1 The role of environmental factors

A number of environmental exposures have been postulated as contributing to the development of IBD. This section summarises the evidence supporting the role of environmental exposures in the development of IBD, the mechanisms by which environmental exposures contribute to the development of IBD is at present largely unknown.

1.4.1.1 Tobacco smoking

The association of tobacco smoking with the development of IBD is well established and discordant for the two diseases. Active tobacco smokers are at reduced risk of developing UC where as in CD active smokers and former smokers are at increased risk of developing the disease.(28, 198) Patients with CD who smoke have a tendency towards a more severe disease course and higher risk of flares compared to non-smokers.(199, 200)

1.4.1.2 Appendectomy

Appendectomy for appendicitis or lymphadenitis is associated with a reduced risk of UC when the surgery is carried out before 20 years of age.(201) The risk of CD following appendectomy has previously been observed to be increased (81) This observed risk is at its highest soon after the surgery and falls thereafter and may therefore be explained by diagnostic inaccuracies of acutely presenting CD being mistaken for appendicitis rather than a true association.(202)

1.4.1.3 Breastfeeding

Several studies have reported that breastfeeding protects infants against the development of IBD.(203, 204) A number of theories have been postulated to explain this association. These include acquiring oral tolerance to microflora and food antigens as well as the presence of lactoferrin in breast milk which is absent in formula milk and may have antiviral and antibacterial properties.(205)

1.4.1.4 Diet

There has been much interest in the role of diet in the pathogenesis of IBD, especially given that the affected intestine in IBD has direct contact with food antigens, the geographical variation observed in IBD prevalence and the emerging trend of rising IBD prevalence in areas adopting a western lifestyle. Identifying disease association with diet however is notoriously difficult due to inherent recall bias of retrospective studies. At present there is no convincing evidence of

particular dietary associations with the development of IBD (206) though it is hoped that prospective studies currently in progress will be a able to answer these questions.

1.4.1.5 Antibiotic exposure

Antibiotic exposure, particularly repeated courses in childhood has been identified as a risk factor for the development of IBD in a number of studies.(71, 207, 208). A recent large Swedish cohort study found an association between foetal exposure to antibiotics during pregnancy and early onset IBD they did not however find an association between antibiotic exposure in infants and subsequent early onset IBD.(209) It is likely that the mechanism of this association is antibiotic induced disturbance of the intestinal microbiome, as discussed in more detail in **section 1.4.2**.

1.4.1.6 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory (NSAID) drug use is associated with number of gastrointestinal complications including gastrointestinal ulcers, erosions, bleeding, perforation, small bowel enteropathy and complicated diverticular disease.(210, 211) These complications may mistakenly be diagnosed as IBD however several studies have demonstrated that NSAIDS increase the risk of new onset IBD and can precipitate flares in those with the disease.(212)

A number of potential biological mechanisms for this association have been postulated. The most popular is the effect of NASIDS on prostaglandin synthesis. NSAIDS inhibit the enzyme cyclooxygenase which reduces prostaglandin production which have important roles in mucosal barrier and immune defence.(213)

1.4.2 The role of the intestinal microbiota

The term “intestinal microbiota” refers to the entire population of the microorganisms that inhabit the intestine, including bacteria, fungi, archaea, viruses an protazoans.(214) The intestinal microbiota is the largest reservoir of microbes in the body and plays an important role in the development of the immune system, energy metabolism and the supply of key nutrients.(215, 216)

In health the intestinal microbiota consists of a vast and diverse community of microbes with over 1000 different species colonising the gut.(217) Metagenomic data of the human intestinal microbiota suggest that the majority of genes are of bacterial origin.(218) Colonisation of the intestine occurs immediately after birth. It has become apparent that the microbiota evolves from birth to the age of around 3 years after which the microbiota achieves an adult like

pattern.(219) A number of factors such as ageing, diet and antibiotic use have been shown alter the intestinal microbiota.(220, 221)

Changes in the intestinal microbiota termed dysbiosis have been implicated as an aetiological factor in a number of diseases including irritable bowel syndrome (222), obesity (223), diabetes mellitus (224) and neuro-developmental disorders.(225) In recent times there has been significant interest in the role of the intestinal microbiota in the development of IBD, much of which has focused on the bacterial component. It is now beyond doubt that the intestinal microbiota plays and important role in IBD.

Many studies utilising DNA sequencing technologies have identified changes in the intestinal microbiome of those with IBD compared to healthy controls. A pattern of reduced bio-diversity with reduced abundance of several taxa within the Firmicutes phylum and an increase in the Gammabproteobacteria has been established by a number of studies.(226) In IBD there appears to be an increase in bacteria belonging to the phylum Proteobacteria which most pathogenic bacterial belong to.(227)

There is evidence to support the theory that groups of intestinal bacteria have a protective role against IBD. Mouse studies have demonstrated that experimental colitis is more severe in germ free compared to conventionally reared mice in the DSS colitis model.(228) Commensal microbiota may protect the host from infection with pathogenic bacteria through colonisation resistance and there is evidence that intestinal commensal bacteria are able to directly dampen the virulence of potential pathogens.(226) Lactobacillus casei has been shown to down regulate inflammatory cytokines in terminal ileal tissue explants from patients with CD.(229)

Manipulation of the microbiome as a therapeutic approach for IBD is under current investigation from a number of different approaches. Current clinical trial evidence for the role of antibiotics in IBD is complex and conflicting due to the diversity of patient phenotype and antibiotics investigated. A systematic review has tentatively suggested that antibiotic therapy may induce remission in IBD.(230) There is however concern that antibiotic therapy may negatively alter the intestinal microbiome to a pro-inflammatory state.(231) A more attractive approach that has gained increasing support in recent years is the repopulation of the intestine with a healthy microbiota. This approach through faecal microbiota transplant (FMT) has been shown to be effective in the treatment of Clostrium difficile associated diarrhoea (232), which is utilised, in clinical practice. The results of clinical trials of FMT in IBD have been varied; a recent systematic review estimated the remission rate in patients treated with FMT to be 45%.(233) Further work in this area to identify the attributes that make “good” donors and the optimal delivery method is required but early results are promising.

1.4.3 The role of the innate immune system

The inflammatory bowel diseases are mediated by the immune system. In order to understand the role immunology of in the pathogenesis of IBD, the function of immune system in health must be understood. The intestine along with the skin and mucosal surfaces of the lung represent the few organs of the body, which have continuous direct contact with the external environment and thus exposure to many foreign antigens in health. The human intestine has the largest surface area of any mucosal surface that is exposed to the external environment.(234) As a consequence of this the immune cells of the gastrointestinal tract are exposed to a huge number of foreign antigens. In health there must therefore be mechanisms to block continuous activation of the immune system to non-pathogenic antigens.

The cells of the innate immune system provide the initial rapid response to luminal microbes. The response of the innate immune system to pathogens is non-specific and no long lasting immunity is developed. Intestinal macrophages and dendritic cells continuously sample the luminal bowel contents. Collectively these cells are termed lamina propria mononuclear cells (LPMCs). These cells display receptors called pattern-recognition receptors (PPRs) that recognise general microbial patterns known as pathogen associated molecular patterns (PAMPs). Recognition of PAMPs by PPRs, triggers release of a range of cytokines via nuclear factor (NF) $\kappa\beta$ activation, which orchestrates the early host resistance to infection, and ultimately activation of the adaptive immune system.(235, 236)

Recognition of PAMPs by the PPRs is also important for immunological homeostasis within the bowel and provides an important epithelial protective function.(237)

In health, the LPMCs generate an immune response to pathogenic insult but also exhibit tolerance to dietary proteins and commensal bacteria, loss of this tolerance has been postulated and is supported with some evidence as a potential trigger for the development of inflammatory bowel disease.(186, 238)

Recent studies particularly GWAS, have highlighted the important role that LPMCs play in the pathogenesis of IBD through the numerous susceptibility single nucleotide polymorphisms (SNPs) that have been identified as being related to the innate immune system in IBD.(239)

The autophagy pathway has been identified as a possible pathogenic mechanism in CD. Autophagy is a cellular process vital for homeostasis. It is a mechanism by which cells remove, damaged or mis-folded cellular proteins, aged proteins and invading organisms.(240) Defective autophagy in CD was first identified by GWA studies, the first associated autophagy gene identified was the ATG16L1 gene (241) but several other genes involved in autophagy associated

with CD have now been implicated.(242) There have been two knock out ATG16L1 mice strains studied though neither developed spontaneous colitis.(243, 244) In one of these studies it was noted that macrophages deficient for ATG16L1 produced increased amounts of the inflammatory cytokines IL1beta and IL-18 when stimulated with LPS.(244)

1.4.4 The role of the adaptive immune system

The key cells of the adaptive immune system in IBD are the T-Cells. T-helper cells (CD4) when activated can differentiate into Th1, Th2, Th17 or Treg cells. Th1 cells have an essential role in the elimination of intracellular pathogens, Th2 cells severe to protect against parasites as well as mediate allergic reactions.(245) The Th17 cells seem to have a role in the clearance of extracellular bacteria and fungi that require a massive inflammatory response which is inadequately produced by Th1 and Th2 responses.(246)

Until recently it was widely believed that CD was characterised by a Th1 response driven by IL-12, while UC was a predominantly Th2 response with excess production of IL-5 and IL-13. Recent studies however have shown that the IL-23/Th17 axis is implicated in both CD and UC. Th17 cells produce IL-17 and their development is dependent on TGF-β and IL-6, whilst IL-23 is important for their maintenance and expansion.(247)

It is unlikely that T-cell dysfunction is the initiating trigger of IBD, however there is now substantial evidence that dysregulated T-helper cells play a central role in driving the mucosal inflammation seen in IBD.(248)

Regulatory T cells (Treg) are a sub set of CD4 T-cells that have important immune suppressive properties.(249) These cells are characterised by the expression of forkhead box PR transcription factor (Foxp3) and express the surface marker CD25.(250) Patients with non-functioning or absent Treg cells have severe intestinal inflammation.(248) The potent anti-inflammatory effects of Treg cells has been shown in both mouse models of experimental colitis (251, 252) and humans with IBD.(253)

1.4.5 Cytokines in IBD

A number of cytokines have been identified as being important in the propagation of the intestinal inflammation in IBD and have with some success been targeted for therapy in both animal models and humans.(254) The excess cytokine production seen in IBD is likely to represent a down stream consequence in a susceptible host rather than an initiating factor for the disease. It has been shown that in active IBD the intestinal antigen presenting cells of the innate (dendritic

cells and macrophages) secrete large quantities of pro-inflammatory cytokines including IL-1β, IL-6, IL-18 and tumour necrosis factor.(255) These pro-inflammatory cytokines released by the innate immune cells have a number of cellular targets including the adaptive immune system such as mucosal T-cells leading to their activation and release of further pro-inflammatory cytokines from these cells.(254) Intestinal antigen presenting cells in both CD and UC secrete increased levels of IL-12 and IL-23, these cytokines share the p40 subunit.(256) The IL-12 cytokine promotes maturation of T-cells to the Th1, IFN-γ producing phenotype. The IL-23 cytokine however does not promote Th1 differentiation owing to the absence of IL-23 receptors on naïve T-cells, though IL-23 does stimulate the matured Th1 cells to release IFN-γ.(257) IL12 and IL23 are also responsible for stimulation of the Th17 response and release of type 17 cytokines IL17, IL22 and granulocyte-macrophage colony-stimulating factor.(256) These Th17 cytokines are increased in patients with IBD.(254) Whilst Th17 cytokines have been shown to meditate a pro-inflammatory microenvironment through the stimulation of other pro-inflammatory cytokines such as IL1 and IL6, there is also data to suggest that Th17 cells also produce anti-inflammatory cytokines such as IL22, which has a role in wound healing.(258)

Cytokines therefore play an important role in IBD pathogenesis, they link the activation of the innate immune system to activation of the adaptive immune system. Pro-inflammatory cytokines work in synergy to set up positive feedback loops. There are anti-inflammatory cytokines such as IL-10 and TGFβ that have been shown to have a protective role in experimental models of colitis.(254)

Targeting of pro-inflammatory cytokines is well established in the treatment of IBD through anti-TNF-alpha medications such as Infliximab, and the anti IL-12/23 medication Ustekinumab, however as of yet there no therapeutic drug that targets the promotion of anti-inflammatory cytokines.

1.4.6 Intestinal vasculature and cell adhesion molecules

The intestinal vasculature and endothelium facilitate the entry of leucocytes into the gut. This has become another target for IBD immune therapy. Leukocytes are known to continuously re-circulate between the blood and tissues. Adhesion molecule is a general term used to describe the molecules such as integrins involved in leukocyte recruitment. Leucocyte extravasation is a 3 stage process; (i) tethering and rolling of the leukocytes on the endothelium, (ii) activation of integrins and (iii) firm adhesion and transmigration.(259) The integrins are a family of adhesion molecules, which play a key role in the firm adhesion of leukocytes to the vascular endothelium. The integrins important in lymphocyte migration include the β2-integrins and two α4 integrins

($\alpha4\beta1$ and $\alpha4\beta7$). These integrins bind to specific endothelial ligands called addressins, the $\alpha4\beta1$ binds to vascular cell adhesion molecule-1 (VCAM-1) and $\alpha4\beta7$ interacts with mucosal addressin-cell adhesion molecule 1 (MAdCAM-1)(260). The $\beta2$-integrins are all expressed on monocytes and neutrophils. The $\alpha1\beta2$ integrin is however expressed on lymphocytes the ligands for which are the intracellular adhesion molecules (ICAM)-1,2 and 3.(261) Selectins are cell adhesion molecules that are important in the early stages of extravasation and appear to be a prerequisite for the role of $\beta2$-integrins but not $\alpha4$ integrins as these can initiate leukocyte adhesion. (262, 263)

T-cells that have been activated for action in the intestine express the integrin $\alpha4\beta7$ which is specific for the gut and allows targeted migration of T-cells.(264)

1.4.7 The intestinal epithelium barrier function

The intestinal epithelium is exposed to a myriad of commensal bacterial and dietary antigens. The intestinal epithelial cell layer provides the physical barrier preventing entry of these foreign antigens.

The functional intestinal mucosal barrier however has several components; secreted mucin and antibacterial products, luminal microbiota as well as the innate and adaptive immune cells of the inner sub-epithelial region.(265)

Abnormal small intestine permeability has been demonstrated in CD (266) and has been shown to be predictive of relapse in both UC and CD.(267, 268) The epithelial cells are held together by intracellular junctions that contribute to the epithelial barrier integrity. The three major intracellular junctions are tight junctions, adherens junctions and desmosomes(269). Defects in intracellular junctions have been demonstrated in IBD.(270-272)

In recent times much debate has centred on whether epithelial dysfunction represents a cause or consequence of intestinal inflammation. Recent evidence from GWA studies suggests that epithelial barrier defects represents a primary pathological mechanism in IBD.(273)

1.4.8 The role of genetics

The role of genetics in the development of IBD was initially highlighted by population based studies in the early 1990s which identified an increase in prevalence of IBD among the relatives of individuals with the diseases.(74) Subsequent twin studies further highlighted and began to quantify the contribution of genes in the development of IBD. Three large European twin studies estimated the concordance rate for CD in monozygotic twins to be between 20-50% with a more modest concordance rate for UC in the region of 16%. This compared with a concordance rate in

dizygotic twins raised in the same environment of 10% and 4% for CD and UC respectively.(30-32) Whilst these studies identified that genetics played an important role in the development of IBD they did not identify any specific genes involved.

The first step towards the identification of IBD susceptibility genes came in the form of genetic linkage studies, the first of its kind was reported in 1996, linking a section of chromosome 16 initially described as IBD1 with CD (274). Further studies identified that the IBD1 region of chromosome 16 was due to 3 risk alleles in the NOD2 gene.(275) Three NOD2 polymorphisms have been identified as occurring with higher frequency in Europeans with CD.(276) NOD2 is expressed by many immune cells including, macrophages, lymphocytes, paneth cells as well as fibroblasts and epithelial cells. Activation of NOD2 by microbial ligands triggers inflammation through the activation of the transcription factor NF $\kappa\beta$ and therefore downstream inflammatory pathways. The loss of NOD2 function alone however is not sufficient to cause the CD phenotype.(277)

The next advance in the understanding of the genetic basis of IBD came in the form of genome wide association studies (GWAS). These studies utilised a number of advances including the development of public databases of common genetic variant, single nucleotide polymorphism (SNPs) by projects such as the SNP consortium and HapMAP (278, 279). These public databases together with an understanding of linkage disequilibrium, technological advances in microarray with reducing costs made these studies feasible on a large scale. (280) CD was one of first diseases studied using these techniques, the first of which was conducted in Japan in 2005 and identified SNPs in the TNFSF15 gene associated with Japanese patients with CD.(281) These early GWA studies identified a number of gene associations with CD in the innate immune system (TLR4, CARD9, IL23R, STAT3) as well as the adaptive immune system (HLA, TNFSF15, IRF5, PTPN220).(282) Ulcerative Colitis GWA studies were also performed confirming the previously identified association with the human leukocyte antigen locus as well as loci implicating intercellular interactions and therefore epithelial barrier defects in the pathogenesis of UC.(280) Meta-analysis of GWAS studies in IBD led by the international IBD genetics consortium increased the number of identified IBD risk loci. At present, 163 loci have been identified, the majority of which (110) are common to both UC and CD (283) highlighting shared pathological pathways for the two diseases.

Although genetic studies in IBD to date have only explained a small fraction of the identified heritability of IBD the GWA studies have identified molecular targets and cellular pathways for further investigation. GWA studies continue to guide on going research and increase our understanding of IBD, such as the recent study demonstrating ileal CD as genetically distinct from

colonic CD and that these should be considered as separate disease entities.(284) This has important implications on both clinical and basic science studies of IBD patients.

1.5 Macrophages

1.5.1 Overview

Macrophages are immune cells of myeloid origin that are characterised by avid phagocytosis. Metchnikoff is credited with the discovery of macrophages, he visualised such cells in pricked starfish larvae and infected water fleas in the late 19th century, a discovery that earned Metchnikoff the Nobel Prize.(285) Over the course of the beginning of the 20th century the phagocytic activity of tissue macrophages was well appreciated and in the 1960s Ralph Van Furth and colleagues termed these groups of cells the mononuclear phagocytic system (MPS).(286)

In humans macrophages are sub-typed according to their anatomical location and function, as illustrated in **figure 1**. These different macrophage populations differ highly in their transcriptional profile (287) and it can be argued that that they each individually represent unique classes of macrophages highlighting the plasticity of macrophages.

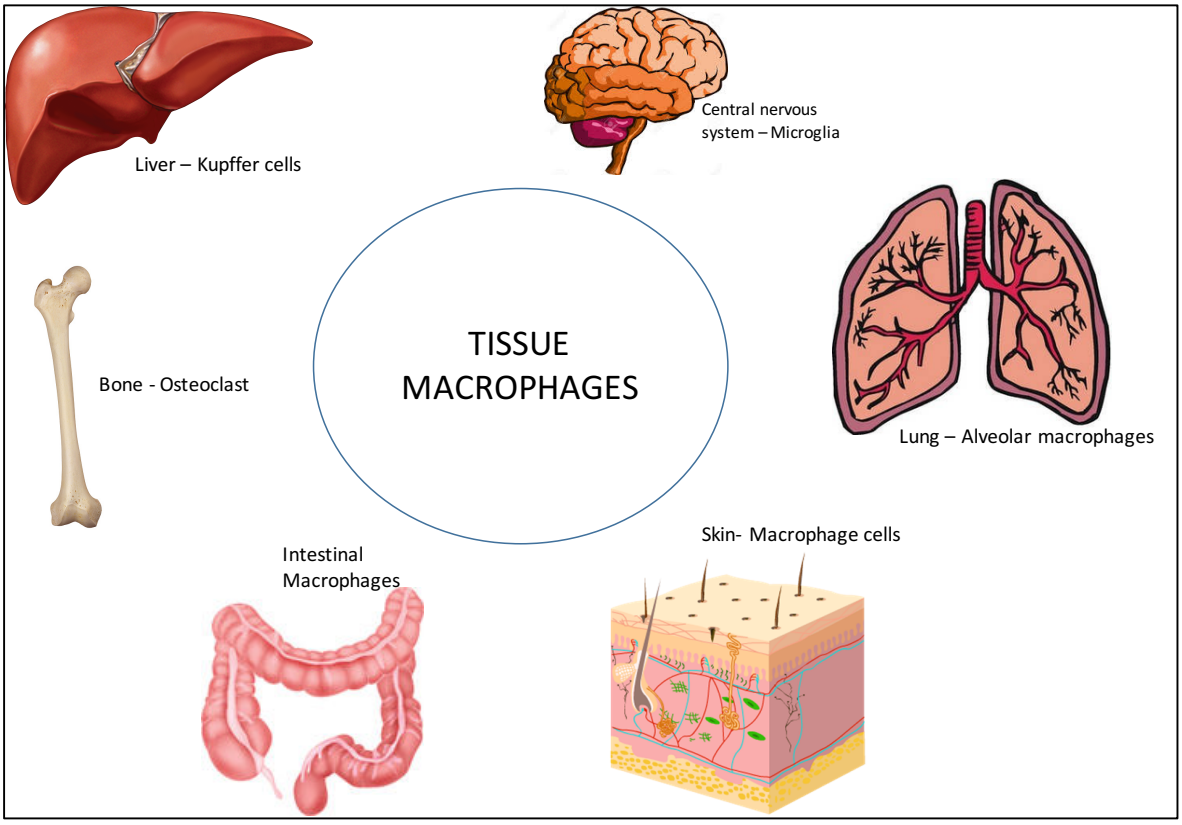


Figure 1 Tissue macrophages by organ

The central role of all these tissue macrophages however is the same, tissue homeostasis and repair.(288) In the most simplistic of understanding, macrophages achieve this by removal of cellular debris and neutralisation of potentially harmful pathogens through phagocytosis. It is however clear macrophages have more complex roles displaying diversity in terms of morphology,

transcriptional profiles, location and functional capabilities.(289) In some tissues such as the kidneys, pancreas and mammary glands macrophages have a tissue re-modelling role both during the embryological state and in the adult. (290, 291) Macrophages are involved in thermogenesis through the promotion of lipolysis of glyceride in white adipose tissue and induction of genes within brown adipose tissue responsible for thermogenesis in response to cold stimuli.(292) In cancer research, there is great interest in the role macrophages play in cancer progression with studies suggesting that tumour associated macrophages promote metastasis and proliferation of malignant tumours.(293) In addition to cancer, defective macrophage function has been implicated in a number of disease processes including atherosclerosis leading to cardiovascular disease, rheumatoid arthritis, fibrotic diseases and demyelinating disease. (292)

1.5.2 The Origin of Tissue Macrophages

The origin of tissue macrophages for several years has been a controversial topic. Until recently it was widely accepted that all tissue macrophages originated from circulating bone marrow derived monocyte precursor cells and hence the mononuclear phagocyte classification system proposed by Van Furth et al.(294) Recent evidence from fate mapping and parabosis studies in mice suggest that the majority of macrophages residing in healthy tissue are established prenatally and are maintained locally independent from haematopoietic input. (295-297) Further supporting tissue macrophage self renewal is the finding that patients with a mutation in GATA2 lack blood monocytes and all subsets of dendritic cells though have normal numbers of macrophages in the skin and lungs.(298)

In the embryo macrophages develop from the mesoderm in independent waves that move between organs, starting in the blood islands of the extra-embryonic yolk sac and eventually moving to the foetal liver which becomes the predominant embryonic site of haematopoiesis.(285) There are however some notable exceptions to this dogma. Mouse studies have shown that yolk sac and foetal liver derived resident intestinal lamina propria macrophages are present during the neonatal period but fail to persist into adult life and are completely replaced by bone-derived monocytes in adulthood.(299) Intestinal tissue macrophages are thus reliant on continuous replenishment from circulating blood monocytes.(300)

Monocytes themselves are a subset of circulating leukocytes that can further differentiate in to tissue macrophages and dendritic cells. In humans they constitute approximately 10% of total leucocytes and remain in the circulation for 1-2 days.(301) Monocytes are derived from haematopoietic stem cells that develop within the bone marrow into pluripotent progenitor precursor cells called common myeloid progenitor (CMP) cells (302) in the bone marrow under

stimulation of IL-1, IL-3 and IL-6. Under the continuous presence of IL-1, IL-13, macrophage colony stimulating factor (M-CSF) and Granulocyte-macrophage colony-stimulating factor (GM-CSF) these stem cells develop into granulocyte/monocyte precursors (303) and then differentiate into monocyte/macrophage and dendritic precursors (common macrophage and dendritic precursor) under the influence of the transcription factor PU.1.(304, 305)

The majority of published data on monocytes is derived from mouse studies. These have identified two major subtypes of circulating monocytes; $CCR2^{hi}$ CX_3CR1^{Low}, which are often referred to as $LY6C^{hi}$ (also termed inflammatory monocytes) and the major other subset is $CCR2^{low}$ CX_3CR1^{high} otherwise known as $LY6C^{low}$. (306) These markers do not exist in humans. The most recent accepted classification divides human monocytes into 3 groups based on expression of the surface markers CD14 and CD16. These three groups are $CD14^{++}CD16^{-}$ which account for 90% of monocytes and are called classical monocytes, $CD14^{low}$ $CD16^{high}$ called the non-classical monocytes and the intermediate monocytes which are $CD14^{high}CD16^{low}$.(307) The classical monocytes seem to be the most similar to the mouse $LY6C^{hi}$ monocytes based on gene expression arrays (308), however it is clear that the subsets between humans and mice are similar but not identical or interchangeable.(309, 310)

1.5.3 Polarisation of Macrophages

The M1 (classic) /M2 (alternatively activated) classification system for mature macrophages has gained prominence over the last two decades. This classification system mirrors that of the T-helper cells. There is interest in identifying whether this system can truly be applied in vivo. Mils and colleagues (311) were the first group to propose the polarisation of macrophages. They noted that macrophages from mice with Th1 and Th2 backgrounds differed in their response to IFNϒ or lipopolysaccharide (LPS). In this paradigm IFNϒ, alone or in concert with LPS activates macrophages in a classical manner. M2 or alternatively activated macrophages result from macrophages exposed to IL-13 and IL-4 (312), M1 and M2 macrophages have distinct cytokine release, chemokine profiles and functionally behave quite differently. Typically M1 macrophages secrete large amounts of IL-12, IL-23, nitric oxides, reactive oxygen intermediates but low levels of the anti-inflammatory cytokine IL-10. The M1 macrophages favour the recruitment of Th1 cells though the release of the Th1 chemokines CXCL9 and CXCL10.(313) The M1 macrophages are therefore pro-inflammatory and play and important role in the host defence against pathogenic viruses, bacteria and protozoan infection. Mouse studies have demonstrated that IFNϒ knock out mice and humans with genetic mutations in these pathways are susceptible to pathogenic infections(314). In contrast M2 macrophages secrete low levels of IL-12 and IL-23 but high levels of IL-10. The M2 macrophages are often referred to as “wound healing” or “anti –inflammatory”

as they have a role in tissue remodelling (315) though they are also known to be involved in the polarisation of Th2 responses and parasite clearance.(316)

Since the initial proposal of this classification system, it has increasingly been recognised that the M1/M2 paradigm is more complex. Mantovani et al (317) further sub-classified M2 macrophages on the basis of different stimuli that induced M2 macrophage polarisation. Namely these are M2a induced by IL-4 or IL-13, M2b induced by immune complexes, agonists of TLRs or IL-1Rα and M2c induced by IL-10 or glucocorticoids **(figure 2)**. These different M2 subtypes had different cytokine profiles and transcriptome signatures, however common to all the M2 subtypes was an $IL10^{High}$ and $IL12^{low}$ cytokine profile and an anti-inflammatory role. The polarisation of macrophages was further refined into a spectrum model by an extensive study undertaken by Xue et al (318) in which in macrophages were stimulated under 28 different conditions and the resulting transcriptional profiles analysed. The 28 conditions utilised were extensive, including pattern receptor ligands, metabolic stimuli as well as cytokines and the chronic activated state was stimulated. This study has provided a useful resource for macrophage transcriptome analysis and hypothesis generation based on the macrophage transcriptome profiles they have described in response to the 28 conditions.

The M1/M2 model in its most basic form has been developed in vitro, ignoring source and context of stimuli.(312) It is unlikely that macrophages in vivo are exposed to a milieu that is dichotomously M1 or M2 stimulatory. It may be that in vivo macrophages develop a mixed M1/M2 phenotype as has been demonstrated with tumour associated macrophages in squamous cell carcinoma (319) and macrophages (microglia) from active lesions in multiple sclerosis.(320) In vitro studies of monocyte-derived macrophages have several limitations and are unable to fully model the complex cytokine environment that tissue macrophages exist in. There is further need to assess the relevance of the M1/M2 paradigm in vivo macrophages with biological context.

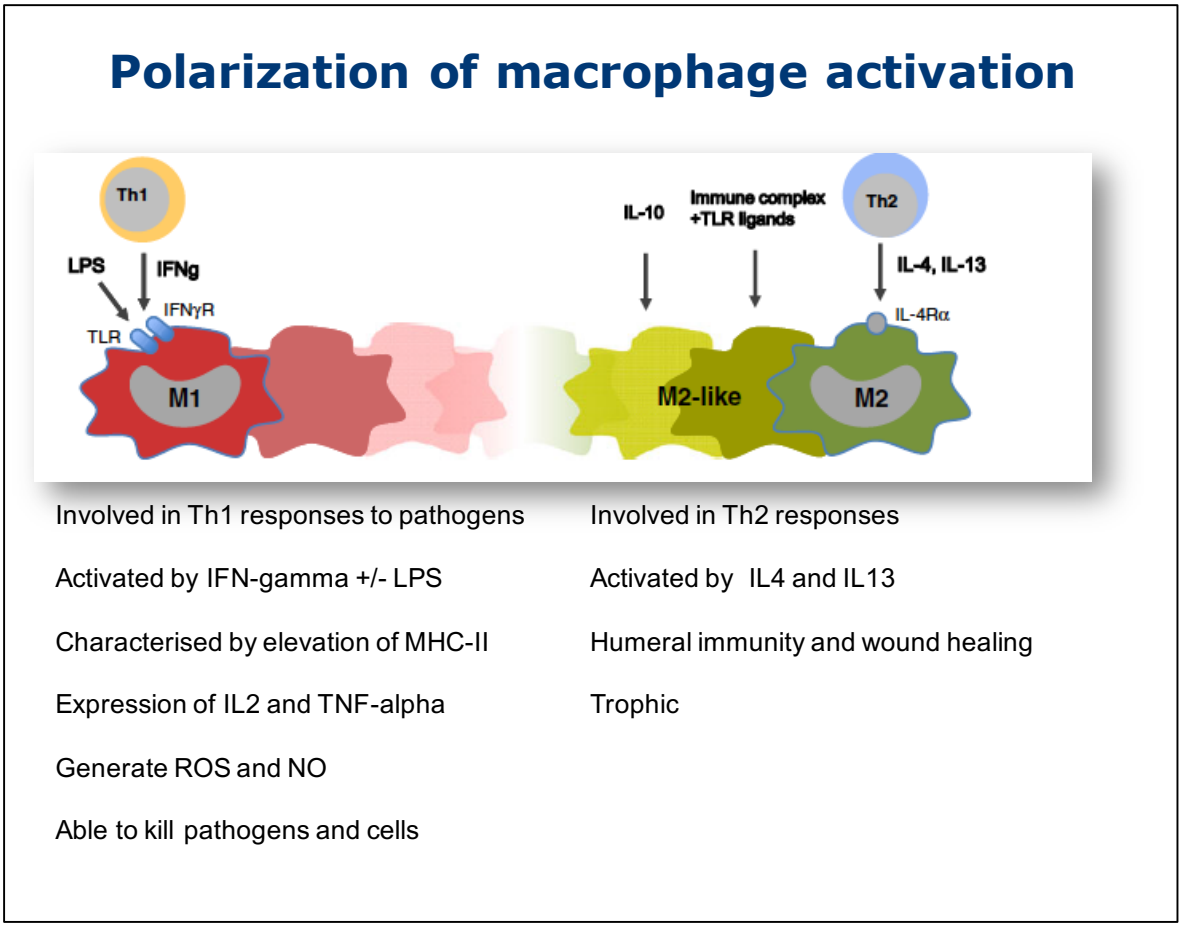


Figure 2 Factors leading to the polarization of macrophages and the functional differences in M1 and M2 macrophages. Figure produced with Dr E Garrido Martin for oral presentation at ECCO congress 2016 (313)

1.5.4 Intestinal Tissue Macrophages

Intestinal macrophages constitute between 10-20% of the mononuclear cells within the lamina propria according to previous immunohistochemistry studies.(321) The majority of the current understanding of intestinal macrophages is derived from mouse studies. The results from the limited number of human studies in collaboration with data from animal models have identified that intestinal macrophages have a number of functions that are essential for intestinal mucosal immune homeostasis. These are explored in detail in this section.

1.5.4.1 The function of Intestinal Macrophages

The major role of intestinal macrophages is as effector cells of the innate immune system to maintain tissue homeostasis. They also play an important role in maintaining the epithelial barrier integrity through the production of prostaglandin E2.(322)

The intestinal lumen contains many foreign antigens including a variety of dietary proteins, in addition to pathogenic and non-pathogenic organisms. In this unique environment the intestinal macrophages must be highly specialised in order to perform their core function of maintenance of tissue homeostasis by only initiating immune responses to potentially harmful foreign antigens whist being tolerant to the many non-harmful antigens they encounter – this is a highly specialised function vital to healthy tissue immune homeostasis.

There remains controversy in the discrimination of intestinal dendritic cells and macrophages. At present there is no clear consensus on the classification of resident macrophages and dendritic cells. The cell surface markers CD11b and CD11c are routinely used to differentiate macrophages from dendritic cells in peripheral lymphoid tissue.(323) This however does not seem to hold true for intestinal tissue macrophages.(324) Traditionally the core function of macrophages has been thought to be phagocytosis whereas the core function of dendritic cells is antigen processing and presentation.(288) It is now clear that intestinal macrophages are antigen presenting but unlike the dendritic cells they do not migrate to lymphoid tissue to present antigens to naïve T-cells located there. Macrophages appear to present processed antigens to effector T-cells already situated within the lamina propria.(325-327) Mouse studies have shown that macrophages are able to sample the luminal antigens by extension of their dendrocytes between the epithelial cells in much the same way that dendritic cells sample the luminal content. (328-330)

In the healthy state intestinal macrophages must respond to pathogenic organisms preventing disease yet exhibit tolerance to commensal gut organisms, dietary proteins and healthy self cells that do not have the potential to cause disease. Intestinal macrophages are able to recognise conserved molecular patterns on microbes known as pathogen-associated molecular patterns (PAMPs). (331) To achieve this, macrophages express a number of surface receptors known as pattern recognition receptors (PRRs) which are functionally categorised into the groups; toll like receptors (TLRs), nucleotide-binding oligomeriation domain (NOD), leucine rich repeat (LRR) receptors, C-type lectin receptors (CLRs) and retinoic acid-inducible gene 1 (RIG-1) like receptors.(332, 333) Activation of these PRRs triggers signalling that culminates in downstream activation of immune response pathways.

In healthy patients, intestinal tissue macrophages are therefore relatively inert, even though they express TLRs they do not respond to specific ligand stimulation.(334, 335) This relative inflammatory tolerance is as of consequence of ineffective TLR signalling responses to bacteria mediated by TGF-β (336) and low expression of co-stimulatory molecules such as CD80, CD86 and CD40.(337, 338) Despite this lack of inflammatory response, these macrophages retain their phagocytic and bactericidal activity.(324, 334) The anti inflammatory role of intestinal

macrophages is supported by studies demonstrating that murine intestinal macrophages secret IL1-10, TGF-β and retinoic acid, which induce differentiation of CD4 T-cells into T-regulatory cells, which have a potent anti-inflammatory role in the gut.(339) It appears that the action of IL-10 on macrophages independent of T-cells is significant; mice with absent macrophage IL-10 receptors develop spontaneous colitis, yet mice with non IL-10 producing macrophages do not.(300) This is supported by clinical cases in which patients with mutations in the IL-10 receptor develop early and aggressive inflammatory bowel disease.(340)

Typically, both mononuclear and polymorphonuclear phagocytes express CD14, a glycophosphatidyl inositol-linked glycoprotein. CD14 interacts with LPS (Lipopolysaccharide), which is a major component of gram-negative bacteria cell walls. The interaction between LPS and CD14 results in activation of the toll receptor-4 initiating the release of pro-inflammatory cytokines, IL-1, IL-6, IL-8 and TNF- α.(334) Some groups have reported the absence of CD14 on macrophages isolated from normal small bowel (jejunum).(341) Other groups however have shown that in normal intestinal tissue, macrophages express a low level of CD14 whist in the inflamed tissue of patients with IBD, CD14 expression on macrophages appears to be increased.(342, 343) Identification of macrophages using cell surface markers is complex. Many of the proposed macrophage markers that have been identified through mice experiments have subsequently been discovered to be shared with dendritic cells and there is a lack of cross-over between these markers in mice and humans.(344)

The haemoglobin-haptoglobulin scavenger receptor CD163 is expressed on intestinal macrophages and to lesser extent monocytes.(345, 346) Expression of CD163 on intestinal macrophages does not appear to be influenced by treatment with mesalazine, corticosteroids or immunomodulators. (347) Fonseca et al(348) demonstrated that in patients with rheumatoid arthritis CD163 was more specific for macrophages than CD68, which cannot differentiate macrophages from fibroblasts. Furthermore a large immunohistochemistry study utilising both neoplastic and non-neoplastic tissue form multiple organs demonstrated that in non-neoplastic tissue CD163 expression is restricted to macrophages and monocytes.(349) This is therefore an attractive surface marker for intestinal macrophage identification and isolation in IBD patients.

1.5.5 The Role of Intestinal Tissue Macrophages in Inflammatory Bowel Disease

There is mounting evidence of an important role for macrophages in the development and propagation of the intestinal inflammation seen in IBD. A combination of genetic studies, mouse models and human studies has implicated macrophages in IBD pathogenesis.

The IBD GWA studies have highlighted mutations in genes of the innate immune system involved in microbial identification and processing as susceptibility loci for IBD. These include NOD2, which encodes intracellular pattern recognition receptors for muramyl dipeptide, a component of the peptidoglycan cell wall in bacteria as well as IRGM (immune related GTPase family M) and ATG16L1, which encode proteins involved in autophagy.(339) These findings have focused attention on the role of innate immune cells in IBD pathogenesis.

There are thought to be 3 major mechanisms by which defects in the innate immune system can initiate or propagate the development of IBD; by inappropriately responding to benign antigens such as commensal bacteria, by inefficiently clearing microbes leading to chronic inflammation or by failing to switch from an initial appropriate pro-inflammatory response to an anti-inflammatory response or steady state.(323) Both animal and human studies have attempted to identify which of these aspects are involved in IBD.

Mouse studies have implicated macrophages in the development of IBD.(350) These studies have shown that depletion of phagocytes in IL-10 deficient mice (IL-$10^{-/-}$)(351) and blocking myeloid cell recruitment ameliorates colitis in 2,4,6-trinitrobenzene sulfonic acid (TNBS) induced colitis and the T-cell adoptive transfer colitis models. (352) In contrast however depletion of lamina propria macrophages prior to the induction of experimental colitis in mice exacerbates the disease.(353) This may be explained by the finding that the phenotype expression of macrophages in the intestine modifies the degree of colitis, the M2 polarisation being protective whilst M1 macrophages exacerbates the colitis. If the newly recruited macrophages are therefore mainly of the M1 subtype, depleting these once colitis has been establised would be expected to result in improvement of the colitis as seen in these mouse models. (354, 355) There is evidence from mouse work that manipulation of the macrophages from a M1 phenotype to a M2 phenotype amilorates exiperimental colitis.(356, 357)

Human studies have also identified macrophages in the pathogenesis of IBD. The number of intestinal macrophages is increased in patients suffering with IBD compared to healthy controls (358), granuloma (aggregate of macrophages) are the histological characteristic of CD (359) and human GWA studies have identified loci related to the macrophage function of bacterial processing in IBD.(360) The macrophages of patients with Nieman-Pick disease type C1, a lysosomal lipid storage disorder, conferring an increased susceptibility to early onset CD, have impaired antibacterial autophagy.(361)

Monocytes are the main source of intestinal macrophages, unlike other tissues in which self-renewal is more dominant. Monocytes therefore play an important role in the innate intestinal immune response. It has been demonstrated that the proportion of peripheral classical

monocytes in CD patients with active luminal disease is reduced (362) suggesting that these cells migrate to the intestine as part of the immune response. This is consistent with the finding that macrophages highly expressing CD14 dominate the affected mucosa of patients with active IBD. (342, 343) Furthermore studies have demonstrated in active IBD there is increased recruitment of monocytes to the inflamed mucosa, which mature into pro-inflammatory macrophages (342, 363). These pro-inflammatory macrophages are characterised by $CD14^{hi}CD11c^{hi}$ $HLA\text{-}DR^{dim}$ surface expression and the release of pro-inflammatory cytokines.(364-366)

Within the inflamed intestinal mucosa it therefore appears that monocytes are recruited from the blood and differentiate into inflammatory (M1) macrophages which respond to TLR stimulation and secret inflammatory cytokines.(303) In addition, these inflammatory (M1) macrophages, contribute to the disruption of the epithelial barrier through interruption of the tight junctions and induction of epithelial cell apoptosis further driving inflammatory damage and immune cell activation.(367)

Smith et al (368) postulated that an impaired clearance of microbes from the intestine may be a contributing factor in the development of chronic inflammation in IBD. Using a novel set of experiments, they demonstrated impaired clearance of a subcutaneously injected bacterial load (Escheria Coli) in CD patients when compared to healthy controls and patients with UC.(368) In addition they found a reduction in secretion of pro-inflammatory cytokines from monocyte-derived macrophages of patients with CD, incubated with heat killed Escheria Coli for 24 hours. The transcriptome profiles of these macrophages, exhibited equivalent mRNA expression of the pro-inflammatory cytokines (GM-CSF, IL-6, TNF and INF) to those of healthy controls measured by qPCR. Analysis of the transcriptome of these cells suggests that the deficiency in pro-inflammatory cytokines was due to impaired release by the macrophages. This finding is consistent with a study by Elliot et al (369) demonstrating that monocyte derived macrophages from CD patients had an impaired response to infection with adherent-invasive Escheria Coli. Furthermore human studies have shown that the peripheral blood derived monocytes from IBD patients express reduced amounts of the pro-inflammatory cytokine TNF-α in response to infection. (370-374) It is important however to note that these studies utilised blood-derived cultured monocytes, which were then stimulated in vitro. A key assumption the investigators made was that these cells were analogous to intestinal macrophages. Given the complexity of intestinal macrophages, the influence of the gut microenvironment on immune cell development and function together with the current lack of understanding of the natural course of these cells, studies of this type need to be interpreted cautiously.

It is clear from both mouse and human studies that intestinal macrophages play a role in the pathogenesis of inflammatory bowel disease though complex interaction with the intestinal immune system, **figure 3**. There is currently insufficient evidence to identify specific defects in macrophage function associated with IBD that could be utilised as therapeutic targets.

Intestinal macrophages have been targeted in early therapeutic studies with some success. Fraxinellone a naturally occurring lactone compound ameliorated DSS induced colitis in mice, an effect attributed to the inhibition of macrophage migration.(375) There is also evidence that established treatments of IBD such as anti-TNF alpha therapy mediate some of their activity through action on macrophages.(376-381) In IBD anti TNF alpha therapy appears to induce a shift in macrophages from the M1 phenotype to the M2 phenotype.(382, 383) Clinical studies have established that combination therapy with Infliximab and azathioprine has superior efficacy in the treatment of CD than either drug alone.(172) Interestingly Vos et al observed that combination therapy with azathioprine and infliximab increased expression of M2 type macrophages more than when compared with patients treated with infliximab alone and no such induction of M2 macrophages was seen in the patients treated only with azathioprine.(383)

In this chapter I have demonstrated that indirect evidence from genetic studies, blood derived macrophage experiments and animal studies suggest that the behaviour of macrophages is altered in IBD. No current established therapies for IBD currently target macrophages though recent published data presented the surprising finding that the drug vedolizumab initially thought to act by inhibiting mucosal migration of T-cells through the blocked of the integrin $\alpha 4\beta 7$ appeared to alter the macrophage population.(384) This finding belies a lack of understanding of the role and behaviour of intestinal macrophages in IBD. In order to investigate if targeting macrophages would be a valid strategy in IBD, we first need to gain a better understanding of their role in the disease. In this project I seek to develop the knowledge base of the behaviour of the intestinal macrophages in active inflammatory bowel disease

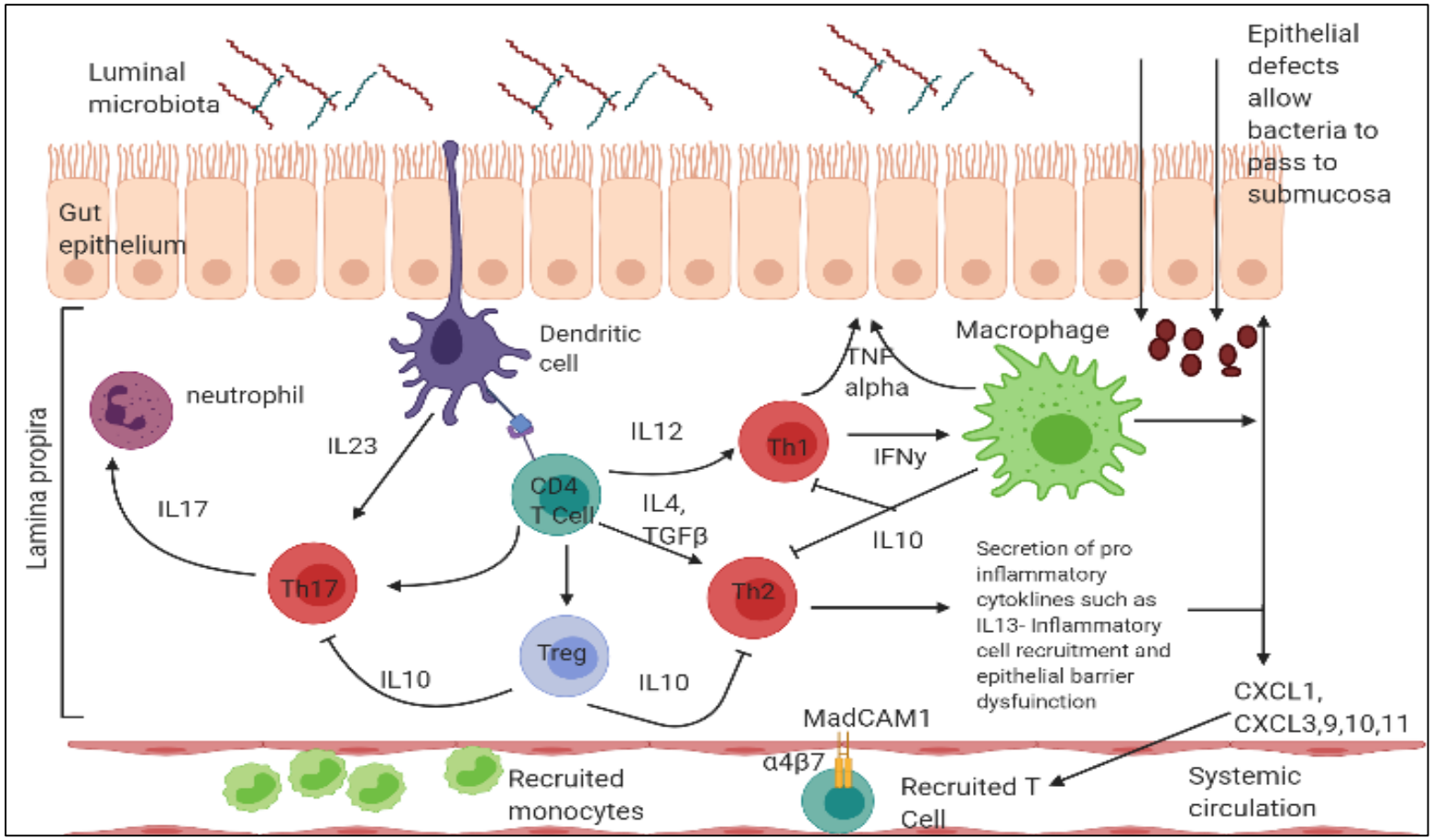


Figure 3 A summary of the mucosal immunology of inflammatory bowel disease. In health there is a balance between the pro inflammatory and anti-inflammatory components within the intestinal mucosa. In active inflammatory bowel disease this balance is lost with dysregulation of pro-inflammatory factors. Activation of the of the innate immune cells, macrophages and dendritic cells leads to the release of pro-inflammatory cytokines and chemokines resulting in immune cell recruitment, activation of the adaptive immune system and epithelial barrier defects further promoting pro-inflammatory signalling and inhibition of immune regulatory control by Treg cells and M2 type macrophages.

Figure produced in Biorender (https://biorender.com)

1.6 Hypothesis and Aims

1.6.1 Hypothesis

We hypothesised that the transcriptome of intestinal macrophages is altered in patients with active inflammatory bowel disease. Furthermore, we hypothesised that the intestinal macrophages isolated from patients with active IBD have an up-regulation of M1 associated genes and are therefore pro-inflammatory.

1.6.2 Objectives

- To develop a method for disaggregation of colonic mucosal biopsies to create cell suspension suitable for flow cytometry analysis
- To isolate intestinal macrophages by fluorescence activated cell sorting
- To perform mRNA high throughput sequencing (RNA-Seq) of isolated intestinal macrophage cells
- To explore the differential expression of genes between disease and normal controls.
- To identify potential macrophage cell targets for novel therapeutic approaches to IBD

I have shown in this thesis introduction that IBD are complex diseases with interactions between genetic factors, the environment and the immune system. Whilst advances have been made in our understanding of IBD there remains much to discover. The understanding of the general biology of macrophages is rapidly advancing though the role they play in IBD is still poorly understood. In the chapters 2,3 and 4 I describe how I approached, designed and optimised experiments to study intestinal macrophages from humans

Chapter 2: Materials and Methods

2.1 Equipment

- Auto-Stainer 4 (Dako, California, USA)
- BD FACSAria™ (BD Biosciences)
- 2100 Bioanalyzer (Agilent, California, USA)
- Eppendorf Centrifuge 5810R (Eppendorf, Stevenage, UK)
- 7900HT Fast Real-Time PCR System (Applied Biosystems, Paisley, UK)
- Micro Start 17R centrifuge (VWR, Leicestershire, UK)
- Nanodrop (Fisher Scientific UK Ltd, Leicestershire, UK)
- Shaking incubator SI500 (Stuart Scientific, Staffordshire, UK)
- Quantifluor dsDNA System (Promega, Wisconsin, USA)
- Zeiss AxioCam MRc5 microscope (Zeiss, Cambridge, UK)

2.2 List of reagents

- AEC - Aminoethyl carbazole (A5754-50G, Sigma-Aldrich Company Ltd. Dorset, UK)
- Agencourt AMPure XP 5mL (#A63881, Beckman Coulter, Buckinghamshire, UK)
- Agilent High Sensitivity DNA Kit (#5067-4626, Agilent Technologies, Cheshire, UK)
- AQIX® RS-I 1xkit (#RSI/KIT, Aqix Ltd., UK)
- BD Pharm Lyse™, lysing buffer (#555899, BD Biosciences, Oxford, UK)
- Bovine serum albumin solution (#A7979, Sigma-Aldrich Company Ltd. Dorset, UK)
- EDTA, 0.5M (#AM9260G, Ambion™, Thermo Fisher, Scientific, Paisley, UK)
- CD45-FITC (Biolgend/H130)
- CD4-PE (BD Biosciences/RPA-T4)
- CD163-PE – Biolegend/RM3/1)
- CD163-PerCP/Cy5.5 (Biolegend/RM3/1)

- CD3-PE/Cy7 (Biolegend/SK7)
- Glycophorin-A-Pacific blue (Biolegend/H1264)
- CD8-PerCP-Cy5.5 (Biolegend/SK1)
- CD14-APC-H7 (BD Biosciences/MφP9
- ERCC RNA Spike-In Mix (#4456740, Ambion™, Thermo Fisher, Scientific, Paisley, UK)
- Deoxyribonuclease (DNase) 1 (#4527-40KU, Sigma-Aldrich Company Ltd. Dorset, UK)
- Dulbecco's Phosphate Buffered Saline, without calcium and magnesium (#D8537-500ml, Sigma-Aldrich Company Ltd. Dorset, UK)
- FcR blocking reagent (#130-059-901, MACS, Miltenyi Biotect, Surrey, UK)
- Liberase™ DL, Research Grade (#5401160001, Sigma-Aldrich Company Ltd. Dorset, UK)
- miRNeasy Micro Kit (#217084, Qiagen, Manchester, UK)
- Penicillin/streptomycin/Glutamine (#10378016, Gibco, Thermo Fisher, Scientific, Paisley, UK)
- Poly(A)Purist MAG kit(#AM1922, Ambion™, Thermo Fisher, Scientific, Paisley, UK)
- RPMI medium 1640, GlutaMAX™-I (#72400-054, Invitrogen Ltd, Paisley, UK)
- SePlex RNA Amplification kit (#SEQR-10RXN, Sigma-Aldrich Company Ltd. Dorset, UK)
- TaqMan™ Universal PCR Master Mix (# 4304437, Thermo Fisher, Scientific, Paisley, UK)
- TE Buffer (#93283, Sigma-Aldrich Company Ltd. Dorset, UK)
- TREM1-PE (BD Biosciences/6B1)
- Tri Reagent® (T3934-100ML, Sigma-Aldrich Company Ltd. Dorset, UK)
- TruSeq Nano DNA library prep kit (#FC-121-4001, Illumina, California, UK)
- Vectastain Eitie ABC HRP Kit peroxidase, standard (#PK-4000, Vector Laboratories, Peterborough, UK)
- 4',6 Diamidino-2-phenylinodole dihydrocholride (#D95442-5MG, Sigma-Aldrich Company Ltd. Dorset, UK)
- ZR96 DNA clean up kit (#D4017, Zymo Research, California, USA)

2.3 Buffer preparation

MACCS buffer

- Bovine serum albumin solution (35%) – 7.14ml
- Dulbecco's Phosphate Buffered Saline – 500ml
- EDTA 0.5M – 2ml

cRPMI

- Penicillin/streptomycin/Glutamine (100x) – 5ml
- RPMI – 500mls

2.4 Ethics approval and consent

This study was given favourable ethical opinion for conduct in the NHS by the Southampton Research Ethics Committee (Reference Number 10/H0502/69). This approval permitted up to 24 intestinal biopsies to be collected at endoscopy for this research in addition to the routine biopsies required for standard clinical care.

The study was conducted according to good clinical practice (GCP) standards. All investigators involved in patient recruitment and sample collection were GCP certified. All patients recruited to the study had an opportunity to read the patient information sheet and ask questions regarding the study prior to signing the informed study consent form. For all the subjects recruited to the study the informed study consent form was signed prior to any study procedures being instigated.

2.5 Patient recruitment and sample collection

Over a 12-month period patients with IBD attending a weekly IBD endoscopy list were approached to participate in the study if they met the study inclusion criteria and none of the exclusion criteria **(table 9)**. This cohort of patients constituted the "RNASeq Cohort". Subsequently there was a further 6-month period of patient recruitment, which formed the "validation cohort". For the patients who agreed to participate and signed an informed consent form prior to their procedure, biopsies of inflamed sections of tissue were taken as per the ethical approval. Samples were placed in AQIX® and transferred to the laboratory on ice for immediate processing.

At the time informed consent was taken, demographic and clinical disease activity data was collected by myself or another appropriately trained research fellow (Dr R Harris) to facilitate the calculation of the MAYO score for UC and the HBI score for CD. In collaboration with the endoscopist, each patient was given an endoscopic disease mucosal activity score; normal/quiescent (A/B), mild (C), moderate to severely inflamed (D) and those with UC were given an endoscopic MAYO score.

The control group were recruited from general gastroenterology endoscopy lists. Patients undergoing endoscopy for surveillance of previous cancer/polyps or asymptomatic iron deficiency anaemia were approached if they met the study criteria **(table 9)**. As with the disease groups patients read the PIS prior to signing the ICF before endoscopy and collection of biopsies.

Inclusion and Exclusion Criteria for Patient Recruitment

Inclusion criteria (Disease group)	Inclusion criteria (Normal control group)
Diagnosis of UC or CD confirmed both clinically and histologically Capacity to consent to study Age >18	No active gastrointestinal symptoms Age >18 Capacity to consent to study
Exclusion Criteria (Disease group)	**Exclusion criteria (Normal control group)**
On treatment with biological therapy Active cancer Diagnosis of IBDU Treatment with trial medication Intravenous corticosteroids	On treatment with biological therapy or immunosuppressant Diagnosis of irritable bowel syndrome Active cancer Diagnosis of IBD previously Treatment with trial medication Mucosal abnormalities such as ulceration identified at endoscopy or previously

Table 9 Study inclusion and exclusion criteria for recruitment of subjects

2.6 Disaggregation of Colonic Biopsies

A minimum of 8 biopsies were utilised for disaggregation. On arrival to the laboratory the samples were immediately removed from the AQIX®, by emptying the sample vial onto a petri dish. All 8 biopsies were then transferred individually using a pair of sterile tweezers into a 50ml universal tube with 1ml of cRPMI, 100µL DNase and 20µL of Liberase. The 50ml universal tube containing the sample with the digestion cocktail was then transferred to the Shaking incubator, for 15 minutes at 37^{o}C. Using a Pasteur pipette the sample was then passed through a 70µm cell strainer into a 50ml falcon tube. The plunger from a 5ml syringe was used to apply mechanical pressure to the remaining sample in the cell strainer to maximise the cell yield. The resulting cell suspension in the 50ml falcon tube was made up to 15mls with cRPMI and centrifuged at 1700rpm at 4^{o}C for 20 minutes. The supernatant was then discarded and the remaining cell pellet disrupted. FcR blocking agent was added to the disrupted pellet at 100µL per 1ml and left for 10 minutes on ice. Red cell lysis was performed at this stage by the addition of BD Pharm Lyse™ at a volume 10 times that of the cell suspension for 3 minutes at room temperature. This mixture was then washed by the addition of 15mls of MACS buffer and centrifuged for 20 minutes at 1700rpm at 4^{o}C. The supernatant was discarded and the pellet re-suspended in 300µL of MACCS buffer. A 50µL aliquot was taken and diluted with 250µL of MACCS buffer to be used an unstained control. The remainder of the re-suspended pellet was measured and then transferred to 1.5ml Eppendorf tubes ready for staining.

2.7 Staining of disaggregated tissue for FACS

For macrophage isolation each disaggregated sample was stained with the following antibodies CD45-FITC (1 in 66 dilution), CD4-PE (1 in 40 dilution), CD163-PE (1 in 40 dilution), CD3-PE/Cy7 (1 in 100 dilution), Glycophorin-A Pacific-Blue (1 in 100 dilution), CD8-PerCP-Cy5.5 (1 in 100 dilution) and CD14-APC-H7(1 in 20 dilution). For the TREM1 validation experiments the following antibodies were added to the disaggregated samples; CD45-FITC (1 in 66 dilution), CD163-PerCP/Cy5.5 (1 in 20 dilution), CD3-PE/Cy7(1 in100 dilution), Glycophorin-A Pacific-Blue (1 in 100 dilution), TREM1-PE (1 in 50 dilution), and CD14-APC-H7(1 in 20 dilution). Each sample was incubated with the antibody cocktail for 30 minutes on ice protected from light. Excess antibody was then removed with a further wash by the addition of 1mL of MACS buffer and centrifuged at 1300 rpm for 5 minutes. The supernatant was discarded and the pellet re-suspended in 300µL of MACS buffer.

2.8 Fluorescence activated cell sorting (FACS)

Once stained, the samples were passed into 5ml FACS tubes through a 35µm cell strainer cap in preparation for analysis and cell sorting on the flow cytometer. The unstained sample was loaded first to set correct baseline gates for each sample particularly the live dead gate and to assess for auto-fluorescence. Each sample was first analysed without the live/dead stain and then with the addition of the live/dead stain; 4',6-diamidino-2-phenylindole (DAPI) at 10µL of 200 µg/ml. Once the gates were set for each sample, the flow cytometer was calibrated for 4 way cell sorting and cells sorted into Eppendorf tubes filled with 750µL of Trizol LS Reagent®. Once complete the Eppendorf tubes containing the sorted cells were labelled and stored in a secure -80 freezer.

2.9 RNA extraction

RNA extraction from the isolated macrophages along with the subsequent amplification of the RNA and cDNA library preparation was performed by of Dr E Garrido-Martin with my assistance. The RNA was extracted using the miRNeasy RNA extraction kit (Qiagen) following the manufacturer instructions. Although specific microRNA extraction was not required for this project this kit was utilised, as in our pervious experience, this kit was effective in the extraction of RNA from a small number of cells, which was a major challenge in this project. The small quantities of RNA extracted also created challenges in measuring the total RNA after extraction. The RNA concentration was estimated using the quantitative real time PCR method. This involved creating a standard curve using serial dilutions of RNA of known concentrations isolated from human peripheral blood monocytes (PBMCs) isolated by Ficoll gradients and using $CD14^+$ isolating MACS columns. We then performed quantitative real time PCR on RNA extracted from these cells for beta-2-microglobulin at serial dilutions to create the standard quantification curve using SyBR-Green primers. The quality of the extracted RNA was assessed on the bioanalyzer using RNA 6000 pico chips.

2.10 RNA pre-Amplification

For pre-amplification, a starting total of 15ng of total RNA for each sample was taken. ERCC spike in mix was added for control RNA. Ribosomal RNA was eliminated from total RNA by Poly-A RNA selection using Poly(A)Purist MAG kit following the manufacturer instructions.

The enriched Poly-A RNA was then pre-amplified using SePlex RNA Amplification kit for whole transcriptome amplification. Annealing mix was added to the Poly-A RNA, which was then denatured by heating to 70^{O}C for 5 minutes. The reverse transcriptase enzymes along with the library synthesis buffer were added and the reverse transcription completed. The number of cycles required for optimal amplification was determined using a small aliquot of the sample and Amplification Mix with enzyme in a real time qPCR using ROX dye and GelGreen. The optimal number of amplification cycles was taken as that which preceded 2 cycles into the amplification plateau. The remainder of the sample was then amplified using the optimal number of amplification cycles in a mix of Amplification Mix and DNA amplification enzyme. The amplified sample was then cleaned with ZR96 DNA clean up kit, following the manufacturers instructions. The final DNA was measured with the Nanodrop and dsDNA measured with dsDNA Quantifluor. During the amplification stage a number of quality controls measures were taken. A key measure at this stage was the ratio of double stranded DNA (dsDNA) to single stranded DNA. A low ratio would have suggested excess single stranded DNA and therefore over amplification. 1ug of each sample was taken for post adaptor removal by enzyme de-activation. Agencourt AMPure XP beads were used for size selection and clean up. Finally, the amplified DNA samples were measured on the Nanodrop and their size assessed with the bioanalyzer using High Sensitivity DNA chips.

2.11 DNA library preparation for high throughput sequencing

In order to perform the messenger RNA sequencing, DNA libraries were prepared from the amplified dsDNA using the TruSeq Nano DNA library prep kit. The first stage was End-Repair to remove “overhangs” and create blunt ended dsDNA. Agencourt AMPure XP beads were used for size selection in two steps, removal of large DNA fragments (>450bp) with a ratio of 1.6:1 of diluted beads: sample and then removal of small DNA fragments (<150bp) with a ratio of 1:3.1 of undiluted beads: sample. DNA of interest was recovered in TE buffer. 3’ ends of 150-450bp DNA fragments were adenylated using A-Tailing Mix during 30 minutes at 37^{o}C following by enzyme deactivation. The Illumuna adaptors were then ligated. This was followed by a further two sets of clean up using the AMPure XP Beads once again, first in a ratio 1.2:1 and then 1.1:1. The DNA was eluted with TE buffer and PCR amplified using PCR Primer Cocktail and Enhanced PCR Mix.

The final amplified libraries were cleaned up using Sample Purification Beads and the size of the library determined by HS-DNA Agilent Bioanalyzer (150bp-430bp). The quantity of the library was determined by Nanodrop and dsDNA Quantifluor.

2.12 RNA high throughput sequencing

The cDNA libraries prepared onsite by Dr E Garrido-Martin were submitted to Ocean Ridge Biosciences (ORB), Florida for mRNA sequencing. The methods utilised by ORB are briefly outlined here. The quality and size distribution of the amplified libraries were confirmed by chip-based capillary electrophoresis (labChip, GX microfluidic system- Caliper Life Sciences) and quantified using the KAPA Library Quantification Kit (Kapa Biosystems; Boston, MA).

For the mRNA sequencing the libraries were pooled at equimolar concentration and diluted prior to loading onto the flow cell of the Illumina cBot cluster station. The libraries were extended and bridge amplified to create sequence clusters using the Illumina HiSeq PE Cluster Kit v4 and sequenced on an Illumina HiSeq Flow Cell v4 with 100-bp pared-end reads plus index read using the Illumina HiSeq SBS Kit v4. Real time image analysis and base calling were performed on the instrument using the HiSeq Sequencing Control Software version 2.2.58.

2.13 mRNA Data Processing

The initial processing of the raw RNA-Seq data was performed by ORB, a brief description of the methods they utilised is provided here.

The raw FASTQ files were spilt into files containing 4,000,000 reads and quality checked using FASTX toolbox.(385)

The sequence alignment to the hg38 human reference genome was performed using TopHat v2.1.0 (386) with fr-unstranded as the library type. The Bioconductor easyRNASeq (387) c2.4.7 package running on R version 3.2.2 was used, with the setting in **table 10** for exon and gene level counting and Ensembl Human Release 83 was used for gene annotation.

Setting Name	Exon Counting	Gene Counting
Organism	Hsapiens	Hsapiens
Read Length	100L	100L
Annotation Method	RDA file	RDA file
Count	Exons	Genes
Summarisation	-	geneModels

Table 10 easyRNASeq settings for read count

To allow for differential gene expression analysis the gene-level read counts were adjusted for library size and gene length by calculating the reads per kilobase transcript length per million mapped reads (RPKM). The RPKM filter cut off filter used was the 50 read RPKM equivalent for each sample. The 50 read RPKM equivalent value for a given sample was calculated as;

$$RPKM = \frac{\left({}^{50\ reads}/_{2Kbases}\right)}{{}^{\#total\ mapped\ reads}/_{100{,}000}}$$

Where 2 Kbases is the average length of a gene and the #total mapped reads is the number of uniquely aligned reads from that sample. The average number of reads aligned was 34 million, which corresponded to an average cut-off value of 0.75 RPKM. A gene was therefore considered detectable if its RPKM value was greater than 0.75 in at least one sample. ORB utilise the 50 read RPKM value in their mRNA sequencing pipeline because the RPKM values of a gene represented by 50 reads should be reproducible in technical replicates.

The 50 read RPKM filter identified 29,403 detectable human genes; the RPKM values of these genes were log2-transformed and used for statistical analysis. The fold changes were calculated as the ratio of the average values from the indicated groups. If the mean changes of both groups under comparison were below the Detection Threshold (10 reads/gene) the fold change was reported as "NA". The statistical analysis was performed using R version 3.2.2. ANOVA and Tukey P values across all genes were adjusted to control the false discovery rate (FDR) which was corrected with the Benjamin and Hochberg method.(388)

This results of this analysis was provided by ORB as an excel spread sheet with all the identified genes together with fold changes, P-values, corrected FDR and normalised gene read counts. ORB also provided a set of principal component analysis for this output.

I performed an initial analysis of the fold changes given by the above methods. It was clear that from this, samples NAC528, CC593 and CDC594 were outliers (further information in results section). These were removed and a further analysis using the R package DESeq2 package (389) to provide estimated log2 fold changes, which account for the distribution in fold changes across all genes. This analysis was performed by ORB and the results of which were provided to myself as an excel spread sheet with log2 fold change, p-value, corrected FDR (388) and normalised gene read counts for each gene. This data output was subsequently used for all differential gene and pathway analysis.

2.14 Real-time polymerase chain reaction

Real-Time polymerase chain reaction (PCR) was used for quantitative analysis of mRNA transcripts from the total RNA extracted from intestinal macrophages. The total RNA was extracted and subjected to pre-amplification using the whole transcriptome amplification kit (Sigma Aldrich) as described in sections 2.10 and 2.11. The resulting cDNA was used as template for Real-Time PCR following the manufacturer's protocol using TaqMan® Universal Master Mix, no AmpErase® (Applied Biosystems). The mRNA transcripts were quantified with TaqMan® Gene Expression Assays (Applied Biosystems). The reactions were completed in triplicate using 5ng of cDNA for each reaction in a final volume of 5µL per well on 384 well plates using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The data was analysed using the ΔΔCt method for normalisation. GAPDH was utilised as an internal control for normalisation.

2.15 Immunohistochemistry

Glycol methacrylate (GMA) and paraffin embedded intestinal tissue were used for immunohistochemical interrogation. A range of non-paired inflamed and non-inflamed tissue samples were embedded in GMA and stored at -20^{O}C securely ready for cutting and staining. The paraffin embedded samples were stored securely at room temperature.

2.15.1 Staining of GMA embedded tissue

Samples were collected in acetone on ice and embedded in GMA within 24 hours. The GMA embedded samples were stored at -20^{O}C ready for sectioning and staining.

The first stage of GMA section staining was inhibition of the endogenous peroxidase with a solution of 5mls of 0.1% sodium azide and 50µ of 0.3% hydrogen peroxide for 30 minutes before washing with Tris-buffered saline (TBS, **table 11**), three times in 5 minute intervals. The slides were then drained the blocking medium applied (**table 12**) and left for 30minutes. The slides were then drained and the primary antibodies applied (CD163 antibody, Cambridge bioscience, at 1 in 150 dilution), a cover slip placed over the section and left to incubate overnight at room temperature.

The next day the cover slips were carefully removed and the sections washed with TBS three times in 5-minute intervals. The slides were then drained and the biotinylated second stage antibodies added and left for 2 hours. The sections were then washed a further three times with TBS with 5 minute intervals and then the avidin-biotin complexes (vectastain Elite ABC HRP Kit) added at the appropriate manufacturer given concentrations and left for 2 hours. This was followed by a further three washes with TBS and the application of amino ethyl carbazole (AEC) for 20 minutes before rinsing with TBS and then washing under a running cold tap for 5 minutes. The slides were then placed in the counter stain Mayer's haematoxylin and blue for between 60 to 90 seconds followed by a wash under a cold tap for a further 5 minutes. To preserve the stained sections, they were all then mounted in pertex.

Reagent	Quantity	Source
Sodium Chloride	80g	Fisher Scientific
Tris	6.05g	Fisher Scientific
1M Hydrochloric Acid	38mls	Fisher Scientific
Distilled water	10L	
Method: Mix buffer salts and acid in 1L of distilled water, adjust pH to 7.65 and add to remaining 9L of water to give a final pH of 7.6		

Table 11 Tris Buffered Saline pH 7.6 composition

Reagent	Quantity	Source
Dulbecco's Modified Eagles Medium (DMEM) *DMEM - 5 bottles* *ROW- 4l* *NaHCO3 – 18.5g (3.7g/l final dilution)*	80mls	Sigma
Foetal calf serum	20mls	IPAA
Bovine serum albumin	1g	Sigma

Table 12 GMA blocking agent

Antibody	Concentration	Source
CD163, clone RM3/1	1 in 150	Hycult Biotech

Table 13 Antibody concentrations for GMA staining

2.15.2 Staining of Paraffin Embedded Samples

The NHS diagnostic laboratory at Southampton General Hospital performed the staining of the paraffin embedded samples. The expression of CXCL9, MMP12 and CD40 was assessed using IHC in colonic biopsy samples from UC, CD and healthy controls. Optimisations staining runs were completed to identify suitable antibodies and concentrations. The tissue sections were stained for CXCL9 (41906, Novus Biologicals, dilution 1/20), MMP12 (ab137444, Abcam dilution, 1/100) and CD40 (ab1345, Abcam, dilution 1/100). Detection and visualisation of the antibody was performed using low pH heat induced epitope retrieval with the ENVision FLEX+ system and DAB as the chromogenic substrate. Pictures of the stained slides were taken on a Zeiss AxioCam MRc5 microscope and Zeiss Axiovision software (version 4.8.1.0; Zeiss).

2.16 Statistical Methods

The software package Graphpad Prism version 7 for mac OS (La Jolla Calafornia, USA) was utilised for statistical analysis of the flow cytometry data, patient demographics and qPCR validation analysis. The ANOVA test with Kruskal-Wallis and subsequent Dunn's test or Tukey for multiple comparisons testing was utilised for the comparisons made between the disease and control groups made in Graphpad Prism. (390)

The RNA-Seq raw data was analysed by Ocean Ridge Biosciences as described in section 2.13. ORB utilised the R-Package DESeq2 (389) to provide fold change analysis with a corrected FDR using the Benjamini-Hochberg procedure (388) this was to control for multiple hypothesis testing given the nature of high throughput RNASeq.

Pathway analysis was performed using IPA (391) which utilises the Fisher's exact test to calculate P-values for comparison of pathways. The pathway analysis utilising GSEA (392) provided q values for the gene set associations which is the FDR and is calculated using the Benjamini-Hochberg procedure (388).

Chapter 3: Study Cohorts

3.1 Patient demographics

A total of 10 patients with active UC, 11 with active colonic CD and 10 normal control patients were recruited to the study for FACS sorting of cells from mucosal biopsies and subsequent high throughput RNA-Seq of the isolated macrophages as per the study inclusion criteria.

For the UC macrophage RNA-Seq group the average age was 44 years (range 24-76), 60% were males and the average duration of disease was 1699 days (range 0-5222). A disease duration of 0 days was given for patients who were diagnosed with IBD at the endoscopy where samples were collected for this project (all these subjects subsequently had clinical and histologically confirmed IBD). Four patients in the UC group were not on any IBD medications, four were on 5ASA preparations, two were on oral steroids and a further two were taking Immunomodulators. The average age of the CD group was 48.6 (range 23-80), 55% of the group were males and the average duration of disease was 3,311 days (range 0-10,298). Within the CD group 5 patients were taking an immnomodulator, 2 were on oral steroids, 1 was taking a 5ASA preparation and 3 were on no IBD medications. The average age of the normal group was 65.8 (range 50-78), again the majority were males (70%). The indication for the endoscopic examination for the normal group was polyp surveillance in all but one patient in which asymptomatic iron deficiency anaemia was the indication for the procedure. In all three groups all subjects were of Caucasian ethnicity except one subject in the UC group who was of Afro-Caribbean ethnicity. This reflects the geographical location from which the study was conducted and the higher prevalence of IBD in those of European ancestry.(393)

The average age of the subjects in the normal group was higher than that in the CD and UC group, this was statistically significant for the comparison of UC Vs N ($p=0.03$). The demographics of this group are similar to other published IBD cohorts in age and duration of disease.(394-396) There were however no patients recruited who were on biologic medications and the smoking rate is lower than that seen in the previously mentioned IBD study cohorts. Due to the nature of the study it was never intended that a fully representative sample of IBD patients was recruited. The study inclusion criteria were designed to recruit IBD patients that would allow the best opportunity to study intestinal macrophages whist at the same time recruit patients at a reasonable rate to the study due to the time limitation on the study. Patients on multiple drug therapies particularly those that target particular inflammatory pathways would have made the study results difficult to interpret and delineate drug effect from disease pathology. The low level of smoking in patients recruited to this study is marked. This does raise the possibility of recall

and patient reporting bias. At the time of recruitment the patients completed a questionnaire with the study investigator and this was the only source of the smoking data, whereas the other demographic data such as drug history was validated with the local hospital records. Another possibility is recruitment bias and that the cohort is skewed towards a motivated group of patients, (IBD patients are encouraged to stop smoking by their clinicians). Given that entry to the study was voluntary, this is possible and a bias that all observational studies of this type suffer from. Whist the study cohort was not fully representative of the general IBD patient population, this was not detrimental to the study quality and does not impact on the ability to generalise the results.

In addition to the patients recruited to the RNA-Seq arm of the study, further cohorts of patients were recruited for validation of the RNA-Seq results, 6 normal control, 5 UC and 6 CD. The full detailed individual patient demographics for all the patients recruited to the study can be found in **Appendix A**. The demographics and disease activity of the RNA-Seq and validation cohort are summarised in **table 14**, both groups were found to be similar. The UC validation cohort had a higher average total MAYO score compared to the UC RNA-Seq cohort suggesting that the patients in the UC validation cohort had more severe disease. This difference in this direction is unlikely to have an impact on the results, had the reversed occurred validation may have been unsuccessful due to lower disease activity in the validation cohort. There were more patients in the CD RNA-Seq cohort taking immunomodulators than the CD validation cohort, 54.5% Vs 33.3% though not statistically significant, the CD validation cohort also had a marginally higher HBI score (7.17 Vs 6.7) suggesting that they may have collectively been an undertreated group of patients, again this is unlikely to have had any impact on the results of the present study.

	Crohn's disease		Ulcerative Colitis		Normal	
	RNA-Seq	**Validation Cohort**	**RNA-Seq**	**Validation Cohort**	**RNA-Seq**	**Validation Cohort**
Mean age (Std. Deviation)	48.64 (17.74)	50.5 (13.28)	44 (17.56)	41.6 (18.93)	65.8 (8.72)	67 (14.32)
Males (%)	54.5%	50%	60%	60%	70%	66.67%
Mean duration of disease in days	3313	5554	1699	948.4	NA	NA
Smokers (%)	18%	0	0	0	20%	0
Taking immnomodulator therapy (n)	54.5 % (6)	33.3 % (2)	1	NA	NA	NA
Mean HBI in CD (range)	6.7 (0-15)	7.2 (3-11)				
Mean full MAYO score (range)			6.2 (2-10)	9 (7-10)		
Mean endoscopic MAYO score (range)			1.9 (1-3)	2.2 (2-3)		

Table 14 Summary of patient demographics across the RNA-Seq and validation Cohorts. Comparisons were made between the two cohorts and also between the disease groups within the cohorts. Within the RNA-Seq cohort, there was no statistical difference in age between the disease groups UC, CD and N , P<0.05 (ANOVA with Tukey test) except for UC Vs N (P=0.03). Comparing the CD RNA-Seq group with the CD validation group, the UC RNA-Seq with the UC validation group and normal RNA-Seq group with the normal validation group showed no statistical difference in age, p<0.05 (ANOVA with Tukey test). Comparing the duration of disease between the validation and RNA-Seq cohorts there was not statistical difference between the two UC cohorts and the two CD cohorts, P<0.05 (ANOVA with Tukey's test). There was no significant statistical difference in the HBI score between the CD RNA-Seq group and the validation group, P<0.05 (two tailed t-test). Although there is was no significance difference in endoscopic MAYO score between the two UC groups there was a statistically significant higher mean full MAYO score for the UC validation cohort compared to the UC RNA cohort, p=0.043 (two tailed t-test).

Chapter 4:

Results 1: Optimisation of cell isolation from whole tissue colonic biopsies

4.1 Introduction

The technology of FACS has progressed significantly since its first inception by Herzenberg and colleagues (397) in 1969. FACS is a powerful tool in the isolation of cells from complex heterogeneous mixtures both in everyday clinical practice and research. FACS relies on the identification of fluorescent cellular "tags" and therefore cells must be fluorescent labelled prior to sorting. Commonly cellular surface molecules are targeted in this process but intracellular staining is also possible. FACS compares favourably with the alternative techniques of cellular isolation such as magnetic bead separation in terms of both efficacy and speed. FACS has the additional benefit of providing quantitative data on cellular composition and sorting yield.(398)

A target of isolating 10,000 macrophages was set, as the amplification and RNA-Seq pipeline in place within the Dr Sanchez-Elsner laboratory required this quantity to ensure sufficient quality. The protocols were optimised with these parameters for macrophage numbers as a minimum target.

4.2 Disaggregation of colonic tissue

A number of methods have been described for the isolation of immune cells from human and mouse intestines. Many of these protocols, first treat with EDTA followed by enzyme digestion with collagenase for up to 180 minutes and have often been applied to resection samples rather than mucosal biopsies as is the case in this project.(399, 400) Mechanical methods of colonic tissue disaggregation as well as combined approaches of enzyme followed by mechanical disruption have also been described.(401-403) Mechanical enzyme free methods of tissue disaggregation tend to yield fewer immune cells than enzyme methods(404) but potentially have the benefit of avoiding enzyme associated surface molecule cleavage.

In this study we wanted the isolated cells to be sorted as soon as possible after harvest to avoid any ex-vivo induced changes of these cells and their gene expression, particularly as macrophages are known to be highly plastic and able to rapidly change function in response to their microenvironment.(333) We had the logistical advantage that the endoscopy theatres where I

harvested the colonic biopsies is in the same building and floor as Dr T Sanchez-Elsner's laboratory where the samples were processed. The time from harvest to start of processing was less than 30mins for all samples and often less than 5 minutes. In developing the protocol for disaggregation I did not want to loose the opportunity to process the samples as fast as possible and minimise and ex-vivo changes in expression profiles. Previous work in the Dr T Sanchez-Elsner laboratory had led to the development of an established protocol for the disaggregation of lung tissue for sorting of cells by FACS utilising the enzyme mixture Liberase™ DL with a short incubation period. This was an attractive protocol to apply to this study because of the speed at which the tissue could be disaggregated. Liberase™ enzymes consist of highly purified collagenase I and collagenase II and as there is less lot-to-lot variability, the manufacturers claim a higher degree of experimental reproducibility compared with collagenase-based experiments. Liberase™ tissue disaggregation has been shown to better at maintaining the integrity of cells in pancreatic islet isolation and the isolation of follicles from ovarian biopsies compared to collagenase protocols.(405) It is therefore considered a more gentle enzyme approach to tissue disaggregation than pure collagenase.

The lung resection tissue however is structurally quite different to the small colonic biopsies harvested in this project and therefore the protocol required alteration and optimisation for this application. I took the opportunity to disaggregate some lung tissue with the lung section of the Dr T Sanchez-Elsner group, to learn the protocol as applied to lung tissue. I then set about determining the optimal duration of incubation with the Liberase™ DL at 37.5^{0}C for the colonic mucosal biopsies. It was apparent that the incubation times used for the lung protocol were too long for the colonic biopsies with the majority of cells appearing non viable under the microscope. I assessed the viability of the cells isolated using trypan blue exclusion at various incubation times with Liberase™ the results of which are shown in **table 15**.

Incubation time	Percentage dead cells	Comment
1 hour	100%	No live cells seen
45 minutes	69.6%	
30 minutes	55.6%	
15 minutes	25%	
10 minutes	Not counted	Very few cells. Tissue not adequately disrupted and difficult to pass sample through filter
5 minutes	Not counted	Very few cells. Tissue not adequately disrupted and difficult to pass sample through filter

Table 15 The effect of incubation time with Liberase™ DL and cell death

The target number of macrophage cells for isolation by FACS was 10,000. Initially during the optimisation phase four colonic biopsies from each recruited patient were used, the macrophage cell yield from theses experiments were insufficient in the region of 2,000 to 4,000 cells. I therefore increased the number of biopsies taken from each patient to a minimum of 8 and this increased the yield of macrophages to consistently above 10,000 for both inflamed and non-inflamed samples.

In order to assess if the Liberase™ had an effect on the surface molecules utilised for cell sorting the protocol was compared with an established collagenase D protocol as described by Vossenkamper et al.(406) It was a challenge to compare the two methods because tissue from the same patient had to be divided up equally between the two disaggregation protocols. The biopsies are generally quite heterogeneous so when making comparisons between the two methods I focused on percentage expression rather than total cell yield as it was not possible to split the tissue equally with confidence. In this experiment the focus was expression of the macrophage markers CD163 and CD14 that were to be used in FACS sorting. **Figure 4** demonstrates the results of this experiment. The collagenase protocol was significantly longer with an incubation period with enzyme of 1 hour compared to the 15 minutes with the Liberase™. As the experiments had to be run along slide each other this did delay the time to the FACS machine for the Liberase™ sample and may explain the higher than expected number of dead

cells for this particular experiment for the Liberase™, though there was greater cell death for the collagenase treated sample (live cells for collagenase 7.4% Vs 35% for Liberase). The expression of CD163 was similar between the two protocols but there appeared to be some loss of the CD14 expression for the collagenase treated sample although this difference was not large. These findings are in keeping with a recent study, which assessed various methods of disaggregating colonic tissue. This study compared a Liberase™ TL protocol directly with a collagenase D protocol finding that expression of monocyte derived surface markers did not differ between collagenase D and Liberase™ TL.(404) In our protocol we have opted to use the less aggressive Liberase ™ DL which has a lower protease activity than Liberase™ TL and is the least aggressive of the Liberase ™ mixtures, in order to minimise any cleavage cell surface molecules. Extrapolating the results of the aforementioned study together with our findings, it seems reasonable to assume that the Liberase DL will not have the effect of altering macrophage surface marker molecules.

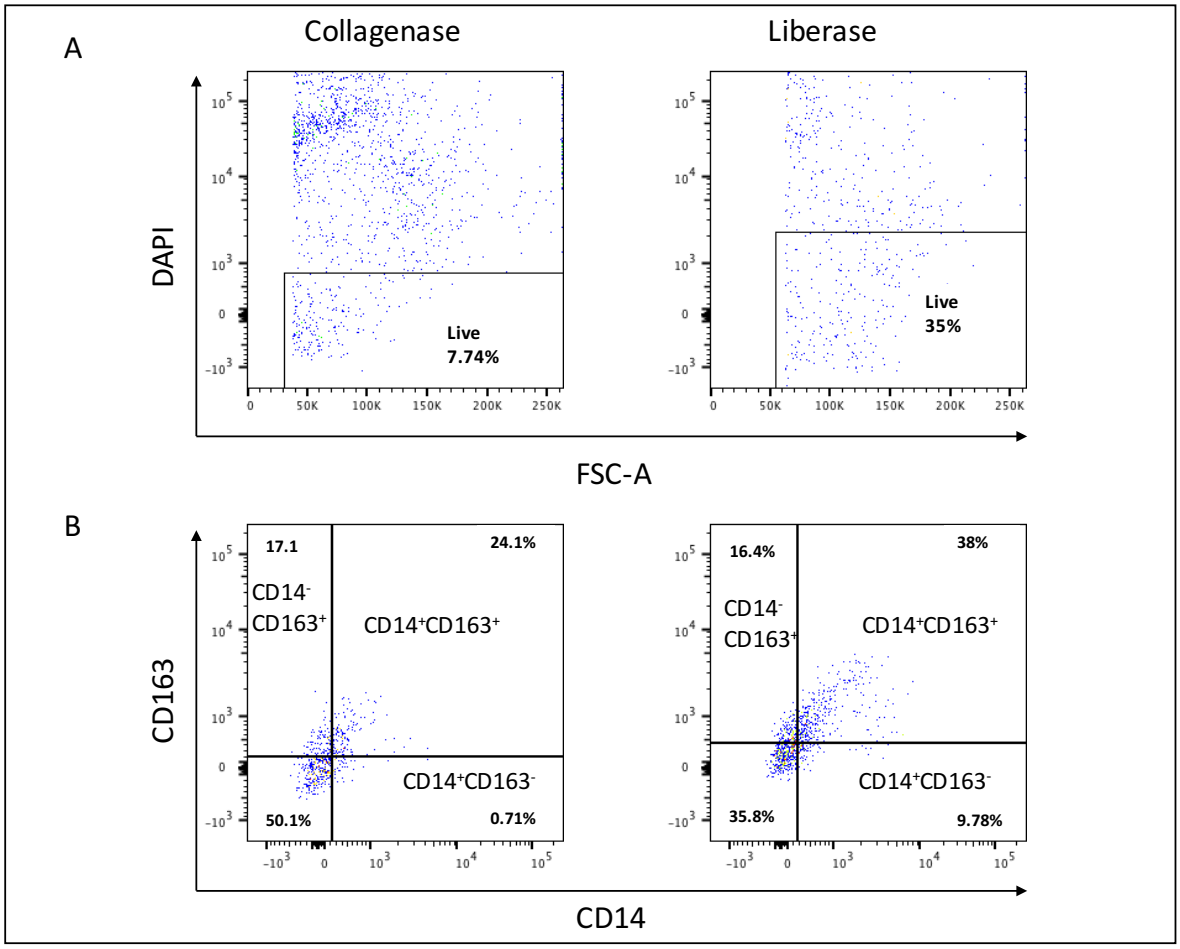


Figure 4 Comparison of collagenase D and Liberase™ protocols for optimisation sample 620. (A) Shows the live dead gate for unstained control samples for treatment with collagenase D and Liberase™ following the addition of the live/dead stain DAPI. Cell survival was less with the collagenase protocol. (B) Shows the expression of the macrophage markers CD163 and CD14. Expression of CD163 was similar between the two samples but there was less expression of CD14 in the collagenase treated sample

Once the disaggregation and flow cytometry protocol had been optimised and we were able to isolate greater than 10,000 macrophages consistently, I utilised IHC to assess CD163 staining in samples embedded in GMA that were also used for the optimisation of flow cytometry protocol in order to visualise the cells isolated by FACS. **Figure 5** shows representative sections of this staining which confirmed the presence of CD163 surface positive cells in the same samples that FACS was utilised to isolate CD163 positive cells.

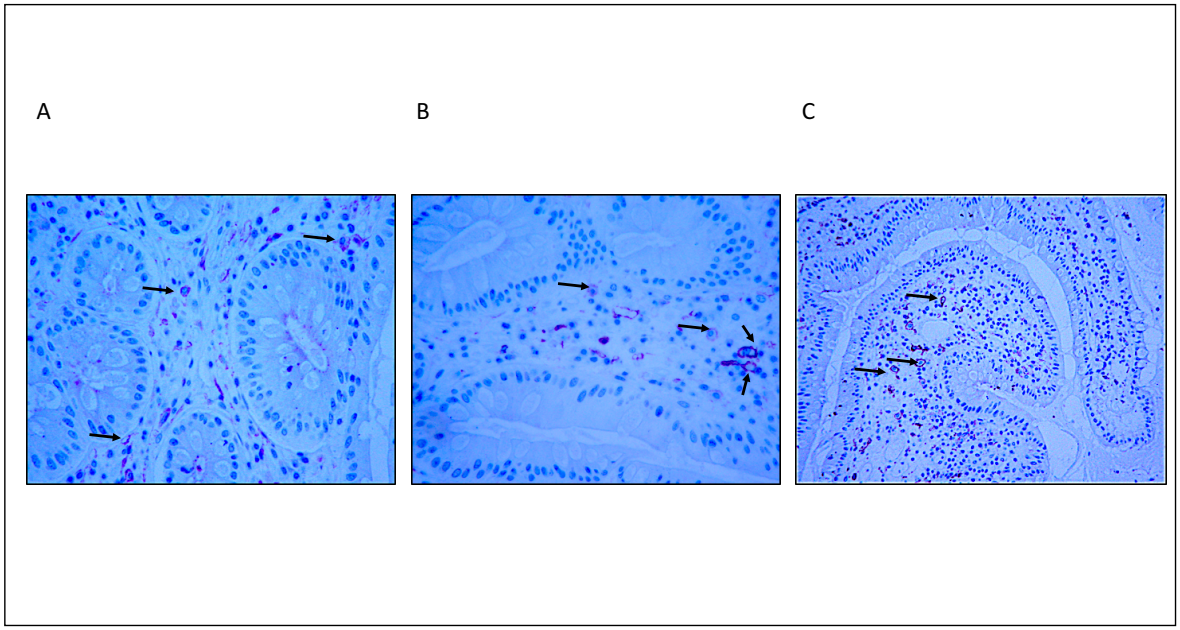


Figure 5 GMA embedded sections of inflamed intestine stained with CD163 antibody. Positive cells are stained red. (A) 20x magnification, (B) 20x magnification, (C) 10x magnification to show overview of tissue. (B) and (C) show the same tissue at different magnification. The arrows point to examples of positively stained cells with the CD163 antibody.

4.3 Flow cytometry

Once the disaggregation and flow cytometry protocols were optimised all recruited samples were processed according to the established protocol without deviation.

In order to set the gates for each sample, a 50ul aliquot of the sample was kept unstained and utilised as a control sample to set the gates. An example of the typical flow cytometry plots for the macrophage gaiting with an unstained control sample, stained inflamed and non-inflamed samples is shown in **figures 6 and 7**.

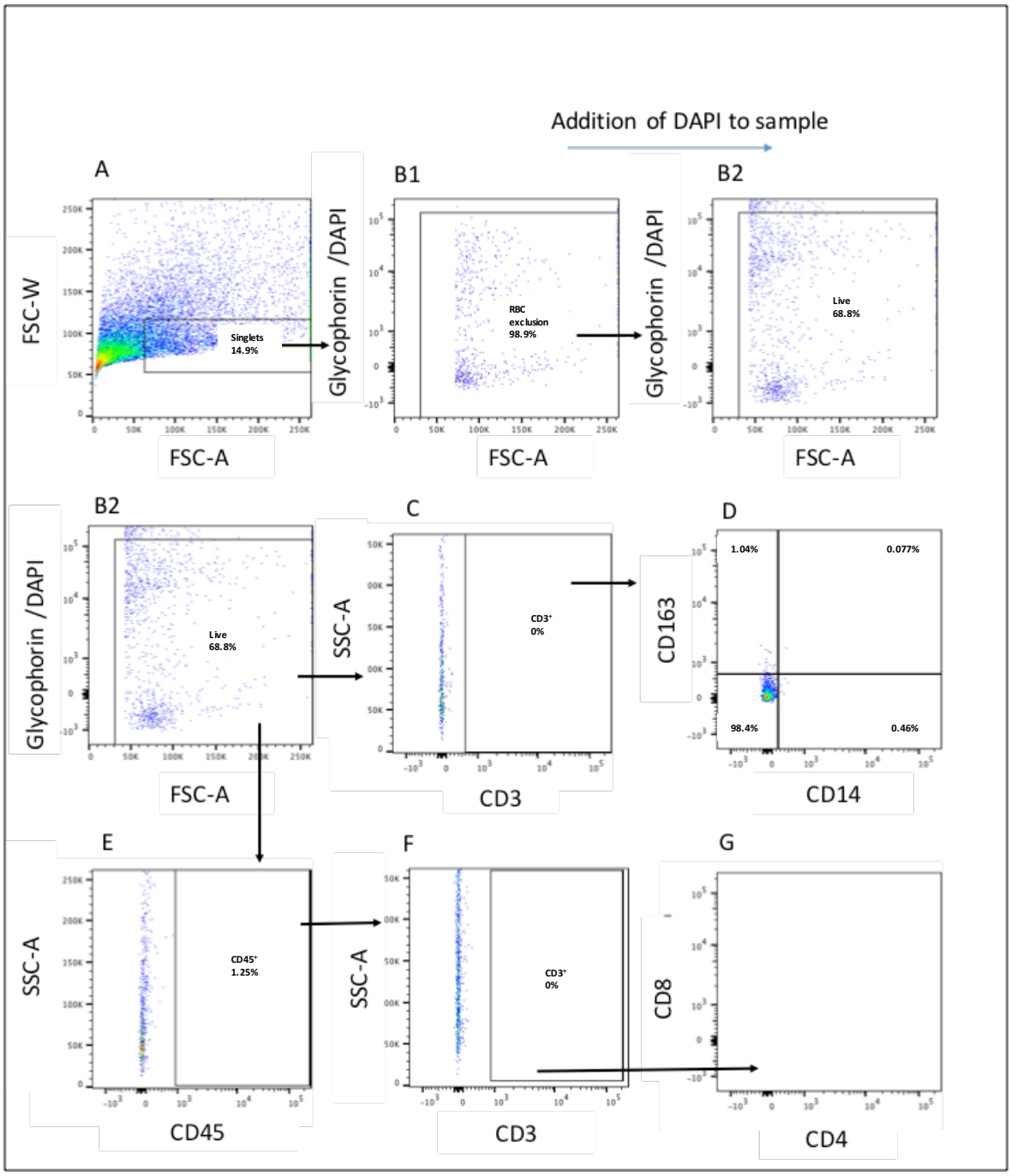
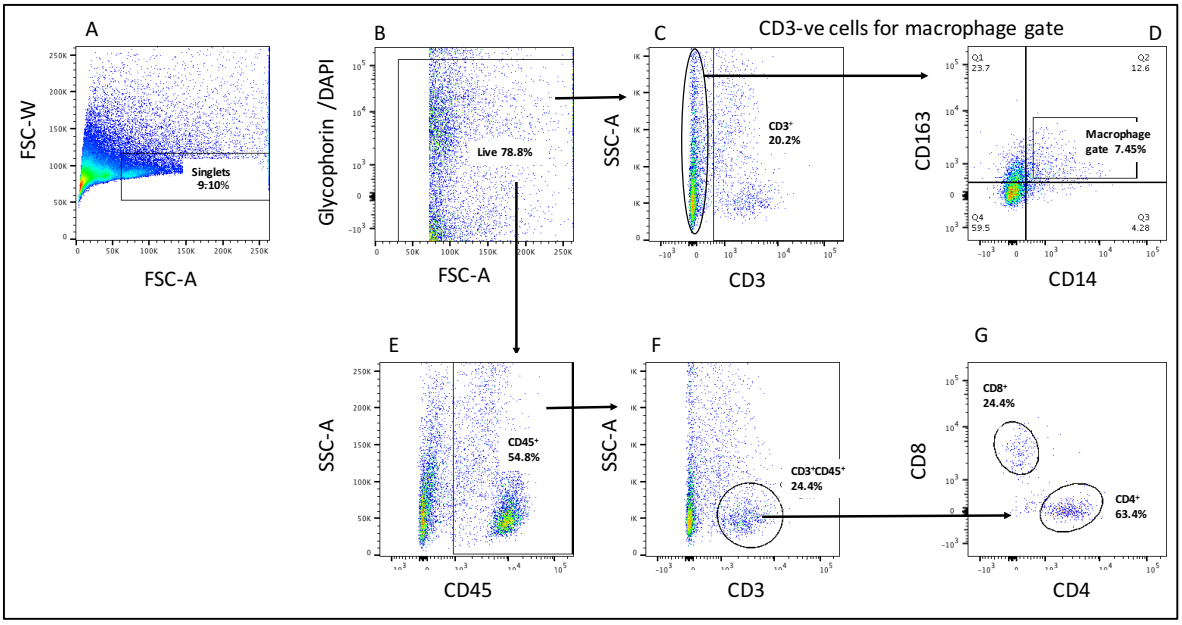


Figure 6 Gating strategy for unstained control sample of CC593. An unstained sample was first passed through the flow cytometer. (A) Singlets were first identified prior to setting (B1) the live/dead gate. DAPI used as the marker for dead cells was then added, and the sample passed again through the flow cytometer excluding the dead cells (B2). The live cells were then utilised to set the gates for (C) CD3, (D) CD163-CD14, (E) CD45.

Inflamed Sample



Non –Inflamed sample

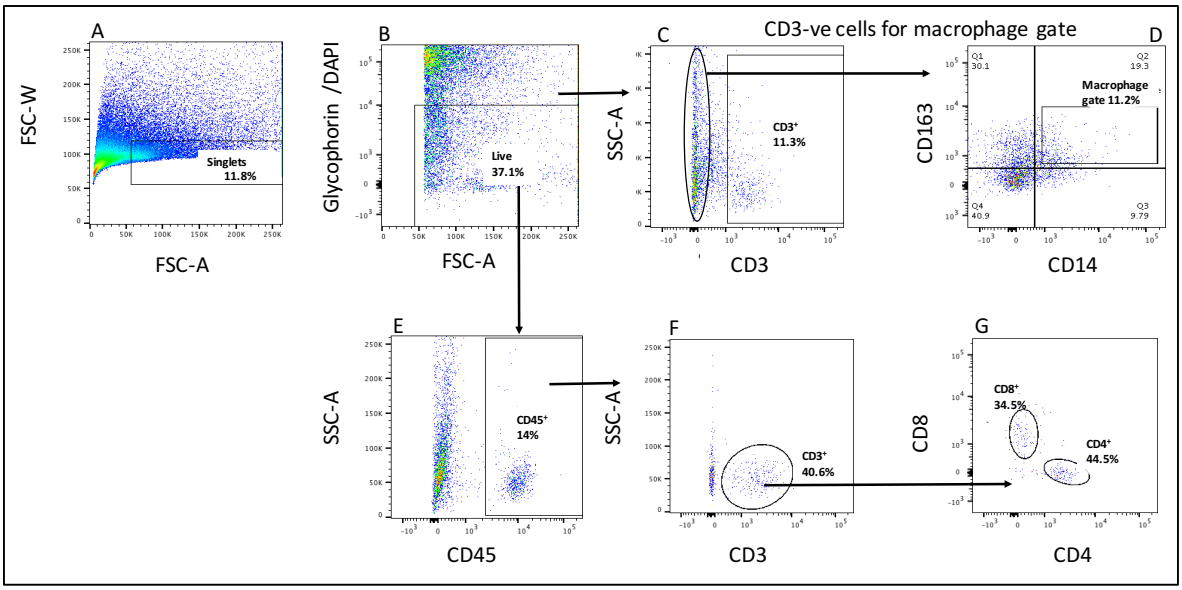


Figure 7 Gating strategy showing sample CCC593 and NAC580 as inflamed and non-inflamed examples. The gates were set using an unstained sample as in Figure 7. Singlets were first selected (A) and from this dead cells positive for DAPI and red blood cells positive for glycophorin A (CD235a) were excluded (B). For the isolation of macrophages, the live cells were gated for CD3+ cells (C), the negative of this gate (CD3 negative cells), were further gated for CD14 and CD163 (D). The macrophages were selected from quadrant two of the CD14 and CD163 plot (D) avoiding cells on the border of the quadrant and selecting a clear population if possible. In order to analyse the lymphocyte population, the live cells were gaited for CD45 (E), the CD45 positive cells were further gaited for CD3 (F) and subsequently CD8 and CD4 plot G.

4.3.1 Isolation of immune cells from colonic tissue biopsies by FACS: RNA-Seq Cohort

The cell numbers obtained from the harvested biopsies for each recruited subject are given in **tables 16-18**. The number of biopsies taken from each patient was standardised to 8 pinch biopsies for the inflamed samples and 10 for the non-inflamed samples. It was however impossible to control for the size of each individual biopsy. It can clearly be seen that the cell yields vary considerably between patients. Although I suspected that the number of immune cells isolated corresponded with severity of inflammation, analysis of this data along this enquiry was not possible as there are too many unmeasured variables that cannot be controlled for to generate meaningful data and analysis.

Sample code	Date of Sample	Macrophage cell Count	CD4 cell count	CD8 cell count
CDC527	14/04/2015	9222	1058	614
CCC536	12/05/2015	11838	2133	216
CDC541	26/07/2015	33836	2035	445
CDC565	21/07/2015	14528	884	132
CCC579	11/08/2015	17936	6462	1073
CDC594	06/10/2015	66371	946	161
CCC593	06/10/2015	24726	10121	3433
CDC606	19/11/2015	18210	844	160
CCC607	24/11/2015	20710	1973	621
CDC609	24/11/2015	25310	1795	198
CDC610	22/12/2015	45264	8179	1402

Table 16 Crohn's Disease sorted cell yields by sample along with eh date of acquisition.

Sample Code	Date of sample	Macrophages cell count	CD4 cell count	CD8 cell count
UCC535	12/05/2015	10572	159	34
UDC546	02/06/2015	57256	11762	612
UCC583	18/09/2015	10986	594	302
UCC588	01/09/2015	39636	10427	1421
UCC595	06/10/2015	12680	1126	181
UCC602	10/11/2015	9109	147	94
UCC604	17/11/2015	31940	3373	747
UDC608	24/11/2015	14703	761	158
UDC612	05/01/2016	44807	12528	0
UDC613	12/01/2016	29336	46457	0

Table 17 Ulcerative Colitis sorted cell yields by sample

Sample Code	Date Of Sample	Macrophage cell number	CD4 cell number	CD8 cell number
NAC528	21/04/2015	13681	208	61
NAC564	15/07/2015	26578	245	74
NAC573	04/08/2015	11590	1122	389
NAC580	13/08/2015	21629	3429	2337
NAC599	12/10/2015	38804	1382	116
NAC601	29/10/2015	37617	363	363
NAC603	17/11/2015	33353	1693	254
NAC605	17/11/2015	33160	678	112
NAC597	12/10/2015	6758	11	99
NAC572	29/7/2015	5012	169	382

Table 18 Normal Controls sorted cell yield by sample

4.3.2 Isolation of immune cells from colonic tissue biopsies by FACS – Validation Cohort

The colonic biopsy samples harvested from the patients recruited to the validation cohort section of the study were handled using the same FACS protocols as those utilised for the original RNA-Seq cohort of patients to isolate, macrophages, CD4 and CD8 T-lymphocytes. In this section **tables 19-21** show the number of cells that were isolated for each sample. As with the RNA-Seq cohort the cell numbers that were isolated vary considerably between samples. This was most likely due to the variations in the biopsy sampling and inter-patient differences rather than experiment methods, as these were kept constant for every sample.

Sample Code	Date of sample	Macrophages cell count	CD4 cell count	CD8 cell count
CDC618	16/02/2016	41346	513	1872
CCC622	07/06/2016	10300	7097	3138
CCD623	28/06/2016	10218	276	96
CCC624	28/06/2016	17266	5158	2552
CCC640	16/01/2017	19586	411	76
CDC646	24/01/2017	32642	1543	0

Table 19 Cell yields isolated by FACS from the Crohn's disease patients recruited to the validation cohort.

Sample Code	Date of sample	Macrophages cell count	CD4 cell count	CD8 cell count
UCC614	26/01/2016	17148	640	3717
UDC615	26/01/2016	7851	1322	1412
UDC619	16/02/2016	24325	184	904
UDC636	13/12/2016	50981	2249	14514
UDC637	21/12/2016	49673	5607	14117

Table 20 Cell yields isolated by FACS from the ulcerative Colitis disease patients recruited to the validation cohort.

Sample Code	Date of sample	Macrophages cell count	CD8 cell count	CD4 cell count
NAC638	10/01/2017	50424	189	260
NAC639	11/01/2017	50338	0	0
NAC641	16/01/2017	51259	157	196
NAC642	16/01/2017	50844	30	301
NAC643	17/01/2017	42166	374	157
NAC644	17/01/2017	25418	324	686

Table 21 Cell yields isolated by FACS from the normal control participants recruited to the validation cohort.

4.3.3 Macrophages – the most abundant immune cells isolated from mucosal colonic biopsies

Intestinal macrophages ($CD163^{+}CD14^{+}$) were the most abundant immune cells isolated using the FACS protocol in this study of the immune cells that were collected, **figure 8**. This was replicated in the validation cohort of patients **figure 9**. This finding is consistent with a human histological study (407) demonstrating a large number of macrophages in the mucosa of the colon compared to other regions of the gut and the observation in mouse studies that the intestine is the largest reservoir of macrophages in the body.(408)

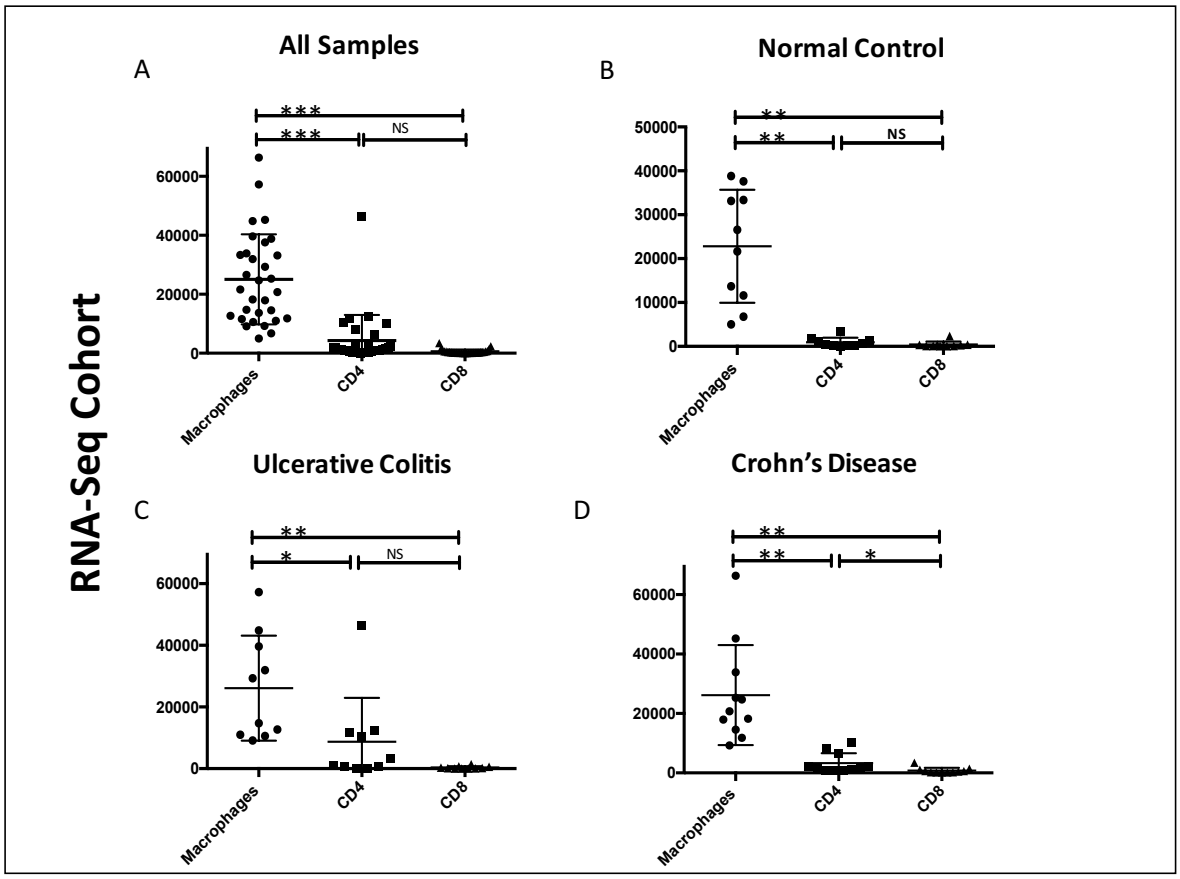


Figure 8 Cell yields by FACS sorting for the RNA-Seq cohort. Showing the raw number of cells isolated for all samples combined 9A (n=31) and then divided in to disease group, Normal control **9B** (n-=10), Ulcerative colitis **9C** (n=10) and Crohn's disease **9D** (n-11). The error bars represent the standard deviation and the mean values. *P< 0.05, ** P<0.001, NS – non significant (All statistical significant tests by the ANOVA test with Tukey multiple comparison test calculated in Graphpad Prism).

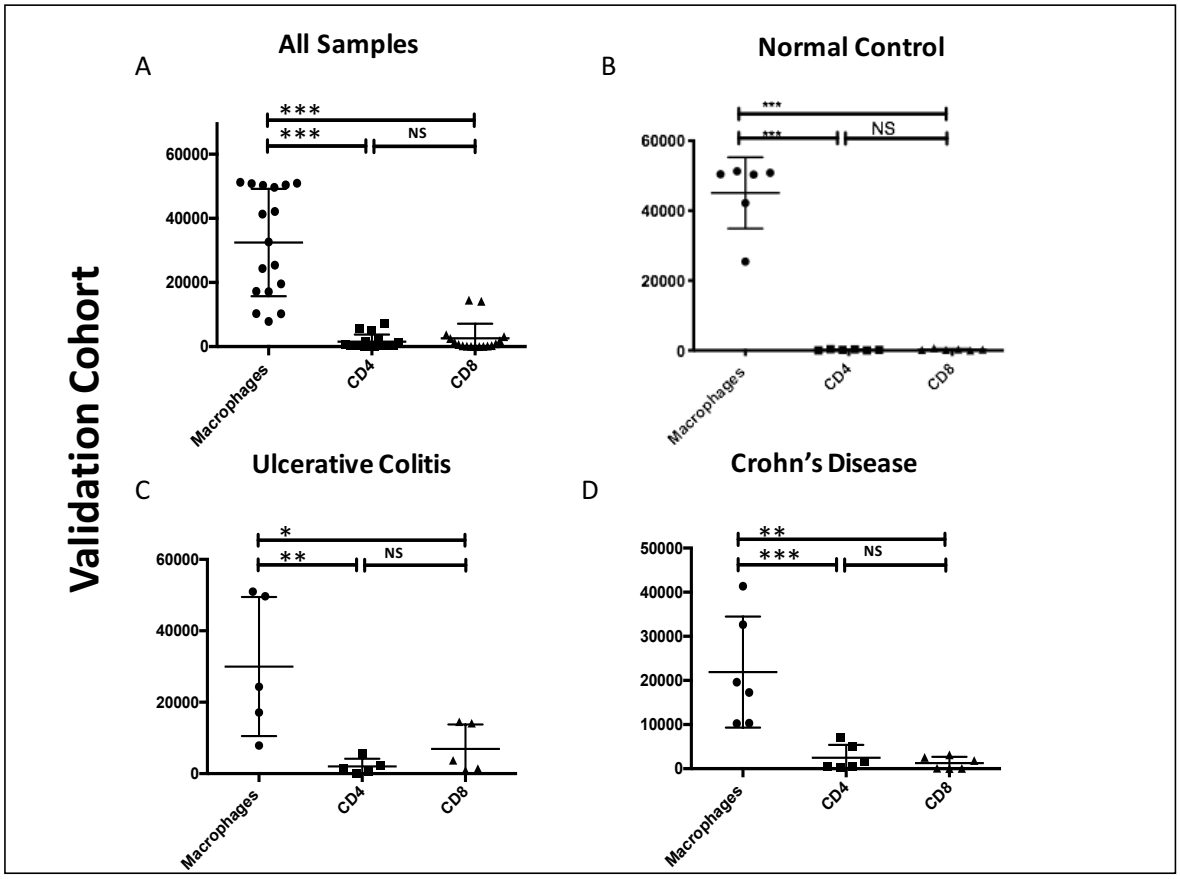


Figure 9 Cell yields by FACS sorting for the validation cohort. Showing the raw number of cells isolated 10A, for all samples combined (n=17) and then divided in to disease group, 10B Normal control (n=6), 10C Ulcerative colitis (n=5) and 10B Crohn's disease (n=.6). The error bars show the standard deviation and mean, *P< 0.05, ** P<0.001 and NS – non significant (All statistical significant tests by the ANOVA test with Tukey multiple comparison test calculated in Graphpad Prism).

4.3.4 Mucosal T-Cell subtypes

It has been well established by animal and human studies that T-lymphocytes particularly CD4 T-helper cells; Th1, Th2, Th17 and FoxP3 T-regulatory cells pay an important role in the pathogenesis of IBD.(409)

I have analysed the flow cytometry data to assess the subsets of CD3 according to disease in comparison to the normal control samples. The average percentage of CD4 cells as a proportion of CD3 cells was increased compared the normal control group in both the UC and CD patients and the CD8 proportion reduced when compared with the health controls **(figure 10)**, although neither comparison was statistically significant for the RNA-Seq cohort of patients.

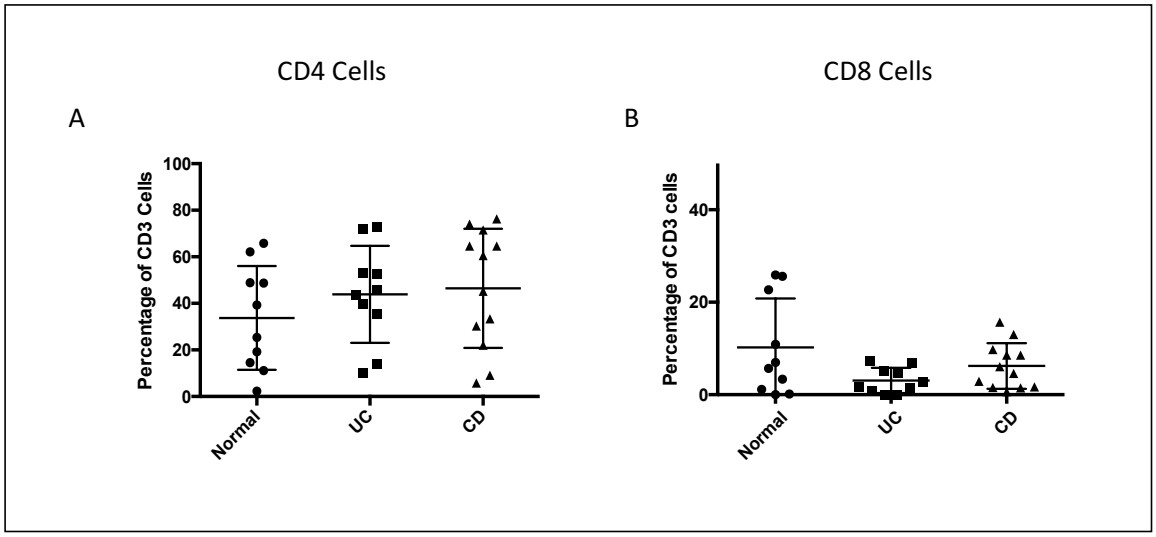


Figure 10(A) CD4 cells and 10(B) CD8 cells as a percentage of CD3 cells identified from mucosal biopsies using flow cytometry for the RNA-Seq cohort. Divided by disease group Normal (n= 10), UC (n=10) and CD (n=11). The error bars represent the standard deviation and mean. No comparison between groups was statistically significant ($p>0.05$, between all group comparisons) ANOVA test with Tukey multiple comparison test calculated in Graphpad Prism.

This analysis was replicated in the validation cohort of patients **(figure 11)**. As with the RNA-Seq cohort, the average number of CD4 cells as a percentage of CD3 cells was greater in both the CD and UC patients and was statistically significant. Unlike the RNA-Seq cohort however the number of CD8 cells as a percentage of CD3 cells was increased in both the CD and UC patients although this was not statistically significant. An increase in percentage of CD8 and CD4 cells could be explained by, changes in proportion of double negative CD3 cells (410) (CD8 &CD4 negative), double positive cells (411) (CD4 and CD8 positive) or staining and flow cytometry artefact. The protocol was not optimised to investigating T-cell subsets, there were too few samples, gated CD3

events and the cells were not stained for further subtypes such at T-reg cells with CD25 to make any conclusions, although this data is useful for hypothesis generation.

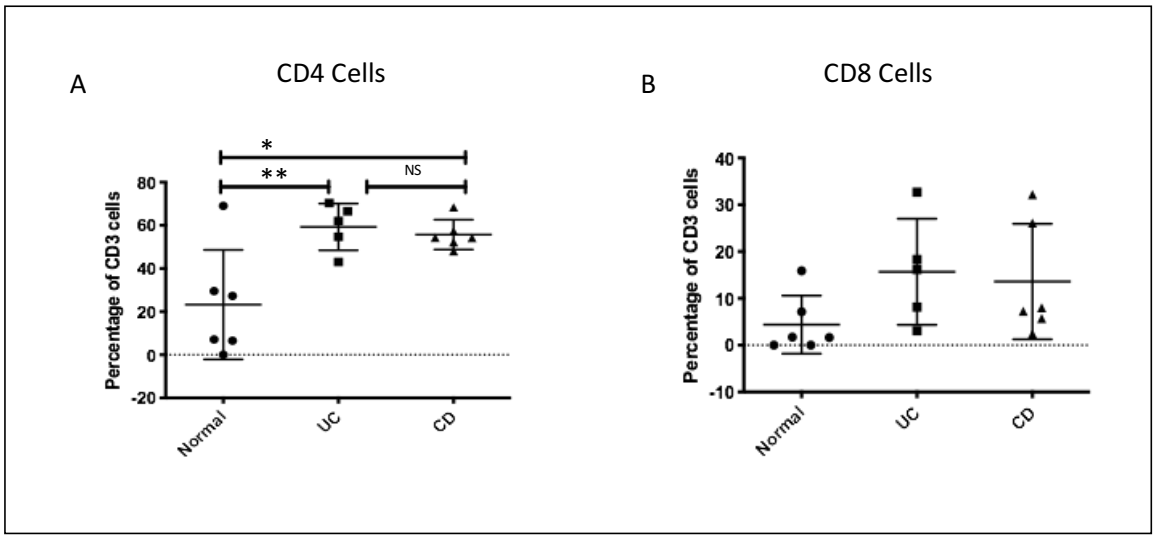


Figure 11(A) CD4 cells and figure 11(B) CD8 cells as a percentage of CD3 cells identified from biopsies mucosal biopsies using flow cytometry for the validation cohort. Divided by disease group. Normal control (n=6), UC (n=5) and CD (n=6). The error bars show standard deviation and means. * P<0.05, **p<0.01, ANOVA test with Tukey multiple comparison test calculated in Graphpad Prism.

4.3.4.1 Leukocyte common antigen expression of isolated macrophage cells

Leukocyte common antigen (CD45) is a transmembrane protein expressed on the cell surface of almost all haematopoietic cells except for mature erythrocytes. CD45 acts as an important regulator of T-cell and B-cell antigen receptor mediated signalling.(412, 413) The ubiquitous expression of CD45 on the cell surface of leucocytes has led CD45 to commonly be used in FACS protocols that isolate leucocytes since the advent of multicolour flow cytometry. This has led to reduced intralaboratory and interlaboratory variations in the investigation of T-cell subtypes by flow cytometry.(414) In the present study CD45 was not utilised in the strategy to isolate macrophages by FACS, although it was used to identify the T-cell subtypes CD8 and CD4. Therefore all samples were stained with CD45 and it was possible using the recorded flow cytometry data to determine the expression of CD45 on the gated macrophage population as shown in **figure12**.

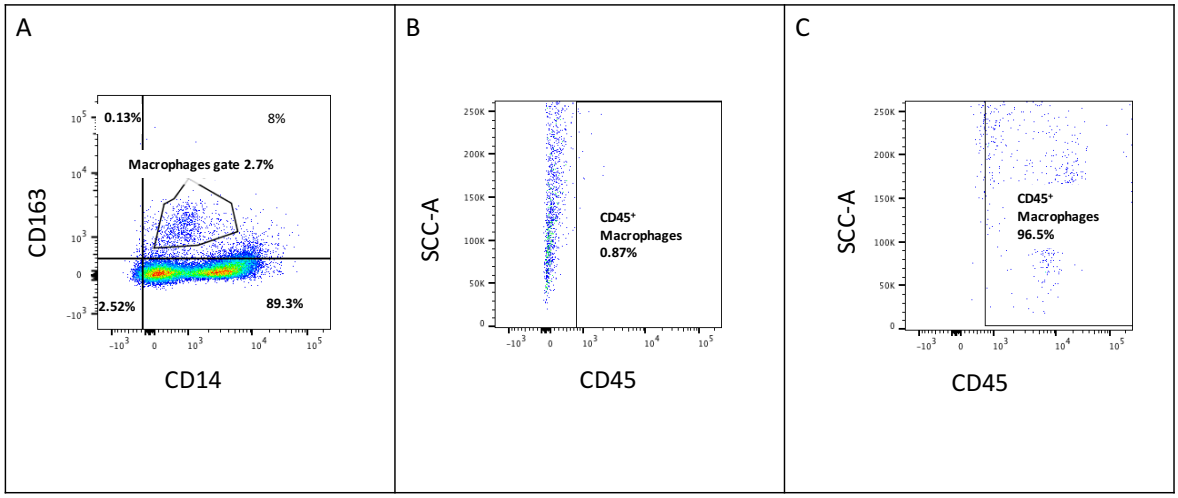


Figure 12 Macrophage CD45 expression FACS plots show high CD45 expression. These plots show (A) the sort gate used for macrophages $CD163^{+}CD14^{+}CD3^{-}$, (B) is the plot for the unstained control sample for the CD45 gate demonstrating how the gate has been set up. The final plot (C) shows the expression of CD45 for the macrophage sorted cells identified in (A) demonstrating for this sample 96.5% of the isolated $CD163^{+}CD14^{+}CD3^{-}$ cells described as macrophages are positive for CD45.

The percentage CD45 expression on the gated macrophage cells was calculated for each of the samples that were sent for RNA-Seq, the results of this analysis are shown in **table 22.** The range of CD45 expression on the macrophages was 82.96 to 100 per cent, with a mean CD45 expression of 89.65% (CI 87.93 to 91.36) across all the groups. There was no statistical significant difference in the average CD45 expression between the three groups, normal control, UC and CD (ANOVA with Tukey test).

Normal Controls	CD45 expression (%)	Crohn's Disease	CD45 expression (%)	Ulcerative Colitis	CD45 expression (%)
NAC528	86.6	CCC536	90	UCC535	95.6
NAC564	100	CCC579	83	UCC583	84.8
NAC572	87.5	CCC593	91.6	UCC588	90.8
NAC573	NA*	CCC607	84.5	UCC595	85.2
NAC580	93.5	CDC527	96.5	UCC602	84.9
NAC597	82.96	CDC541	87.6	UDC546	90.5
NAC599	86.4	CDC565	92.1	UDC604	84.9
NAC601	89.5	CDC594	93.9	UDC608	89.5
NAC603	87.5	CDC606	87.3	UDC612	86.2
NAC605	96.8	CDC609	98	UDC613	91.7
		CDC610	90		

Table 22 Expression of CD45 on the gated macrophage cells shown as a percentage. *****Unfortunately little data was recorded for sample NAC573 and it was therefore not possible to give an accurate value for CD45 expression, as there were too few cytometry events to analyse**.**

4.3.5 FACS analysis demonstrates a population of cells positive for the macrophage marker CD163 and negative for CD14

The post sort analysis of the flow cytometry data revealed a population of cells that were positive for CD163 and negative for CD14 (quadrant 1, plot D, **figure 7**). Intestinal macrophages negative for CD14 have been well described in the literature.(341) A number of studies have demonstrated that newly recruited macrophages in the intestine resemble their monocyte precursors though do show transcriptional changes early on.(415) Newly recruited macrophages then undergo a process of maturation through a number of intermediaries. The eventual phenotype of the fully matured intestinal macrophage is dependant on the gut molecular environment, in health a population of resident macrophages loose CD14 expression in this process becoming hyporesponsive to antigenic stimulation and therefore promote mucosal immune homeostasis.(416)

Given that CD163 appears to be a specific marker for monocytes and macrophages, it is possible that the $CD163^{+}CD14^{-}$ cells represent a cohort of macrophages.(349) This cohort of cells were not isolated and therefore not subjected to RNA-Seq or any other further gene expression analysis. In hindsight it would have been interesting to study this group of cells further and elucidate through analysis of gene expression profiles if their behaviour differs from the $CD163^{+}CD14^{+}$ cells that we have studied. It would be wise to assess these $CD14^{-}$ $CD163^{+}$ cells for additional macrophage markers such as CD64 and CD33. It may be that the $CD14^{-}$ cells represent a specific macrophage sub-population with a particular function, however the lack of the CD14 surface marker may simply be transient or even an experimental artefact. In order to further understand and elucidate if the $CD14^{-}$ population identified on FACS was experimental artefact we attempted IHC co-localisation. In the stored GMA samples double staining of the slides was not possible as no recognised protocol or expertise existed locally. We attempted co-localisation in paraffin embedded samples, though this was unsuccessful with the resources and time available. We then attempted to sequentially stain GMA sections with CD163 and CD14, digitally orientated the slides and utilise digital counting software to elicit if there was a discrepancy for CD163 and CD14 staining. Unfortunately, the software was unable to perform this automated task on these slides. An example of an orientated stained slide set is given in **figure 13**. Although it appears similar areas are stained for both CD163 and CD14 its difficult manually to assess and compare the individual cells. In the slide set shown the CD14 staining seems stronger than that of the CD163, even though the same areas stain. This could be caused by differing primary antibody performance in GMA, as the CD163 antibody was not previously validated in GMA (none exist on the market). This method therefore was not taken further as interpretation of these images is subjective and unlikely to robustly confirm or refute the presence of $CD14^{-}CD163^{+}$ cells.

The flow cytometry data for the $CD163^{+}CD14^{-}$ cells were analysed across all the disease groups and normal controls for the RNA-Seq cohort and the validation cohort. There was no significant difference in the relative numbers of these cells between the groups, suggesting that omission of the $CD163^{+}CD14^{-}$ cells form the RNA-Seq is unlikely to have biased the results of this study for disease comparison, **figure 14**.

Macrophages characteristically are heterogeneous group of cells with marked plasticity.(417) There is currently no consensus on a panel of macrophage surface markers that will identify all intestinal macrophages. This is partly due to the plastic nature of macrophages, the likelihood that not all macrophages express all surface markers and that murine macrophage markers do not translate to humans well.(418) In this project we have taken a pragmatic approach and studied cells expressing the macrophage markers $CD14^{+}CD163^{+}$. We accept that this approach will mean that there are populations of macrophages that have not been included in our analysis. The benefit of our approach in this hypothesis generating study is that we have analysed a relatively large cell group and compared like with like, which will aid RNA-Seq analysis in forming a coherent signal and therefore hypothesis generation for further testing. The $CD14^{+}CD163^{+}$ cells are referred to as macrophages for the purposes of this thesis with the acknowledgment that this is simplifying the issue and these macrophages may represent a subset.

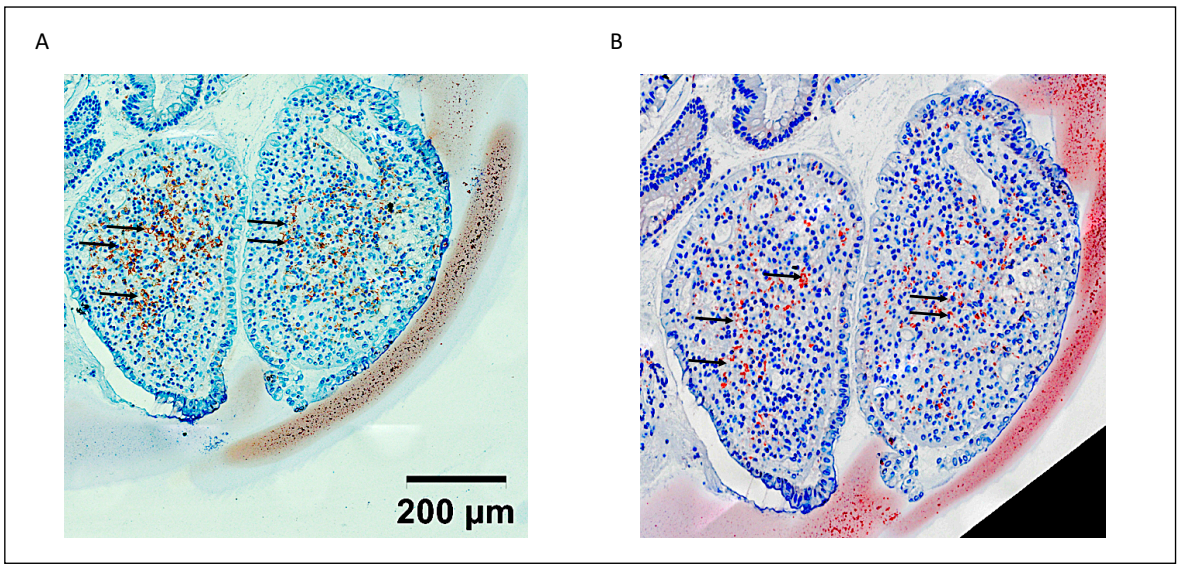


Figure 13 Two sequentially cut sections of a GMA embedded intestinal biopsy stained for (A) CD14 and (B) CD163. The slides have been digitally rotated so that the orientation matches. Arrows highlight examples of positively stained cells.

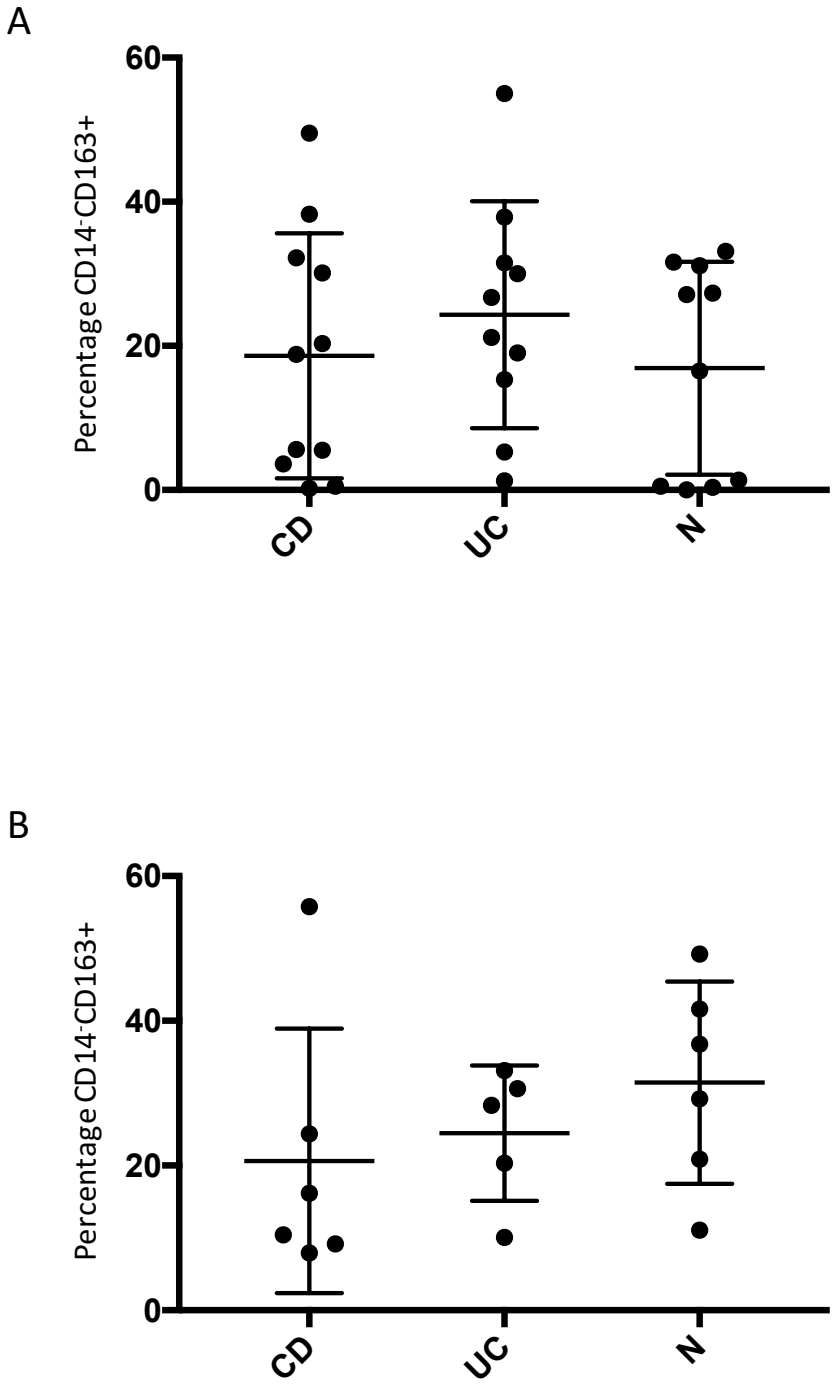


Figure 14 Graphs showing $CD14^{-}CD163^{+}$ cells as a percentage of the total $CD163^{+}CD3^{-}$ cells across all disease groups and normal controls in the (A) RNA-Seq cohort and (B) the validation cohort. For the RNA-Seq cohort, CD (n=11), UC (n=10) and Normal control (n=10). For the validation cohort CD (n=6), UC (n=5) and N (n=6). The error bars show standard deviation and mean. Comparisons were made between all groups using the ANOVA test with Tukey multiple comparison test calculated in Graphpad Prism, no group comparison was significant ($p>0.05$).

4.4 Chapter Discussion

The FACS data illustrates that the experimental protocol was sufficiently optimised to collect the required minimum 10,000 macrophages for whole transcriptome amplification and subsequent high throughput RNA-Seq. The macrophages were the most abundant immune cells identified and collected of those analysed, consistent with observations in mice that macrophages are one of the most abundant leukocytes found in the intestine and intestinal macrophages represent the largest population of macrophages in the body.(238) In humans intestinal macrophage numbers have been seen to increase with intestinal inflammation in IBD patients suggesting that there is recruitment of monocytes to the intestine in active IBD.(358) The data collected from the flow cytometry analysis in this project provides raw numbers of macrophages identified from each patient. Comparison of macrophage number between different patient samples and investigating any association of macrophage number with inflammation was not possible because of a number of uncontrolled variables. The number of biopsies from each patient was standardised, however the size of each individual biopsy was not known creating an important variable that was not controlled for or measured. Simply weighing the biopsies taken from each patient is unlikely to be adequate and prone to inaccuracy. A possible option would be to use a reference cell type that is not affected by inflammation to normalise the cell counts. This would most likely be a non-immune cell, epithelial cells unfortunately are not a good candidate as they are well know to have a role in IBD and breakdown of the epithelial barrier is seen with active inflammation.(419) This will be an important consideration for future work. A literature review, revealed no studies that have reported absolute numbers of intestinal macrophages. Mouse studies show that macrophage numbers within the intestine correlate with relative bacterial load and are thus most concentrated in the colon where the bacterial concentration is highest.(408) This is supported by the finding or reduced number of intestinal macrophages in germ free mice.(420)

The isolated macrophages were further characterised using the flow cytometry data generated by the FACS sorting. The isolated macrophages were found to have a mean expression of 90% of CD45 across all groups and there was no significant difference in CD45 expression between the disease groups and normal controls. Although CD45 was not utilised in the gating strategy for macrophage isolation due to the high specificity of CD163 for macrophages, this analysis provides a degree validation that the cells isolated and studied were myeloid cells.

We have also identified a population of cells that were positive for CD163 yet negative for CD14; it is likely that these represent a sub-population of macrophages. Attempts using various IHC methods including double staining were unsuccessful in elucidating if the $CD14^{-}CD163^{+}$ population was true finding or represented experimental artefact with any degree of certainty. There are

however several studies that report intestinal macrophages lacking CD14 expression(334, 335, 343) in the literature and it is accepted that populations of intestinal resident macrophages loose CD14 expression in the maturation process. This population of cells have not been subjected to further analysis in this project, clearly this a population of cells that would be interesting to study in comparison to the $CD14^{+}CD163^{+}$ macrophages that we have analysed.

We have identified an increase in the CD4 compartment this is consistent with published literature.(421) Mucosal CD4 effector cells are known to play an vital role in the pathogenesis of IBD and is supported by the numerous animal studies and the observation that there is an influx of $CD4^{+}$ into the inflamed mucosa in IBD.(422) There is some evidence to suggest that in those with IBD and concomitant HIV infection (resulting in CD4 depletion) have a less aggressive course of IBD. Further supporting the role of CD4 cells in the inflammatory process are drugs targeting the T-cell trafficking to the intestinal mucosa that have been shown to be effective in IBD and now are used in routine clinical practice.(423)

The gaiting strategy utilised for this project was selected with the view of balancing the two completing pressures of being inclusive of as many intestinal macrophages as possible whist not being too general as to include a large number of heterogeneous cells and cells that were not macrophages, which would have impaired the signal from the RNA-Seq analysis. As shown earlier in this chapter CD45 was not utilised within the macrophage gaiting strategy but post sorting analysis has shown that there is high level CD45 expression on the cells isolated to form the macrophage cohort. The gaiting strategy would have been strengthened had CD45 been included to isolate the macrophages. I have shown in this chapter that there is a subset of CD14 negative cells that are positive for CD163. It is not clear if these are macrophages. The addition of further macrophage markers such as CD11c, CD86, CD206 and HLA-DR (364) would strengthen the gaiting strategy, allowing for more in depth analysis of the flow cytometry data and also for identification of subsets of macrophages, which then could undergo comparison transcriptomic analysis. Intestinal macrophages are difficult to differentiate from dendritic cells within the intestine, they have similar functional ability to sample the lumen content with dendrocytes but it is only the dendritic cells that present antigens to local lymphoid tissue.(416) The addition of dendritic cell markers to the gaiting strategy would allow the dendritic cells to be positively identified and separated from the macrophages for further study and comparison with the macrophages. This data could then be utilised in future studies as reference point for the differentiation of macrophages from dendritic cells. This would also improve the purity of the isolated macrophages strengthening subsequent RNA-Seq analysis.

The optimisation of the flow cytometry and FACS enabled the isolation of a cohort of cells with macrophage surface markers (CD14 and CD163) for patients with active UC and CD as well as a control group with biopsies taken from healthy colonic mucosa. The RNA from these cells was extracted and DNA libraries produced which were sent to ORB for RNA-Seq. In the next chapter I describe how the data output from ORB was initially handled and an overview of the initial analysis, which subsequently informed the analysis described in the later chapters of this thesis.

Chapter 5:

Results 2: RNA Sequencing Data Analysis and Overview

5.1 Introduction

High throughput RNA sequencing has been demonstrated to be superior to the much utilised and established method of microarray in the study of the transcriptome.(424) RNA-Seq has been shown to be accurate in quantifying gene expression with highly reproducible results.(425) Studies that have directly compared the methods of RNA-Seq with microarray have demonstrated the former to produced fewer false positive results and perform better at the extremes of gene expression.(426) RNA-Seq is therefore able to detect small changes in gene expression previously not recognised using microarray technology.

The analysis and interpretation of the data generated using high throughput RNA-Seq technique raises a major challenge for statistical modelling and analysis due to the large data sets that are generated. Prior to disease association analysis the data was assessed for validity and clear outliers identified and further investigated. This chapter describes the processes and tools that were utilised to perform the initial validity assessment and provide an overview of the data. An overview of the data was vitally important to guide the focus of the subsequent detailed analysis.

5.1.1 Primary analysis for validity

The primary analysis was performed by ORB according to their established mRNA high throughput analysis pipeline. A total of 60,504 known transcripts were detected after alignment to the reference genome hg38 and annotated using Ensembel version 83 prior to filtering. A total of 29,403 detectable transcripts passed though the 50 read RPKM filter in at least one sample. ORB on request supplied a Principal Component Analysis (PCA) and correlation plot on these detectable genes across all the sequenced samples **figures 15 & 16**. The PCA analysis was performed on the RPKM data for all the 29,403 detectable genes. The RPKM values were log10-transformed, and the first 5 principal components calculated. In order to find genes that substantially contribute to each principal component value the correlation and fold-change in expression of each gene with the first five principal components was calculated using the methods described by Sharo et al.(427)

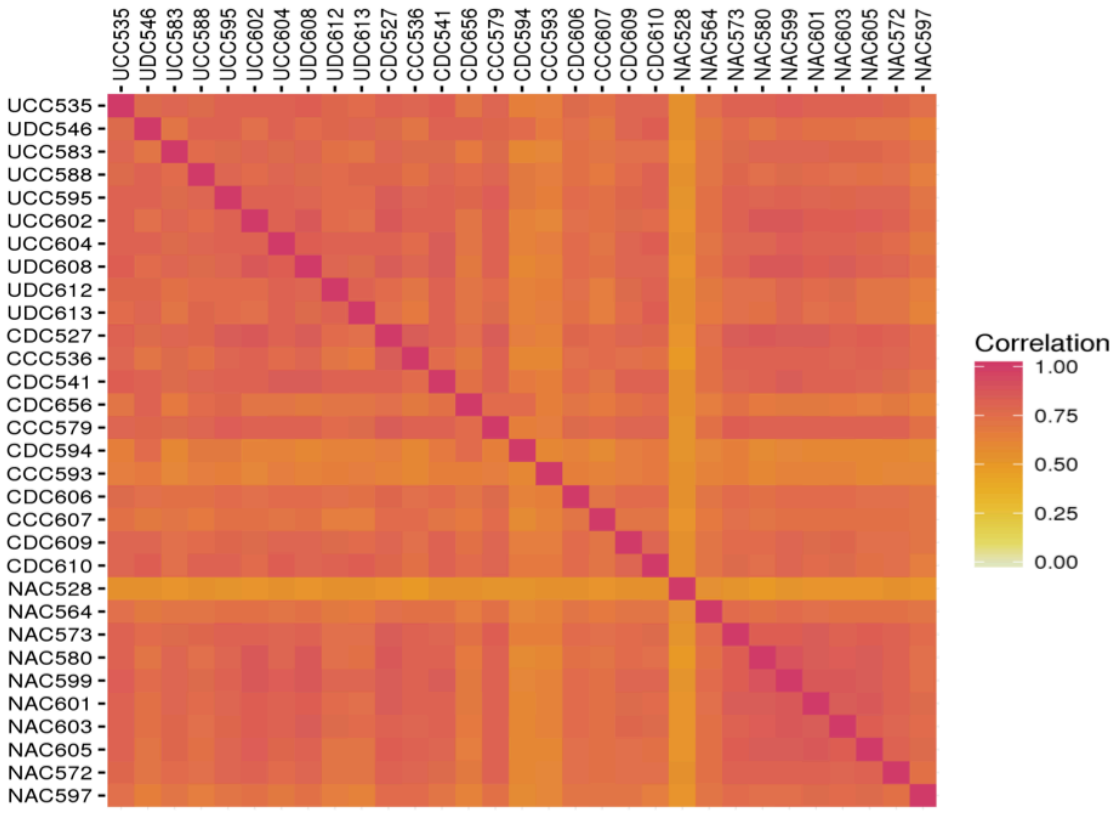


Figure 15 Correlation matrix calculated by using the log10 transformed RPKM values from the 29, 403 detectable genes produced by ORB as presentation of original mRNA-Seq analysis. NAC528, CDC594 and CCC593 have lower correlation in gene expression to the other sequenced samples compared to all the samples sequenced. This identifies these samples as outliers in their gene expression.

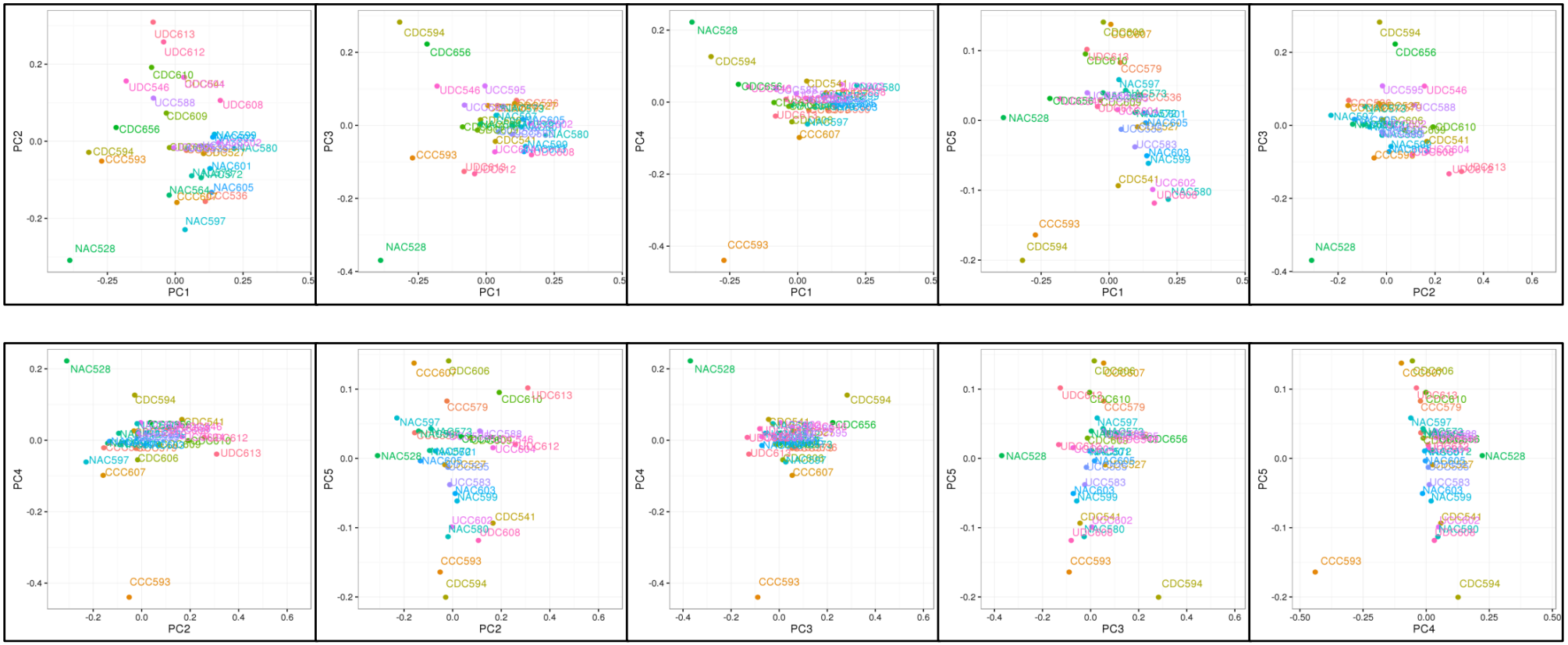


Figure 16 PCA Plots for all samples for the first 5 components. PCA analysis was performed on the RPKM values from the 29,403 detectable genes. Genes with a positive or negative correlation of at least 0.8 and a fold change of at least 2 with the principal components are shown. Samples NAC528, CDC594 and CCC593 across the 5 principal components are outliers.

Both the PCA and correlation analyses show that there are three samples that are outliers; NAC528, CCC593 and CCC594. The patient medical history, circumstances of biopsy collection and laboratory processing notes were reviewed for these patients. No clinical reason or laboratory processing issue for these samples being outliers could be identified. These samples were excluded from all further analysis.

The RNA-Seq data was then re-analysed using the DESeq2 normalisation to provide binary comparisons of Normal Vs CD, Normal Vs UC and CD Vs UC as this form of analysis is known to perform well for high throughput RNA-Seq data comparisons with small replicate numbers.

DESeq2 is a method for performing analysis of differentially expressed genes, available as an R/Bioconductor package. The DESeq2 method utilises shrinkage estimators for dispersion and fold change. The DESeq2 method has been shown to perform consistently over a range of data types and for small studies with few replicates.(389)

The DESeq2 analysis was performed by ORB and provided to myself as an excel file with normalised read counts for each identified gene for each sample and also fold changes in Log2 format. The fold change comparisons were CD Vs N, UC Vs N and CD Vs UC controlled for with a false discovery rate (FDR) value.

The DESeq2 data was then further analysed locally using R version 3.2.2 and Graphpad software. In this analysis I used the criteria of a fold change of 1.5 fold up or down and a False Discovery Rate of <0.1 for statistical significance to identify the differentially expressed genes (DEGs). This relatively relaxed criteria for statistical significance was employed because the aim of this project was hypothesis generation and therapeutic target discovery. Starting with more stringent statistical criteria would have reduced the number of identified DEGs potentially resulting in less information being obtained from the experiment and ultimately a reduced chance of novel discovery.

5.2 The macrophage transcriptome shows a profound re-programming in IBD

5.2.1 Comparison of Differentially Expressed Genes

The normalised read counts for each identified gene were utilised to identify the differentially expressed genes. The differentially expressed genes were identified as those with a fold change of greater than or equal to 1.5 fold up or 1.5 fold down and a corrected false discovery rate of <0.1 for all comparisons. The comparison of CD and UC macrophages to the healthy colon control macrophages revealed that the gene expression was profoundly reprogrammed in the IBD macrophages indicating that the intestinal macrophages in IBD patients with active disease are behaving quite differently to those found in the healthy colon.

The PCA plot and heatmap for these comparisons **(figure 17)** show that the intestinal macrophages from healthy controls can be clearly differentiated from the IBD macrophages by the differential gene expression however the macrophages from CD and UC patients cannot be easily distinguished using the expression of these genes. The reason for this is that the majority of the genes that are differentially expressed for the comparison of UC Vs N are also differentially expressed for the comparison for CD Vs N.

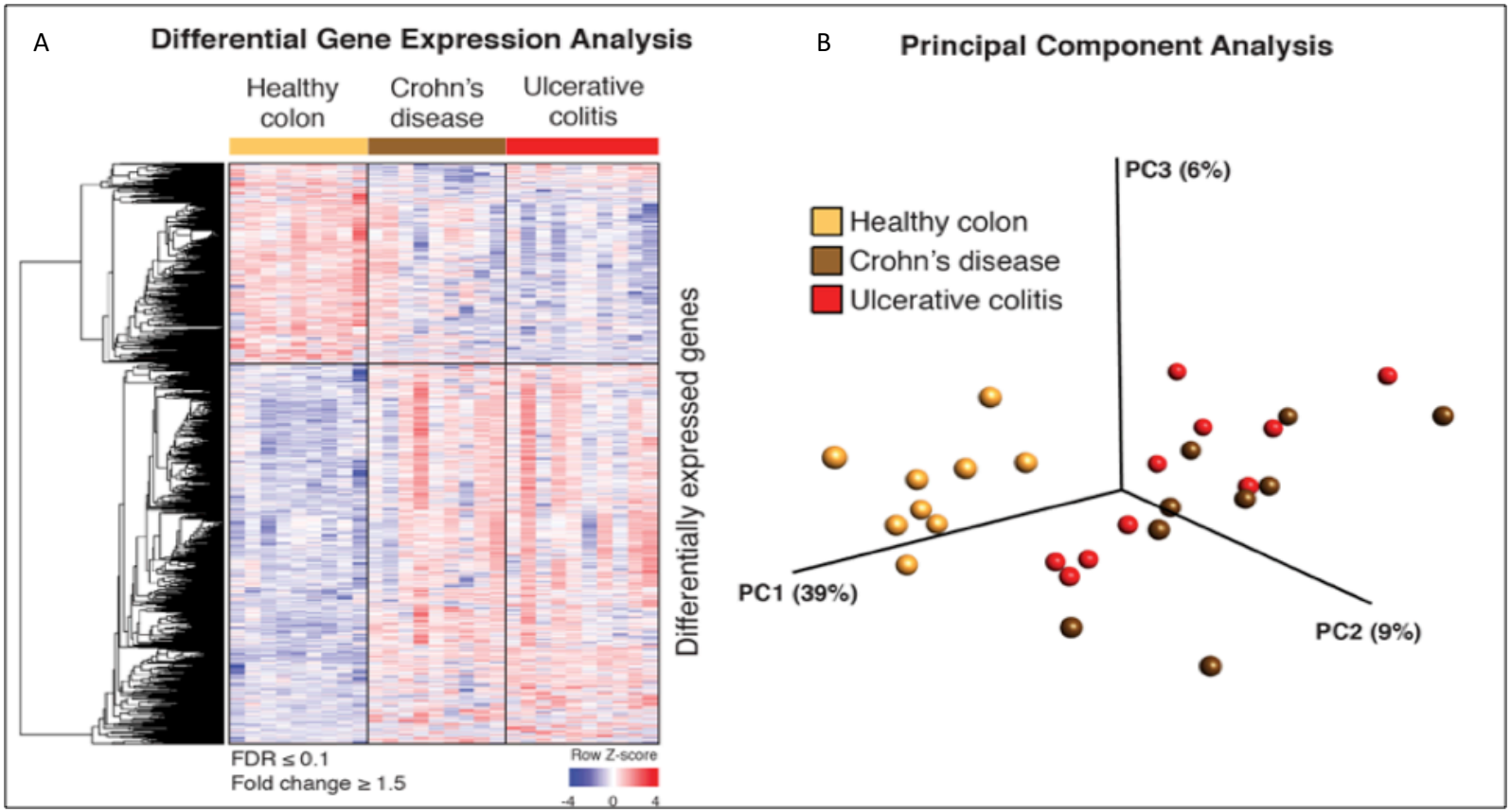


Figure 17 (A) Heatmap (with hierarchical clustering) and (B) PCA plot of the differentially expressed genes (FDR ≤0.1, Log2FC ≥|0.58|) for at least one comparison (CD Vs N or UC Vs N) demonstrating that the intestinal macrophages from the healthy control patients can be distinguished from the IBD macrophages based on the expression of this set of genes. CD (n=9, UC (n=10) healthy colon (n=9). The Heatmap was produced using the R software environment (428), plotting the gene expression of the DEGs as log2 counts. The row Z-score represents relative gene expression for each gene across all samples and groups (i.e for each row) but not compared to other genes. Red colour signifies up regulation and blue down regulation for that gene relative to all other samples. The PCA plot was produced using Qlucore Omics Explorer.(392)

The profound change in gene expression of macrophages isolated from IBD patients compared with the healthy controls is highlighted by the large number of DEGs identified for the individual diseases UC and CD. A total of 1287 differentially expressed genes (DEGs) between the macrophages from UC patients and healthy controls were identified and of these 450 genes were down regulated and 837 up regulated **(figure 18).** For the comparison of macrophages from CD patients with healthy controls 840 DEGs were identified, of these 147 genes were down regulated and 693 up regulated **(figure 19)**.

The transcriptome of the macrophages from UC and CD are similar and the majority of the genes that are differentially expressed compared to normal are so for both CD and UC. There were just 20 DEGs for the comparison CD Vs UC suggesting that the macrophages from CD and UC patients are quite similar. The PCA plot for this comparison suggests that using this small set of DEGs there is a tendency for separate clustering between the CD and UC macrophages **(figure 20)**. A manual analysis of the common up-regulated genes for CD and UC highlighted up-regulation of a number of pro-inflammatory genes such as TREM1, IL1β, IL6, as well as genes involved in antigen presentation including CD86, CD74 and CD40. Gene expression of chemokines involved in recruiting the adaptive immune system including CXCL9, 10 and 11 are up-regulated in both the UC and CD macrophages. Increased expression of chemokines such as CXCL10 and CCL25 within the intestinal mucosa is described in IBD.(429) This data identifies that macrophages are a source of the increased chemokine expression seen in IBD and also suggest that intestinal macrophages play an important role in coordinating the inflammatory process seen in IBD. NOS2 gene expression was up-regulated in both the UC and CD macrophages further suggesting a generalised inflammatory phenotype to the macrophages. Inflammatory M1 macrophages have been described as characteristically expressing NOS2.(430) These findings of an activated mucosal pro-inflammatory macrophages is not surprising given that the biopsy samples were taken from areas of colonic tissue where there was clear evidence of macroscopic active inflammation.

The common down regulated genes for CD and UC show that a number of these genes are involved in mitochondrial function including GFM1, ACAA2 and CPT1A. This is an interesting finding, mitochondrial dysfunction has been identified in infective conditions of the gastrointestinal tract such as enteropathogenic E.Coli and salmonella typhimurium that result intestinal inflammation and similar symptoms to IBD.(431) It has been shown that within inflamed colon of humans and mice the mitochondrial activity within colonic epithelial cells and the levels of mucosal ATP produced is reduced and it has been proposed that mitochondrial dysfunction within epithelial cells contributes to the inflammatory process in IBD.(432) The data I have presented here suggest that mitochondrial dysfunction in IBD is not limited to the epithelial cells

but also present within the macrophages. A role for altered autophagy has been established in IBD through a number of GWAS studies.(433) An excess of reactive oxygen species (ROS) has been proposed as a further contributory factor in the propagation of inflammation in IBD supported by data from both animal and human studies.(434) It is possible that an imbalance of ROS and dysfunctional autophagy could be a result of mitochondrial dysfunction. Our data suggests that there are alterations in the mitochondrial gene expression within the IBD patient macrophages. Further experiments would be required to assess if these changes in gene expression functionally have the effect of impairing the function of the mitochondria, resulting in impaired autophagy and increased ROS leading to epithelial damage.

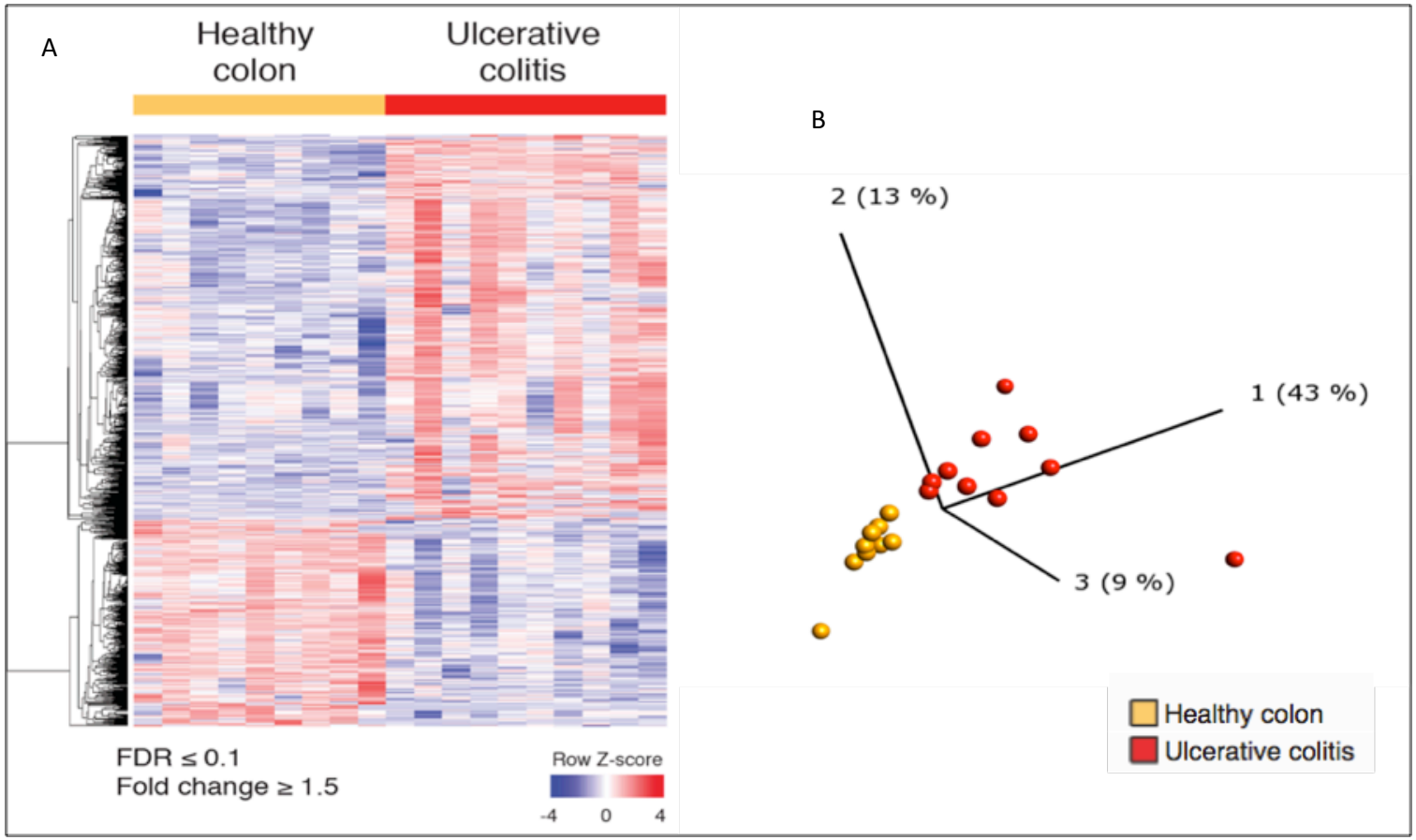


Figure 18 (A) Heatmap (with hierarchical clustering) and (B) PCA plot of the differentially expressed genes (FDR ≤0.1, Log2FC ≥|0.58|, 1287 genes) for the comparison of UC Vs N, showing that the UC macrophages can be differentiated from the healthy controls on the bases of these DEGs. UC (n=10) and healthy colon (n=9). The Heatmap was produced using the R software environment (428), plotting the gene expression of the DEGs as log2 counts. The row Z-score represents relative gene expression for each gene across all samples and groups (i.e. for each row) but not compared to other genes. Red colour signifies up regulation and blue down regulation for that gene relative to all other samples. The PCA plot was produced using Qlucore Omics Explorer.(392)

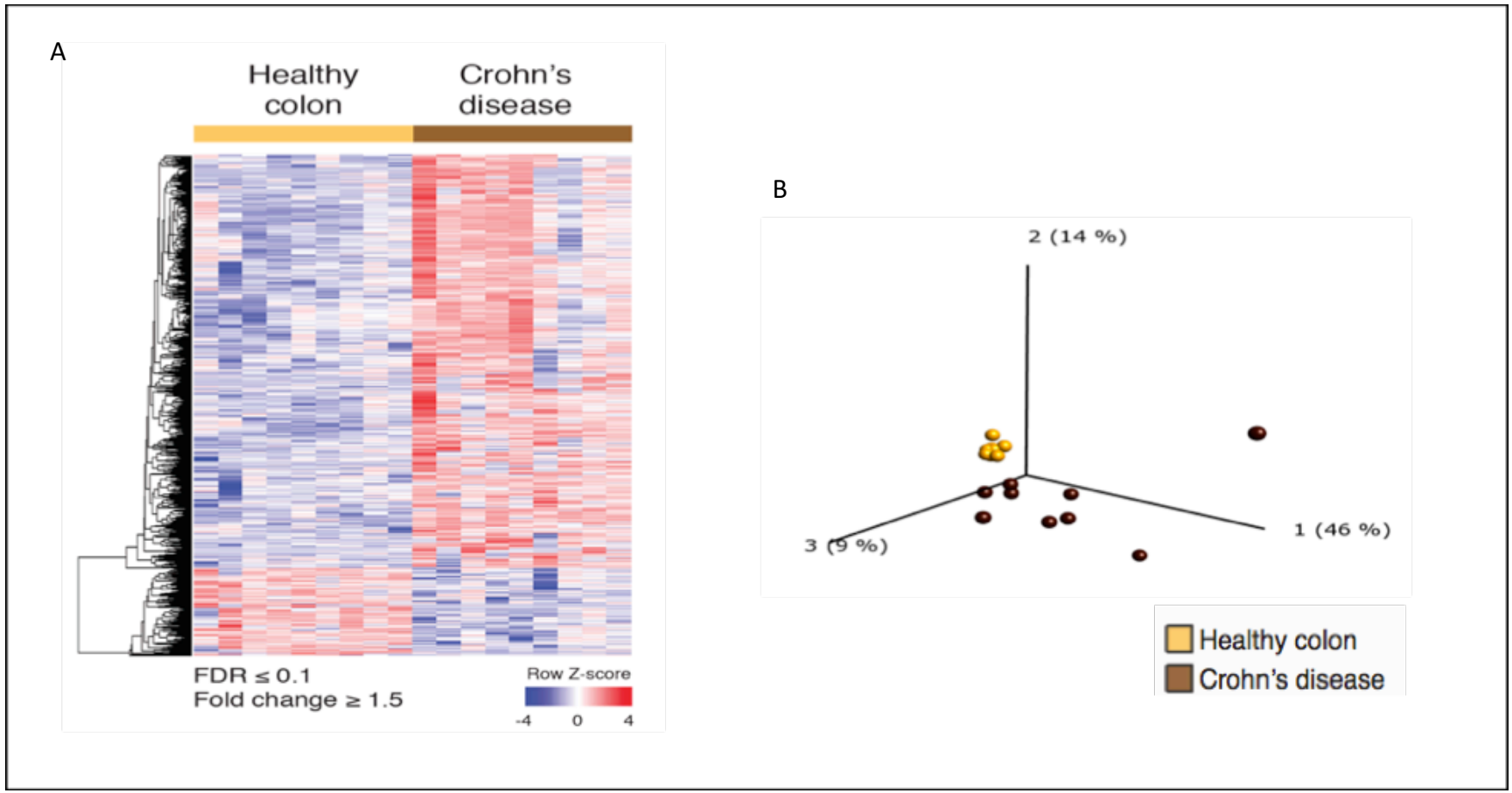


Figure 19(A) Heatmap (with hierarchical clustering) and (B) PCA plot of the differentially expressed genes (FDR ≤0.1, Log2FC ≥|0.58|, 840 genes) for the comparison of CD Vs N, showing that the CD macrophages can be differentiated from the healthy controls on the bases of these DEGs. CD (n=9) and healthy colon (n=9) The Heatmap was produced using the R software environment (428), plotting the gene expression of the DEGs as log2 counts. The row Z-score represents relative gene expression for each gene across all samples and groups (i.e. for each row) but not compared to other genes. Red colour signifies up regulation and blue down regulation for that gene relative to all other samples. The PCA plot was produced using Qlucore Omics Explorer.(392)

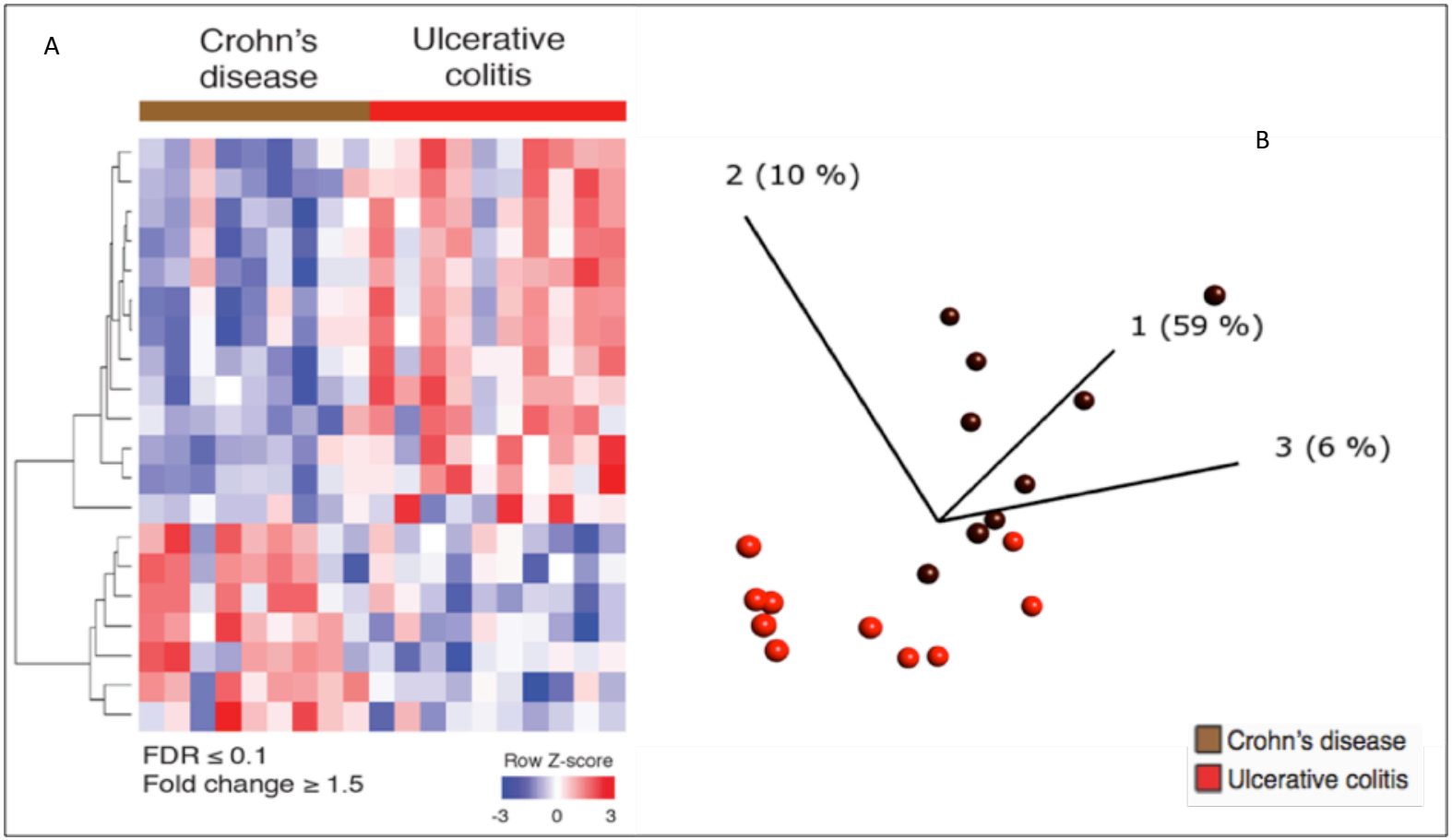


Figure 20 (A) Heatmap (with hierarchical clustering) and (B) PCA plot of the 20 differentially expressed genes (FDR ≤0.1, Log2FC ≥|0.58|) for the comparison of UC Vs CD. For UC (n=10) and CD (n=9). The Heatmap was produced using the R software environment (428), showing the gene expression of the DEGs as log2 counts. The row Z-score represents relative gene expression for each gene across all samples and groups (i.e. for each row) but not compared to other genes. Red colour signifies up regulation and blue down regulation for that gene relative to all other samples. The PCA plot was produced using Qlucore Omics Explorer.(392)

5.3 Pathway Analysis reveals that a number of cellular pathways are altered in IBD intestinal macrophages

5.3.1 Dysregulation of cellular pathways in CD and UC Macrophages

Pathway analysis was performed utilising Ingenuity Pathway Analysis (IPA) software.(391) The DEGs for CD Vs N and UC Vs N were uploaded onto the software for analysis. **Figure 21** shows the top associated pathways by P-value for the UC and CD macrophages. A comparison of the identified dysregulated pathways for CD and UC was made using the IPA software.

The significant pathways for both CD and UC **(figure 22)** were broadly similar further suggesting similarities between the CD and UC macrophages in this cohort with similar activation Z-scores. The activation Z score provided in IPA canonical pathway analysis is a statistical measure of the match between the expected relationship from the ingenuity knowledge base (literature utilised by IPA) and the observed gene expression. A Z-score of greater than 2 (activated) or less than -<2 (inhibited) is considered significant.

The identified associated pathways were further investigated in more depth to assess their biological importance in IBD. A number these pathways were found to be of interest. In this chapter, three of these pathways are explored in more depth; *TREM1* signalling, communication between the innate and adaptive immune systems and *PPARγ* signalling. I have selected these pathways to discuss further because I believe that they demonstrate an overall theme of macrophage behaviour in IBD and represent areas of translational interest for therapeutic targeting. The leukocyte extravasation-signalling pathway is also discussed further to highlight the importance of interpreting the pathway analysis in the context of the experimental origin of the data and illustrates the risk of misinterpretation with reliance on analytical software.

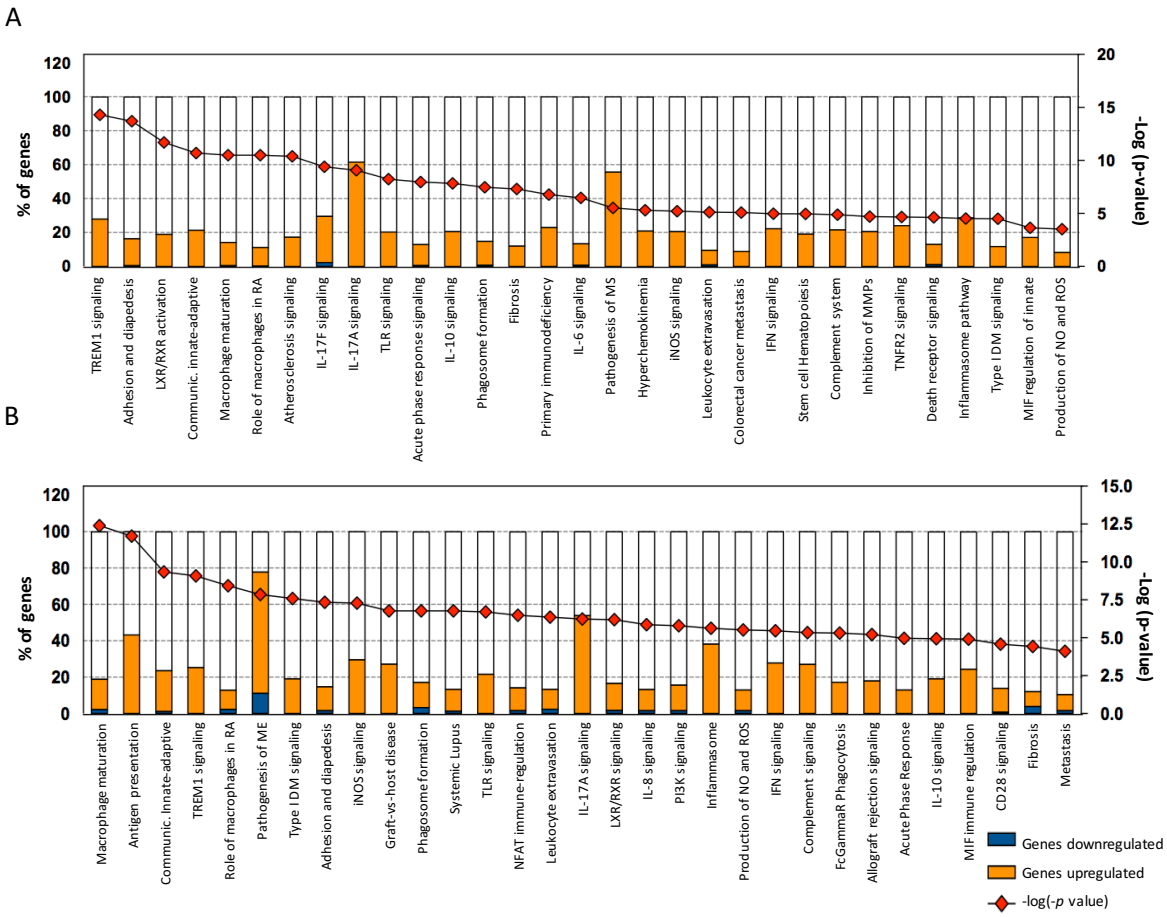


Figure 21 Top identified canonical pathways by p-value for (A) UC and (B) CD. The columns show the percentage of genes in the pathway that are either up or down regulated for the DEGs identified by the DESeq2 analysis and uploaded on to IPA, the left Y-axis corresponds with this. The Diamond line shows the p-value as –log (p-value), calculated in IPA using right-tailed Fisher's exact test. A threshold of $p<0.05$ was considered significant. This graph was originally produced in IPA, the data exported and then modified in Graphpad Prism version 7 for Mac OS (435) as there were too many identified significant pathways to illustrate them all (171 for UC and for 120 CD).

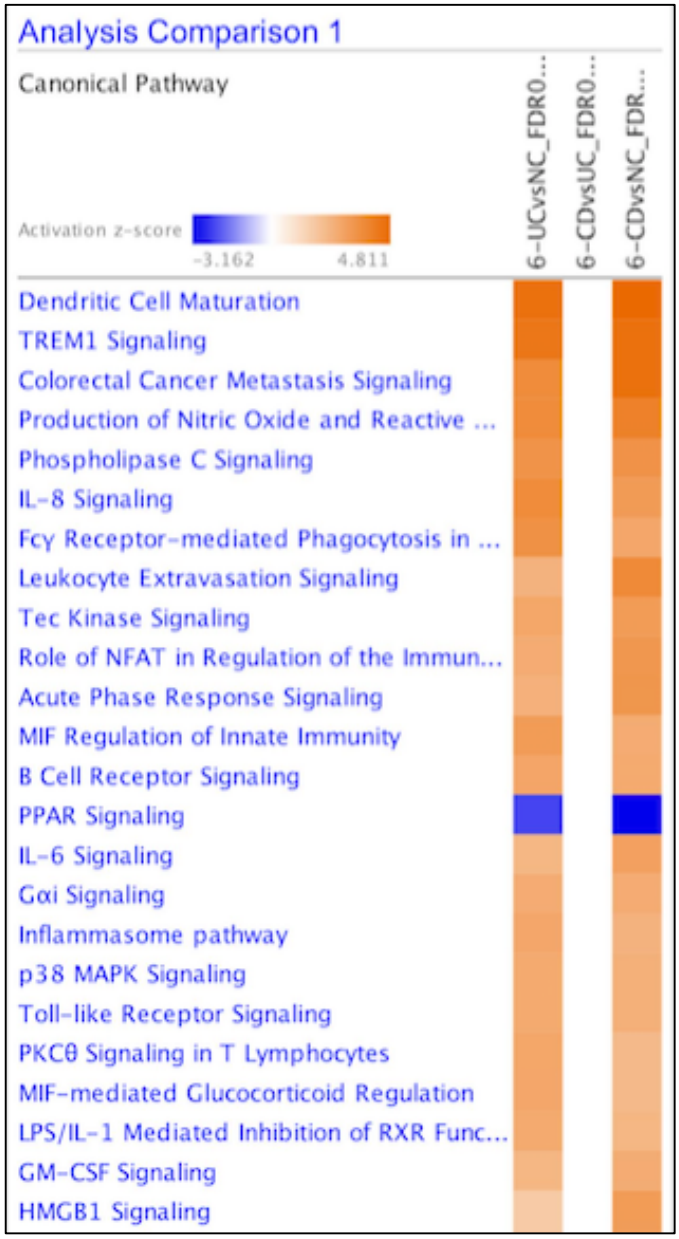


Figure 22 Comparison of significant canonical pathways for UC Vs CD. The pathways considered significantly associated with the DEGs uploaded to IPA were those with a p-value of <0.05. P-value calculated by IPA using right-tailed Fisher's exact test. The pathways are ordered by magnitude of Z-value, only the top pathways by Z-value are shown. The direction of activation of the pathways can be seen to be very similar for the UC and CD macrophages.

5.3.2 *TREM1* signalling is up regulated in CD and UC Macrophages

Triggering receptor expressed on myeloid cells-1 (*TREM-1*) belongs to the immunoglobulin superfamily. *TREM-1* is expressed on the cell surface of the majority of monocytes, macrophages and neutrophils. In the healthy intestine however only a small number of resident macrophages express the *TREM-1* receptor.(436) Expression of *TREM-1* is up regulated by microbial components, TLR stimulation and TNF alpha.(437, 438) Activation of *TREM-1* leads to amplification of down stream inflammatory pathways.(439)

The IPA pathway analysis found the *TREM-1* signalling pathway to be up regulated in both the CD and UC macrophages. For the UC cohort, 19 of the identified DEGs overlapped with the 75 genes **(table 23)** of the IPA *TREM1* pathway ($p=6.81 \times 10^{-4}$, right-tailed Fisher exact test) and for CD 21 of the DEGs **(table 24)** overlapped with the *TREM1* pathway ($p=3.84 \times 10^{-15}$, right-tailed Fisher Exact Test). The Z values for these pathway associations were 4.359 and 4.583 for UC and CD respectively.

Gene symbol	Gene name
CASP1	Caspase 1
CASP5	Caspase 5
CD40	CD40 molecule
CD86	CD86 molecule
CIITA	Class II MHC transactivator
CXCL3	C-X-C motif chemokine ligand 3
CXCL8	C-X-C motif chemokine ligand 8
DEFB4A/DEFB4B	Defensin beta 4A
IL1B	Interleukin 1 beta
ITGAX	Integrin subunit alpha X
JAK2	Janus kinase 2
MYD88	Myeloid differentiation primary response 88
NFKB2	Nuclear factor kappa B subunit 2
NLRC5	NLR family CARD domain containing 5
RELA	RELA proto-oncogene, NF-kB subunit
TLR1	Toll like receptor 1
TLR4	Toll like receptor 4
TLR8	Toll like receptor 8
TREM1	Triggering receptor expressed on myeloid cells 1

Table 23 The 19 DEGs for the UC cohort that overlap with the 75 genes of the IPA TREM1 signalling pathway. This list was generated in the IPA software. $P=6.81 \times 10^{-4}$, right-tailed Fisher exact test.

Gene symbol	Gene name
CASP1	Caspase 1
CASP5	Caspase 5
CCL2	C-C motif chemokine ligand 2
CCL3	C-C motif chemokine ligand 3
CCL7	C-C motif chemokine ligand 7
CD86	CD86 molecule
CXCL8	C-X-C motif chemokine ligand 8
DEFB4A/DEFB4B	Defensin beta 4A
ICAM1	Intercellular adhesion molecule 1
IL6	Interleukin 6
IL1B	Interleukin 1 beta
ITGAX	Integrin subunit alpha X
NFKB2	Nuclear factor kappa B subunit 2
NLRC5	NLR family CARD domain containing 5
STAT3	Signal transducer and activator of transcription 3
TLR1	Toll like receptor 1
TLR2	Toll like receptor 2
TLR4	Toll like receptor 4
TLR8	Toll like receptor 8
TNF	Tumour necrosis factor
TREM1	Triggering receptor expressed on myeloid cells 1

Table 24 The 21 DEGs for the UC cohort that overlap with the 75 genes of the IPA TREM1 signalling pathway. This list was generated in the IPA software. $P=3.84 \times 10^{-15}$, right-tailed Fisher Exact Test

A number of the *TREM1* pathway genes listed in **tables 23 & 24** such as *CASP1, IL1B, STAT3, TLR2, TLR3, TLR4, MYD88* and *JAK2* have been established has having a role in IBD pathogenesis.(440-444) This supports our finding that *TREM1* signalling is important IBD pathogenesis. The large number of pro-inflammatory and IBD implicated genes involved in the *TREM1* signalling suggests a central or coordinating role for *TREM1*. I was able to confirm that *TREM1* is acting as an upstream regulator in the IBD macrophages using the upstream regulator function in IPA **(figures 23 & 24).** The aim of the upstream regulator analysis in IPA is to take the uploaded gene expression data and use this information to identify upstream regulators and given the gene expression data predict if these upstream regulators are activated or not. IPA carries out a Fisher's Exact test to determine if the overlap is significant. IPA uses 4 data points to calculate the Fisher's Exact p-value.(445)

- Genes Known to be regulated by the regulator and are in the dataset
- Genes known to be regulated by the regulator but are not in the dataset
- Genes not regulated by the regulator but are in the dataset
- All genes regulated by some regulator in the Ingenuity Knowledge Base but not in the dataset and not regulated

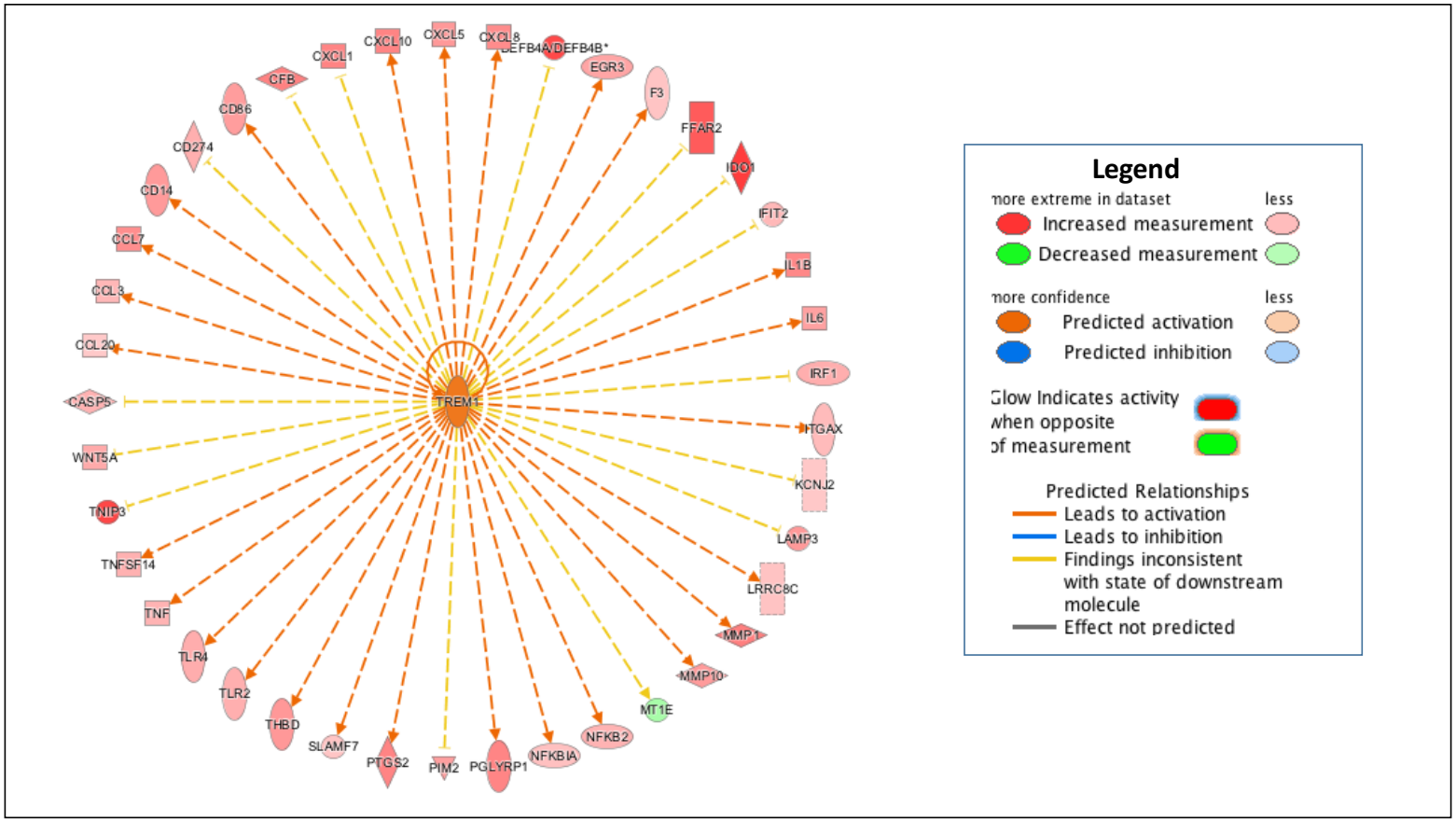


Figure 23 *TREM-1* identified as an upstream regulator in the CD macrophages using IPA. P-value = 16.92 e^{-23} (Fisher's Exact test). *TREM1* at the centre of the wheel is predicted to be activated based on the genes that it is regulating, these are represented as shapes around the circle, the different shapes represent functional categories of the protein that these genes encode For full key of shapes see Appendix B.

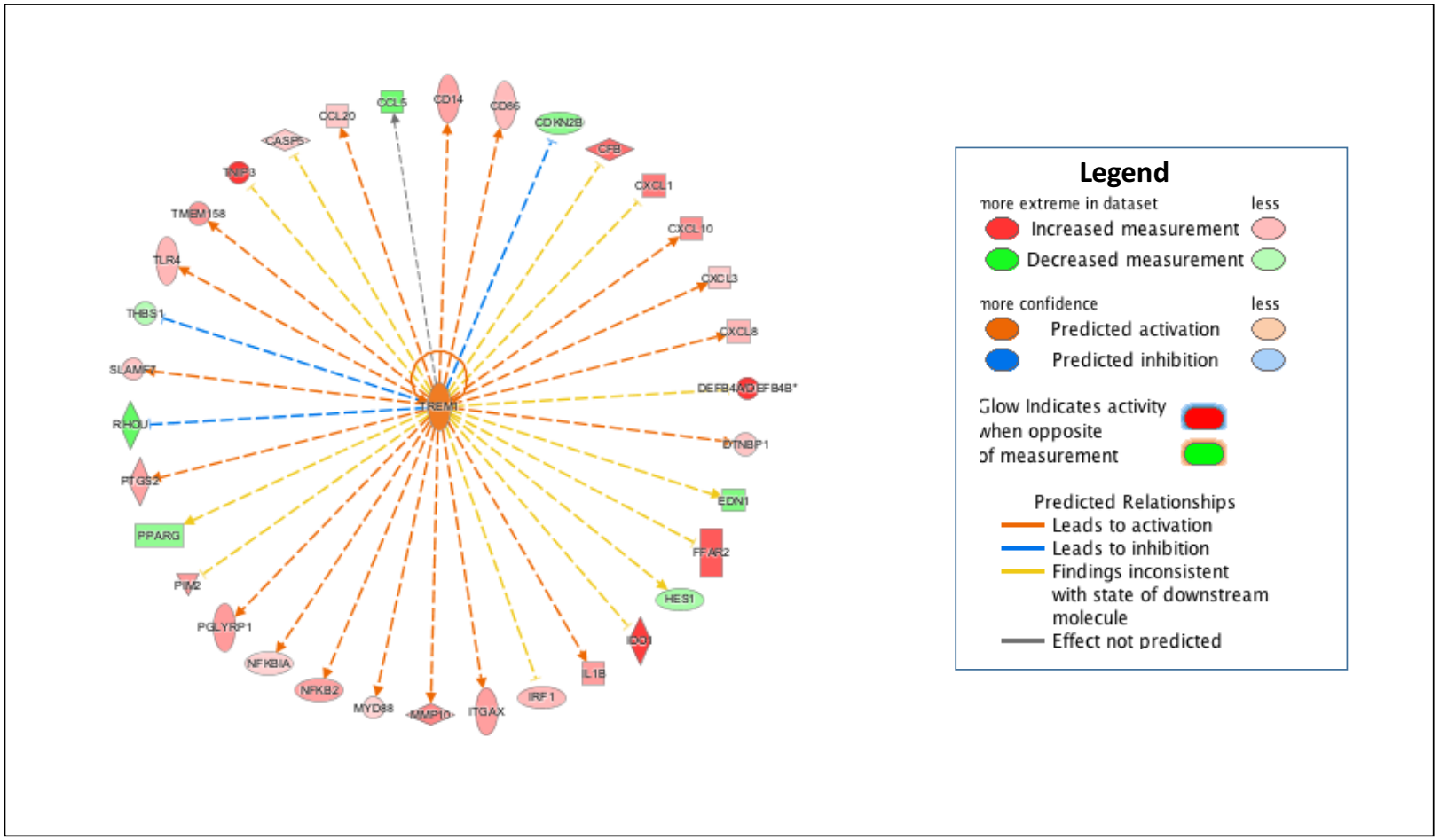


Figure 24 *TREM-1* identified as an upstream regulator in the UC macrophages using IPA. P-value = 1.96 e^{-11} (Fisher's Exact test). *TREM1* at the centre of the wheel is predicted to be activated based on the genes that it is regulating, these are represented as shapes around the circle, the different shapes represent functional categories of the protein that these genes encode For full key of shapes see Appendix B.

The IPA upstream regulator analysis uses the DEGs expression data to predict if the identified regulator is activated. The RNA-Seq data are consistent with the predicted activation state. *TREM1* is significantly up regulated in both CD and UC (**figure 25**).

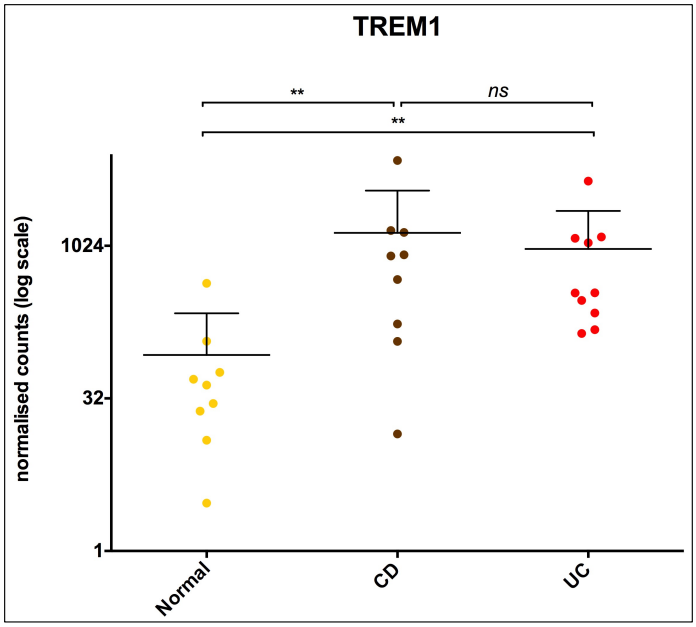


Figure 25 Normalised read counts from the RNA-Seq data for *TREM1* in CD and UC compared with the normal control group. The error bars show standard deviation and mean, p-value <0.01, ANOVA with Tukey test.

5.3.2.1 *TREM1* validation using flow cytometry confirms increased TREM1 expression in inflamed and non-inflamed colonic tissue from IBD patients

A further cohort of patients was recruited to assess *TREM1* expression using FACS **(figure 26)**, in patients with active IBD (biopsies taken from inflamed colonic mucosa), patients with IBD in remission (biopsies taken from non-inflamed mucosa of previously affected areas i.e. quiescent areas) and a group of healthy controls (biopsies taken form non inflamed sigmoid colon). A total of 5 patients with active IBD, 8 with IBD in endoscopic remission and 5 healthy controls were recruited for this part of the study. The biopsies for these patients were disaggregated as per the RNA-Seq cohort of patients and stained as per the *TREM1* panel described in the methods section. The percentage of *TREM1* expressing cells identified as macrophages $CD45^{+}CD14^{+}CD163^{+}$ and $CD3^{-}$ was determined and compared across the three groups. Given that the RNA-Seq data demonstrated increased gene expression of *TREM1* for both UC and CD, these patients were grouped together for the purpose of this analysis.

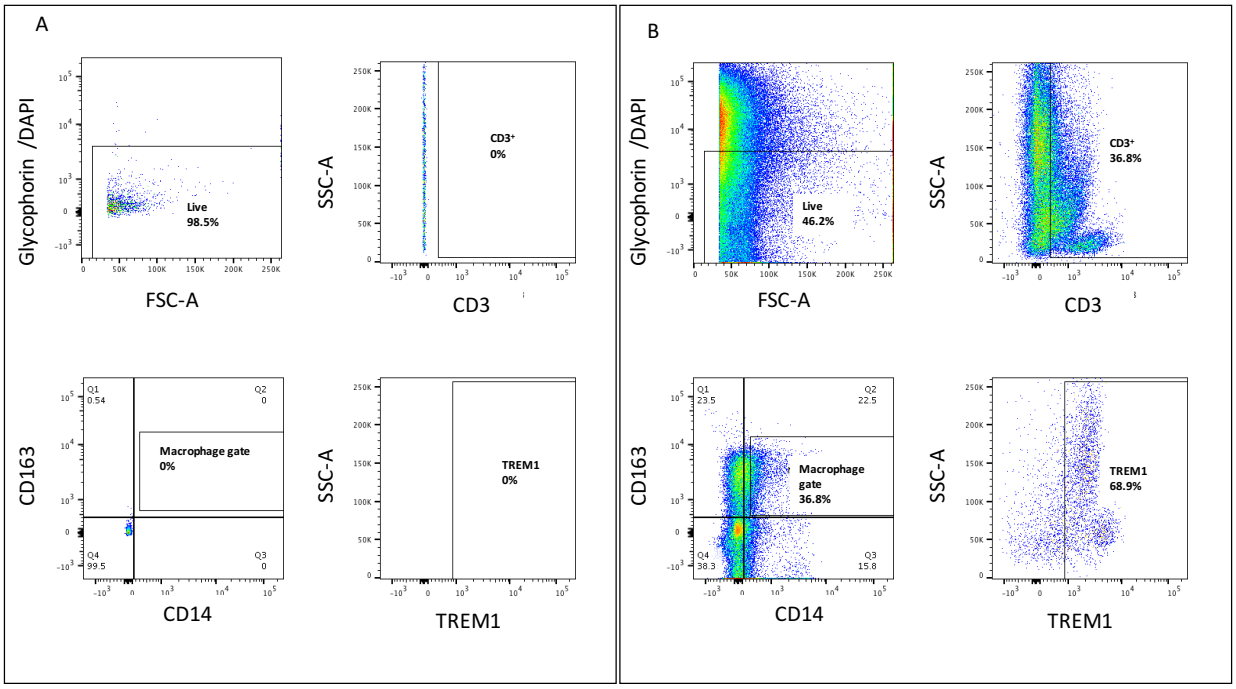


Figure 26 FACS plots for showing representative sample UDC647 TREM1 validation. (A) Unstained sample utilised to set gaits and (B) stained sample. The percentage of cells in the TREM1 gate with stained samples was measured

As shown in **figure 27,** *TREM1* expression was increased in the inflamed and non-inflamed IBD groups when compared to the healthy controls, though the non-inflamed group did not reach statistical significance when compared to the inflamed or normal groups. Interestingly this is suggestive that the increased *TREM1* expression observed in the RNA-Seq cohort is not purely explained by active inflammation. Therefore, an underlying increased *TREM1* expression on the intestinal macrophages of patients with IBD may be a predisposing or triggering factor for the development of chronic intestinal inflammation. Another implication of this finding is that it raises the possibility of using *TREM1* as a predictive or diagnostic marker for the development of IBD in patients who have no other evidence active disease. Although this validation cohort was small, it supports the finding of the RNA-Seq data that the increased *TREM1* gene expression does translate to increased protein expression.

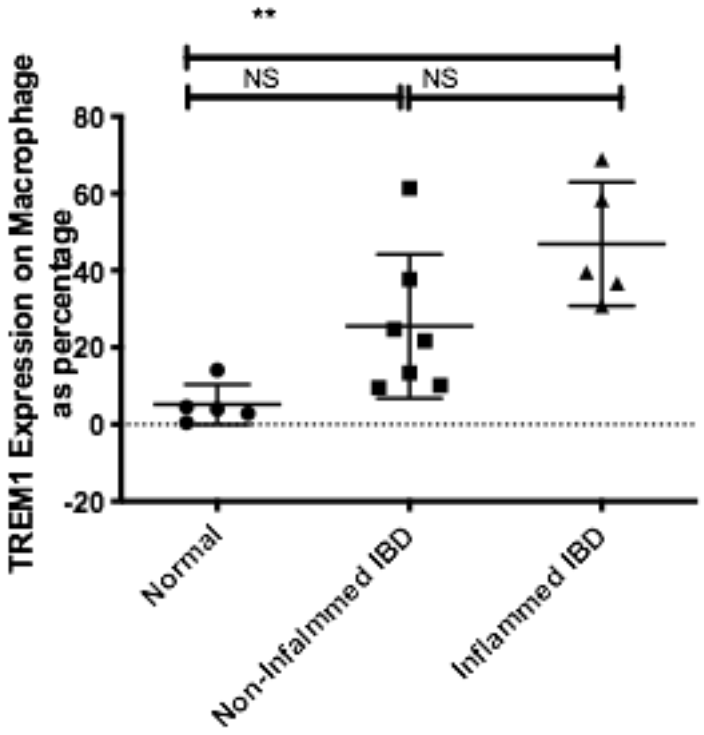


Figure 27 Graph showing percentage of *TREM1* expressing macrophage cells across 3 groups. Normal Vs Non-inflamed IBD p=0.0088, Normal Vs Inflamed IBD p= 0.004, inflamed IBD Vs non-inflamed IBD. Normal (n= 5), Non inflamed IBD (n=7) and inflamed IBD (n=5). The error bars show standard deviation and the mean. **p<0.05 ANOVA with Tukey test.

5.3.3 Innate immune system communication with the adaptive immune system pathways are up-regulated in CD and UC macrophages

The innate immune system is the first line defence mechanism against infection. As the major effector cell of the innate immune system, macrophages have a key role in the identification of pathogens through the recognition of pathogen-associated molecular patterns (PAMPs) utilising pattern recognition receptors (PRRs) expressed on their membrane surface. Activation of PRRs leads to release of inflammatory cytokines such as IL-1, TNF-α and IL-6, chemokines and increased expression of co-stimulatory molecules. This leads to antigen presentation and activation of the cells of the adaptive immune system. Effective antigen presentation to the adaptive immune cells requires expression of co-stimulatory molecules by antigen presenting cells such as CD80 and CD86.(446). This provides a further layer of regulation preventing inappropriate excess activation of the adaptive immune system. The CXC chemokines released by the cells of the innate immune system such as macrophage inflammatory protein 1α (CCL3) and inducible protein-1 (IP-10) have an important role in recruiting components of the immune system, they appear to preferentially attract monocytes and lymphocytes.(447)

The IPA pathway analysis I conducted identified that 25 of the DEGs identified in the UC cohort of macrophages overlapped with the genes of the IPA *communication between innate and adaptive immune system pathway* **(table 25)** which has a total of 94 genes listed (p-value for this association was 4.23×10^{-13}, right-tailed Fisher Exact Test). For the CD macrophage cohort, 20 DEGS **(table 26)** overlap with the 94 genes of the *communication between the innate and adaptive immune system pathway* (p-value $4.81 \times 10^{-12,}$ right-tailed Fisher Exact Test). The activation of these pathways suggests that the intestinal macrophages in these IBD patients are "activated", recruiting components of and activating the adaptive immune system. This pathway analysis therefore supports the theory that the intestinal macrophages in IBD play a role in the propagation of the intestinal mucosal inflammation and are likely to have a role in promoting the dysregulated immune response seen in IBD.

Gene Symbol	Gene name
CCL4	C-C motif chemokine ligand 4
CCL5	C-C motif chemokine ligand 5
CCL3L3	C-C motif chemokine ligand 3 like 3
CD40	CD40 molecule
CD86	CD86 molecule
CD79A	CD79a molecule
CD79B	CD79b molecule
CXCL8	C-X-C motif chemokine ligand 8
CXCL10	C-X-C motif chemokine ligand 10
HLA-B	Major histocompatibility complex, class I, B
HLA-C	Major histocompatibility complex, class I, C
HLA-E	Major histocompatibility complex, class I, E
IGHA1	Immunoglobulin heavy constant alpha 1
IGHD	Immunoglobulin heavy constant delta
IGHG1	Immunoglobulin heavy constant gamma 1
IGHG2	Immunoglobulin heavy constant gamma 2
IGHG3	Immunoglobulin heavy constant gamma 3
IGHG4	Immunoglobulin heavy constant gamma 4
IGHM	Immunoglobulin heavy constant mu
IL33	Interleukin 33
IL1B	Interleukin 1 beta
IL1RN	Interleukin 1 receptor antagonist
TLR1	Toll like receptor 1
TLR4	Toll like receptor 4
TLR8	Toll like receptor 8

Table 25 The 25 DEGs of UC macrophage cohort that significantly overlap with the 94 genes of the IPA pathway: communication between the innate and adaptive immune system. P= 4.23x 10^{-13}, right-tailed Fisher Exact Test.

Gene symbol	Gene name
CCL3	C-C motif chemokine ligand 3
CCL3L3	C-C motif chemokine ligand 3 like 3
CD86	CD86 molecule
CXCL8	C-X-C motif chemokine ligand 8
CXCL10	C-X-C motif chemokine ligand 10
HLA-B	Major histocompatibility complex, class I, B
IGHA1	Immunoglobulin heavy constant alpha 1
IGHG1	Immunoglobulin heavy constant gamma 1
IGHG2	Immunoglobulin heavy constant gamma 2
IGHG3	Immunoglobulin heavy constant gamma 3
IGHG4	Immunoglobulin heavy constant gamma 4
IGHM	Immunoglobulin heavy constant
IL6	Interleukin 6
IL1B	Interleukin 1 beta
IL1RN	Interleukin 1 receptor antagonist
TLR1	Toll like receptor 1
TLR2	Toll like receptor 2
TLR4	Toll like receptor 4
TLR8	Toll like receptor 8
TNF	Tumour necrosis factor

Table 26 The 20 DEGs of the CD macrophage cohort that significantly overlap with the 94 genes in the IPA pathway Communication between the innate and adaptive immune system. P-value= 4.81 x $10^{-12,}$ right-tailed Fisher Exact Test.

5.3.3.1 PPAR signalling pathway is down-regulated in intestinal macrophages in active IBD

Peroxisome proliferator-activated receptor gamma (*PPARγ*) belongs to a family of nuclear receptors which once activated interact with nuclear proteins. *PPARγ* itself is responsible for regulation of a number of genes involved in lipid metabolism, insulin sensitisation as well as inflammation.(448) PPARγ is principally expressed in adipose tissue, though it is now recognised that *PPARγ* is also highly expressed in the intestinal mucosa.(449) Data from mouse models of colitis suggest that *PPARγ* plays an important anti-inflammatory role, T-regulatory cells appear to require *PPARγ* to carry out their anti-inflammatory functions.(450) Mice with macrophage specific deletion for *PPARγ* have a more aggressive inflammatory response to DSS colitis compared to the wild type controls.(451) In humans it has been demonstrated that expression of *PPARγ* in intestinal tissue is reduced in patients with UC compared with those with CD and healthy controls.(452)

Pathway analysis using IPA identified that the *PPARγ* pathway is down regulated for both the UC and CD macrophage cohorts. For the UC cohort 12 of the identified DEGs **(table 27)** overlapped significantly with the 95 molecules included in this IPA pathway, p= 1.2×10^{-3} (right-tailed Fisher Exact Test).

Gene symbol	Gene
IL33	Interleukin 33
IL1B	Interleukin 1 beta
IL1RAP	Interleukin 1 receptor accessory protein
IL1RN	Interleukin 1 receptor antagonist
MAP4K4	Mitogen-activated protein kinase 4
NFKB2	Nuclear factor kappa B subunit 2
NFKBIA	NFKB inhibitor alpha
PDGFA	Platelet derived growth factor subunit A
PPARG	Peroxisome proliferator activated receptor gamma
PTGS2	Prostaglandin-endoperoxide synthase 2
RELA	RELA proto-oncogene, NF-kB subunit
SCAND1	SCAN domain containing 1

Table 27 The 12 DEGs in the UC cohort that overlap with the 95 genes of the PPARγ IPA pathway. P= 1.2×10^{-3}, right-tailed Fisher Exact Test.

The analysis of the CD cohort identified that 10 of the DEGs **(table 28)** overlap significantly with genes of the PPARγ pathway, P= 5.83 x 10^{-4} (right-tailed Fisher Exact Test).

Gene Symbol	Gene Name
IL1B	Interleukin 1 beta
IL1RAP	Interleukin 1 receptor access ry protein
IL1RN	Interleukin 1 receptor antagonist
MAP4K4	Mitogen- tivated protein kinase 4
NCOR2	Nuclear receptor co-repressor 2
NFKB2	Nuclear factor kappa B subunit 2
NFKBIA	NFKB inhibitor alpha
PTGS2	Prostaglandin-endoperoxide synthase 2
TNF	Tumour necrosis factor
TNFRSF1B	TNF receptor superfamily member 1B

Table 28 The 10 DEGs in the CD cohort that overlap with the 95 genes of the PPARγ IPA pathway. P= 5.83 x 10^{-4} right-tailed Fisher Exact Test.

The down regulation of the *PPARγ* pathways suggests a role in the development of chronic intestinal inflammation in IBD. It may be that "switching off" the *PPARγ* pathways propagates an inflammatory phenotype of macrophage that impairs wound healing through the reduction of regulatory and anti-inflammatory cells such as T-reg cells. It therefore seems that drugs that activate the *PPARγ* pathways such as the Thiazolidinediones, already utilised as insulin-sensitising agents in the treatment of type 2 diabetes mellitus, are a therapeutic strategy worth exploring in IBD. The efficacy of Thiazolidinediones has been previously been investigated in several clinical trials. Further investigation for a role of these drugs in IBD, despite randomised controlled evidence demonstrating superiority both for clinical response and remission over placebo has been curtailed by cardiovascular safety issues associated with the Thiazolidinediones.(453) The 5-ASA drugs, which are the first line therapeutic option for UC are thought to exert some of their anti-inflammatory effect through the activation of *PPARγ*.(118) This has led to the development of 5-ASA analogue drugs, which have a greater affinity for *PPARγ* resulting in a 100-150- fold greater intestinal anti-inflammatory properties. These drugs have shown promising results in animal models of colitis.(454) The data I have presented supports the on-going investigation of drugs for IBD that target activation of the *PPARγ* pathway but also goes one step further and suggest that it may be of benefit to target this pathway specifically in intestinal macrophages.

5.3.3.2 Leukocyte extravasation signalling pathway appears up-regulated in both the CD and UC macrophage cohorts

The migration of leukocytes to the site of injury and infection is a vital component in the immune system's response to such insults. Leucocyte migration is governed by a number of elaborate adhesive interactions between the leukocytes and the endothelium which help to ensure the migration of the leukocytes to the correct site at the correct time.(455) Inappropriate recruitment of leucocytes to the site of chronic inflammation occurs in inflammatory bowel disease and similarly in other chronic inflammatory disorders such as rheumatoid arthritis, psoriasis and multiple sclerosis.(456) Lymphocyte recruitment to the site of inflammation is largely regulated by the microvascular endothelium which is responsive to activation by inflammatory cytokines and other inflammatory mediators.(457) The migration of T-lymphocytes has been successfully targeted in IBD therapy by drugs targeting the interaction of integrins with vascular adhesion molecules. Vedolizumab, a drug targeting the $\alpha_4\beta_7$ integrin is now licenced for use in the UK for the treatment of both CD and UC.(158, 194)

The IPA pathway analysis demonstrated that for both the UC and CD macrophages there is up-regulation of the *leukocyte extravasation pathway*. For the UC cohort 28 of the identified DEGs **(table 29)** were associated with the *leucocyte extravasation-signalling pathway* of a total of 211 molecules in this pathway (p-value for association = 3.78×10^{-7}, right-tailed Fisher Exact Test). For the CD cohort 20 of the identified DEGs **(table 30)** are known molecules in the *leucocyte extravasation-signalling pathway* of a total of 211 (p-value for association = 3.78×10^{-7}, right-tailed Fisher Exact Test)

Gene symbol	Gene
CLDN2	Claudin 2
CLDN8	Claudin 8
CLDN18	Claudin 18
CTNND1	Catenin delta 1
CYBA	Cytochrome b-245 alpha chain
FGFR2	Fibroblast growth factor receptor 2
GNAI2	G protein subunit alpha i2
ICAM3	Intercellular adhesion molecule 3
ITGAM	Integrin subunit alpha M
ITGB2	Integrin subunit beta 2
MMP3	Matrix metallopeptidase 3
MMP7	Matrix metallopeptidase 7
MMP9	Matrix metallopeptidase 9
MMP10	Matrix metallopeptidase 10
MMP12	Matrix metallopeptidase 12
MMP25	Matrix metallopeptidase 25
NCF1	Neutrophil cytosolic factor 1
NCF2	Neutrophil cytosolic factor 2
NCF4	Neutrophil cytosolic factor 4
PIK3R5	Phosphoinositide-3-kinase regulatory subunit 5
PRKCB	Protein kinase C beta
RAC2	Ras-related C3 botulinum toxin substrate 2
SIPA1	Signal-induced proliferation-associated 1
TIMP1	TIMP metallopeptidase inhibitor 1
VASP	Vasodilator-stimulated phosphoprotein
VCL	Vinculin
WAS	Wiskott-Aldrich syndrome
WASL	Wiskott-Aldrich syndrome like

Table 29 The 28 DEGs from the UC macrophage cohort that overlap with 211 genes of the leukocyte extravasation pathway. P-value = 3.78 x10^{-7}, right-tailed Fisher Exact Test**.**

Gene symbol	Gene
ACTN1	Actinin alpha 1
CD44	CD44 molecule (Indian blood group)
CLDN2	Claudin 2
CLDN8	Claudin 8
EDIL3	EGF like repeats and discoidin domains 3
ICAM1	Intercellular adhesion molecule 1
ITGA2	Integrin subunit alpha 2
ITGA4	Integrin subunit alpha 4
ITGAM	Integrin subunit alpha M
MMP1	Matrix metallopeptidase 1
MMP3	Matrix metallopeptidase 3
MMP7	Matrix metallopeptidase 7
MMP9	Matrix metallopeptidase 9
MMP10	Matrix metallopeptidase 10
MMP12	Matrix metallopeptidase 12
MMP25	Matrix metallopeptidase 25
NCF2	Neutrophil cytosolic factor 2
NCF4	Neutrophil cytosolic factor 4
PIK3R5	Phosphoinositide-3-kinase regulatory subunit 5
TIMP1	TIMP metallopeptidase inhibitor 1

Table 30 The 20 DEGs from the CD macrophage cohort that overlap significantly with the 211 genes of the leukocyte extravasation pathway. P-value= 3.78 x10^{-7}, right-tailed Fisher Exact Test

This data however needs to be interpreted carefully. The RNA-Seq data utilised for this pathway analysis is derived from intestinal macrophage cells. Whist this analysis is suggestive that generalised leucocyte extravasation is up regulated it is clear from careful analysis of the pathway **(figure 28)** that many of genes such as the integrin sub-units would only logically have the effect of extravasation of the leukocyte if it was up-regulated in that cell type. As we only have data for macrophage gene expression in this study, the correct interpretation is to limit this finding to macrophages. There are however secreted proteins such as the MMPs that may well increase the extravasation of other leukocyte cell types such as lymphocytes; this association however is over interpreting the pathway analysis.

In the context of IBD, activation of leukocyte extravasation pathway even if restricted to macrophages is a finding worth exploring. A therapeutic strategy in IBD of blocking the migration of leukocytes to the intestinal mucosa has so far focused on lymphocytes, with effective drugs in this area already licenced for use. In this approach so far various mechanisms have been explored. In the case of vedolizumab this has been blockade of the interaction between the integrin $\alpha_4\beta_7$, expressed on T-cells that home to the gut specifically and the integrin receptor MAdCAM-1. Other drugs such as vercirnon block chemokine receptors on lymphocytes (CCR9), or trap the lymphocytes within the lymph nodes as in the case of the specific sphingosine-1-phosphate (S1P) -1 agonist, RPC 1063.(458) The expression data from the current study would suggest that inhibiting intestinal cells such as macrophages from releasing factors promoting leukocyte trafficking or neutralising these released factors could be beneficial in reducing inflammation. The drug BMS-936557, an antibody to the lymphocyte chemokine CXCL10 has taken this approach with some success in early clinical trials.(459, 460) The blocking of lymphocyte trafficking to the intestinal mucosal has therefore been shown to be beneficial in treating IBD in both human and mouse models.

The role the inhibiting monocyte (and therefore macrophage) recruitment to the intestinal mucosa is less clear. Data presented by at the 2018 ECCO congress raised the possibility of adverse results in blocking the migration of monocytes to the intestinal mucosa. Zundler et al (461) showed that blocking the integrin $\alpha_4\beta_7$ on monocytes in their experiments in humans and mouse models, reduced the presence of M2 macrophages surrounding mucosal wounds and in mice resulted in impaired healing. Interestingly they were also able to demonstrate that human $CD16^+$ intermediate and non-classical monocytes expressed high levels of $\alpha_4\beta_7$. Recent published data has demonstrated that the anti-integrin drug vedolizumab which targets blockade of recruitment of T-lymphocytes to the intestinal mucosa also seems to have an important effect on the innate immune system including alteration of macrophage populations.(384) I could find no other studies investigating manipulation of

monocyte migration in IBD. The analysis I have presented here suggests that these are targets that should be explored further.

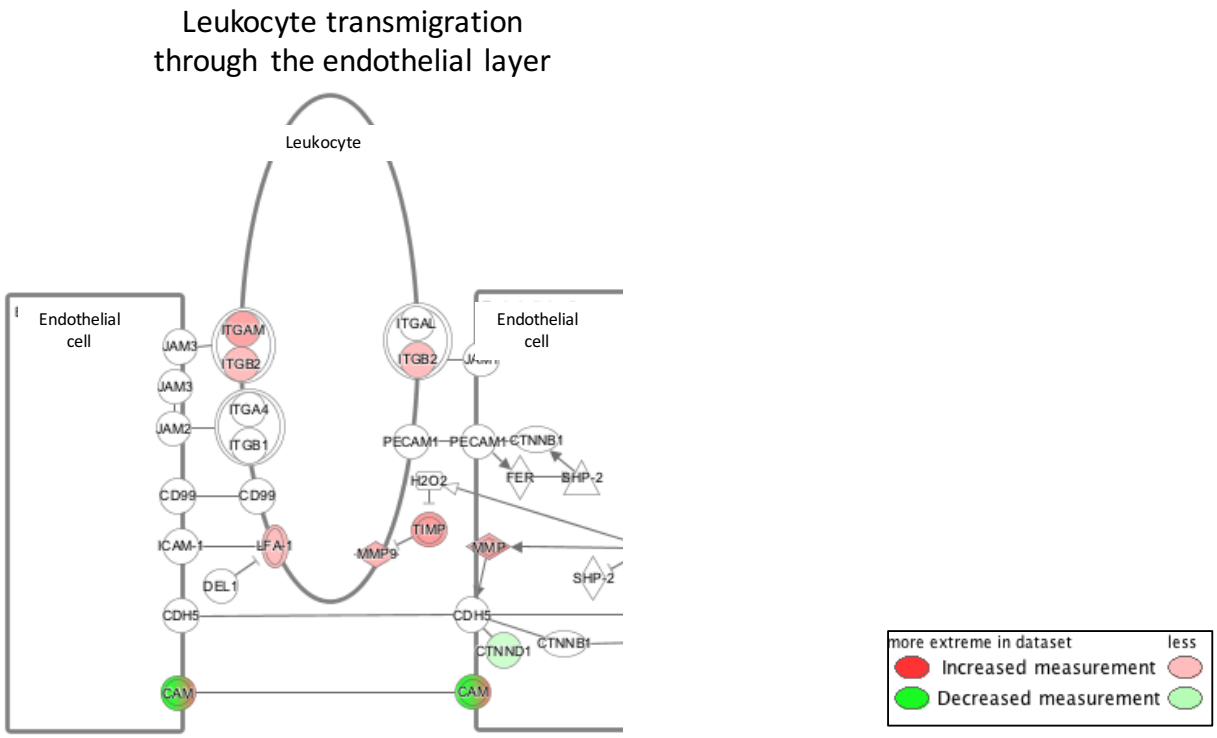


Figure 28 A section of the leukocyte transmigration pathway for the UC macrophage gene expression data

The shapes on the schematic shapes represent genes and the coloured shapes show those genes that hav up regulated (red) or down regulated (green) expression relative to the normal macrophages. The figur illustrates that genes such as *ITGAM, ITGB2* are located on the cell surface and therefore the association o this pathway for both CD and UC needs to be restricted to intestinal macrophages where the data originate and cannot be extrapolated to other intestinal leukocytes confidently. Image created in IPA.(391)

5.4 Exploration of RNA-Seq data with alternative statistical criteria

The primary analysis for this study was made with the aim of hypothesis generation to find avenues that warrant further investigation and to search for potential macrophage specific therapeutic targets. It was therefore important in this study that we captured as much biological information as possible rather than prove associations with a high degree of statistical certainty. For this reason, relaxed statistical criteria were employed for the identification of the DEGs. In this section of the thesis the effect of stricter statistical criteria for the identification DEGs and the associations so far described is explored.

5.4.1 Assessing data with stricter FDR and fold change

A relaxed FDR of <0.1 was utilised in this project for all the major analyses presented so far in this thesis. In this section the RNA-Seq data is explored using a variety of more stringent statistical criteria to assess the strength of the associations explored earlier in this chapter.

The data was assessed with an FDR of < 0.05, <0.01 and different cut off values of fold changes (FC) to explore how these impacted the number of DEGs observed and the significant pathways identified by the IPA pathway analysis. Applying the stricter FDRs of <0.05 or <0.01 markedly reduced the number of DEGs identified (**Table 31**). Applying an FDR of <0.05 and keeping the FC cut off at 1.5 fold up or down resulted in a 31.86% and 32.6% reduction in the number of DEGs identified for UC Vs N and CD Vs N respectively compared with the number of DEGs identified with an FDR of <0.1. At an FDR of <0.05 no DEGs were identified for the comparison of CD Vs UC. Furthermore, applying a FDR of <0.01 resulted in a 62% and 65% reduction in the number of DEGs for UC Vs N and CD Vs N respectively at a fold change of 1.5 up or down. In contrast adjusting the fold change cut off to greater than 2 did not have as large an impact on the number of DEGs. There was a reduction of 6.29% and 5.6% in the number of DEGs identified for UC Vs N and CD Vs N respectively with a FC of greater than 2 when compared with a FC of greater than 1.5 at an FDR<0.1.

Comparison	FDR <0.1 FC -1.5			FDR<0.1 FC -2			FDR<0.05 FC-1.5			FDR<0.05 FC- 2			FDR <0.01 FC-1.5			FDR<0.01 FC -2		
	Total DEGs	Up	Down	Total DEGs	Up	Down	Total DEGs	UP	Down	Total DEGs	Up	Down	Total DEGs	Up	Down	Total DEGs	Up	Down
UC Vs N	1287	837	450	1206	811	395	877	578	299	854	571	283	477	295	182	423	294	129
CD Vs N	840	693	147	793	667	125	575	509	66	556	497	59	293	276	17	291	274	17
UC Vs CD	20	7	13	12	6	6	0	0	0	0	0	0	0	0	0	0	0	0

Table 31 The number of DEGs identified utilising a range of FDRs and fold change (FC) cut off points for comparisons of UC Vs N, CD Vs N and UC Vs CD. The fold change cut off values were used to identify for both up and down regulated genes.

5.4.2 Pathway analysis using alternative FDR

The effect of the reduced numbers of DEGs resulting from using a more stringent FDR cut off on the IPA pathway analysis was assessed. The fold change cut off was kept at 1.5 up or down, as this did not appear to change the number of DEGs by a large amount. The data was analysed once again on IPA comparing FDR cut off points of <0.1, <0.05 and <0.01.

5.4.2.1 The UC RNA-Seq Cohort

The number of significantly associated canonical pathways for UC Vs N (P-value<0.05, set by IPA) were 170, 152 and 123 for FDR cut of values of <0.1, <0.05 and <0.01 respectively.

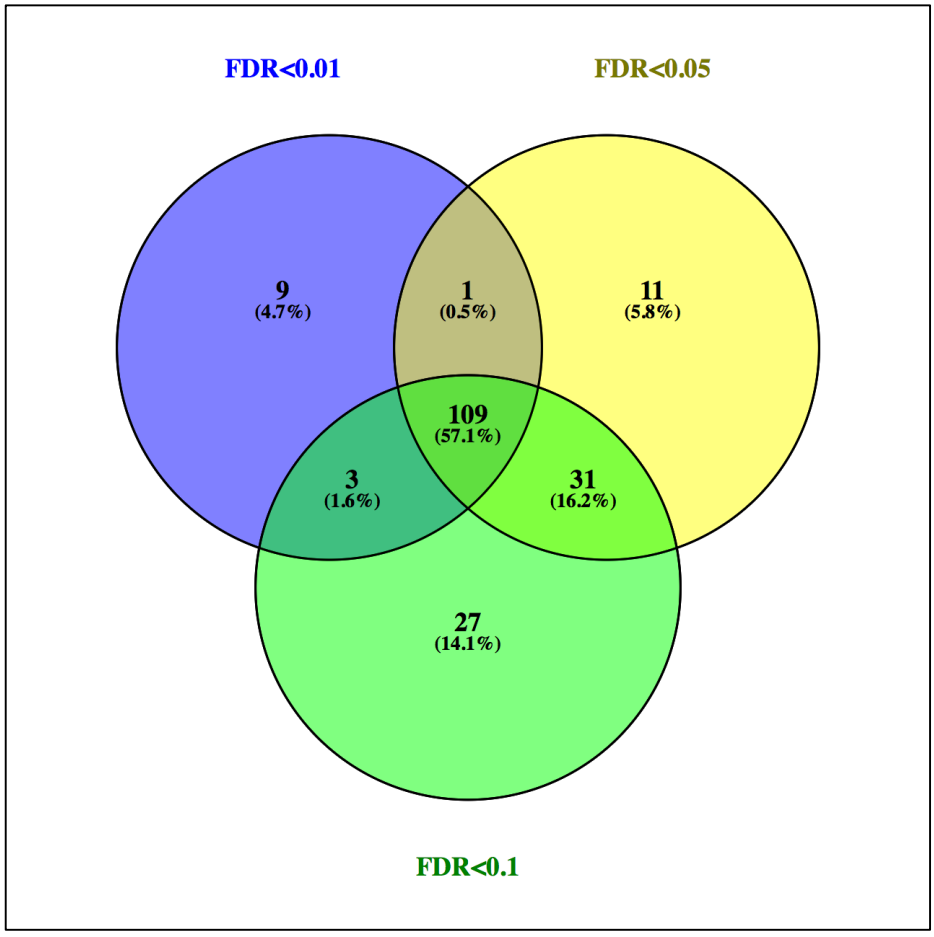


Figure 29 Venn diagram illustrating the number of common significant pathways identified by IPA analysis when using different FDR values to identify differentially expressed genes. Shown FDR<0.1, <0.05 and <0.01 for the UC Vs N analysis. *Diagram produced using Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venney.htm*

The Venn diagram in **figure 29** shows that 57.1% of the significant canonical pathways that were identified for UC Vs N with an FDR cut off value of <0.1 were also identified as significant with FDR values of <0.05 and <0.01. There were 27 pathways that were only significantly associated when an FDR of <0.1 was used to identify DEGs.

Interestingly there were 21 pathways that were significant when an FDR of <0.05 or <0.01 was used but not when an FDR<0.1 was used to identify the DEGs. Eleven pathways were identified only with an FDR<0.05 and a further 9 were only identified as significant with an FDR<0.01. As shown in **tables 32 & 33,** the ratio of associated genes in these pathways is low and it is difficult to interpret the biological significance of these pathways.

Pathway	-log(p-value)	Ratio of genes affected in pathway
Sphingosine and Sphingosine-1-phosphate Metabolism	1.53	0.222
Fc Epsilon RI Signalling	1.52	0.0672
tRNA Splicing	1.51	0.103
CXCR4 Signalling	1.51	0.0606
NGF Signalling	1.5	0.0667
Tight Junction Signalling	1.48	0.0599
Role of PI3K/AKT Signalling in the Pathogenesis of Influenza	1.47	0.0759
Macropinocytosis Signalling	1.42	0.0741
RhoGDI Signalling	1.39	0.0578
GNRH Signalling	1.35	0.062
FLT3 Signalling in Hematopoietic Progenitor Cells	1.34	0.0706
Glycogen Degradation II	1.3	0.167

Table 32 The Significantly associated canonical pathways identified using the DEGs identified using an FDR of <0.05 that were not identified with using the DEGs identified with an FDR of <0.1 for the comparison UC Vs N. Significant pathways identified by –log(p-value) >1.3 (i.e. p value <0.05) and as calculated using Fisher Exact Test.

Pathway	-log(p-value)	Ratio of genes affected in pathway
Intrinsic Prothrombin Activation Pathway	1.62	0.0714
Gαi Signalling	1.48	0.0417
FLT3 Signalling in Hematopoietic Progenitor Cells	1.42	0.0471
Thyroid Hormone Biosynthesis	1.36	0.333
Triacylglycerol Degradation	1.36	0.0566
Role of Cytokines in Mediating Communication between Immune Cells	1.34	0.0556
Unfolded protein response	1.32	0.0545
HMGB1 Signalling	1.32	0.0376
IL-22 Signalling	1.31	0.0833
Tryptophan Degradation III (Eukaryotic)	1.31	0.0833

Table 33 The Significantly associated canonical pathways identified using the DEGs identified using an FDR of <0.01 that were not identified with using the DEGs identified with an FDR of <0.1 for the comparison of UC Vs N. Significant pathways identified by –log(p-value) >1.3 (i.e. p value <0.05) calculated using Fisher Exact Test.

The biologically interesting canonical pathways discussed in detail earlier in the chapter; Communication between innate and adaptive immune cells, TREM1 signalling, PPAR signalling and leukocyte extravasation signalling were found to be statistically significantly associated with UC at the more stringent FDRs of <0.05 and <0.01. Thus suggesting that the association with these pathways and IBD macrophages is reasonably robust.

A direct comparison of the canonical pathways identified with the 3 different stringency levels of FDR was made within the IPA software in order the asses the degree and direction of predicted activation of these pathways across the different FDR analyses, **figure 30**. This analysis demonstrated for the pathways associated with a high degree of statistical significance, the direction of predicted activation was the same across all the tested FDR stringency levels. The degree however of predicted activation (magnitude of Z-score) does appear to be altered depending on the FDR utilised to identify the DEGs.

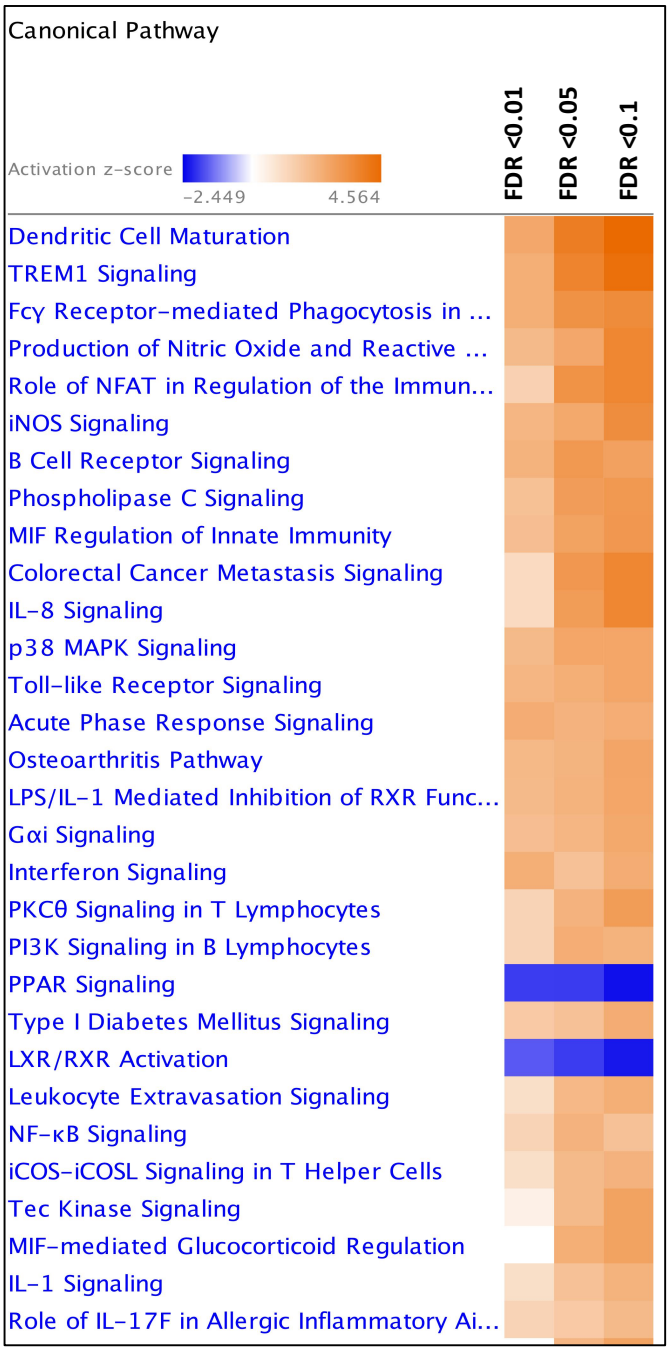


Figure 30 Heatmap produced in the IPA software showing comparison of the top identified associated canonical pathways arranged by the activation (z) score for UC Vs N at the three different FDRs. A z-score of > than 2 or less than<-2 is specified as significant by IPA. A positive Z score signifies a pathway that is predicted to be activated based on the DEGs and a negative Z score predicts that the pathway is inhibited. In this figure the comparison has been ordered according to Z-score, for which IPA calculates the combined Z-score for all comparisons for each pathway and lists the pathways from high Z score to low (without taking account of the direction of score).

5.4.2.1.1 The CD RNA-Seq Cohort

The RNA-Seq data for the comparison CD Vs N was analysed in a similar way using IPA. For this comparison the number of significantly associated pathways were 118, 121 and 131 for an FDR of < 0.1, <0.05 and <0.01 respectively. The Venn diagram, **figure 31** shows the overlap in pathways as the FDR varies. The more stringent FDR values were therefore associated with a greater number canonical pathways significantly overlapping with the DEGs.

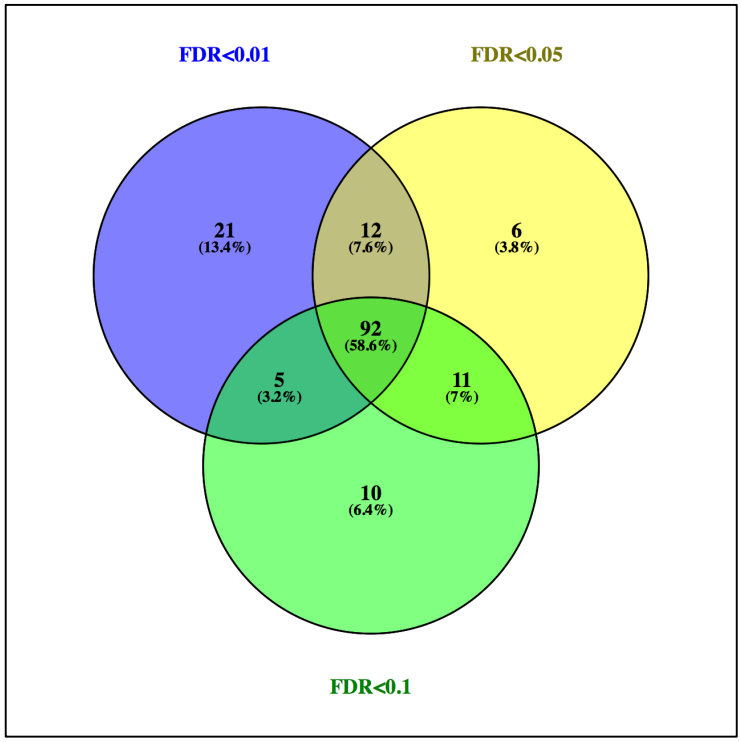


Figure 31 Venn diagram illustrating the number of significant canonical pathways identified by IPA analysis when using different FDR values to identify differentially expressed genes. Shown is FDR<0.1, <0.05 and <0.01 for the CD Vs N analysis. *Diagram produced using Oliveros, J.C. (2007-2015) Venny. An interactive tool for comparing lists with Venn's diagrams. http://bioinfogp.cnb.csic.es/tools/venney.html*

There were 21 and 6 pathways that were only identified in the FDR <0.01 and <0.05 respectively as well as 12 canonical pathways that were identified by both the FDR<0.01 and <0.05 analyses but not the FDR<0.1 analysis. There were a total of 39 pathways that were significantly associated in the analyses with FDR<0.01, <0.05 or both that were not significantly associated with the analysis using an FDR<0.1. As with the UC cohort of patients the additional pathways found with the DEGs gene set obtained using a more stringent FDR, showed low ratio of number of genes involved in those pathways, generally less than 10%, as shown in **tables 34 & 35**. Importantly as per the UC cohort; *Communication between innate and adaptive immune cells TREM1, PPAR signalling and leukocyte extravasation pathways* remained statistically significantly associated with CD at the more stringent criteria of FDR<0.05 and <0.01 for DEGs identification.

Pathway	-Log(p-value)	Ratio of DEGs involved in pathway
IL-1 Signalling	1.96	0.0652
Th2 Pathway	1.94	0.0533
fMLP Signalling in Neutrophils	1.9	0.0569
Hypoxia Signalling in the Cardiovascular System	1.77	0.0676
Protein Ubiquitination Pathway	1.71	0.0415
Gαs Signalling	1.63	0.055
Huntington's Disease Signalling	1.57	0.0412
Ovarian Cancer Signalling	1.56	0.0486
Circadian Rhythm Signalling	1.54	0.0909
Signalling by Rho Family GTPases	1.52	0.0403
Glycogen Degradation III	1.5	0.143
LPS-stimulated MAPK Signalling	1.5	0.0575
Relaxin Signalling	1.43	0.0455
Endothelin-1 Signalling	1.4	0.0421
Vitamin-C Transport	1.39	0.125
Mitochondrial L-carnitine Shuttle Pathway	1.34	0.118
Type II Diabetes Mellitus Signalling	1.34	0.0469
Lymphotoxin β Receptor Signalling	1.33	0.0597

Table 34 Canonical pathways associated with the CD Vs N analysis at FDR<0.05 but not the analysis with an FDR<0.1. P values give as –log(p-value), identified as significant if –log(p-value) was greater than 1.3 which corresponds to an actual p-value of less than 0.05. P-values in IPA are calculated using Fisher Exact Test.

Pathway	-log(p-value)	Ratio of DEGs involved in pathway
IL-1 Signalling	3.29	0.0652
Hypoxia Signalling in the Cardiovascular System	2.89	0.0676
Huntington's Disease Signalling	2.84	0.037
fMLP Signalling in Neutrophils	2.63	0.0488
Endothelin-1 Signalling	2.31	0.0368
Lymphotoxic β Receptor Signalling	2.21	0.0597
Relaxin Signalling	2.16	0.039
Role of JAK1 and JAK3 in γc Cytokine Signalling	2.12	0.0563
Glycogen Degradation III	2.01	0.143
Erythropoietin Signalling	1.92	0.0494
Role of RIG1-like Receptors in Antiviral Innate Immunity	1.91	0.0682
Type II Diabetes Mellitus Signalling	1.88	0.0391
Small Cell Lung Cancer Signalling	1.85	0.0471
CD28 Signalling in T Helper Cells	1.83	0.0379
LPS-stimulated MAPK Signalling	1.82	0.046
PEDF Signalling	1.82	0.046
PKCθ Signalling in T Lymphocytes	1.81	0.0376
Docosahexaenoic Acid (DHA) Signalling	1.72	0.0577
ILK Signalling	1.68	0.0306
Prostate Cancer Signalling	1.68	0.0417

Gαs Signalling	1.5	0.0367
T Cell Receptor Signalling	1.5	0.0367
Gαq Signalling	1.5	0.0311
p53 Signalling	1.48	0.036
Pancreatic Adenocarcinoma Signalling	1.39	0.0339
Glioma Invasiveness Signalling	1.39	0.0429
Acetate Conversion to Acetyl-CoA	1.37	0.25
Natural Killer Cell Signalling	1.35	0.0328
Ephrin B Signalling	1.34	0.0411
Gαq Signalling	1.5	0.0311
p53 Signalling	1.48	0.036
Pancreatic Adenocarcinoma Signalling	1.39	0.0339
Glioma Invasiveness Signalling	1.39	0.0429

Table 35 Canonical pathways associated with the CD Vs N analysis at FDR<0.01 but not the analysis with an FDR<0.1. P values give as –log(p-value), identified as significant if –log(p-value) was greater than 1.3 which corresponds to an actual p-value of less than 0.05. P-values in IPA are calculated using Fisher Exact Test.

The commonly associated pathways at an FDR<0.1, <0.05 and <0.01 were then further analysed to assess if there were any clear differences between the prediction of activation (described as the Z-score in IPA). As with the comparison of UC Vs N, for the CD cohort of patients this analysis shown in **figure 32**, shows that the direction of predicted activation of the top associated canonical pathways by statistical significance does not seem to be differ across three FDR levels compared. There does appear to be some variation of the degree of activation with FDR used though this is unlikely to impact on the conclusions made from the canonical pathway analysis.

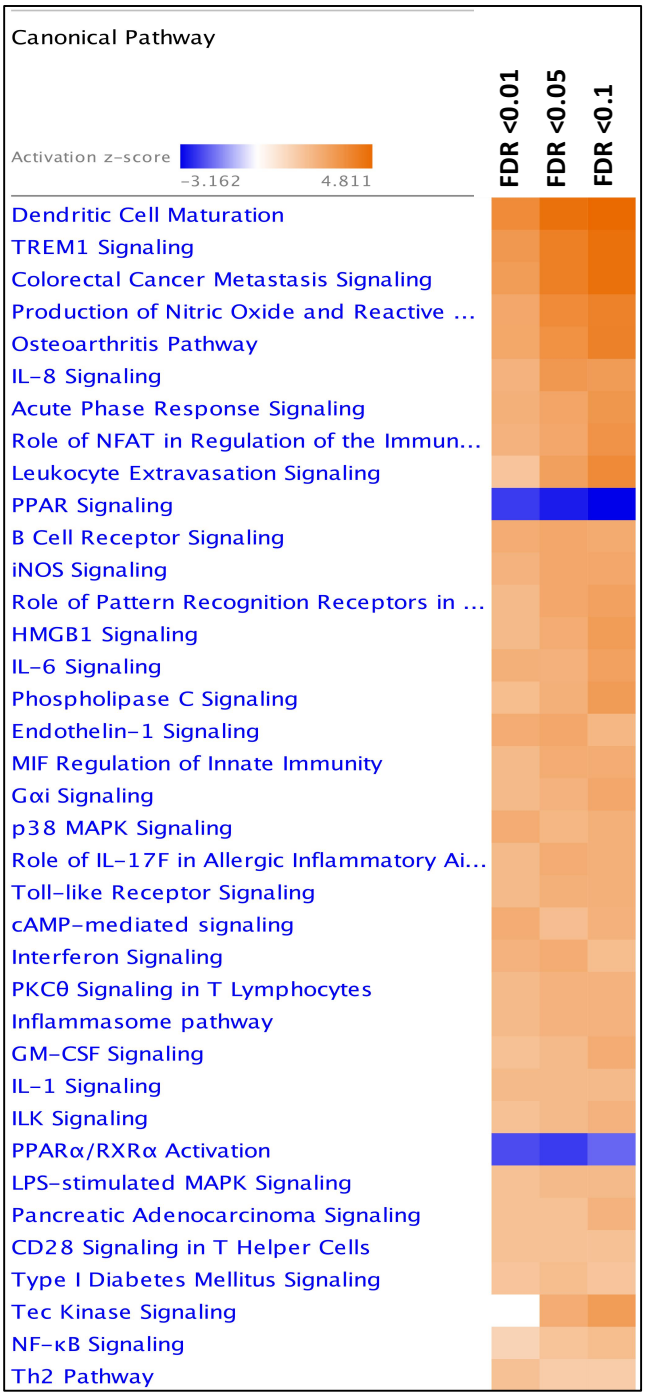


Figure 32 Heatmap produced in the IPA software showing comparison of the top identified associated canonical pathways according to the activation (z) score for CD Vs N at the three different FDRs. A z-score of > than 2 or less than<-2 is specified as statistically significant by IPA. A positive Z score signifies a pathway that is predicted to be activated based on the DEGs and a negative Z score predicts that the pathway is inhibited. In this figure the comparison has been ordered according to magnitude of Z-score, for which IPA calculates the combined Z-score for all comparisons for each pathway and lists the pathways from high Z score to low (without taking account of the direction of score).

5.5 Validation of RNA-Seq findings utilising qPCR

A second cohort of patients with active colonic CD, UC and healthy controls were recruited as a validation cohort. The intestinal macrophages from these patients were isolated using the same methods as the RNA-Seq cohort of patients. The RNA was extracted from these cells and amplified again utilising the exact same methods as the RNA-Seq cohort. The resulting cDNA was utilised in Real-Time PCR in an attempt to replicate the findings in an independent cohort of patients. In addition, we had remaining cDNA from the original RNA-Seq cohort, which we also subjected to Real-Time PCR to validate the RNA-Seq results.

There were unfortunately technical issues with the pre-amplification step for the validation cohort. A larger than expected number of amplification cycles were required to achieve satisfactory quantities of cDNA. It was suspected at this time that as a consequence the Real-Time PCR on the validation cohort would be un-reliable. This proved to be the case, with poor intra-experimental replicates. Therefore, the replication cohort could not be used to validate the results using real time PCR. The cDNA from the original RNA-Seq cohort however did remain viable and allowed for validation real time PCR experiments. Whist the inability to validate the findings of the RNA-Seq experiment on an independent cohort of patients using qPCR was a failure in this project it does not prevent the achievement of the project objectives. The core objective of this project was to generate hypotheses for the role of intestinal macrophages in IBD. It was therefore understood at the outset that any identified associations would need rigorous validation in a further project. It should also be noted that validation with an independent cohort would be best served using the same experimental methodology, RNA-Seq. The time and resource limitations of this project did not allow for this.

A selection of appropriate DEGs were identified from the RNA-Seq data for Real-Time PCR validation.

5.5.1 *TREM1* Pathway genes

The RNA-Seq data demonstrated up-regulation of *IL1B, CASP1 and TREM1.* These genes are all part of the *TREM1* signalling pathway that I demonstrated to be associated with the CD and UC macrophages earlier in the chapter using IPA canonical pathway analysis. **Figure 33** shows that the data obtained from the Real-Time PCR experiments support the findings of the RNA-Seq experiment

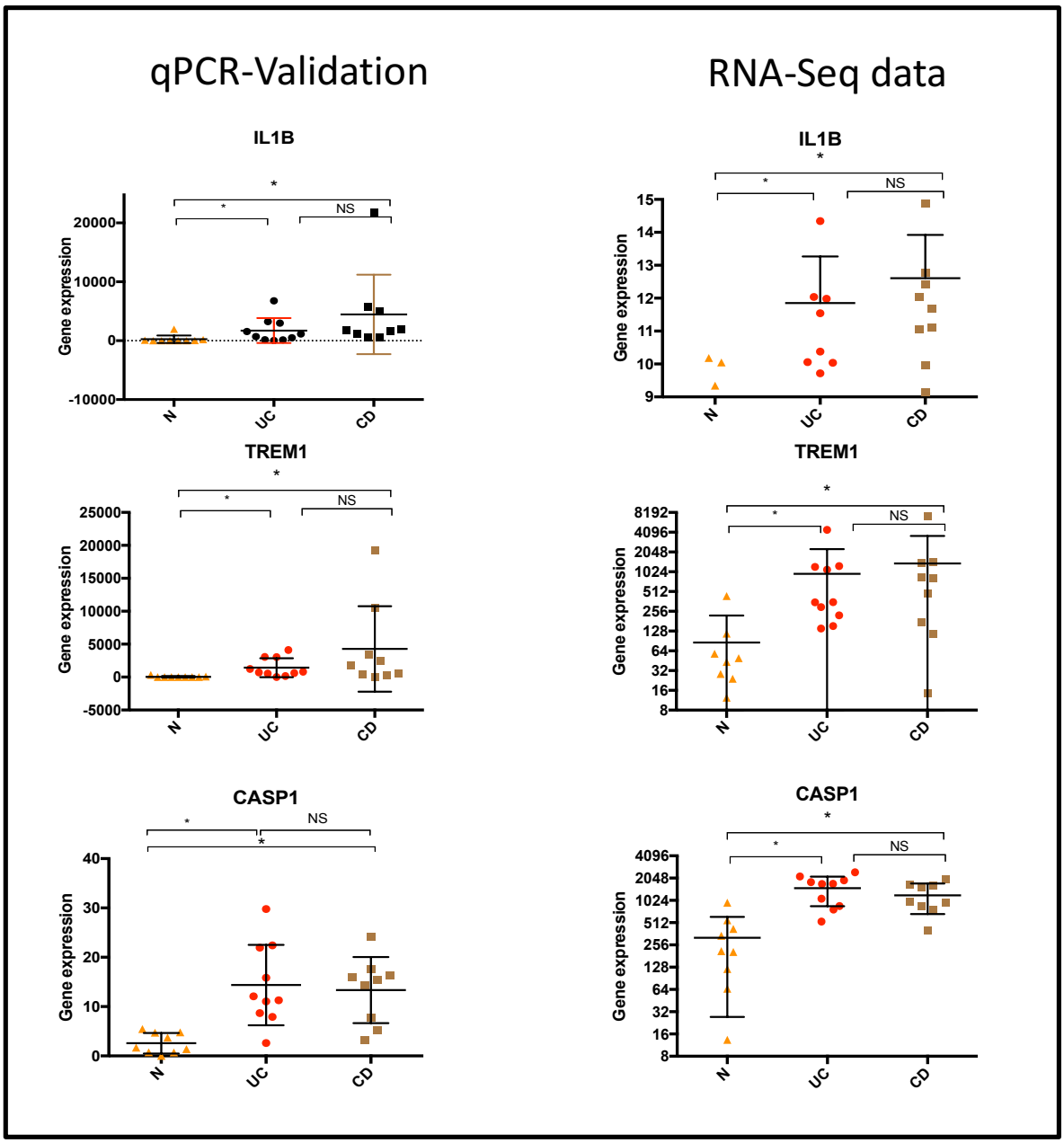


Figure 33 Comparison of the Real-Time PCR (qPCR) data and RNA-Seq data for the original cohort of patients for *ILB1, TREM1 and CASP1* genes. The Y-axis is in linear scale for the qPCR validation and shows normalised gene expression using the ΔΔCt method for normalisation. The Y-axis for the RNA-Seq data shows gene expression, as normalised read counts in Log scale. Normal (n=9), UC (n=10) and CD (n=9). The error bars show mean and standard deviation. P values for qPCR data calculated using the Kruskal Wallis test with Dunn's test in Graphpad prism, *indicated P<0.05. RNA-Seq significance test is as per the DESeq2 analysis for differentially expressed genes * indicates FDR<0.1.

5.5.2 T-Cell migration

The DEGs identified by the RNA-Seq analysis show that the macrophages are active in the recruitment of T-cells to the sites of colonic inflammation through up-regulation of chemokines such as CXCL9, CXCL10 and CXCL11. We sought to validate these findings with qPCR. The qPCR experiments confirmed the increased expression of CXCL9 and CXCL11 in CD and UC compared to normal macrophages. There was however no significant statistical difference for CXCL10 for these comparisons and therefore the RNA-Seq results could not be verified for this using qPCR for CXCL10, **figure 34**.

5.5.3 Antigen presentation and communication between the innate and adaptive immune systems

The pathway analysing using IPA demonstrated that communication between the adaptive and innate immune system and well as antigen presentation was up regulated in both the CD and UC cohort of macrophages. We selected genes involved in these pathways to validate the RNA-Seq data that these findings were based on. Some of these genes were not significantly differentially expressed in the RNA-Seq data (i.e. FDR<0.1) for both CD and UC. The qPCR validation results are were consistent with the RNA-Seq data except for CD74 which was found to be significantly differentially expressed from the qPCR data but not for the RNA-Seq analysis for the comparison of CD Vs N. In addition the increased expression in the qPCR experiment of CD86 was not significant for the comparison of CD Vs N but was for the RNA-Seq data as shown in **figure 35**. The is likely to underlie the difference in sensitivity between the two methods rather than a true difference.

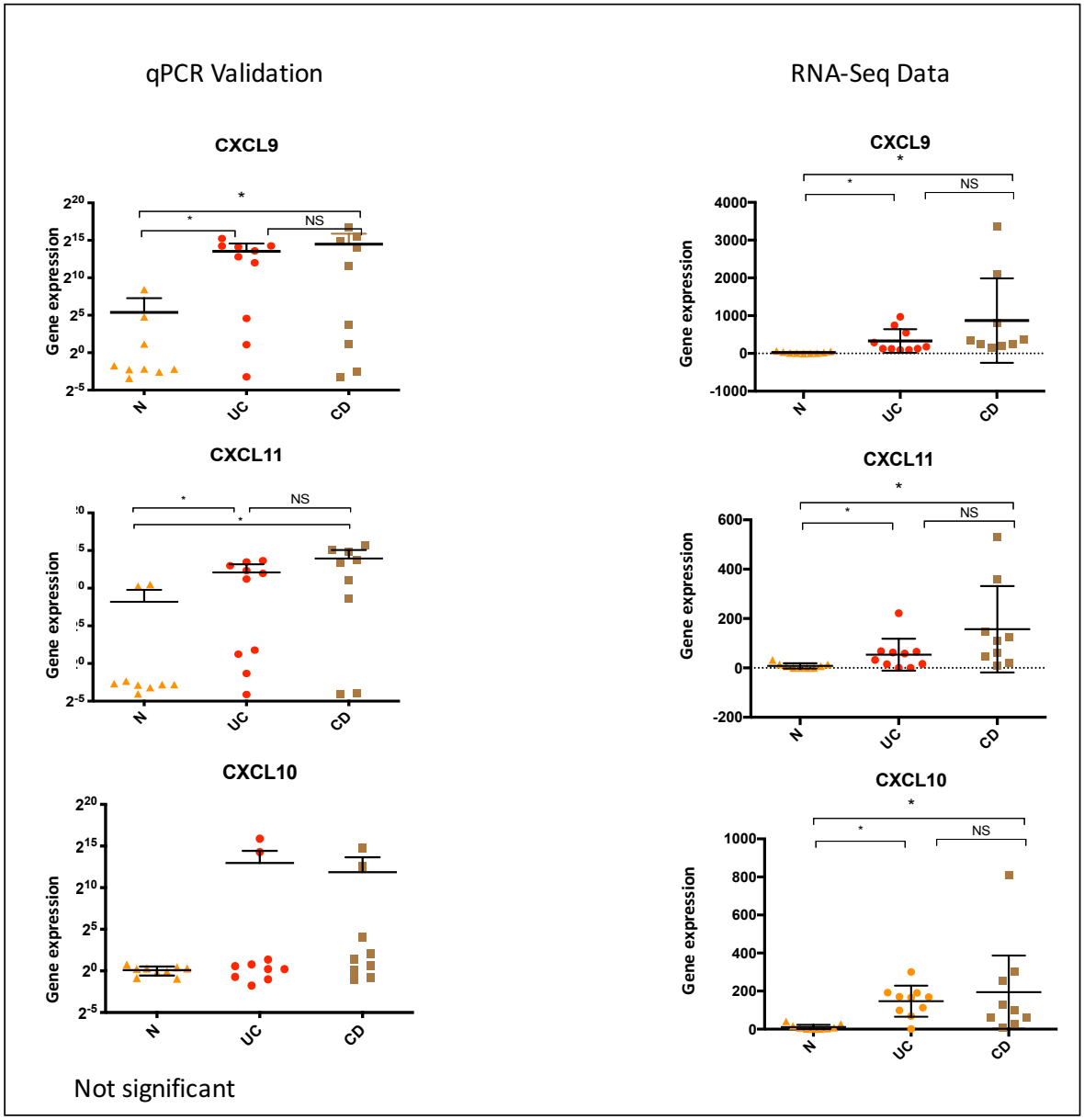


Figure 34 Comparison of the Real-Time PCR (qPCR) data and RNA-Seq data for the original cohort of patients for CXCL9, CXCL10, and CXCL11. The comparison of CXL10 expression of the qPCR data did not reveal any statistical difference between CD VS N or UC Vs N. The Y-axis is in Log scale for the qPCR validation and shows normalised gene expression using the ΔΔCt method for normalisation. The Y-axis for the RNA-Seq data shows gene expression, as normalised read counts. Normal (n=9), UC (n=10) and CD (n=9). The error bars show the mean and standard deviation. P values for qPCR data calculated using the Kruskal Wallis with Dunn's test in Graphpad Prism, *indicated P<0.05. RNA-Seq significance test is as per the DESeq2 analysis for differentially expressed genes * indicates FDR<0.1.

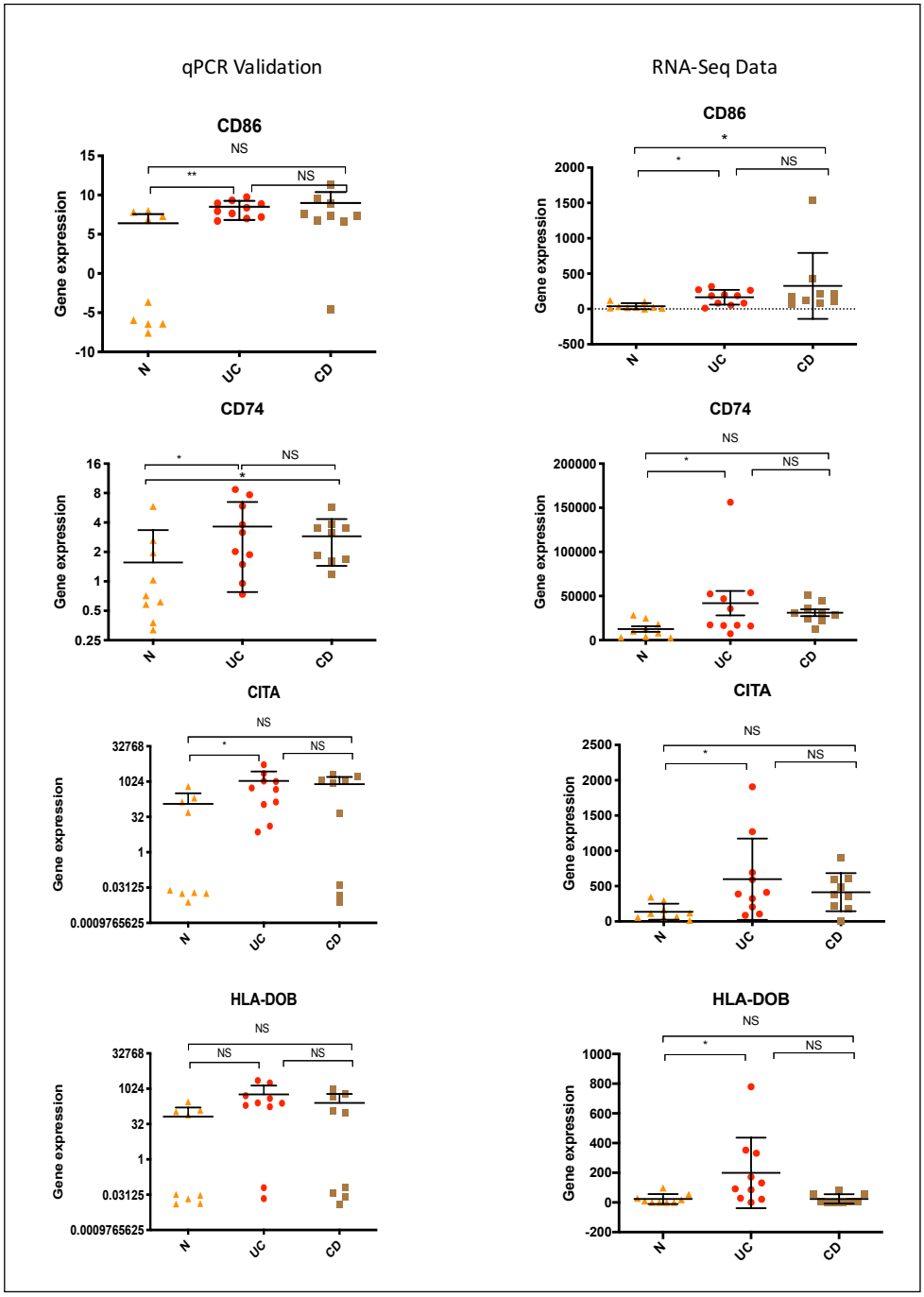


Figure 35 Comparison of the Real-Time PCR (qPCR) data and RNA-Seq data for the original cohort of patients for CD86, CD74, and HLA-DOB. The Y-axis is in Log scale for the qPCR validation and shows normalised gene expression using the ΔΔCt method for normalisation. The Y-axis for the RNA-Seq data shows gene expression, as normalised read counts. Normal (n=9), UC (n=10) and CD (n=9). The error bars show mean and standard deviation. P values for qPCR data calculated using the Kruskal Wallis with Dunn's test in Graphpad Prism, *indicated P<0.05. RNA-Seq significance test is as per the DESeq2 analysis for differentially expressed genes * indicates FDR<0.1.

5.6 Validation of RNA-Seq findings utilising Immunohistochemistry

We further validated the findings of the RNA-Seq using IHC on colonic biopsies embedded in paraffin. We used the qPCR validation cohort where these samples were available and in order to boost the number of samples a further cohort of patients were included in this section including healthy controls. Three reviewers independently scored the slides following a moderation process for each stain. The reviewers were blinded to the disease but were aware of the stain used for the slides. Each reviewer gave a score of 1-5 for each slide, 1= minimal staining, 5 = heavily stained. We selected three targets for staining; CD40 a co-stimulatory molecule that plays and important role communication between the innate and adaptive immune system (462), CXCL9 an important chemokine for T-cells through interaction with the CXCR3 receptor (463) and MMP12 which belongs to a group of metalloproteinases which are involved in remodelling of the extracellular matrix.(464) These three molecules were up regulated in the RNA-Seq data and identified as potential therapeutic targets through the pathway analysis.

We found that the in CD and UC samples these molecules stained more significantly than the healthy controls. **Figure 36** shows the average histological scores across the disease groups and **figure 37** shows representative examples of the histological staining that was scored.

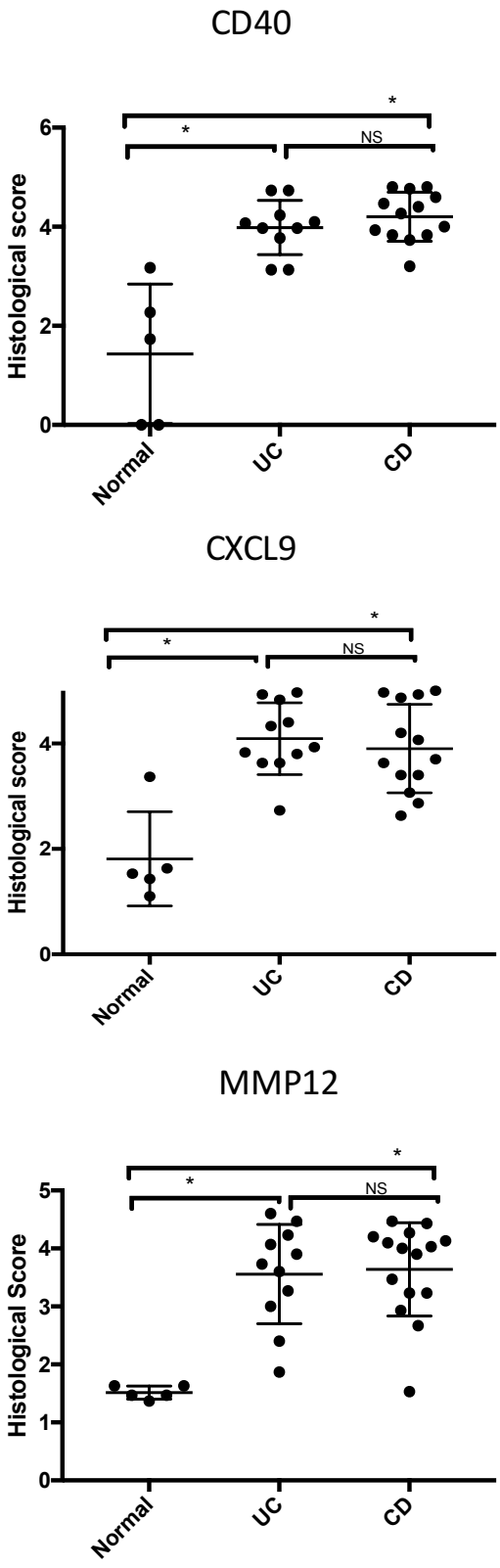


Figure 36 Average histological scores for CD40, CXCL9 and MMP12 IHC staining (scores taken from all 3 reviewers). Normal (n=5), UC (n=11) and CD (n=15). The error bars show standard deviation and mean values. * P-value <0.05 by ANOVA with Kruskal-Wallis and Dunn's test in Prism.

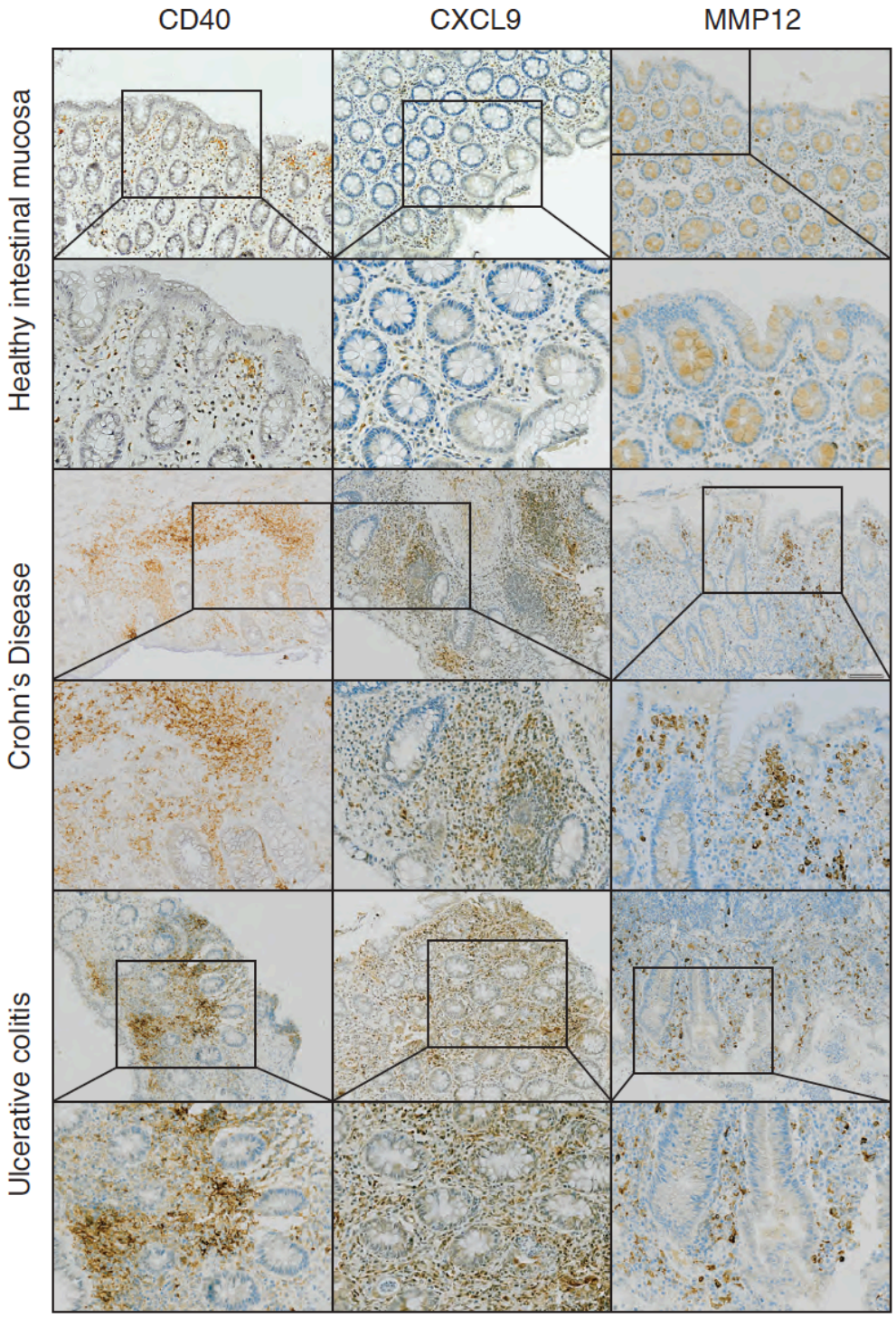


Figure 37 Validation IHC for CD40, CXCL9 and MMP12 using Immunohistochemistry, showing representative examples form healthy subject, active CD and UC in 10x and 20x magnification. The small inserts show the areas of magnification used for 20x images

5.7 Chapter Discussion

The two major forms of IBD, Crohn's disease and Ulcerative colitis can be difficult to differentiate clinically in patients who present with isolated colonic disease. In such patients the symptoms, endoscopic and histological findings can be the same for both CD and UC. The histological and clinical patterns that can differentiate the two diseases, principally that CD may affect any part of the gastrointestinal tract in a discontinuous manner, whereas UC is limited to the colon, CD is a transmural inflammatory process and histologically granulomas are seen in CD but not UC, which are well described differences between the two diseases. (465) In addition to these clinical differences between CD and UC, there are a number of differences in pathogenesis and genetic basis. Whist the majority of susceptibility loci identified by GWAS are common to both CD and UC, there a number that are only associated with one or the other of these diseases. For example the solute carrier family 9 member gene that has been implicated in UC but not CD (283) and the autophagy genes ATG16L1 and IRGM that have been associated with CD but not UC.(466) The data presented so far in this thesis however suggests that the intestinal macrophages of CD and UC patients are highly similar and it is difficult to differentiate between the two diseases on the basis of the macrophage transcriptional profile. For the comparison of CD Vs UC there were just 20 DEGs with our relaxed statistical criteria utilised for discovery purposes and there were no DEGs for this comparison when the stricter FDRs of <0.05 or <0.01 were applied. In order to minimise the heterogeneity of the CD group I only recruited and harvested biopsies from patients with colonic CD. A large well conducted multinational genetic study by Cleynen et al (467) demonstrated that ileal CD, colonic CD and UC represent three distinct genetic entities on a genetic continuum, such that colonic CD is more similar to UC than to ileal CD. It is therefore not surprising that the macrophages from the colonic CD and UC patients were similar in terms of gene expression and on this basis I would expect the gene expression of ileal CD to differ from both colonic CD and UC.

The quality analysis of the RNA-Seq data identified through principle component and correlation plot analysis that there were the three samples, NAC528, CCC593 and CCC594, which were clear outliers. The medical records including endoscopy reports were analysed to assess for any biological cause for this but none was apparent. It is likely that this variation represents experimental artefact and probably RNA damage at the amplification stage. Due to the nature of RNA-Seq analysis outliers in these experiments may also arise form data processing.(468) This is an emerging field, with software available that is able to help identify and deal with these issues such as OUTRIDER (469) and iSeqQC.(470) At the time of the RNA-Seq analysis these automated

methods were not routinely used and we did not have the local expertise to reliably use these methods. The PCA and correlation analysis however were sufficient to identify the outliers given the small sample size of the project. The outliers were removed from all subsequent analysis and calculation of differential gene expression; this was an important step as removal of outliers can significantly improve the detection of differential gene expression.(471)

The RNA-Seq data from the isolated macrophages of IBD patients demonstrates that the gene expression of these cells is markedly altered when compared to the macrophages from healthy controls. This analysis therefore supports the hypothesis that macrophages play a role in the intestinal inflammation described in IBD patients. I identified 1287 and 837 DEGs for the comparison of UC Vs N and CD Vs N respectively at an FDR <0.1. The hierarchical clustering shown in the heatmaps in **figures 17-20** demonstrate clear differences between the gene expression of the IBD macrophages compared to the health control group. Hierarchical clustering orders the rows and columns of the heatmap putting similar observations close together, so in this case the genes with reduced gene expression are grouped together as are the genes of increased relative expression. This method of presentation of the data was selected because differences in the groups are easily visualised despite the large number of genes that were identified. The data in the heatmaps were supervised for disease groups, so that the results of the disease groups are presented together, there was no other supervision or cluster learning applied for the generation of these heatmaps. Supervision by disease state was selected because we know that both CD and UC each represent complex groups of diseases that have heterogeneous pathological aetiologies with some shared features (472), non-supervised clustering would have as a consequence been challenging to interpret and difficult to gain useful information from. We have shown by utilising disease supervision as a class for the heatmaps we were able to clearly identify differences between the IBD transcriptome and the healthy controls and therefore effectively test the hypothesis of the project.

On the early analysis the IBD macrophages demonstrated that the CD and UC macrophages were very similar expressing a predominantly inflammatory transcriptome. I identified up-regulation of pro-inflammatory genes such as TREM1, IL1β and IL6, which have previously been recognised to be up-regulated in IBD.(473, 474) The IBD macrophages also demonstrate increased expression of chemokine genes *CXCL9*, *10* and *11*. This is in keeping with previous data in IBD that has shown an increase in these chemokines but also the corresponding chemokine receptor *CXCR3* on Th1 cells (475) thus promoting the recruitment of T-cells to the intestinal mucosa. The additional value of the present study is that the chemokines can be identified as originating from the isolated

macrophage cell population. The CD and UC macrophages showed increased expression of the NOS2 gene, which is associated with a pro-inflammatory M1 phenotype of macrophage.(476) Although there were fewer down regulated genes, 450 for UC and 147 for CD, I identified that a number of the common down regulated genes were mitochondrial related including *GFM1*, *ACAA2* and *CPT1A*. This could also be contributing to the inflammatory process in IBD through altered mitochondrial activity leading to impaired autophagy, increased release of ROS leading to oxidative stress and epithelial cell injury. Defects in autophagy and excess ROS production has been described as an important as aspect in the pathology of IBD.(477)

I have shown that the DEGs allow macrophages from patients with active IBD to be differentiated from healthy intestinal macrophages in this cohort of patients. This analysis however provided little additional information regarding the functional and biological outcomes of these changes in the gene expression identified in IBD macrophages beyond a predominantly inflammatory phenotype. We utilised IPA pathway analysis to understand how the DEGs were interacting and the biological consequences of these changes. We initially used a relaxed FDR of <0.1 to identify DEGs for the IPA pathway analysis, as the aim of this project was hypothesis generation. This identified a number of significant canonical pathways for the comparison of CD Vs N and UC Vs N. The canonical pathways were arranged by P-values. The top 50 pathways were reviewed for biological significance in IBD pathogenesis and translational therapeutic opportunity. In this chapter I have discussed in further detail four of these canonical pathways because they have previously been highlighted as potentially significant in IBD and/or there appeared to be a potential therapeutic target within these pathways. These pathways were: *TREM1 signalling, PPARγ signalling, communication between the innate and adaptive immune system* and *the leukocyte extravasation pathways*. These pathways were significantly associated with both the macrophages from the CD and UC cohorts when compared to the healthy controls. These pathways remained in the top 50 canonical pathways (arranged by P-value) when the stricter FDRs of <0.01 and <0.05 were applied to DEGs identification, suggesting that these associations are reasonably robust.

The TREM1 signalling pathway was found to be activated in both the UC and CD macrophages and the TREM1 receptor was identified as an up-stream regulator. The TREM1 receptor is found on the surface of the majority of monocytes, macrophages and neutrophils. Intestinal macrophages however express very little TREM1 in the healthy state.(478) Activation of the TREM1 receptor leads amplification of pro-inflammatory down stream pathways and therefore has a pro-inflammatory role.(439) In the present study I have identified that there is up-regulation of the

TREM1 receptor on the intestinal macrophages of patients with IBD. I validated these finings using flow cytometry and was able to show that the expression of TREM1 was also increased compared to healthy controls on macrophages of patients with quiescent IBD. This suggests that therapeutic targeting of the TREM1 receptor is a treatment strategy that should be considered not only for treating active IBD but also for the maintenance of remission once further validated in a larger study. Another role of TREM1 that is of interest given that in the present study the TREM1 expression seemed to be related to the degree of inflammation is as a biomarker of disease activity. One of the major challenges in the management of patients with IBD is determining if a patient is having a true flare of the disease and the degree to which the disease is active. The gold standard to assess IBD disease activity is direct visualisation by endoscopic assessment as symptoms can poorly correlate with disease activity often as a consequence of co-existing irritable bowel syndrome which is more common in IBD patients. (479) It is not desirable to perform endoscopic procedures regularly given that they are invasive, carry risk of significant complications, are expensive and time consuming for patients.(480, 481) Several stool markers have been investigated for roles in the assessment of disease activity in IBD, there is good evidence for faecal calprotectin in this context and is currently widely utilised in routine clinical practice. (482) Whist this is a very useful marker of disease and offers an alternative to endoscopic assessment of disease activity, patients find providing stool samples challenging and as consequence compliance with faecal calprotectin testing is poor, estimated as low as one-third in some studies.(483) Therefore there is still a role for potential blood serum biomarkers for IBD disease activity as blood tests seem more acceptable to patients with IBD who often require regular blood test monitoring for the treatments they are on. Although we have demonstrated that intestinal macrophage expression of TREM1 is elevated this is unlikely to be as useful as a standalone biomarker as measurement of intestinal TREM1 levels would require endoscopy with biopsies unless it could be shown to be excreted in the stool. There is data however to support the use of soluble TREM1 in the serum both as a non-invasive measure of IBD disease activity and response to therapy with Anti TNF.(484) There may still however be a role for measuring the levels of TREM1 in intestinal biopsies obtained from endoscopic assessment. There is operator variability in the reporting of disease activity seen at endoscopy although validated scoring systems do help (485) and there are similar issues with the reporting of histological samples in IBD patients to assess the disease severity.(486) The measurement of mucosal TREM1 therefore potentially offers an objective quantitative measurement of the disease activity seen at endoscopy and perhaps scoring systems that incorporate this would be more transferable and

better able to accurately record response to treatment. Further validation in large cohorts of patients would be required before moving this to the clinical setting.

Interestingly the PPARγ signalling pathway was down regulated for the CD and UC macrophages. Reduced expression of PPARγ has been identified previously in the colon of patients with UC but not CD when compared to healthy controls, it is important to note that the reduced expression of PPARγ was also seen in inactive UC. (449) Thus suggesting that reduced PPARγ is not a consequence of inflammation but perhaps predisposes to developing IBD and therefore is another potential target for maintenance therapy. This has been considered previously, PPARγ ligands such as the thiazolidinediones which are utilised in the treatment of diabetes mellitus, have shown efficacy in the treatment of the intestinal inflammation seen in IBD and also mouse models of IBD.(487, 488) Progress with this line of treatment however stumbled due to cardiovascular concerns associated with the thiazolidinediones and there now seems little interest in the area.(489) There are already established treatment strategies that target the PPARγ pathway, given that there is now evidence from mouse studies confirmed with human organoids that 5-aminosalicylic drugs have their efficacy owing to targeting of PPARγ.(490)

We were able to validate our findings using a combination qPCR, flow cytometry and IHC. We had hoped to replicate the RNA-Seq results of a selection of genes using qPCR on a new independent cohort of patients. Unfortunately we encountered problems at the stage of macrophage RNA amplification that prevented us from performing the qPCR accurately on this cohort of patients. The financial and time constraints of the project did not allow for a further cohort of patients to be recruited and samples harvested for a further attempt at validation qPCR on a fresh cohort. We were however able to perform validation qPCR on the original cohort of patients used for the RNA-Seq as we had sufficient amplified RNA remaining. Whist the validation in this study supports further exploration of the findings, more robust validation of the therapeutic targets in a large, ideally multicentre recruiting study should be the next step prior to developing new therapies directed at the targets we have found.

The analysis of the RNA-Seq data shown in chapter 5 provides an overview and clearly shows that there are marked changes in the transcriptome of intestinal macrophages of IBD patients compared to healthy controls but suggests that the macrophage transcriptome of UC and CD patients are similar. In the next chapter the RNA-Seq data is analysed using additional tools to assess the data to provided a multi-modal analysis of the transcriptome of the macrophages searching for subtle differences that a single modality analysis approach may have missed.

Chapter 6:

Results 3: Intestinal Tissue Macrophage Phenotypes in active IBD

6.1 Introduction

Intestinal macrophages represent a diverse heterogeneous group of cells. As with macrophages found elsewhere in the body, efforts to phenotype intestinal macrophages have focused on the M1 (pro-inflammatory) /M2 (anti-inflammatory) polarisation classification system first described by Mills (311) and then further refined by Mantovani. (317) Xue et al in their extensive in vitro study of macrophage expression profiles under 28 different conditions demonstrated the plasticity and complexity of macrophage phenotypes based on gene expression and proposed a spectrum model for macrophage phenotype.(318) It has been recommended that macrophages produced in vitro from monocytes should be named based on the activating conditions.(491) Firmly classifying macrophages in tissue based on the described characteristics of M1 and M2 macrophages derived from in vitro macrophages, even with the current refined models is challenging.(492) Generally a pragmatic approach has been taken in classifying in tissue macrophages, those that express features, behaviour or gene expression that is predominantly inflammatory are described as M1.

In healthy colonic tissue, macrophages have been shown to be relatively inert, resistant to Toll-like receptor stimulation, secret high levels of the anti-inflammatory cytokine IL-10 and are thought to be provide a central regulatory function preventing inappropriate inflammatory responses to commensal luminal microbes.(363) This inert property of intestinal macrophages has led to the hypothesis which is now supported with some evidence that “resident” intestinal macrophages are predominantly of the M2 phenotype and during instances of mucosal inflammation a trigger results in the recruitment of M1 inflammatory macrophages from circulating monocytes.(493) The mechanisms for this are not yet well understood. This hypothesis assumes that an M2 phenotype of intestinal macrophage is of benefit in the healthy state and is protective against the development of the dysregulated intestinal inflammation seen in IBD. There is evidence that in the normal healthy state signals from the intestinal mucosa such as TGFβ and IL-10 polarise newly recruited monocytes into an M2 (anti-inflammatory) phenotype.(336) The majority of research investigating intestinal macrophage phenotype has focused on cell

surface receptor expression on these cells, with little of this research completed using human tissue macrophages.

In this chapter I explore the phenotype of intestinal macrophages in patients with active CD and UC using the RNA-Seq data described in the previous chapter. This chapter builds on the pathway analysis described previously, explored further using Gene Set Enrichment Analysis (GSEA) to gain a more in-depth understanding of the macrophage phenotype described by the RNA-Seq data in the biological context of active IBD.

6.2 Transcriptome analysis demonstrates that intestinal macrophages from patients with active colonic CD and UC express both M1 and M2 genes

The gene expression profiles of macrophages induced to M1 or M2 polarisation from monocytes in vitro has been explored in several studies generating an accepted gene expression profile for M1 and M2 macrophages.(494-496) These studies have generally taken the classic approach to stimulating macrophage polarisation. In the case of M1 polarisation this has involved using IFNγ alone or together with LPS or TNFα. M2 macrophage stimulation in these experiments was generally achieved using IL-4 and IL-13.(317) Xue et al studied macrophage activation under several more conditions to develop a spectrum model for macrophage activation and described the transcripts of the resulting macrophages.(318)

In this section, macrophage gene expression signatures for M1 and M2 macrophages described in the literature are compared with the RNA-Seq data set generated from the present study from IBD intestinal macrophages with reference to healthy controls.

6.2.1 M1 gene expression in CD and UC macrophages demonstrates up-regulation of known M1 signature genes

The gene expression for UC and CD for a curated list of known M1 signature genes **(Appendix C)** that were differentially expressed in our RNA-Seq data were plotted on a hierarchical heatmap supervised by disease group, **figure 38**. This analysis demonstrates that there is up-regulation of M1 genes in both the CD and UC cohorts of macrophages. This finding is not surprising given that the macrophages from the IBD patients were harvested from colonic areas of active inflammation. The presence of macrophages with an M1 phenotype in these areas of active inflammation in IBD does however confirm that macrophages are actively involved in the intestinal inflammation, which forms the major organ damage seen in IBD.

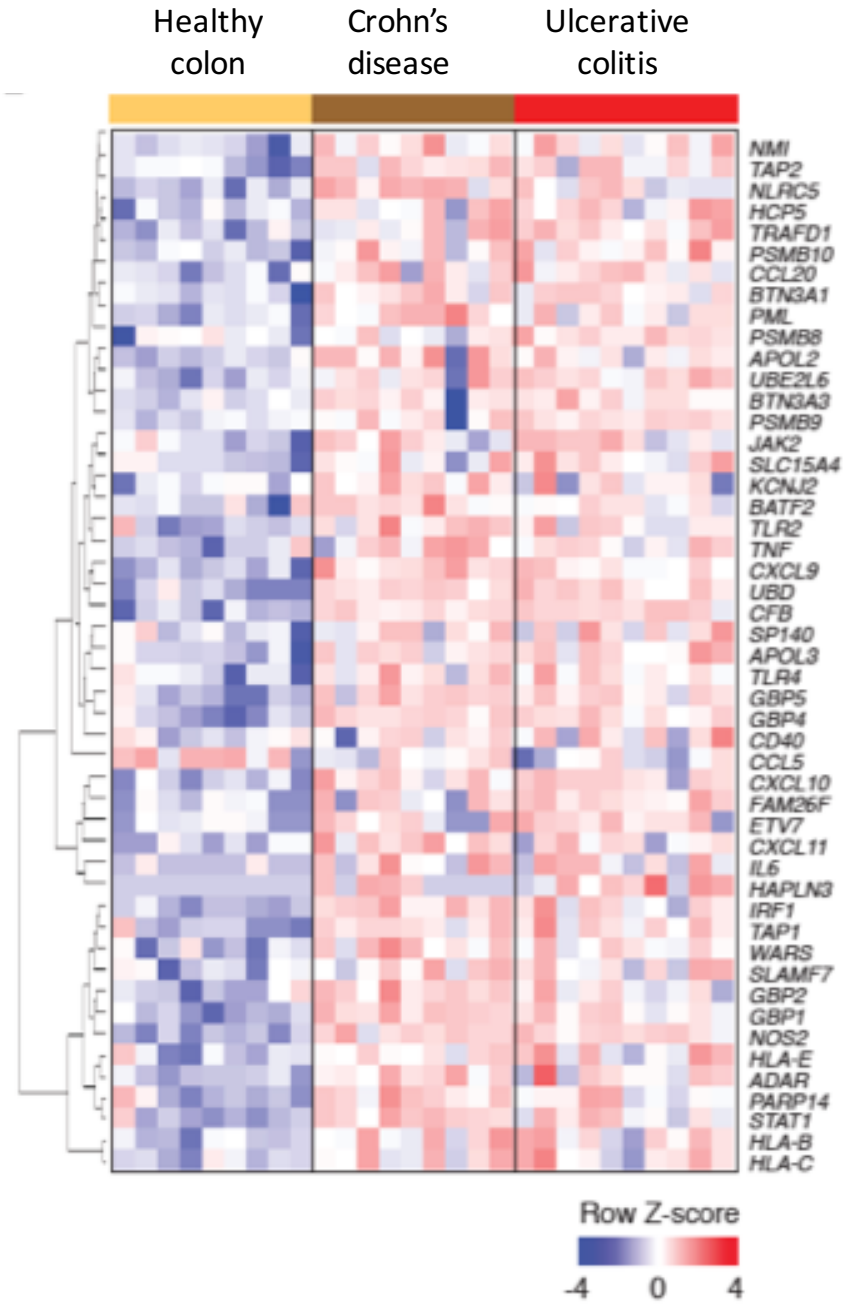


Figure 38 Hierarchical clustering of the 41 M1 differentially expressed genes (Fold change ≥ 1.5 and FDR ≤ 0.1) as log2 counts arranged by disease group. The row Z-score represents relative gene expression for each gene across all samples and groups (i.e. for each row) but not compared to other genes. Red colour signifies up regulation and blue down regulation. Healthy colon (n=9), CD (n=9) and UC (n=10).

6.2.2 M2 gene expression in CD and UC macrophages show up-regulation of a selection of M2 signature genes

As with the M1 signature genes, the DEGs generated from the RNA-Seq data for the IBD macrophages compared with healthy control patients were assessed for expression of M2 signature genes **(Appendix C)**. The DEGs that were known to be associated with the M2 phenotype were plotted on a heatmap according to disease group with hierarchical clustering. This heatmap demonstrates that there does appear to be up-regulation of M2 genes but the pattern is not as homogeneous as that seen with the M1 signature genes, **figure 39**.

The presence of both M1 and M2 gene expression in this cohort of IBD macrophages may be due to the co-expression of M1 and M2 genes in individual macrophage cells or this finding may be due the to a mixed population of M1 and M2 macrophage cells, that exclusively express M1 or M2 genes. The experimental methods of this study do not allow for differentiating between the two possible explanations for this finding.

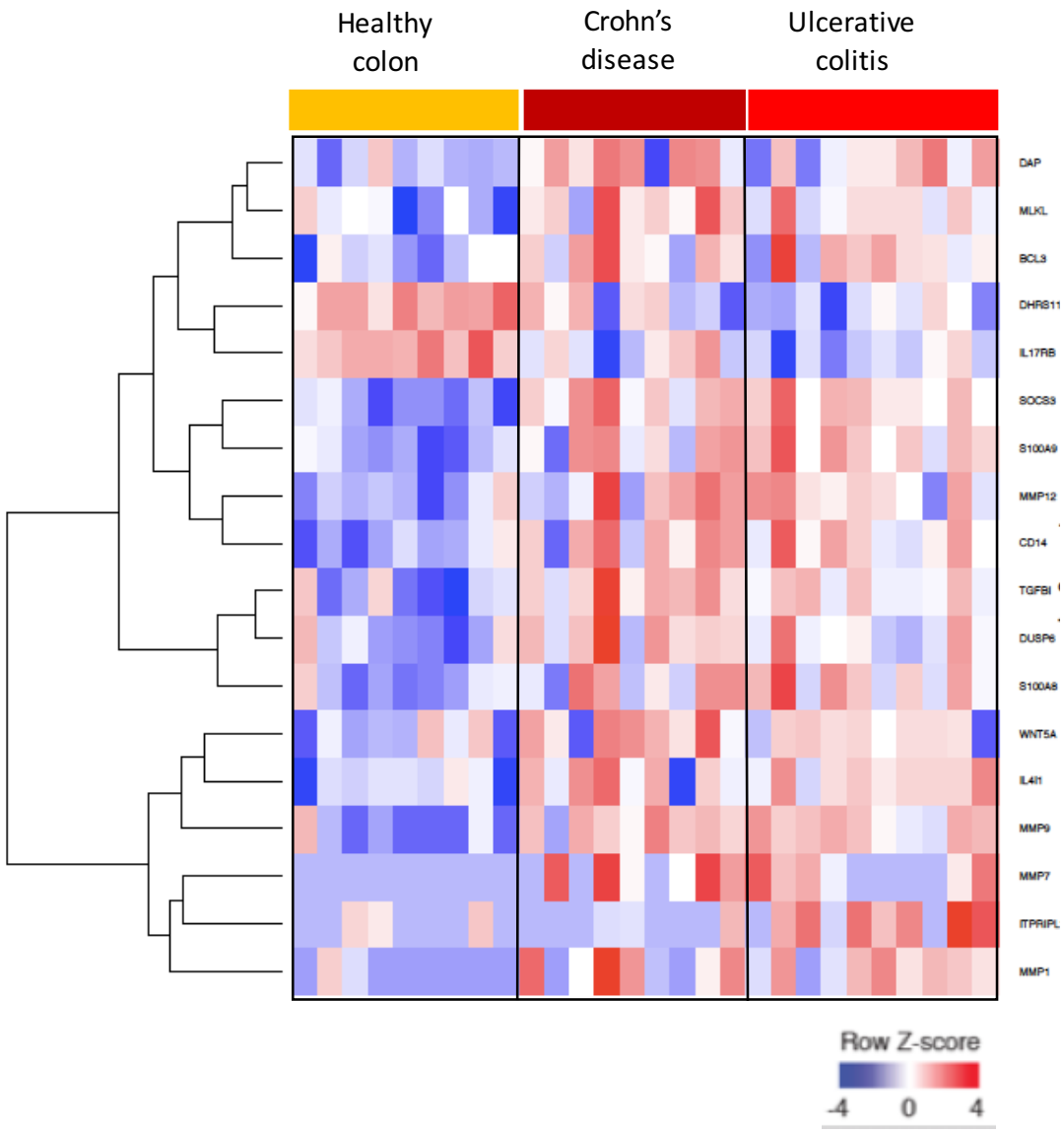


Figure 39 Hierarchical clustering of the 18 M2 differentially expressed genes (Fold change ≥ 1.5 and FDR ≤ 0.1) as log2 counts arranged by disease group. The row Z-score represents relative gene expression for each gene across all samples and groups (i.e. for each row) but not compared to other genes. Red colour signifies up regulation and blue down regulation. Healthy colon (n=9), CD (n=9) and UC (n=10).

6.3 Pathway Analysis exploration of intestinal macrophage phenotype in IBD

Pathway analysis has become an essential tool in omics research for the interpretation of large data sets created by high throughput sequencing experiments. Pathway analysis algorithms bring together biological knowledge from the literature held in databases (in the case of IPA this is the Ingenuity Knowledge Base) with statistical analysis using computational algorithms. (497) There are several different tools using a range of methods for pathway analysis, each with their own benefits and drawbacks.

The methods for pathway analysis utilised in this project can be broadly categorised as:

Over representation analysis; takes a list of (user) defined differentially expressed genes and identifies significant pathways as those that contain a greater proportion of these genes than could be randomly expected.(498) The ingenuity pathway analysis utilised in this project is an example of over representation pathway analysis. The major advantage of this form of analysis is the speed and ease with which large data sets can be given biological meaning. There are limitations to this approach however. As only the user defined DEGs are considered, there is a large amount of potentially biologically important data created from experiments that are not considered, just because a gene expression does not reach a pre-defined statistical significance level in isolation. This therefore does not mean when considered in network with other genes that this information is not valuable or statistically important. This also has an impact on result stability as different thresholds of DEGs yield different results (499) as was demonstrated in the previous chapter with repeated analysis at different FDRs for identification of DEGs.

The second-generation pathway analyses are also known as **functional class scoring**. These methods consider that in addition to large changes in gene expression having significant effects in a pathway, smaller changes in gene expression that are coordinated are able to impact on the overall pathway.(497) Functional class scoring methods are not reliant on arbitrary user defined cut off thresholds to divide data into significant and not significant categories. As with the over expression method, the functional class approach analyses each pathway independently and does not recognise pathway interaction. In this project pathway analysis is explored with both IPA and GSEA (an example of functional class scoring analysis) as analysis utilising different modalities offers a greater depth of understanding of the RNA-Seq data set generated in this project.

6.3.1 Ingenuity Pathway Analysis demonstrates that IBD Macrophages are activated and pro-inflammatory

The pathway analysis using IPA identified over 100 statistically significantly associated canonical pathways for the both the CD and UC macrophages respectively. In this section the top 30 canonical pathways (by z scores) that were statistically significant across all 3 FDR thresholds (<0.1, <0.05, <0.01) used to identify DEGs were investigated for function. The biologically relevant of these pathways are listed in **tables 36 & 37**. The predicted activation direction of each pathway along with the biological activity of those pathways is also listed. The aim of illustrating these pathways in this way is to demonstrate the overall theme of activity revealed by this pathway analysis.

Analysis of the canonical pathways demonstrates an overwhelming pro-inflammatory phenotype to the macrophage population from both the UC and CD patients. The activation of pathways such as *"Fcγ Receptor mediated phagocytosis in macrophages and monocytes",* "*Production of Nitric Oxide and Reactive Oxygen Species in Macrophages"* and *"Toll-like receptor signalling"* further suggests that the macrophages in these disease states are in an activated rather than in the inert state that intestinal macrophages are thought to be in within the healthy colon.

Pathway	Predicated activation state	Pathway category	Biological consequence of activated pathway
TREM1	Activated	Cellular immune response	Pro-inflammatory immune response
Fcγ Receptor mediated phagocytosis in macrophages and monocytes	Activated	Cellular immune response	Promotion of phagocytosis
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	Activated	Cellular immune response	Pro-inflammatory response
Role of NFAT in Regulation of the Immune Response	Activated	Cellular immune response Humoral immune response Intracellular and Second messenger signalling	Pro-inflammatory response
iNOS signalling	Activated	Cellular immune response	Pro-inflammatory Direct inhibition of pathogen replication by NO generation
Phospholipase signalling	Activated	Intracellular and second messenger signalling	Signalling leading to activation of PLC-δ
MIF Regulation of Innate Immunity	Activated	Cellular immune response	Pro-inflammatory
IL-8 Signalling	Activated	Cellular immune response	Pro-inflammatory
p38 MAPK Signalling	Activated	Cellular immune response , Cellular stress and injury, Cytokine signalling, Humoral immune response, Intracellular and second messenger signalling	Pro-inflammatory Apoptosis
Toll-like Receptor Signalling	Activated	Cellular immune response, Apoptosis, Humoral immune response, Pathogen-influenced signalling	Pro-inflammatory
Acute Phase Response Signalling	Activated	Cytokine signalling	Pro-inflammatory
LPS/IL1 Mediated inhibition of RXR function	Activated	Nuclear receptor signalling, Pathogen-influenced signalling	Impaired lipid metabolism, transport and biosynthesis
Interferon Signalling	Activated	Cellular immune response, Cytokine	Pro-inflammatory

		signalling	
PPAR Signalling	Inhibited	Nuclear Receptor signalling	Fatty acid metabolism
LXR/RXR Activation	Inhibited	Nuclear receptor signalling	Lipid metabolism
Leukocyte Extravasation Signalling	Activated	Cellular immune response	Pro-inflammatory
NF-κB Signalling	Activated	Cellular immune response, Cytokine Signalling, Humoral immune response, organismal growth and development	Pro-inflammatory
MIF-mediated Glucocorticoid Regulation	Activated	Cellular immune response, Nuclear receptor signalling	Pro-inflammatory
IL-1	Activated	Cytokine signalling	Pro-inflammatory
Role of IL-17F in Allergic Inflammatory Airway Diseases	Activated	Cytokine signalling	Pro-inflammatory

Table 36 The biologically relevant pathways from the top 30 pathways arranged by Z (activation) score that were statistically significantly associated with all 3 FDR threshold levels tested for the UC RNA-Seq macrophage data set.

Pathway	Predicated activation state	Pathway category	Biological consequence of activated pathway
TREM1	Activated	Cellular immune response	Pro-inflammatory immune response
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	Activated	Cellular immune response	Pro-inflammatory response
Role of NFAT in Regulation of the Immune Response	Activated	Cellular immune response Humoral immune response Intracellular and Second messenger signalling	Pro-inflammatory response
iNOS signalling	Activated	Cellular immune response	Pro-inflammatory Direct inhibition of pathogen replication by NO generation
Phospholipase C signalling	Activated	Intracellular and second messenger signalling	Signalling leading to activation of PLC-δ
MIF Regulation of Innate Immunity	Activated	Cellular immune response	Pro-inflammatory
IL-8 Signalling	Activated	Cellular immune response	Pro-inflammatory
p38 MAPK Signalling	Activated	Cellular immune response, Cellular stress and injury, Cytokine signalling, Humoral immune response, Intracellular and second messenger signalling	Pro-inflammatory Apoptosis
Toll-like Receptor Signalling	Activated	Cellular immune response, Apoptosis, Humoral immune response, Pathogen-influenced signaling	Pro-inflammatory
Acute Phase Response Signalling	Activated	Cytokine signalling	Pro-inflammatory
Interferon Signalling	Activated	Cellular immune response, Cytokine signalling	Pro-inflammatory
PPAR Signalling	Inhibited	Nuclear Receptor signalling	Fatty acid metabolism

LXR/RXR Activation	Inhibited	Nuclear receptor signalling	Lipid metabolism
Leukocyte Extravasation Signalling	Activated	Cellular immune response	Pro-inflammatory
IL-1	Activated	Cytokine signalling	Pro-inflammatory
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	Activated	Cellular immune response, Pathogen-Influenced Signalling	Pro-inflammatory
IL-6 Signalling	Activated	Cellular immune response, Cytokine signalling	Pro-inflammatory
HMGB1 Signalling	Activated	Cellular immune response, Cellular stress and injury Cytokine signalling, Humoral immune response	Pro-inflammatory
Inflammasome Pathway	Activated	Cellular immune response, Cellular stress and injury Cytokine signalling, Pathogen-induced signalling	Pro-inflammatory
GM-CSF Signalling	Activated	Cellular growth, Proliferation and development, Cellular immune response, Cytokine signalling, Growth factor signalling	Cell survival and proliferation
Role of IL-17F in Allergic Inflammatory Airway Diseases	Activated	Cytokine signalling	Pro-inflammatory

Table 37 The biologically relevant pathways from the top 30 pathways arranged by Z (activation) score that were statistically significantly associated with all 3 FDR threshold levels tested for the CD RNA-Seq macrophage data set.

6.3.2 Gene set enrichment analysis demonstrates intestinal macrophage activation in both CD and UC but also identified differences between CD and UC intestinal macrophage gene expression

The DESeq2 analysis identified 20 DEGs for the comparison of CD Vs UC with FDR<0.1 and FC of 1.5 up or down, **table 38**. In chapter 5 it was shown that at a stricter FDR of <0.05 and <0.01 no DEGs for the comparisons CD Vs UC were identified. This suggests that the gene expression of macrophages from active CD and UC are quite similar. This however is probably an over simplified approach to looking at the differences between CD and UC intestinal macrophages. Applying pre-defined statistical cut off points for this data set excludes a large quantity of potentially relevant biological information and disregards the concept that genes generally work in complex networks rather than individually in most circumstances.

There are clear biological and clinical differences between CD and UC. It was therefore important to explore in further detail for any potential differences between the CD and UC intestinal macrophages that could explain these differences that we see clinically. The Qlucore Omic Explorer 3.2 software package (Qlucore AB, Lund, Sweden) was used for Gene Set Enrichment Analysis (GSEA) (500) to further assess whether specific biological pathways or signatures were significantly enriched between the two groups. The GSEA method ranks genes from expression experiments bases of their expression and the class distinction using a suitable metric. It is then determined if a pre-defined set of genes of interest (e.g., cellular pathway or phenotypic signature) are randomly distributed throughout the ranked list of genes (from expression data) or predominately found at the top or bottom and thus demonstrate association with that pre-defined gene set.

This section describes the analysis of the RNA-Seq data using GSEA. Several published sources of gene lists for cellular pathways and functions were utilised in this analysis: IPA (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis), Reactome Pathway Knowledgebase (501, 502) and the GSEA Molecular Signatures Database (500)

This GSEA demonstrated that pathways associated with macrophage activation such as antigen presentation and interferon pathways were activated in both CD and UC (**figure 40)** again suggesting a pro-inflammatory state to the macrophages. This analysis also however revealed that the gene expression of the CD macrophages was significantly associated with the M2 phenotype whist the UC macrophages were not **(figure 41)**. Furthermore the gene expression for CD was significantly associated with the gene panels for fibrosis and granuloma formation but no such association was seen in UC (**figure 42**). These subtle differences between the CD and UC macrophage gene expression correlated with the clinical differences observed in the two diseases.

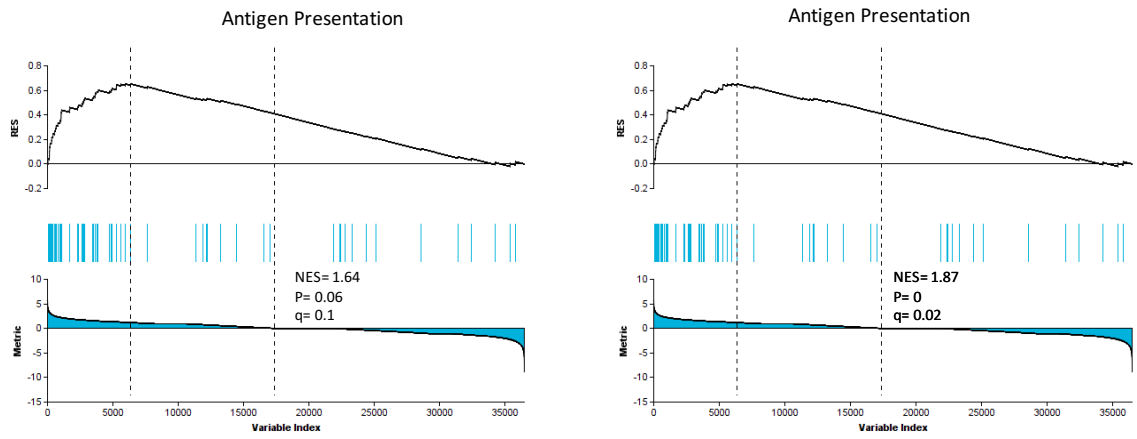
CD is associated with fibrotic stenotic complications and granuloma formation neither of which is common in UC. Furthermore these findings raise the possibility that the M2 phenotype of intestinal macrophages may not necessarily be entirely protective but rather it is the correct balance of M1 and M2 phenotype that is important. One hypothesis on this basis is that too much M2 type macrophage activity is responsible for the fibrotic complication seen in CD. This would be important to appreciate in any treatment strategy that targets moving intestinal macrophages from an inflammatory M1 phenotype to a wound healing M2 phenotype.

Associated Gene Name	Description
HPGD	Hydroxyprostaglandin dehydrogenase 15-(NAD)
BHLHE40	Basic helix-loop-helix family, member e40
VDR	Vitamin D (1,25- dihydroxyvitamin D3) receptor
AC016708.2	
PDE9A	Phosphodiesterase 9A
CLEC2D	C-type lectin domain family 2, member D
VPS29	VPS29 retromer complex component
RPL23	Ribosomal protein L23
GAS5	Growth arrest-specific 5 (non-protein coding
RPS27A	Ribosomal protein S27a
FEM1C	Fem-1 homolog c (C. elegans)
RP11-849F2.7	
ERH	Enhancer of rudimentary homolog (Drosophila)
ZCRB1	Zinc finger CCHC-type and RNA binding motif
RP11-92K2.2	
MUC17	Mucin 17, cell surface associated
MRPL24	Mitochondrial ribosomal protein L24
RP11-75L1.2	
RPL26	Ribosomal protein L26
OXR1	Oxidation resistance 1

Table 38 The 20 DEGs for the comparison of CD Vs UC with a FDR <0.1 and fold change 1.5 up or down

Crohn's Disease **Ulcerative Colitis**

A



B

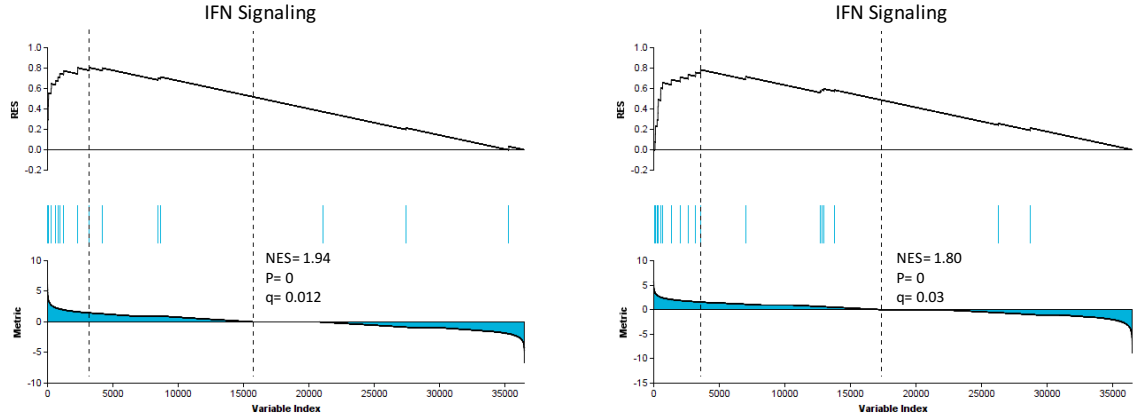
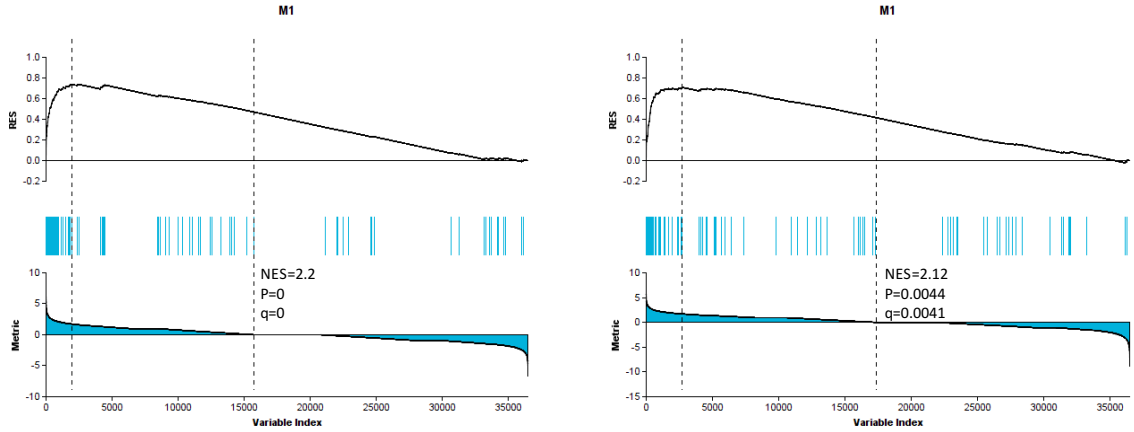


Figure 40 GSEA demonstrates that Antigen Presentation pathway and Interferon (IFN) signalling pathway of these pathways are activated for the CD and UC macrophages. GSEA for CD Vs N and UC Vs N for (A) Ingenuity Knowledge based Antigen Presentation pathway and (B) Interferon (IFN) signalling pathway. Normalised enrichment score (NES) is the enrichment score corrected for gene set size. The enrichment score is a numerical representation of the degree to which a gene set is overrepresented (if positive) and down regulated (if negative). q is the false discovery rate (FDR) estimating the that the NES is a false positive finding. Ingenuity knowledge base antigen presentation pathway, 22 genes and the Interferon signalling pathway 37 genes, appendix C for full gene lists.

Crohn's Disease **Ulcerative Colitis**

A



B

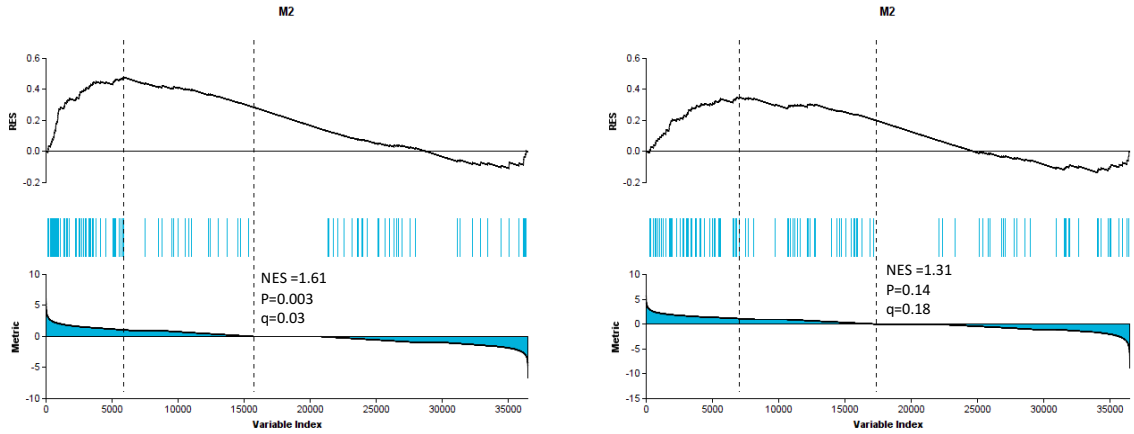
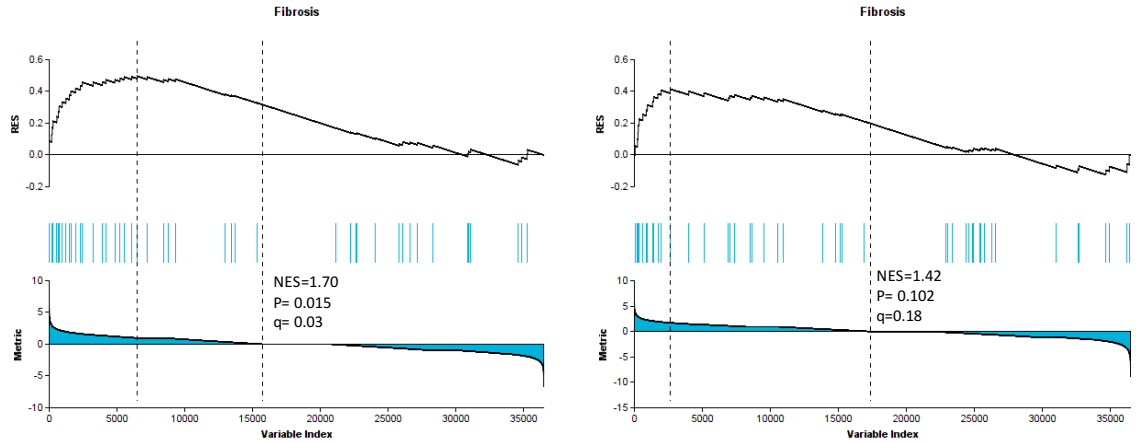


Figure 41 GSEA demonstrates that for CD Vs N there is statistical significant activation of the M1 and M2 gene sets. For UC Vs N there is statistical significance of activation of the M1 gene set but not the M2 gene set. GSEA for CD Vs N and UC Vs N for (A) M1 signature genes and (B) M2 signature genes. Normalised enrichment score (NES) is the enrichment score corrected for gene set size. The enrichment score is a numerical representation of the degree to which a gene set is overrepresented (if positive) and down regulated (if negative). q is the false discovery rate (FDR) estimating the that the NES is a false positive findings M1 gene set utilised 109 genes and M2 gene set consisted of 121 genes, appendix C for full gene lists.

Crohn's Disease **Ulcerative Colitis**

A



B

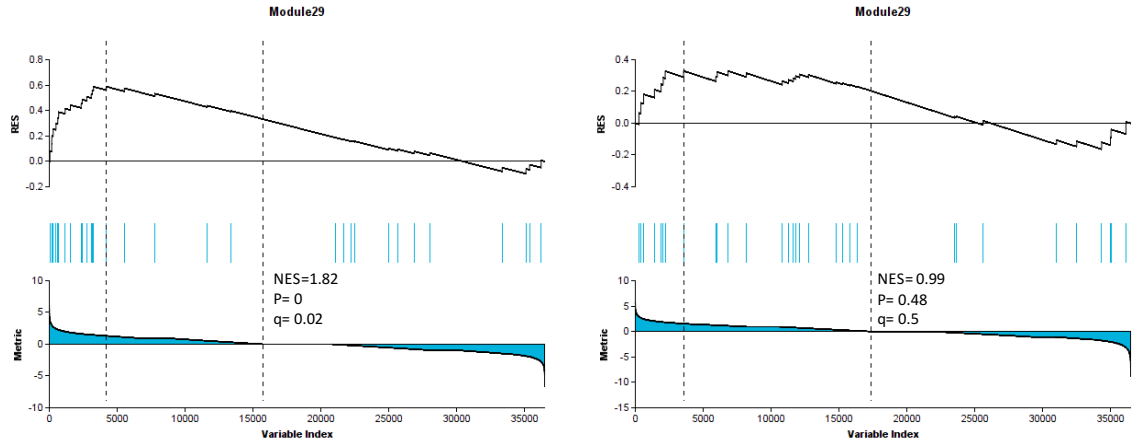


Figure 42 GSEA shows that fibrosis and granuloma pathways were found to be activated In the CD cohort of macrophages but not the UC macrophages. GSEA for CD Vs N and UC Vs N for Fibrosis panel of genes and Module 29 from Xue et al, which describes gene expression of granuloma forming macrophages. Normalised enrichment score (NES) is the enrichment score corrected for gene set size. The enrichment score is a numerical representation of the degree to which a gene set is overrepresented (if positive) and down regulated (if negative). q is the false discovery rate (FDR) estimating the that the NES is a false positive finding. Module 29 consisted of 38 genes, fibrosis pathway 154 genes, appendix C for full list of genes.

6.3.3 IBD intestinal macrophages harvested from areas of active colitis have down regulation of gene involved in cellular metabolic processes.

The majority of the identified differentially expressed genes for the comparisons of CD Vs N and UC Vs N were up-regulated. I have demonstrated that analysis of this gene expression suggests overall that the macrophages in active IBD lesions are activated and promote inflammation. The signal from these up-regulated genes however appears to "drown out" the signal of the down regulated genes of which there are far fewer, a total of 549 across CD and UC compared to 1069 up-regulated genes **(figure 43)**. We identified 48 genes that were found to be commonly down regulated in both CD and UC. The heatmap **(figure 44)** shows that the gene expression of these commonly down regulated genes is homogeneous for the two diseases. The down-regulated genes were analysed in further detail in order to investigate their biological importance.

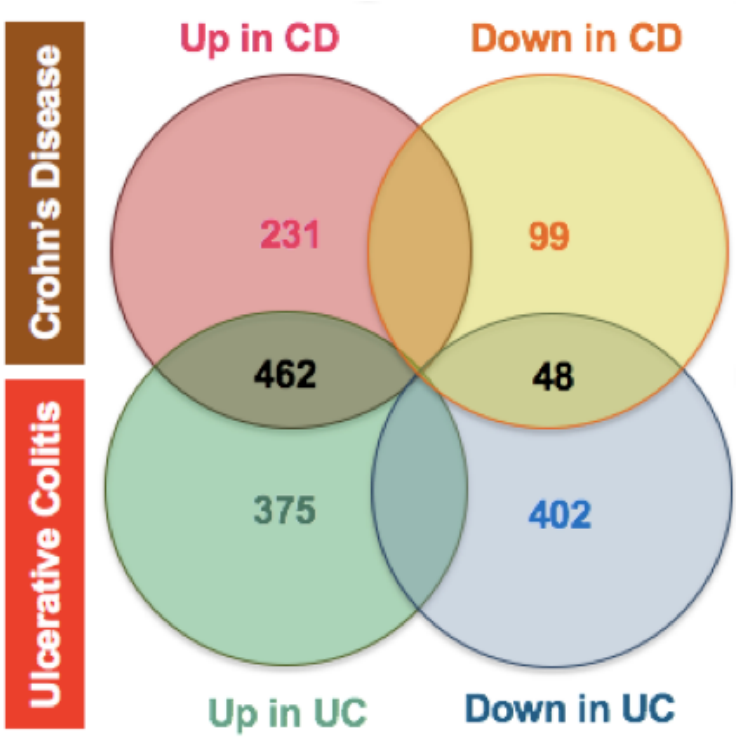


Figure 43 Venn diagram demonstrating the overlap of up and down regulated genes for CD Vs N and UC Vs N for a FDR <0.1. Gene expression was consider significantly up-regulated or down regulated when they were a factor 1.5 up or down respectively when compared with the gene expression of the normal controls. This Venn diagram shows the number of the genes that were up-regulated or down regulated in both diseases or one disease only.

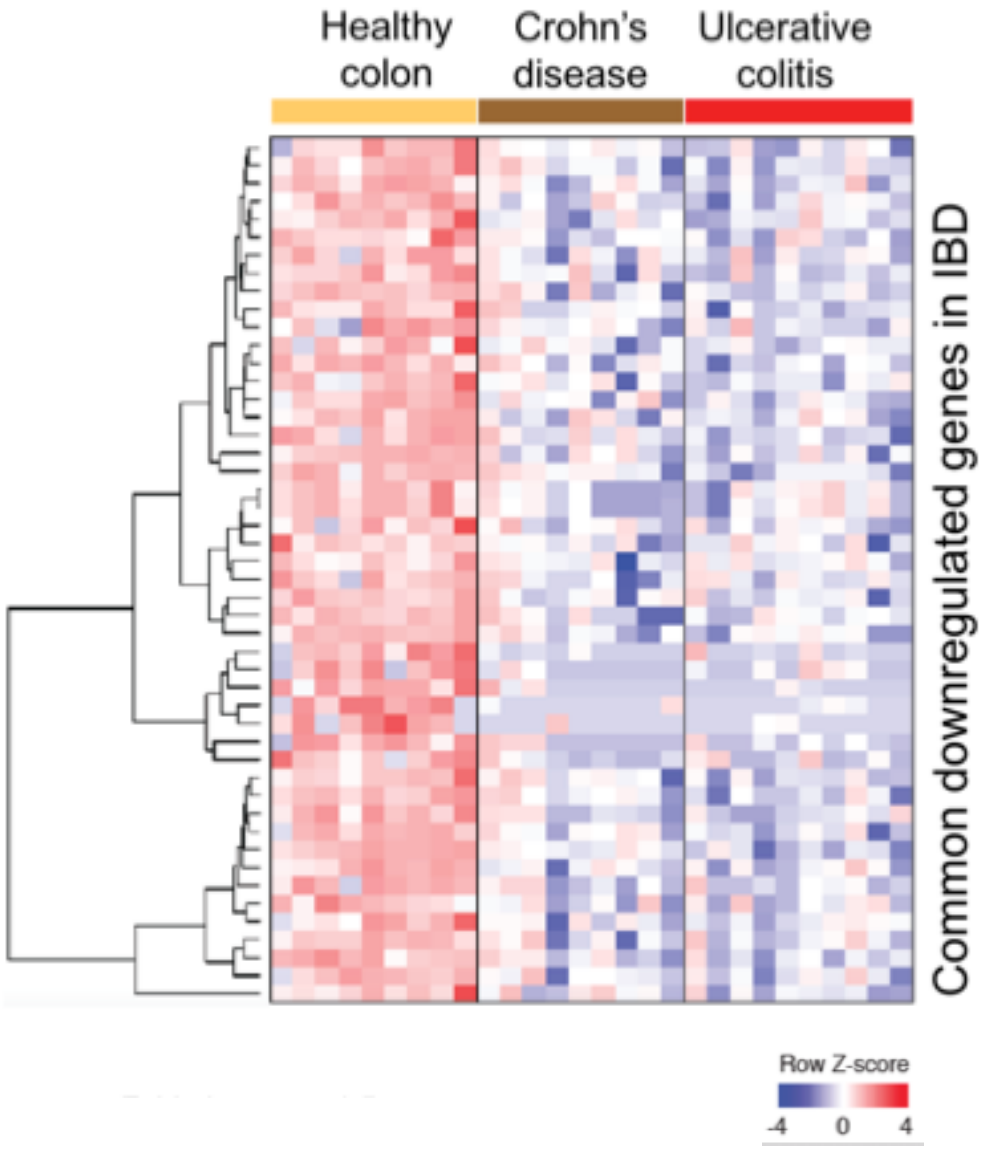


Figure 44 Hierarchical clustered heatmap supervised by disease group showing the relative gene expression for the 48 commonly down regulated genes for CD Vs N and UC Vs N. The row Z-score represents relative gene expression for each gene across all samples and groups (i.e. for each row) but not compared to other genes. Red colour signifies up regulation and blue down regulation. Healthy colon (n=9), CD (n=9) and UC (n=10).

In order to gain an overview of the functional consequences of the down-regulated genes they were uploaded onto the ConsensusPathDB (503) platform. This is a web based tool (http://consensuspathdb.org/) that utilises statistical methods together with information form 32 major public repositories for human, mouse and yeast molecular interactions to enrich genome and mechanistic network analysis of high throughput genomic data.(504) I used the ConsensusPatDB to perform an over-representation analysis on the down regulated genes, specifically for gene ontology categories level 3, in order to see if there was a pattern of function for these down regulated genes. The results of this analysis is summarised in **tables 39-41**. The number of down regulated DEGs for UC Vs N was much greater than that for the CD Vs N analysis, 450 and 147 genes respectively. This translated to the identification of a much larger number of gene ontology terms for UC compared to CD. A total of 138 significant gene ontology terms were found for UC, which was not suitable for table representation. **Table 40** lists the top 14 gene ontological terms by q-value rather than the full list for UC. A world cloud **(figures 45-47)** of all the gene ontology terms associated with the down regulated genes is also included as this gives a better overview of the ontology of these genes when a large number of terms are identified.

The pattern that emerged from this analysis together with manual inspection of the role of each of these individual down regulated genes is that of down regulation of metabolic processes in the IBD macrophage cells. There is also down regulation of genes that are involved in the neutralisation of oxygen free radicals, suggesting an impaired ability to handle reactive oxygen species.

Gene ontology term	Set Size	% Candidate Contained	p-value	q-value
Extracellular Vesicle GO: 1903562	2826	1.4%	1.19e-6	3.36e-5
Mitochondrial part GO:0044429	972	2.2%	1.6e-6	3.36e-5
Small molecule metabolic process GO:0044281	2049	1.4%	5.63e-5	0.0106
Cytoplasmic Part GO:0044444	8019	0.9%	0.00011	0.00162
Organelle inner membrane GO:0019866	543	2.2.%	0.00026	0.0028
Organic acid metabolic process GO:0006082	1087	1.7%	0.00029	0.0275
ESCRT complex GO:0036452	27	11.1%	0.00074	0.00597
Vesicle GO:00311982	4005	1.1%	0.00085	0.00597
Lipid metabolic process GO:0006629	1315	1.4%	0.00103	0.0538
Sulphur compound metabolic process GO:0006790	390	2.3%	0.0014	0.0538
Mitochondrial membrane GO:0031966	674	1.8%	0.00176	0.0106
Oxidative reduction process GO:0055114	979	1.5%	0.00207	0.00714
Cellular oxidant detoxification GO:0098869	83	4.8%	0.00227	0.0714
Cellular lipid metabolic process GO:0044255	1034	1.5%	0.00348	0.0939

Table 39 ConsensusPatDB over-representation analysis of down regulated genes for gene ontology level 3 for CD Vs N. P-value calculated by the Fisher's exact test and the q value is the p-value corrected for multiple hypotheses testing using the false discovery rate procedure.

Gene ontology term	Set Size	% Candidate Contained	p-value	q-value
Small molecule metabolic process GO:0044281	1315	4.2%	8.00E-13	2.99E-10
Lipid metabolic process GO:0006629	2826	4.6%	3.34E-11	6.25E-09
Extracellular vesicle GO: 1903561	31	3.5%	5.84E-11	6.31E-09
Flavonoid metabolic process GO:0009812	97	30.0%	2.60E-09	2.84E-07
Xenobiotic metabolic process GO:0006805	107	14.6%	3.04E-09	2.84E-07
Brush border GO:0005903	4005	13.1%	1.28E-08	4.81E-07
Vesicle GO:0031982	110	13.0%	1.33E-08	4.81E-07
Response to xenobiotic stimulus GO:0009410	2626	12.8%	1.63E-08	1.22E-06
Cellular response to chemical stimulus GO:0070887	1034	3.3%	4.12E-08	2.30E-06
Cellular lipid metabolic process GO:0044255	41	4.5%	4.30E-08	2.30E-06
Isoprenoid binding GO:0019840	4614	22.0%	5.31E-08	5.36E-06
Transport GO:0006810	1118	2.8%	5.79E-08	2.55E-06
Organophosphate metabolic process GO:0019637	3174	4.3%	6.13E-08	2.55E-06
Phosphorus metabolic process GO:0006793	39	3.0%	4.99E-07	1.77E-05
Drug metabolic process GO:0017144	1315	20.5%	5.20E-07	1.77E-05

Table 40 ConsensusPatDB over-representation analysis of down regulated genes for UC for gene ontology level 3, top 14 terms by q-value shown. The p-value is calculated by the Fisher's exact test and the q value is the p-value corrected for multiple hypotheses testing using the false discovery rate procedure.

Gene ontology term	Set Size	% Candidate Contained	p-value	q-value
Small molecule metabolic process GO:0044281	2049	0.8%	1.45e-06	0.00018
Organic acid metabolic process GO:0006082	1087	1.1%	4.7e-06	0.00024
Lipid metabolic process GO:0006629	1315	1.0%	5.78e-06	0.00024
Extracellular vesicle GO: 190356	2826	0.7%	6.34e-06	0.00015
Cellular lipid metabolic process GO:0044255	1034	1.0%	1.05e-04	0.00325
Oxidation-reduction process GO:0055114	979	0.9%	3.59e-04	0.0089
Vesicle GO:0031982	4005	0.5%	8.38e-04	0.00964
Cellular oxidant detoxification GO:0098869	83	3.6%	9.59e-04	0.0198
Cytoplasmic part GO:0044444	8019	0.4%	1.43e-03	0.011
Organic hydroxyl compound metabolic GO: 1901615	459	1.1%	4.13e-02	0.0732
Single-organism biosynthetic process GO:0044711	1415	0.6%	4.73e-03	0.0733
Mitochondrial part GO:0044429	972	0.7%	6.86e-03	0.0386
Organelle inner membrane GO:0019866	543	0.9%	8.40-3	0.0386

Table 41 ConsensusPatDB over-representation analysis of common down regulated genes for CD &UC for gene ontology level 3. The p-value is calculated by the Fisher's exact test and the q value is the p-value corrected for multiple hypotheses testing using the false discovery rate procedure.

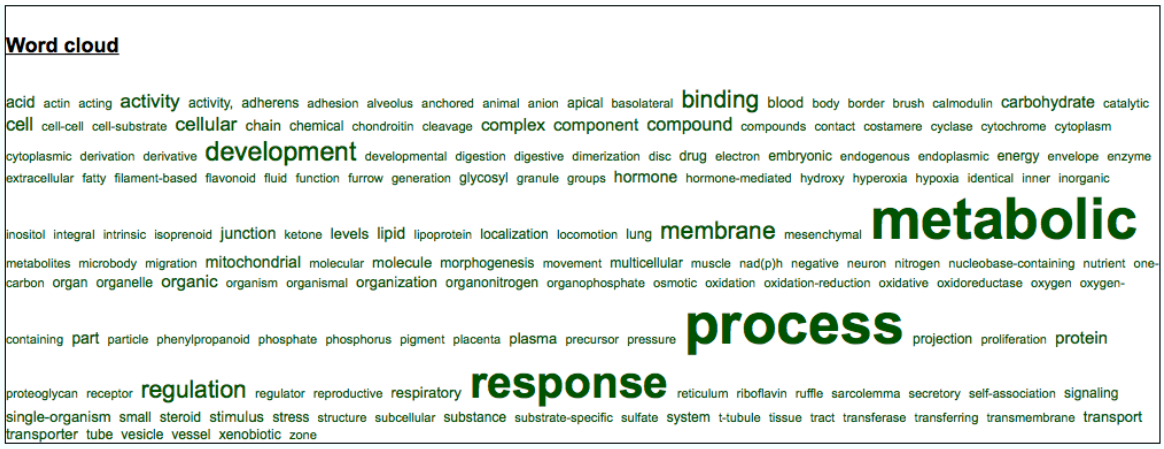


Figure 45 Word cloud for the gene ontology terms for the down-regulated genes for the analysis of UC Vs N. Figure produced in ConsensusPathDB web tool (503), the gene ontology function found within the over-representation analysis function was utilised and gene expression together with FDR values of the down regulated genes for the comparison of UC Vs N were uploaded, gene ontology category was set at level 3.

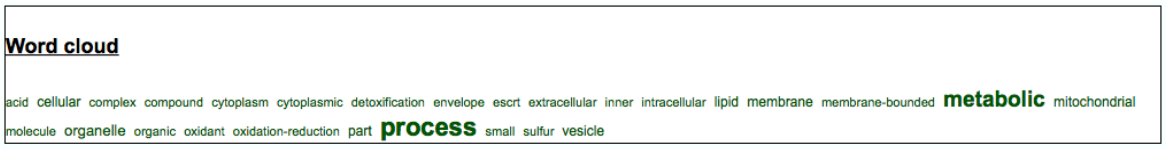


Figure 46 Word cloud for the gene ontology terms for the down-regulated genes for the analysis of CD Vs N. Figure produced in ConsensusPathDB web tool (503), the gene ontology function found within the over-representation analysis function was utilised and gene expression together with FDR values of the down regulated genes for the comparison of CD Vs N were uploaded, gene ontology category was set at level 3.

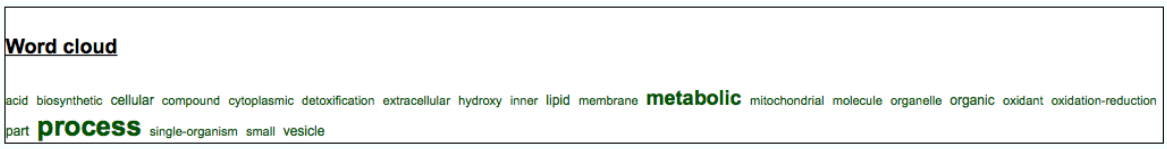


Figure 47 Word cloud for the gene ontology terms for the common down-regulated genes for the CD &UC. Figure produced in ConsensusPathDB web tool (503), the gene ontology function found within the over-representation analysis function was utilised and gene expression together with FDR values of the common down regulated genes for the comparison of CD & UC when compared with healthy controls were uploaded, gene ontology category was set at level 3.

6.3.3.1 Validation of identified commonly down regulated genes for CD and UC intestinal macrophages

A selection of the genes identified to be down regulated in the IBD macrophages were selected for validation using qPCR. The RNA that was extracted and amplified from the original cohort of patients as described in the methods section was utilised for these experiments. The data from these experiments support the findings of the RNA-Seq experiment, **figure 48**.

6.3.3.2 Down regulation of metabolic genes of intestinal macrophages in active IBD

Altered metabolism in activated macrophages is well described. It has been established for some years that pro-inflammatory macrophages, now described as M1 macrophages switch from oxidative phosphorylation to glycolysis for cellular energy production.(505) Subsequently it was discovered that for M1 macrophages, expression of the enzyme inducible nitric oxide synthase (iNOS), which generates nitric oxide (NO) from arginine catabolism, is increased. NO a reactive nitrogen species is capable of inhibiting mitochondrial respiration and there is evidence to suggest that this is the mechanism by which activation of M1 macrophages leads to a switch to glycolysis energy production.(506) This change in energy metabolism forms some of the defining differences between M1 and M2 macrophages. M1 macrophages have up-regulation of iNOS which metabolises arginine to NO and citrulline. As a consequence M1 macrophages have high levels of NO and citrulline. M2 macrophages on the other hand have reduced expression of iNOS and as a consequence arginine is preferentially metabolised to urea and ornithine by glarginase.(311) The analysis of the down regulated genes for UC and CD, identified down regulation of genes involved in mitochondrial function and cellular oxidant detoxification. These observations are likely to represent the switch from oxidative metabolism to glycolysis in what we have already demonstrated are predominately inflammatory macrophages.

We validated using qPCR a selection of genes that were down regulated in the RNA-Seq data set in CD and/or UC macrophages. The PCK1 gene was confirmed to be down regulated in both CD and UC, **figure 48**. The PCK1 gene in humans encodes an enzyme called cytosolic phosphoenolypyruvate carboxykinase, which is involved in gluconeogenesis and glyceroneogenesis, variants in the PCK1 gene have been associated with type 2 diabetes mellitus in UK resident South Asian populations. (507) There is no published data of any association with IBD at present. PADI2 belongs to a family of enzymes that promote the conversion of arginine residues to citrullines. PADI enzymes have been associated with a range of auto-inflammatory conditions such as rheumatoid arthritis and multiple sclerosis, but in these associations there is

up-regulation of the PADI enzymes (508) rather than the down regulation seen in the present study both in the RNA-Seq data and the subsequent qPCR validation but this was only significant for UC Vs N for the qPCR experiment. Interestingly a small study of patients with colorectal tumours which also included a separate cohort of 11 patients with UC found PADI2 gene expression to be down regulated in colonic biopsies from patients with colorectal cancer and also patients with UC, the investigators postulated that PADI2 could be considered as a potential biomarker for dysplasia in UC, a pre-cursor of cancer.(509) The final down-regulated enzyme that I validated with qPCR was SLC26A2, which codes sulphur transporter responsible for supplying cellular SO_4^2, required for cellular and tissue survival.(510) Two previous studies have identified down-regulation of SLC26A2 in UC patients but unlike our study they found SLC26A2 to be up regulated in CD patients.(511, 512)

These findings suggest that therapy targeted at metabolic reprogramming of the M1 intestinal macrophages towards a wound healing M2 macrophage phenotype could be beneficial. Such strategies are currently being investigated in cancer, where pro-metastatic M2 tumour associated macrophages (TAMs) have been targeted for metabolic reprogramming towards an M1 phenotype known to reduced tumour progression (the reverse of what is required in IBD).(513)

In addition to alterations in energy metabolism highlighted by the down-regulated genes, the gene ontology analysis shows down regulation of genes involved in anti-oxidant defences. Evidence of oxidative stress contributing to the pathogenesis of IBD has been established from experimental models and clinical trials.(477) Oxidative stress is linked with glycolytic metabolism through the generation of the oxygen free radical NO. The current treatment strategies for IBD already target reactive oxygen species (ROS). Immunomodulators such as azathioprine and corticosteroids have free radical scavenging abilities.(514) Mesalazine, the first line agent in the treatment of UC appears to have some of its beneficial effects through the reduction of oxygen free radicals.(514) Although previous studies have identified a contributory role of ROS in IBD pathogenesis, it has not been established if this is due to altered cellular metabolism, impaired mitochondrial activity or decreased local scavenging capacity. We have identified in this cohort of IBD intestinal macrophages that genes involved in all these areas appear to be down regulated.

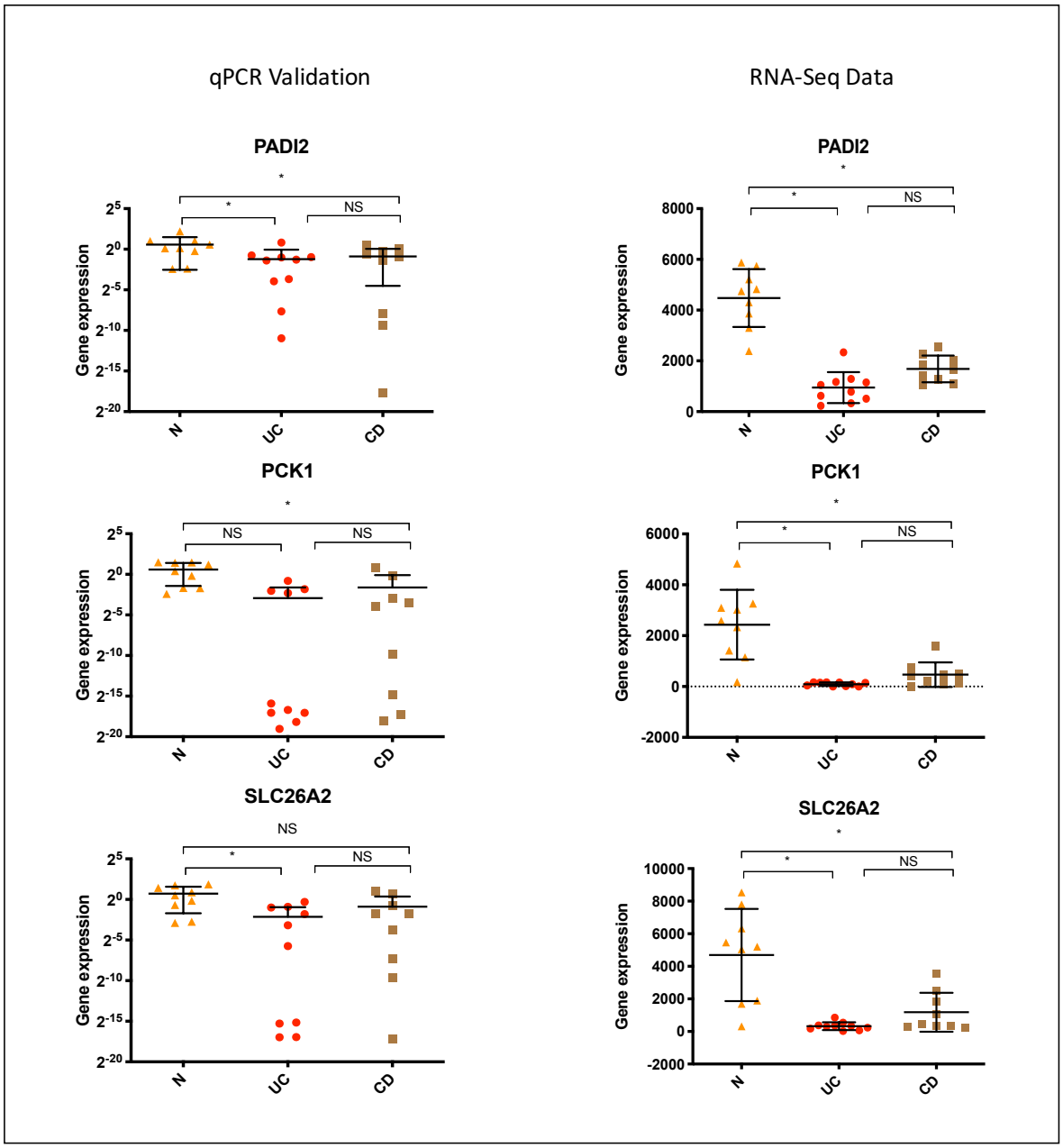


Figure 48 Validation by qPCR of a selection of down regulated genes identified by RNA-Seq. The Y-axis is in Log scale for the qPCR validation and shows normalised gene expression using the ΔΔCt method for normalisation. The Y-axis for the RNA-Seq data shows gene expression, as normalised read counts. Normal (n=9), UC (n=10) and CD (n=9). The error bars show mean and standard deviation. P-values for qPCR data calculated using the Kruskal-Wallis test with Dunn's test in Graphpad Prism. *indicated P<0.05. RNA-Seq significance test is as per the DESeq2 analysis for differentially expressed genes * indicates FDR<0.1.

6.4 Chapter Discussion

The analysis described in this chapter has utilised the RNA-Seq data to describe the phenotype of the intestinal macrophages from the IBD patients recruited to this study. We have demonstrated that the macrophages from the IBD patients are activated and in a pro-inflammatory state. We have found evidence that antigen presentation pathways are activated, PPRs receptors such as Toll-like-receptors and their pathways are up-regulated and that classical inflammatory pathways including IL1, IL6 and INOS related pathways are activated. This is an expected finding given that these intestinal macrophages were harvested from areas of active colonic inflammation. The overwhelming pro-inflammatory picture of these macrophages raises challenges in analyses beyond an inflammatory hypothesis. In particular, this strong pro-inflammatory signal from the up-regulated DEGs creates difficulty in the identification of therapeutic targets beyond classical pro-inflammatory pathways many of which are already targeted directly or indirectly by current therapeutic strategies. The analysis in this chapter has allowed the DESeq2 analysis to be further analysed looking beyond the pro-inflammatory signal.

The M1/M2 classification of macrophages has gained much traction in recent years yet it is now understood that this model is an over simplification of the tissue macrophage phenotype. Our RNA-Seq data set demonstrated that the intestinal macrophages from the IBD patients express both classical M1 and M2 genes. A limitation of the design and methods utilised in this study is that it was not possible to determine if this is due to macrophages expressing both M1 and M2 genes simultaneously, a mixed population of M1 & M2 macrophages or a combination of these factors. Various methods including further flow cytometry analysis with specific M1 and M2 markers as well as IHC with confocal imaging (515) could aid in identifying whether individual macrophages express both M1 and M2 genes or if the biopsies from the IBD patients contain mix populations of M1 and M2 macrophages. A conclusive answer however is most likely to be achieved with well designed single cell RNA-Seq experiments which at the time of developing this project were not available but now with rapid developments in the area would be feasible.

Xue et al (318) described an extensive spectrum model of macrophage phenotypes, which I have used as a reference for phenotypes of macrophages in addition to the classically described M1/M2 gene expression signatures. Whist the GSEA did identify once again common pro-inflammatory signatures for both the UC and CD macrophages (antigen presentation and Interferon signalling shown but several other pro-inflammatory pathways identified as active) important differences were identified between CD and UC macrophages. The GSEA demonstrated that there was up-regulation of the M2 signature genes for the CD macrophages but not the UC macrophages. Similarly, the fibrosis and granuloma pathways were found to be up-regulated for

the CD macrophages but not the UC macrophages. These are subtle but important differences that correlate with the clinical and behavioural differences seen between CD and UC. Although granulomas are not found in the majority of patients with CD, the presence of granulomas on microscopic examination is an important finding, often used clinically to differentiate CD from UC as granuloma formation is not described in the latter.(516) Intestinal fibrosis is an important complication of IBD, most commonly complicating CD in up to a third of patients but much less frequently affecting those with UC in 5% of cases.(517) These findings implicate a role for intestinal macrophages in the clinico-pathogenic differences seen in CD and UC. It has been assumed that an M2 phenotype of intestinal macrophage is protective in IBD; whist it may be that M2 macrophages play an important role in healing of intestinal inflammation the picture from this data is one that is more complex. It seems possible that a phenotype of the M2 intestinal macrophage promotes fibrosis and therefore the stricturing complications seen in IBD. This is an important finding as any treatment targeted at promoting the M2 macrophage phenotype risks developing fibrotic complications. In this study the findings of the gene analysis have not been correlated with the disease phenotype of the patients, principally because this was set up as a hypothesis generating study and ongoing prospective data on patient disease course was therefore not collected. As a consequence I can only hypothesise based on the gene expression findings and analysis what the phenotypic correlation and results are. It would be useful to have prospective data to evaluate if those patients with high levels of M2 macrophages showing increased relative expression of fibrosis and granuloma related genes ultimately suffered increased fibrotic complications as we would expect from our findings. We have demonstrated changes in gene expression and pathways but we have not confirmed these changes translated at the protein level or the functional consequences of these changes. A major limitation therefore of the analysis in this chapter is that no association has been truly confirmed and this form of analysis generates further hypothesis that then need further studies and experiments to confirm on the protein and functional level the findings.

In this chapter the framework of the M1 and M2 macrophage paradigm was utilised to aid description of the macrophage phenotype as this is the current convention and language utilised widely in the science community. This paradigm however is problematic particularly when applied to complex in vitro systems such as the intestine immune system. This paradigm was introduced by Charlie Mills (311) based on in vitro experiments and conveniently mirrored the Th1/Th2 concept. It is therefore not surprising that in complex biological systems including the cardiovascular (518) and pulmonary (515) systems that the M1/M2 paradigm does not hold true and limits rather than furthers the understanding of macrophage biology. The data from the experiments presented in this chapter supports this opinion for the intestinal environment. Whist

it is helpful to ascribe a functional macrophage phenotype it is clear that the M1/M2 model is an over simplification. A standardised framework for macrophage phenotype that accommodates the complexities of macrophage biology does not currently exist though some have been proposed such as utilising the stimulatory conditions to classify macrophages. (518) Now that high throughput RNA-Seq is widely utilised as well as single cell techniques, we are in a position where we should consider describing what we see rather than attempting to fit what is discovered into rigid pre-defined frameworks for convenience and oversimplification.

The number of down regulated genes in this RNA-Seq data set is small compared to the up-regulated genes, though the information gained from these down regulated genes provided another avenue of understanding the macrophage pathology in IBD. In this chapter I have demonstrated that the down regulation of these genes is likely to have a functional consequence. I utilised the gene ontology software web tool ConsensusPathDB (503), to gain an overview of the biologic function of the down regulated gene expression for the CD and UC macrophages. This identified several metabolic ontological terms including lipid metabolic process, small molecule metabolic process, oxidative reduction process and phosphorus metabolic process highlighting a common metabolic theme. It is known that macrophages along with neutrophils generate ROS during bacterial engulfment (519) and it has been shown that patients who have variants in NADPH oxidases resulting in altered production of ROS are susceptible to IBD (520), however there does not appear to be any direct evidence to support that in IBD, macrophages contribute to alteration of ROS levels. The analysis in this chapter shows that there is down regulation of metabolic processes within the IBD macrophages including oxidative reductive process and cellular oxidant detoxification this is likely to have an impact on the handling of ROS in the intestinal mucosa. This is indirect evidence that in IBD macrophages metabolic dysfunction is likely to lead to altered ROS levels. We validated three down regulated genes identified by the RNA-Seq analysis with qPCR. These were *PADI2*, *PCK1* and *SLC26A2*, which were all confirmed as being down regulated in both CD and UC with the qPCR experiments. *PADI2* promotes the conversion of arginine residues to citrullines, and has been shown to be down regulated in colorectal cancers as well as UC patients. (509) *PCK1* codes an enzyme involved in gluconeogenesis and has not previously been linked with IBD. *SLC26A2* is involved in sulphur metabolism, which is vital for cellular survival and homeostasis. *SLC26A2* has been found to be down regulated in UC in two studies but in contrast to the present study these studies also found this gene to be up-regulated in CD patients. (511, 512) The analysis I have presented in this chapter identifies an interesting avenue in understanding the role of macrophages in IBD. There appears to be down regulation of metabolic processes in these cells. Further work needs to be done to understand these processes and changes more fully and to confirm the findings on a

protein and functional level. The findings of this chapter do however raise possibility of stabilising macrophage metabolic pathways to reduce inflammation and promote intestinal mucosa healing as an avenue for therapeutic targeting. Although altering cellular metabolism is not a new theory in IBD, short chain fatty acids are known to be reduced in the intestinal mucosa of IBD patients and there is a theory that increasing this will improve inflammation, epithelial cell function and have anti-inflammatory effects on immune cells, at present however there is no good evidence of any clinical benefit with this strategy.(521) The data and analysis I have presented has demonstrated that an approach targeted at specific cell types could be used to manipulate the metabolic pathways for benefit in IBD.

A major challenge in interpreting the data from the experiments from this project is segregating the inflammatory signal from that which is specific to IBD pathogenesis, principally because the samples were taken from areas of the colon that were macroscopically inflamed. This explains why the majority of the up-regulated genes were pro-inflammatory. It also unfortunately limits the interpretation of the data; it is not possible to specify if the transcriptomic intestinal macrophage changes described in the present study are IBD specific or a general consequence of intestinal inflammation. To overcome this issue the addition of an inflammatory control group to the study design would be beneficial. The options for this would be enteric infection and diverticulitis, these patients do not routinely have endoscopy, making recruitment to this arm more challenging but given the importance in aiding the interpretation of the data, I believe this would be a worthwhile addition. The inclusion of paired inflamed and non-inflamed samples taken at the same endoscopic procedure would also aid the study design and data interpretation in relation to understanding specific IBD related changes and those that are generic inflammatory changes. Further along these lines an additional group of IBD patients in deep remission, defined by normal faecal calprotecin systematically well and evidence of mucosal healing at endoscopy (522), would add to the usefulness of the data obtained. The challenge of recruiting such a group would be that the majority would be on medications, many on biological medications that inherently alter the inflammatory pathways of immune cells, so the challenge would be understanding which changes are drug related. The addition of these group of patients particularly those not on long term medication would allow for further testing of the hypothesis of whether there are inherent differences in the IBD macrophage transcriptome to be assessed. This would have implications on the role of therapies for maintenance of clinical remission targeting the intestinal macrophages.

In the final chapter I describe the next phases of this line of work that are required to build upon the knowledge gained from this project to bring tangible benefits in the understanding of intestinal macrophages and transfer of this knowledge to the clinical setting.

Chapter 7: Discussion and Future Work

7.1 General Discussion

In this project I have studied the role and contribution of intestinal tissue macrophages in the pathogenesis of the IBD. We elected to utilise high throughput RNA-Seq on intestinal macrophages isolated from patients with IBD and compared these with a cohort of healthy controls in our study. To the best of my knowledge intestinal macrophages have not been studied in this way previously comparing CD with UC. At the study design stage of the project there were no published studies that had utilised RNA-Seq on isolated intestinal macrophage cells. This in itself created major challenges in experimental design and optimisation. The intestinal macrophages were identified and isolated using FACS following disaggregation of the colonic tissue biopsies. The early phase of the project was spent developing protocols for the disaggregation of intestinal colonic biopsies to create cell suspension suitable for FACS. Although the Dr Sanchez-Elsner laboratory had experience and had developed extensive protocols for the disaggregation of lung tissue for FACS, early experiments demonstrated that the colonic biopsies required a significantly modified protocol. This protocol was optimised and proved reliable in extraction of immune cells from colonic biopsies and continues to be used in on going IBD projects in the University of Southampton.

Recruitment of patients to the study began only once we were satisfied that our developed protocols were reliably extracting greater than 10,000 macrophages. Experience from Dr Sanchez-Elsner lab has identified this to be a reliable minimum for the RNA-Seq pipeline that was utilised. Patients invited to participate in the study were carefully selected and characterised. We chose not to recruit patients on biologic medication, as we were concerned that this may alter the behaviour of the macrophages we collected. We know that macrophages are plastic and very sensitive to the microenvironment (523), in the study design we wanted to as far as possible study naïve IBD intestinal macrophages for this reason. This view is further supported by evidence principally from in vitro experiments but also from a small human histological study that anti-TNF alpha medication encourages an M2 macrophage phenotype.(383, 524)

Macrophages demonstrate significant plasticity expressing different cell surface makers depending on their activation states.(418) The FACS antibody panel was carefully considered in an effort to capture macrophages without being so restrictive that any particular subtype of macrophages was excluded. I utilised two cell surface markers to identify the macrophages, CD14 and CD163. It has been previously suggested that CD163 is marker of the M2 macrophage phenotype.(525) The data that we have obtained from this study show that the macrophages

isolated from the colonic intestine identified as $CD163^+$ and $CD14^+$ cells show up-regulation of a number of pro-inflammatory genes and pathways. This data suggests that CD163 is not a marker for M2 macrophages by itself, as Barros et al (526) reported in their immunohistochemical study of tissue macrophage polarisation concluding that CD163 should therefore be considered a general macrophage marker. Interestingly, analysis of the flow cytometry data revealed a population of cells that were positive for the CD163 marker but negative for the CD14 surface marker. These cells were not collected for RNA-Seq and therefore did not contribute the DESeq2 analysis or subsequent pathway analysis. It is well described in the literature that intestinal macrophages can lack expression of the CD14 surface marker.(334, 341, 343) Studies of the maturation of intestinal macrophages suggest that a group of intestinal macrophages loose CD14 expression as part of the maturation process and represent "inert" resident macrophages that contribute to intestinal homeostasis by remaining inactive to the relative high concentration of foreign antigens the intestinal mucosa is exposed to. In this hypothesis it is suggested that in instances of pathological inflammation or infection, newly recruited inflammatory macrophages are found in the intestine and these are the cells that have high levels of CD14 expression. The experiments we have performed have neglected this group of CD14 negative cells which could be argued represent a subset of macrophages. The percentages of these cells were not statistically different between disease groups in both the original RNA-Seq cohort of patients and the validation cohort. It would have been interesting to collect and sequence these cells separately to test the hypothesis that they are a different subset of macrophages to the $CD163^+CD14^+$ macrophages we have sequenced. If it were confirmed that the CD14 negative macrophage represent an inert or anti-inflammatory macrophage type, then clearly this would be an important finding. This would open up a therapeutic approach of expanding this celluar compartment, similar to the approach taken in studies that have focused on the expansion of regulatory T cells to treat CD, these are still currently in the clinical trial stage for example the TRIBUTE study (ISRCTN97547683). Although the flow cytometry strategy for sorting did not capture the $CD14^-$ $CD163^+$ cells, it was one that was broad and inclusive. A consequence of this a relatively heterogeneous set of cells may have been collected. In designing such as an experiment there is a comprise between purity of cell population and being over specific and missing important signals from previously un-recognised subsets of cells. Martin et al (527) in their single cell RNA-Seq study found that the macrophage surface markers CD68 and CD206 were important for macrophage phenotype in particularly differentiating between inflammatory and non inflammatory cells and those that appeared to promoted fibroblasts. Studies such as this suggest more complex sorting strategies isolating multiple subsets of macrophages would be more informative in progressing our understanding of macrophage biology.

Prior to the production of the DNA libraries for sequencing, the RNA was amplified allowing us to work with the relatively small numbers of macrophage cells isolated. At the time of conception of the study, single cell sequencing was not performed locally and therefore not available for this project. The RNA amplification and the DNA library preparation is a highly skilled process that was completed by Dr E Garrido-Martin. The resulting DNA libraries were sent to ORB in Florida to perform the RNA sequencing and initial bioinformatics to provide a DESeq 2 analysis. I have taken a multi-modal approach to understand the effects of the differential gene expression between the disease groups using a number of different software packages and strategies. This proved essential, in the challenge of understanding the biological consequences of the 1287 and 840 DEGs identified for the comparisons of UC Vs N and CD Vs N respectively. We applied relaxed criteria for the identification of the DEGs (FDR<0.1, FC>1.5), this was intentional as the project was exploratory and hypothesis generating in principle. We did not aim to prove any association with a high degree of certainty as we appreciated at the planning stage that with this novel approach this was an unrealistic aim and any associations found would required further validation in a large dedicated study. I did however subject the DESeq2 analysis to stricter statistical analysis (FDR<0.05 and FDR<0.01) to explore the consequences of such an analyses. As expected this did reduce the number of DEGs identified but the therapeutic targets of interest that we identified as clinically relevant remained associated at the stricter statistical analysis. All the subsequent further analysis of the DESeq2 results were conducted using the relaxed criteria (FDR<0.1, FC>1.5) as we were dedicated to keeping this as a discovery and hypothesis generating project. Using these relaxed criteria for identification of differential gene expression does however increase the false positive rate, which must be kept in mind when interpreting the results of this study.

Hierarchical clustering and PCA showed that the gene expressions of the IBD macrophages were profoundly altered compared to the normal macrophages and the gene expression profiles could be used to differentiate the IBD macrophages from controls. The IPA (391) pathway analysis identified a large number of associated canonical pathways, 172 and 120 for UC and CD respectively. Interestingly the majority of the top canonical pathways by P-value were the same for both UC and CD. This is probably a representation that the colonic biopsies were taken from inflamed areas, and therefore an inflammatory signal dominated the data set which this was seen in the pathway analysis in chapter 5.

A number of important canonical pathways were identified by this analysis. The TREM1 gene expression was up-regulated and the TREM1 pathway was found to be activated in both the CD and UC macrophages. The TREM-1 sequencing results were validated using RT-qPCR on the RNA-Seq cohort of patients and flow cytometry on a second independent validation cohort.

The TREM1 receptor belongs to the family of receptors known as triggering receptors expressed on myeloid cells (TREMs).(528) Within this family of receptors there are both activating and inhibiting receptors, TREM2 inhibits the inflammatory response where as TREM1 potentiates the inflammatory response and appears to act synergistically with TLR signalling. (529) Up to recently much of the research on TREM1 has focused on its role in infection and resulting sepsis syndrome.(439) More recently however the importance of TREM-1 in inflammatory conditions such as rheumatoid arthritis, psoriasis, Behçet's disease and IBD has been identified and explored. TREM1 is up-regulated on intestinal macrophages of patients with IBD whereas macrophages from healthy colon express minimal TREM1, furthermore blockade of TREM1 in experimental animal models of colitis attenuates intestinal inflammation.(436) The results of the present study further support an important role for TREM1 expressing intestinal macrophages in IBD patients as a potential target for therapy. The is also interest in the use of serum soluble TREM1 as biomarker for disease activity.(484) The results of the present study would support the use of TREM1 to provide a quantifiable score of disease severity seen on colonic biopsies. This has the potential to improve the inter-rater variability which is currently problematic in histological assessment of disease activity in IBD. (530) Furthermore there is emerging evidence that TREM1 can be utilised as predictor of response to anti-TNFα both in the blood and intestinal biopsies, from the study by Gaujoux et al (531) which utilised deconvolution and meta-analysis methods followed by experimental validation on blood and biopsies in independent cohorts. This is exciting progress. At present a great limiting factory in delivery of precision medicine to IBD patients is the lack of objective measures that allows clinicians to select biological drugs that are likely to work, the current paradigm is essentially one of trial and error where drugs are sequentially utilised and patients assessed for response during which time if they do not, precious time and quality of life is lost. (532) Along these lines of investigation Martin et al (527) utilised single cell RNA-Seq to identify a cellular signature which the authors called GIMATS, (consisting of IgG expressing plasma cells, inflammatory macrophages and activated T and stromal cells) in ileal CD patients which was associated with poor response to anti-TNF therapy independent of disease activity. This finding has important implications for the clinical setting and demonstrates how high throughput RNA-Seq studies such as this and the present study can translate important findings to the clinical setting.

A multi-platform approach was taken to analysing the RNA-Seq data to gain a depth of understanding of this large data set. The IPA pathway analysis identified that there was activation of the pathways responsible for leukocyte extravasation and communication between the innate and adaptive immune systems further supported by the up regulation of chemokines involved in T-cell migration (CXCL9, 10 and 11). The migration of T-cells to the intestinal mucosa is

known to be an in important factor in the establishment of chronic intestinal inflammation in IBD. (533) Holgersen et al (534) in their RNA-Seq study of whole tissue biopsies from inflamed IBD patients and three mouse models similarly identified up-regulation of T cell chemokines CXCL9 &10. T-cell trafficking has been investigated as a target for therapy. The most successful approach in targeting T-cell trafficking so far has been anti-integrin therapy, which targets the $\alpha_4\beta_7$ integrin subunits, interfering with interaction of these T-cell surface molecules with the MAdCAM-1 molecule expressed by intestinal venules. The efficacy of Vedolizumab a drug that blocks the $\alpha_4\beta_7$ integrin in both CD and UC has been confirmed in large phase III trials and is now utilised in routine clinical practice.(194, 535) Other approaches taken to target T-cell trafficking to include blockade of CCR9 which has demonstrated mixed results in clinical trials.(536) Eldelumab an antibody targeting CXCL10 was investigated in a phase IIa clinical trial after promising animal studies, again the results were inconclusive but suggested a modest benefit in a subgroup of patients.(429) The data from our study suggest another approach to reducing pro-inflammatory T-cell trafficking to the intestinal mucosa is modification of the macrophage behaviour particularly the secretion of T-cell chemokines. We have validated our RNA-Seq data of up-regulation of chemokines on the RNA level with qPCR and at a protein level using IHC. Interestingly recent data published by Zeissig et al (384) demonstrated that Vedolizumab is associated with an increased abundance of M2 macrophages relative to M1 macrophages, in those patients achieving clinical remission with the therapy. Furthermore a recently published study has demonstrated that Vedolizumab blocks homing of $\alpha_4\beta_7$ expressing monocytes.(537) This data suggest that the positive clinical effects of Vedolizumab may be as a consequence of an effect on macrophages as well as T-cell migration directly. In current study we have shown that the transcriptome of the CD and UC macrophages is altered to a pro-inflammatory (M1) phenotype. It is now clear from a number of mouse and human studies that in active inflammatory bowel disease there is expansion of the lamina propria macrophage compartment at the site of inflammation and this arises from the monocyte recruitment which is associated with depletion of circulating monocytes.(527, 538) It is these recruited cells that appear to be pro-inflammatory, Bernado et al (364) identified these cells as $CD11c^{high}$. In the normal state these mature into tolerogenic cells that are $CD11c^{-}$ yet in IBD this process is dysregulated leading to increased numbers of the pro-inflammatory $CD11c^{high}$ cells. The CD11c marker should therefore be considered in future flow cytometry strategies of macrophage isolation. A number of groups have considered strategies to target the recruitment of monocytes to the mucosa, mainly focusing around blockage of CCR2 which appears to be the essential chemokine receptor for recruitment of monocytes from the circulation to the mucosa.(539) The consequences of this strategy however remain unclear, the CCR2 dependent monocyte recruitment is also the process by which the tolerogenic resident

macrophages are recruited and therefore CCR2 blockade may deplete their number and worsen the outcome as these cells have a role in healing.(540)

The pathway analysis identified a number of pathways that on analysis looked to be biologically relevant. To test the strength of the association the analysis was run utilising stricter FDR levels of 0.05 and 0.01 for the identification of the DEGs. This resulted in fewer DEGs but a number of important IPA pathways including *Communication between the innate and adaptive immune system, TREM1, PPAR signalling and leukocyte extravasation pathways* remained significantly associated with the dataset. Thus suggesting that the association with these pathways was relatively strong. Communication between the innate and adaptive immune system is a vital pathological step in the development of the dysregulated and non-resolving inflammation seen in IBD but it appears activation of the innate immune system is likely to to be the triggering step.(541) The association of this IPA pathway with the dataset is a representation that the macrophages in the IBD cohorts were predominantly activated and pro-inflammatory. Activated macrophages release pro-inflammatory cytokines such as IL1, IL6 and IL12 which activate the innate immune system and also secret chemokines such as CXCL9,10 and 11 which recruit components of the adaptive immune system from the circulation.(542) There are already treatment strategies in place in clinical practice that target the interaction between the innate and adaptive immune system such ustekinumab that targets IL12 which promotes T-cells differentiation.(543) The more non-specific Anti-TNF drugs are also likely to play a role in breaking the link between the adaptive an innate immune system through the reduction of the epithelial cell apoptosis and release of pro-inflammatory mediators.(541) The finding of activation of the *Communication between the innate and adaptive immune pathway*, further highlights the importance of targeted therapies to the root of the problem which appears to be pathological innate immune system activation to break the vicious cycle of subsequent activation of adaptive immune system and the dysregulated ensuing inflammation. The down regulation of the PPARγ pathway has previously identified as being associated with IBD, colonic PPARγ expression has been shown to be down regulated in mouse models of colitis and patients with UC.(448) PPARγ also appears to have a role in fibrosis and the anti-inflammatory role PPARγ agonists in IBD as well as other inflammatory disorders such as SLE, pancreatitis and animal models of IBD has been know for a number of years.(544) There are current treatment strategies that target the PPARy pathway, the 5-aminosalicylic drugs which are effective treatments for UC an appear to have their efficacy owing to targeting of PPARy though this was not known when they were developed.(490) Further research into the use of PPARy agonists such as the Thiazolidinediones in IBD in recent times has stuttered mainly due on concerns around adverse cardiovascular outcomes.(545)

We found that the analysis of CD Vs UC using the absolute statistical cut off and over representation pathway analysis showed that the CD and UC intestinal macrophages were very similar, with only 20 DEGs between the two. This I believe is partly due to the biopsies being taken from inflamed areas of colon. An inflammatory control would have been useful. The options for inflammatory control in humans would be cases of enteric infection, which would represent a heterogeneous group and cases of diverticulitis. Both of these cases infrequently require endoscopic evaluation as part of routine care. A suitable number of cases would be difficult to generate without asking participants to have an endoscopic procedure that is not part of their routine care. This would of course require further ethical submission of a substantial amendment. Another experimental change that may have allowed for a greater detection of DEGs between CD and UC may have been to take the approach of utilising intestinal biopsies from areas of quiescent disease i.e. areas of colon previously inflamed but now healed. In CD this approach can be technically challenging as the disease can be patchy and in quiescent disease there may be no signs to suggest areas of previous inflammation and thus targeting these areas is difficult. We were however able to discover some subtle but biologically important differences between the CD and UC macrophages using GSEA, a form a functional class scoring pathway analysis. The benefit of this form of pathway analysis is that it is not dependent on user defined statistical cut of points as utilised in over representation analysis, which results in excluding potentially relevant biological information. We found that although both the CD and UC macrophages express a M1 profile only the CD macrophages were significantly associated with the M2 signature genes as described by Xue et al.(318) Furthermore the CD but not the UC macrophages were associated with the fibrotic and granuloma signature gene set by GSEA. These findings are consistent with the clinical picture seen in IBD where granulomas are seen in CD but not UC and fibrotic complications are common in CD but much less so in UC.(516, 517) These findings suggest that the M2 (wound healing) phenotype of macrophage may not be entirely beneficial; un-regulated this healing may lead to fibrosis as a consequence of "excess healing". The detrimental effect of chronic M2 activation as been demonstrated in other diseases such as fibrotic liver disease and heart failure.(546) Chronic unbalanced activation of the M2 phenotype appears to drive fibrosis through the TGFβ and growth factors that promote myelofibroblasts and extra-cellular matrix deposition.(547) Other researchers have pursued a strategy of promoting the M2 intestinal macrophage (548) and indeed Vos et al(383) were able to demonstrate in those responding to anti-TNF alpha therapy (measured by mucosal healing) M2 induction was present yet not so in the non-responders. Our results suggest that this approach should be taken with caution as over promotion of the M2 intestinal macrophage phenotype could unintentionally result in fibrotic complications. I have explained earlier that the M1/M2 phenotype is actually a

spectrum model and it may therefore be that we should be aiming to promote M2 macrophages that have a less pro-fibrotic tendency.

The majority of the DEGs identified for the comparisons of disease Vs healthy control were up-regulated genes. The effect was to "drown out" the signal of the fewer down regulated genes in the generalised pathways analyses. There were a total of 48 commonly down regulated genes for CD Vs N and UC Vs N. The down regulated genes for CD and UC were analysed using gene ontology software, revealing a common theme of down regulation of metabolic processes in the IBD macrophages. In particular, we identified down regulation of genes involved in mitochondrial function and cellular detoxification. Targeting these down regulated metabolic genes therefore appears to be another potential option for therapy. Metabolic reprograming of M1 macrophages may be the mode of moving these macrophages to a potentially more beneficial M2 (wound healing) phenotype. A similar strategy has been utilised in the opposite direction in cancer research where the M1 phenotype in TAMS is beneficial in preventing the progression of cancer.(513) Given the previous discussions on the questionable value of the M1/M2 concept, describing a pro-resolving cohort of macrophages in this context is more logical and has been adopted by some in the area already.(540) These pro-resolving macrophages have been demonstrated to secrete pro-resolving mediators which include long-chain fatty acid mediators.(540) The down regulation of the metabolic processes in macrophages identified in this project would therefore be consistent with a reduction in pro-resolving macrophages and providing further explanation to the dysregulated inflammation seen in IBD.

7.2 Study limitations

"Our Scientific power has outrun our spiritual power.

We have guided missiles and misguided men"

Martin Luther King

The greatest challenge in this project was keeping pace with advancing scientific methods and analysis. When I embarked on the project the methods and study design we selected were innovative, cutting edge and the felt to be the best available techniques given the financial and time limitations of the project. We were however ambitious in our objectives for this project. All the experimental techniques and analysis methodology were new skills I learnt in the course of the project. Whist this was a great developmental process for myself it meant that the project progressed at a slow rate that did not keep up with the technological advancements happing in the related fields. The consequence was that on completing this project the methods utilised were no longer novel or cutting edge. In particular a number of groups have now shown the benefit of single cell RNA sequencing in discovering important aspects of the biology of IBD and importantly how this can be translated to provide real benefits in terms of precision and personalised medicine for these patients.(527, 549, 550) If I was to design this project again now with the technologies available I would utilise single cell sequencing rather than bulk cell sequencing which is unable to account for the potential heterogeneity of the intestinal macrophage cell population.

The sorting strategy that was utilised to identify the intestinal macrophages was designed to be broad to be inclusive but did not account for different subpopulations of macrophage cells. It is clear from recent studies that there are different populations of intestinal macrophage cells and relative abundance is important not just for disease activity but also response to therapy.(364, 527) The addition of further relevant macrophage surface markers would have allowed for the isolation of several different macrophages sub-populations allowing for the transcriptome of these cells to be compared adding further granularity to the information gained from the project and potentially further therapeutic avenues.

The samples we utilised for the active IBD cases were taken from the inflamed colonic areas only. This did create challenges in understanding the data as there was a predominantly inflammatory signal. No inflammatory control was included in this project which does limit the interpretation of the results as it is not possible to determine if the findings are specific to IBD (UC and CD) or that

of colonic inflammation of any cause such as infection or ischaemia. This situation would have been resolved by the inclusion of any inflammatory control such as diverticulitis patients and also harvesting paired samples of inflamed and non inflamed colonic tissue from each disease patient recruited to the study.

We have conducted a small study that was discovery in nature. A larger study with prospective follow up of several years to assess the disease course of the patients recruited with further sample collection would have been desirable and aided the translational aspect of the project. Unfortunately, the budget restraints of the project did not allow for larger cohorts of patients to be recruited and the time and resource limitations did not allow for any prospective disease course data to be collected. A relaxed FDR was used for discovery purposes, so we need to be mindful of the false positive rate of the gene expression associations described, however when the data was assessed at more stringent FDR criteria the important identified associations remained statistically significant.

7.3 Conclusions

To the best of my knowledge this is the first study to utilise high throughput bulk RNA-Seq to assess genome-wide transcription changes of intestinal tissue macrophages comparing CD and UC with a healthy control group. Chapuy et al (550) recently reported a study identifying two subsets of $CD14^+$ intestinal mononuclear phagocytes (MNP) in Crohn's disease patients. In this study they utilised single cell RNA-Seq on subpopulations of MNPs identified by flow cytometry from 3 patients to further define MNP sub-populations. Consistent with our findings they identified a pro-inflammatory sub-set of MNPs that expressed TREM1. The Chapuy et al (550) al study however did not compare CD with UC and the number of patients utilised for sequencing was small, just 3 cases given the potential heterogeneity we see particularly in CD patients this is a significant limitation of their study and subsequent generalizability of their results.. Other studies have utilised high throughput RNA-Seq on whole tissue biopsies in IBD patient cohorts. Haberman et al (551) used RNA-Seq on terminal ileum biopsies from paediatric CD patients to identify gene expression alterations associated with specific gut microbial changes. Hong et al (426) used RNA-Seq on whole tissue intestinal biopsies to compare inflamed CD with non-inflamed CD and healthy controls, identifying transcriptional differences across all three groups and CXCL1 as a potential disease activity marker in the blood. Holgersen et al (534) performed RNA-Seq on whole tissue inflamed intestinal biopsies from IBD patients and three mouse models of colitis, consistent with the present study, they identified up-regulation of T-cell chemokines CXCL9-11 in both the IBD patients and mouse models.

A major strength of the present study was the isolation of a specific cell type for transcriptome profiling. It is increasingly becoming clear that an understanding of the roles played by individual immune cells will open up new therapeutic targets and allow a more personalised approach and better prognostication. Lee et al (552) have demonstrated such an approach, having identified prognostic biomarkers in isolated CD8 T-cells. These results have subsequently been validated and are currently being tested in clinical practice in the PROFILE study.(553) I hope that the results of the present study can be taken further in conjunction with the latest technologies described earlier in this chapter to be utilised in a similar way in clinical practice to bring benefits to patients with IBD.

7.4 Future work

We have identified a number of potential macrophage therapeutic targets in inflamed IBD patients using the RNA-Seq data generated from a cohort of IBD patients with active intestinal inflammation. These findings have been validated in small cohorts using qPCR, IHC and flow cytometry. It would be helpful to validate the results using RNA-Seq in a larger cohort of patients. This would also allow for IBD patients in remission to be recruited so that a comparison of intestinal macrophages from inflamed Vs non-inflamed tissue can be made. Inclusion of IBD patients in remission with mucosal healing would have two important benefits. Firstly, it would deal with the overriding signal of inflammation that appears to "drown out" subtler changes in gene expression, such as that seen with the down regulated genes. Secondly, samples from patients in remission would help to determine if there is an underlying defect in intestinal macrophages in IBD patients and if so a preventative therapeutic approach could be explored rather than the reactionary, as is the current paradigm in IBD therapies. In addition to this it would be helpful to take paired samples from the patients with active disease, from both inflamed tissue and adjacent non-inflamed tissue. This again would help understand if there is an inherent change in the transcriptome of the the macrophages of IBD patients or if the changes we have described in this project are a consequence of established dysregulated inflammation. As described earlier, this project has not been able to determine if the changes in the macrophage transcriptome are IBD specific or are general changes that would be seen in any form of colonic inflammation. This issue would be solved by the addition and recruitment to an inflammatory control group of patient with diverticulitis.

I have identified that there appears to be a group of macrophage cells that are CD14 negative and positive for CD163. These cells may have an inherent role that differs to the macrophage studied in the present study; further work should include these cells as a separate cohort. Furthermore we have seen that recent studies have shown that macrophage sub-populations have different characteristics and can inform response to IBD treatment.(364, 527) Future studies should therefore compare a number of different macrophages populations rather than grouping them together as we have done in this project. This would require extensive development of FACS sorting strategies but these flow cytometry experiments themselves would provide useful information on the relative abundance of intestinal macrophage subpopulations in IBD patients compared to healthy controls. The use of single cell RNA-Seq would further help in understanding the macrophage phenotype in IBD patients.

I would recommend that in future projects more work is done with the patient phenotype data with the analysis for associations with macrophage transcriptomic changes. This would require

both prospective and retrospective data to be collected from patients. The would be a resource and time challenge but this together with new techniques such as single cell RNA-Seq will allow for correlation of macrophage phenotype with disease prognosis but also potentially the development of macrophage biomarkers for predicting response to treatment and aiding a move to personalised medicine.

The immune system of the bowel is complex with the immune cells of the intestine interacting with each other and the intestinal microbiota.(554, 555) Studying single cell types or even the immune cells alone is an over simplification of the whole system. Further work should aim to analyse multiple parameters using advanced high throughput genomic techniques. Rather than studying the macrophages in isolation, single cell RNA-Seq of the major immune cells to include T-cells, dendritic cells and epithelial cells along with microbiome sequencing should be conducted. This would not only be able to validated the findings of the present study but will vastly improve the understanding of IBD pathogenesis and I believe this philosophy offers the greatest opportunity in developing personalised approaches for treating patients with IBD. There are of course major challenges of a project such as this. Wide ranging expertise is required, significant funding and time is essential. Realistically this requires collaboration between different groups disciplines, institutions and countries which is in itself can be a challenge to deliver significant outputs. The European SysmedIBD group (https://www.sysmedibd.eu/) demonstrated that it is possible to conduct projects in this way.

The first major aim of further work from the present project however is to replicate the data in larger independent cohorts, targeting metabolic macrophage processes and T-cell chemokine secretion by macrophages. Once completed the findings would need to be tested in functional models, the human ex-vivo colonic tissue model developed by the Dr Sanchez-Elsner laboratory (556) would be a suitable model for such experiments. These I believe are exciting new avenues for therapy in IBD that are not currently under exploration and would help to move towards the personalised treatment approach that patients and the IBD clinical community alike so desperately crave.

Appendix A Demographic tables

Patient code	Age	Sex	Smoking	Ethnicity	Duration of disease (Days)	Current medication for IBD	Endoscopic MAYO	Full MAYO
UCC535	38	F	N	Caucasian	0	None	1	6
UDC546	69	M	EX	Caucasian	1613	Oral Pentasa, AZA	2	7
UCC583	26	M	N	Caucasian	5222	MXT	2	6
UCC588	44	F	EX	Caucasian	1553	None	2	2
UCC595	76	M	N	Caucasian	1222	Oral prednisolone	2	8
UCC602	24	F	N	Afro-Caribbean	1095	Prednisolone Suppository, Oral Salofalk	1	6
UCC604	39	M	N	Caucasian	0	None	2	6
UDC608	55	F	EX	Caucasian	0	None	2	9
UDC612	39	M	EX	Caucasian	4601	Oral Prednisolone, Oral Pentasa	3	10
UDC613	30	M	N	Caucasian	1686	Oral Salofalk	2	2

Table 42 RNA-Seq demographic data for UC patients

Patient Code	Age	Sex	Ethnicity	Smoker	Duration of disease (Days)	Montreal Classification				Previous surgery	IBD Medications	HBI Score
						A	L	B	P			
CDC527	23	M	Caucasian	N	1552	A2	L2	B1	0	N	AZP	7
CCC536	40	M	Caucasian	N	3997	A2	L3	B1	0	N	Oral Prednisolone Oral Pentasa	0
CDC541	35	F	Caucasian	EX	116	A2	L2	B1	0	N	AZP	7
CDC565	66	M	Caucasian	N	5528	A3	L2	B1	0	Appendectomy	None	15
CCC579	45	F	Caucasian	EX	10298	A2	L3	B1	0	N	None	3
CDC594	54	M	Caucasian	N	0	A3	L2	B1	0	N	Oral prednisolone	5
CCC593	49	M	Caucasian	N	4875	A2	L3	B1	0	N	MP	9
CDC606	67	F	Caucasian	Y	1314	A3	L3	B1	0	N	Methotrexate	4
CCC607	49	F	Caucasian	Y	5654	A2	L2	B1	0	N	AZP	5
CDC609	80	F	Caucasian	N	2002	A3	L2	B1	0	N	Methotrexate	8
CDC610	27	M	Caucasian	N	1095	A2	L3	B1	0	N	None	11

Table 43 CD patient demographics for he RNA-Seq Cohort

Patient Code	Age	Sex	Smoker	Ethnicity	Indication for Endoscopy	Site of biopsy
NAC528	66	M	N	Caucasian	Polyp follow up	Sigmoid colon
NAC564	71	M	N	Caucasian	Polyp follow up	Sigmoid colon
NAC573	74	M	N	Caucasian	Polyp follow up	Sigmoid colon
NAC580	69	M	N	Caucasian	Polyp follow up	Sigmoid colon
NAC597	55	M	N	Caucasian	Polyp follow up	Sigmoid colon
NAC599	60	M	Y	Caucasian	Polyp follow up	Sigmoid colon
NAC601	50	M	Y	Caucasian	Polyp follow up	Sigmoid colon
NAC603	71	F	EX	Caucasian	Polyp follow up	Sigmoid colon
NAC572	78	F	EX	Caucasian	Polyp follow up	Sigmoid colon
NAC605	64	F	N	Caucasian	Iron deficiency anaemia without gastrointestinal symptoms	Sigmoid colon

Table 44 Normal patient demographics

Patient code	Age	Sex	Smoking	Ethnicity	Duration of disease (Days)	Current medication for IBD	Endoscopic MAYO	Full MAYO
UCC614*	30	M	N	Caucasian	2430	Octasa Azathioprine	2	10
UDC615	43	M	N	Caucasian	0	None	3	9
UDC619	30	F	N	Caucasian	656	Pentasa (oral)	2	9
UDC636*	31	F	N	Caucasian	1656	Pentasa (oral) Azathioprine	2	10
UDC637	74	M	N	Caucasian	0	None	2	7

Table 45 UC validation cohort patient demographics. * Samples used for IHC.

Patient Code	Age	Sex	Ethnicity	Smoker	Duration of disease (Days)	Montreal Classification				Previous surgery	IBD Medications	HBI Score
CDC618*	53	F	Caucasian	N	2451	A3	L3	B2	1	Right hemicolectomy	None	11
CCC622*	51	M	Caucasian	N	13155	A1	L2	B1	0	None	None	6
CCD623*	32	F	Caucasian	N	1854	A2	L3	B2	0	Segmental colonic resection	Mercaptopurine Prednisolone (oral)	9
CCC624*	73	F	Caucasian	N	13176	A2	L2	B1	0	None	Asacol (oral)	3
CCC640*	46	M	Caucasian	N	2299	A2	L3	B1	0	None	None	4
CDC646	48	M	Caucasian	EX	389	A3	L2	B1	0	None	Azathioprine	10

Table 46 Patient characteristics of CD qPCR validation cohort *samples used for IHC validation

Patient Code	Age	Sex	Smoker	Ethnicity	Indication for Endoscopy	Site of biopsy
NAC638	82	F	N	Caucasian	Anaemia	Sigmoid colon
NAC639	48	F	N	Caucasian	Anaemia	Sigmoid colon
NAC641	70	M	N	Caucasian	Polyp follow up	Sigmoid colon
NAC642	75	M	EX	Caucasian	Anaemia	Sigmoid colon
NAC643	74	M	N	Caucasian	Polyps	Sigmoid colon
NAC644	56	M	EX	Caucasian	Anaemia	Sigmoid colon

Table 47 Patient characteristics of normal control qPCR validation cohort

Patient code	Disease	Age	Sex	Ethnicity	Smoking status	Current medications
UDC647	UC	22	F	Caucasian	N	Azathioprine
UBC649	UC	55	F	Caucasian	N	Azathioprine
UCC665	UC	42	M	Caucasian	N	Pentasa oral
UDC670	UC	24	F	Caucasian	N	Prednisolone suppository Pentasa suppository
CDC633	CD	62	F	Caucasian	EX	None

Table 48 Patient characteristics of active IBD TREM1 validation cohort

Patient code	Disease	Age	Sex	Ethnicity	Smoking status	Current medications
UAC630	UC	58	M	Caucasian	N	Pentasa oral
UAC634	UC	70	F	Caucasian	N	Pentasa oral
UAC635	UC	52	M	Caucasian	N	Azathioprine
UAC666	UC	30	M	Caucasian	N	Mezevant
CAC629	CD	24	F	Caucasian	N	None
CAC658	CD	33	F	Caucasian	N	Azathioprine
CAC667	CD	38	M	Caucasian	N	Mercaptopurine Asacol

Table 49 Patient characteristics of IBD remission patients for TREM1 validation cohort

Patient Code	Age	Sex	Smoker	Ethnicity	Indication for Endoscopy	Site of biopsy
NAC648	76	F	N	1	Iron deficiency anaemia	Sigmoid colon
NAC661	64	M	N	1	Iron deficiency anaemia	Sigmoid colon
NAC660	81	M	N	1	Iron deficiency anaemia	Sigmoid colon
NAC 664	55	M	N	1	Iron deficiency anaemia	Sigmoid colon
NAC668	55	M	N	1	Iron deficiency anaemia	Sigmoid colon

Table 50 Patient characteristics of normal control TREM1 validation cohort

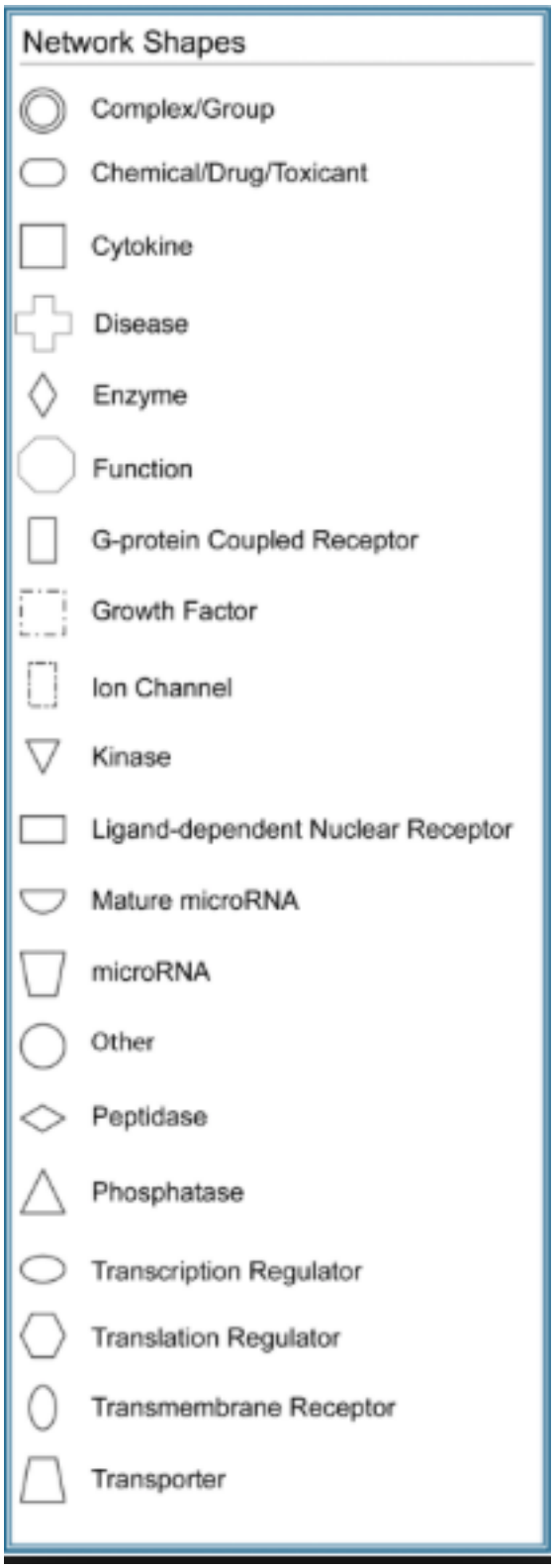
Patient code	Disease	Age	Sex	Ethnicity	Smoking status	Current medications
UCC614	UC	30	M	Caucasian	N	Octasa Azathioprine
CDC618	CD	53	F	Caucasian	N	None
CCC622	UC	51	M	Caucasian	N	None
CDC623	CD	32	F	Caucasian	N	Mercaptopurine
CCC624	CD	73	F	Caucasian	N	Oral Asacol
CDC633	CD	63	F	Caucasian	EX	None
UDC636	UC	31	F	Caucasian	N	Oral Pentasa Azathioprine
UDC637	UC	74	M	Caucasian	N	None
CDC640	CD					
CDC646	CD	48	M	Caucasian	EX	Azathioprine

Table 51 Patient characteristics IBD IHC validation cohort

Patient Code	Age	Sex	Smoker	Ethnicity	Indication for Endoscopy	Site of biopsy
NAC660	79	M	N	Caucasian	Anaemia	Sigmoid colon
NAC661	65	M	N	Caucasian	Anaemia	Sigmoid colon
NAC664	55	M	N	Caucasian	Colorectal cancer family history	Sigmoid colon
NAC668	55	M	N	Caucasian	Colorectal cancer family history	Sigmoid colon
NAC674	78	F	EX	Caucasian	Anaemia	Sigmoid colon

Table 52 Patient characteristics of the normal control group IHC validation cohort

Appendix B Key for Ingenuity Network shapes



Appendix C Gene lists for GSEA

Interferon (IFN) signaling pathway		**Ingenuity Knowledge based Antigen Presentation pathway**	
BAK	IFNγRα	BAK	IFNγRα
BAX	IFNγRβ	BAX	IFNγRβ
BCL-2	IFNAR1	BCL-2	IFNAR1
DRIP150	IFNAR2	DRIP150	IFNAR2
G1P2	IRF1	G1P2	IRF1
G1P3	IRF9	G1P3	IRF9
Glucocorticoid	IFITM1	Glucocorticoid	ISGF3
IFI35	IFITM2	IFI35	JAK1
IFIT1	IFITM3	IFIT1	JAK2
IFIT3	IFNα/β	IFIT3	MX1
IFNγ		IFITM1	NF-κBp65
		IFITM2	OAS1
		IFITM3	PIAS1
		IFNα/β	PSMB8
		IFNγ	SOCS1
		STAT1	TAP1
		Stat1 dimer	TC-PTP
		Stat1-Stat2	TYK2
		STAT2	

Appendix C

M1 Signature Gene List					
ATF	CD69	HCG4	IRF7	OSCAR	SP100
ADAR	CFB	HCP5	IRF9	PARP14	SP140
AIM2	CLDN7	HLA-A	ITGAL	PAXIP1	STAT1
ALPK1	CXCL10	HLA-B	JAK2	PLEKHA7	STAT2
ANKRD22	CXCL11	HLA-C	KCNJ2	PML	TAP1
APOBEC3A	CXCL12	HLA-E	LRRCC1	PRPF3	TAP2
APOL2	CXCL9	HLA-F	MDK	PSMB10	TIFA
APOL3	DCAF6	HLA-G	MFAP1	PSMB8	TLR2
APOL6	DYNLT1	HLA-H	MIA3	PSMB9	TLR4
BATF2	ELF4	IL11	MOV10	RARRES3	TM9SF2
BTN3A1	ETV7	IL12A	MR1	RBCK1	TM9SF3
BTN3A2	FAM26F	IL12B	NFIX	SELT	TMEM140
BTN3A3	GBP1	IL23A	NLRC5	SERPING1	TNF
CCL15	GBP2	IL27	NMI	SERPINI1	TNFSF10
CCL20	GBP4	IL6	NOS2	SLAMF7	TRAFD1
CCL5	GBP5	IRF1	NUP50	SLC15A4	
CD40	HAPLN3	IRF5	OAT	SLC6A12	

M2 Signature Gene List					
ADAM15	CCL22	GALNT12	ITPRIPL1	PPP1R14A	TGFBI
ADAM28	CCL26	GALNTL4	KIAA0182	PPP1R3B	TIGD5
ADORA3	CCRN4L	GLS	KIAA1671	RAB33A	TOR3A
ALDH5A1	CD14	GOLGA8B	KTN1	RAB40B	TRIB2
ALOX15	CD163	GPD1L	LOC284998	RAMP1	TTC9C
ALOX5AP	CD206	GPR65	LYZ	RAP1GAP	UST
ARG1	CD209	HARS2	MACF1	RASL10A	WDR33
ARG2	CLIC2	HEMK1	MAOA	RASSF7	WNT5A
ARHGAP23	CRH	HOMER2	MAP1A	RPL28	WNT5B
ATG7	CTNNAL1	HPS1	MLKL	RRP1B	ZNF317
AUH	CYBB	IL10	MMP1	RTKN	TGFBI
BAP1	DAAM1	IL10RA	MMP12	S100A8	TIGD5
BATF3	DAP	IL10RB	MMP7	S100A9	TOR3A
BCL3	DHRS11	IL13RA1	MMP9	SNX8	TRIB2
C3AR1	DLST	IL17RB	MUTED	SOCS1	TTC9C
C5AR1	DUSP6	IL1R1	NAGPA	SOCS3	UST
CABIN1	EGLN3	IL1R2	NLRP12	SOX8	WDR33
CARD9	ESPNL	IL4I1	NMD3	STAB1	WNT5A
CBR3	EXOC2	IL4R	PALLD	STAT6	WNT5B
CCDC6	FOS	INTS3	PELP1	SUCNR1	ZNF317
CCL17	FOXD2	IRF4	PITPNA	SULF2	TGFBI
CCL18	FOXQ1	ISYNA1	PITRM1	SYT17	TIGD5

Xue at at Module 29	
REPIN1	CD70
CSF2RA	CD80
SOCS2	FOXD4
CMTM6	IDO2
UBXN11	RELB
TGFA	TIAM2
PYGL	MAP3K14
KTELC1	MMP25
LOC727935	SLC27A1
CST7	GRASP
MCOLN2	TBC1D13
NAV1	ALPK2
LOR	CASZ1
GPR64	LYPD3
C1ORF115	EHF
ETV3	
PHF16	
LAMP3	
INSM1	
NCCRP1	
TUBB2B	
BIRC3	
FOXD4L1	

Fibrosis Gene set							
A2M	BMPR2	DCN	ID2	RUNX3	TGFB1	MAGED1	RBX1
ACTA2	CALR	DFTR	ID3	SERPINE1	TGFB2	MAPK1	RHOA
ACVR1	CCL2	DIO2	ID4	SFTPA1	TGFB3	MAPK3	ROCK1
ACVR1B	CCR5	E2F4	IFNG	SFTPA2	TGFBI	MLLT11	ROCK2
ACVR1C	CD14	E2F5	IGFBP4	SFTPC	TGFBR1	MMP1	RPS6KB1
ACVR2A	CDH2	EDN1	IGFBP5	SKP1	TGFBR2	MMP2	RPS6KB2
ACVR2B	CDKN2B	EDNRB	IL10	SMAD1	TGFBR3	MMP9	RUNX3
ACVRL1	CFHR1	EMILIN1	IL1A	SMAD2	TGFBRAP1	MPL	SERPINE1
ALDH1A3	CHRD	ENC1	IL1R2	SMAD3	THBS1	MUC5B	SFTPA1
AMH	CLEC11A	EP300	IL6R	SMAD4	THBS2	MYC	SFTPA2
AMHR2	COL16A1	FBLN1	INHBA	SMAD5	THBS3	MYL6B	SFTPC
BCL2	COL1A2	FHL2	INHBB	SMAD6	THBS4	NODAL	SKP1
BMP2	COL3A1	FLT1	INHBC	SMAD7	THY1	NOG	SMAD1
BMP4	COL6A1	FN1	INHBE	SMAD9	TMED9	PDLIM4	SMAD2
BMP5	COL6A2	FST	JAK2	SMURF1	TNC	PITX2	SMAD3
BMP6	COL9A2	GDF5	LEFTY1	SMURF2	TNF	PPP2CA	RBX1
BMP7	COMP	GDF6	LEFTY2	SP1	TWIST1	PPP2CB	RHOA
BMP8A	CREBBP	GDF7	LEP	TERC	UCHL1	PPP2R1A	ROCK1
BMP8B	CUL1	HGF	LIMK2	TERT	VCAN	PPP2R1B	ROCK2
BMPR1A	CXCL3	ICAM1	LOC728622	TFDP1	VEGFA	RBL1	RPS6KB1
BMPR1B	CXCL9	ID1	LTBP1	TGFA	ZFYVE16	RBL2	RPS6KB2
ZFYVE9							

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