AN INVESTIGATION OF MOLECULAR DEFECTS UNDERLYING IMPAIRED ACUTE INFLAMMATION IN CROHN'S DISEASE

By

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I, Gavin William Sewell confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Mounting evidence suggests the pathogenesis of Crohn's disease (CD) involves an impaired acute inflammatory response. Monocyte-derived macrophages from CD patients release deficient levels of pro-inflammatory cytokines in response to *Escherichia coli*, arising from post-translational trafficking abnormalities. In this thesis, the macrophage responses to microbial stimuli were further characterised, and the underlying molecular lesions investigated.

Macrophage TNF release was attenuated in response to *Escherichia coli*, *Candida albicans* and Toll-like receptor (TLR) stimulation in CD. This deficit was unrelated to use of medication, age, gender and smoking status. Patients with stricturing and colonic disease demonstrated the most profound defects in response to TLR2 and TLR4 stimulation respectively. Genotyping for 34 known CD susceptibility polymorphisms revealed no detectable association between any individual variant and impaired TNF release. However in CD, TNF secretion in response to *Escherichia coli* was weakly correlated with overall genetic risk score.

Macrophage sphingolipid and phospholipid compositions were subsequently investigated, given the prominent role of these molecules in vesicle trafficking. Mass spectrometric analysis revealed no gross abnormalities in CD macrophages, although a reduced percentage of phosphatidylinositol 16:0/18:1 was synthesised over 3 hours; the same species was also present at reduced levels in ileal biopsies.

Given the heterogeneity of CD, a microarray outlier analysis strategy was developed to identify specific gene expression abnormalities in individual patients. A subset of patients had deficient expression of optineurin, a molecule implicated in vesicle trafficking and autophagy. These individuals shared a number of allelic variants,

which were associated with optineurin expression levels. Macrophages from these patients had attenuated TNF secretion downstream of TLR2. Concordantly, depletion of optineurin in THP-1 cells impaired TNF release after TLR2 activation.

This study supports a role for immune deficiency in the pathogenesis of CD and identifies abnormal optineurin expression as a relevant molecular abnormality in a subset of patients.

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Statement of Collaborative Work

A number of aspects of the work presented in this thesis were conducted as collaborative projects. These included

- Macrophage cytokine secretion assays in response to TLR agonist stimulation. L929 bioassays investigating TNF-α release to TLR agonists were performed jointly with Dr Farooq Rahman (FR). Initial optimisation studies were performed by FR. Data presented comparing HC with CD are the combined results for assays performed by FR (~70% of total) and GS (~30%), with the exception of TLR5, which were performed by FR and are presented here for completeness. Subsequent analysis of phenotype and genotype correlations was performed by GS. All assays investigating HkEc and HkCa stimulation in this study were performed by GS.
- Sequenom genotyping and determination of genetic risk scores for the UCLH cohort of CD patients and HC individuals was performed as a collaborative project with Dr Jeffrey Barrett and Mr Luke Jostins at the Wellcome Trust Sanger centre, and Mr Adam Levine.
- Analysis of macrophage lipids in Crohn's disease.

  Macrophage ceramide content was investigated as a collaborative project with the Medical University of South Carolina. All samples were collected and prepared by GS; lipid extractions were performed by GS and Professor Jacek Bielawski (JB). Mass spectrometry was performed by JB with technical support. Subsequent analysis was performed by GS.

  Macrophage phospholipid dynamics were investigated in a collaborative study with the University of Southampton. Samples were prepared by GS; lipid extractions were performed by GS under kind supervision of Dr Vikki Ledger

(VL). Mass spectrometry was performed by Dr Grielof Koster and VL. Spectra and data generated by the in house macro were analysed by GS with helpful advice from members of the University of Southampton lipidomics group.

Shotgun lipidomics experiments were performed as a collaborative project with Dr Xianlin Han (XL) (Washington University, St Louis). Sample preparation was performed by GS and XL. Mass spectrometry was performed by XL. Data were analysed by XL and GS.

- Transcriptomic abnormalities in Crohn's disease

  Sample preparation and RNA extraction was performed by GS (with technical assistance of Dr Carol McDonald). Array hybridisation was performed at the Wellcome Trust Sanger Centre. Analysis and subsequent experiments were performed by GS. The software for customised outlier analysis was developed in collaboration with Dr Daniel Roden and Dr Anna Lobley, Department of Computer Sciences, UCL.

List of abbreviations

5-ASA: 5-aminosalicylate

ADAMDEC1: ADAM-like decysin 1

AIEC: Adherent, invasive *Escherichia coli*

ALS: Amyotrophic lateral sclerosis

ANOVA: Analysis of variance

ASCA: Anti-*Saccharomyces cerevisiae* antibodies

ATG16L1: Autophagy-related 16-like 1

BCA: Bicinchoninic acid

BMI: Body mass index

BSA: Bovine serum albumin

C. albicans: *Candida albicans*

CARD: caspase recruitment domain-containing protein

CCT: CTP: phosphocholine cytidylyltransferase

CD: Crohn's disease

CDAI: Crohn's disease Activity Index

CGD: Chronic granulomatous disease

CL: Cardiolipin

COPA: Cancer outlier profile analysis

DAG: Diacylglycerol

DMEM: Dulbecco's Modified Eagle medium

DSS: Dextran sodium sulphate

E. coli: *Escherichia coli*

EDTA: Ethylenediaminetetraacetic acid

eQTL: expression quantitative trait locus

ER: Endoplasmic reticulum

ESI-MS: Electrospray ionisation tandem mass spectrometry

FBS: Foetal Bovine Serum

FDR: False discovery rate

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GM-CSF: Granulocyte macrophage-colony stimulating factor

GO: Gene ontology

GRS: Genetic risk score

GWAS: Genome-wide association study

HC: Healthy control

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV: Human Immunodeficiency virus

HkCa: Heat-killed *Candida albicans*

HkEc: Heat-killed *Escherichia coli*

HLA: Human leukocyte antigen

HPLC: High performance liquid chromatography

HPLC-MS: High performance liquid chromatography tandem mass spectrometry

IBD: Inflammatory bowel disease

IFN: Interferon

Ig: Immunoglobulin

IL: Interleukin

IL-23R: Interleukin-23 receptor

IRGM: immunity-related GTPase family, M

LB: Luria-Bertani

LC3: microtubule –associated protein 1 light chain 3

LPS: Lipopolysaccharide

M cell: Microfold cell

MAP: *Mycobacterium avium* subspecies *paratuberculosis*

MDP: Muramyl dipeptide

MS: Mass spectrometry

MSD: MesoScale Discovery

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide

NEMO: NF-κB essential modulator

NLR: Nod-like receptor

NOD: Nucleotide oligomerisation binding domain

OPTN: Optineurin

PA: Phosphatidic acid

Pam3CSK4: N-palmitoyl-S-[2,3-bis(palmitoyloxy)-propyl]-(R)-cysteinyl-(lysyl)3-lysine

PAMP: Pathogen-associated molecular pattern

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate buffered saline

PC: Phosphatidylcholine

PCA: Principal component analysis

PCR: Polymerase chain reaction

PE: Phosphatidylethanolamine

PEMT: Phosphatidylethanolamine N-methyltransferase

PG: Phosphoglycerol

PI: Phosphatidylinositol

PLA2: Phospholipase A2

PMA: Phorbol-12-myristate-13-acetate

PRR: Pathogen pattern recognition receptor

PS: Phosphatidylserine

S. cerevisiae: *Saccharomyces cerevisiae*

SAMP: SAMP1/ Yit

SAP: Shrimp alkaline phosphatase

SDS: Sodium dodecyl sulphate

siRNA: Small interfering RNA

SNAP: SNP annotation and proxy search

SNARE: Soluble NSF attachment protein receptor

SNP: Single nucleotide polymorphism

TACE: TNF converting enzyme

TBK: TANK-binding kinase

TGF-β: Transforming growth factor-β

TGN: Trans-Golgi network

Th: T-helper

TIM: T-cell immunoglobulin mucin

TIR: Toll / IL-1 receptor

TIRF: Total internal reflection fluorescence microscopy

TLR: Toll-like receptor

TNF: Tumour necrosis factor

UC: Ulcerative colitis

UCL: University College London

UCLH: University College London Hospitals NHS Foundation Trust

UK: United Kingdom

USA: United States of America

UTS: Urotensin

VAMP: Vesicle-associated membrane protein

WTCCC: Wellcome Trust Case Control Consortium

YPD: Yeast peptone dextrose

Chapter 1: Introduction

1.1 Clinical and epidemiological aspects of Inflammatory Bowel Disease

1.1.1 Clinical presentation and management of Crohn's disease

Crohn's disease (CD) is a common inflammatory bowel disease (IBD) associated with considerable lifelong morbidity. The first descriptions of the disorder may date back to the 18th century, when Giovanni Battista Morgagni described 'erosion' and 'ulceration' of the internal surface of the terminal ileum and caecum in a 20 year old patient with abdominal pain and diarrhoea. There were a number of subsequent descriptions of a CD-like disorder, and notably in 1913 T. Kennedy Dalziel published a case series describing 13 patients with thickening of the terminal ileum and transmural inflammation, some of whom also had colonic involvement and intestinal obstruction. Dalziel also recognised that the disorder strikingly resembled Johne's disease in cattle and human intestinal tuberculosis, but without demonstrable acid-fast bacilli. From 1932 onwards, the disorder became known as Crohn's disease after Burrill B. Crohn, Leon Ginzburg and Gordon Oppenheimer published a case series of patients with 'regional ileitis' (1).

CD currently affects approximately 100 individuals per 100,000 in developed populations, incidence having increased dramatically in the second half of the twentieth century. The majority of cases occur in Western populations, including Europe and North America. Caucasians are more frequently affected than other ethnic groups, and females are slightly more frequently affected than males. The age of onset of CD follows a bimodal distribution, with a first peak of onset in the second and third decade, followed by a second peak in patients 60-79 years of age. The underlying reasons behind the trends in incidence and onset remain incompletely understood (2).

Clinical features of CD include chronic abdominal pain and diarrhoea, weight loss, gastrointestinal bleeding and malnutrition. There are often associated constitutional symptoms, which may include low grade fever, malaise and lethargy. The onset is typically insidious, although some patients may present acutely, either with fulminant symptoms or with complications such as toxic megacolon. Over 25% of patients also experience extra-intestinal manifestations of CD, which typically affect the eyes, joints and skin. Cutaneous manifestations include erythema nodosum and pyoderma gangrenosum; inflammatory ocular manifestations include uveitis, episcleritis and conjunctivitis, and in the joints patients may develop seronegative arthritis, ankylosing spondylitis and sacroiliitis. There is also an association with primary sclerosing cholangitis, a chronic condition affecting the bile ducts, characterised by progressive inflammation and fibrosis. Furthermore, the malabsorptive state experienced by some CD patients may lead to sequelae such as anaemia, metabolic bone disease, cholelithiasis, nephrolithiasis, and in children, failure to grow appropriately. Patients with colonic CD are also at increased risk of colorectal cancer. In most patients, the disease is relapsing and remitting, with flares of active inflammation interspersed with periods of remission ('quiescent disease'). At any one time, approximately half of all CD patients will have quiescent disease (3).

Considerable heterogeneity exists in the clinical presentation of CD between individual patients. Nevertheless, there are definable patterns of disease location, behaviour and age of onset. Although CD may affect any part of the gastrointestinal tract from mouth to anus, typically the terminal ileum (~40% of patients) and colon (~30% of patients) are affected. In approximately one third of patients, lesions occur in both large and small bowel ('ileocolonic disease'). Upper gastrointestinal tract involvement is relatively uncommon, occurring in less than 10% of patients. The region of the bowel affected is a major determinant of the predominant clinical symptoms

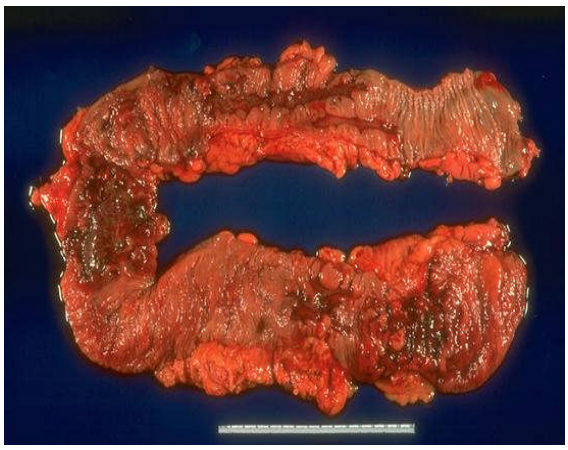
experienced by the patient. Patients may also be subdivided as having either pure inflammatory disease, stricturing, or penetrating disease (where abnormal connections between epithelial surfaces, termed 'fistulas' develop). In a small proportion of patients, a mixed picture of stenotic and fistulating disease is observed. The Montreal system has been developed for classification of CD phenotypes, incorporating age of onset, disease location and behaviour (Figure 1.1A) (4). However, it is also recognised that both disease location and behaviour are dynamic over time; for example, patients with inflammatory disease are at risk of developing stricturing and fistulating complications (5).

The lesions that characterise CD have a number of distinctive features, which help distinguish CD from other intestinal diseases. Typical macroscopic features include mucosal oedema and erythema, aphthous ulceration and discontinuous skip lesions (discrete patches of inflammation separated by normal mucosa). Together these give rise to a characteristic cobblestone appearance of the mucosa. Fistulas and strictures may also be observed in some patients. Additional macroscopic features identifiable from surgical resection specimens include changes in the vasculature (6) and fat wrapping (where the intestinal circumference becomes covered with adipose tissue and there is associated loss of the bowel-mesentery angle), the latter being strongly correlated with the presence of transmural inflammation (7). Histological features of actively inflamed bowel include transmural inflammation with leukocytic infiltration and lymphoid hyperplasia, often with non-caseating granulomata (aggregates of activated macrophages surrounded by a mantle of lymphocytes) (Figure 1.1B, C) (8). The early lesions observed in CD also have a characteristic distribution, tending to overlie Peyer's patches in the small intestine and lymphoid follicles in the colon (9). Peyer's patches are aggregates of lymphoid follicles covered by a dome-shaped 'follicle

A

Age at diagnosis	
A1	16 years or younger
A2	17-40 years
A3	Over 40 years
Anatomical location	
L1	Ileal
L2	Colonic
L3	Ileocolonic
L4	Isolated upper GI disease
Disease behaviour	
B1	Inflammatory
B2	Stricturing
B3	Penetrating
p	Perianal disease modifier

B



C

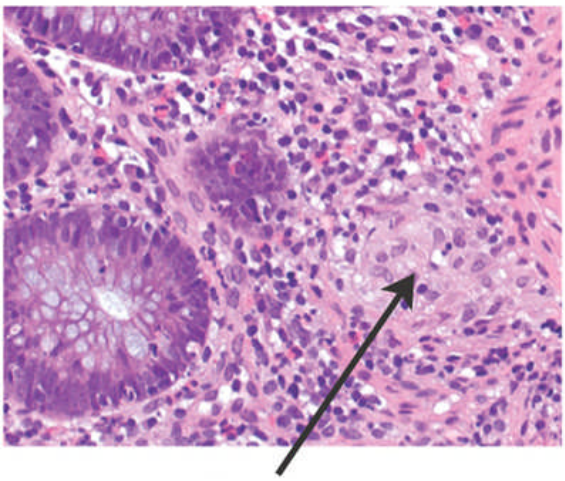


Figure 1.1 Clinicopathological features of Crohn's disease.
(A) Montreal classification of CD phenotypes, based on age at diagnosis, anatomical location and disease behaviour. Adapted from (4). (**B)** Colon from patient with CD, showing areas of ulceration separated by areas of normal mucosa (from http://www.pathology.washington.edu). **(C)** Ileal biopsy from a patient with active disease. The arrow indicates a granuloma. Image was obtained from (8).

associated epithelium' which may have important roles in the control of the immune response to luminal bacteria and antigens.

Currently, no curative treatments exist for CD. Management of CD involves induction of remission from an active flare, and the prevention of recurrence once a patient has entered the quiescent phase of the disease. Immunosuppressants form the basis of current medical management for inducing remission. These include corticosteroids, thiopurines such as azathioprine and its metabolite 6-mercaptopurine, 5-aminosalicylates (5-ASA) and biological therapies such as the anti-tumour necrosis factor (anti-TNF) agents infliximab and adalimumab. Surgical management may be required for patients with disease that is refractory to medical treatment, or in patients who experience complications such as intractable haemorrhage, perforation, recurrent obstruction or cancer (3). Furthermore, an important aspect of CD management is ensuring patients receive adequate nutritional support when indicated. This can be delivered as food or prepared formula feeds (enteral nutrition), and administered orally or via enteral feeding tubes and devices. Poor nutritional status is associated with an increased risk of complications after surgery, and growth retardation in paediatric patients. Nutritional therapy is also helpful in inducing remission from an active flare of CD. Indeed, evidence from randomised trials suggests that enteral nutrition might be as effective as corticosteroids in inducing remission in paediatric CD patients (10).

There is currently no 'ideal' maintenance therapy for preventing recurrence of CD. Systemic corticosteroids are not effective (11) and are associated with significant toxicity, and similarly 5-ASA compounds are poorly efficacious after medically-induced remission. Immunosuppressive agents such as azathioprine and methotrexate, and biological therapies are beneficial, but not without side effects. Azathioprine may cause pancreatitis, bone marrow suppression (12) and is associated with a small

increased risk of lymphoma (13). Potential adverse effects of biological therapies include infusion reactions, deterioration of heart failure (14) and increased susceptibility to infection, including reactivation of latent tuberculosis (15). Neither current medical treatments nor surgical resection of affected bowel can be considered 'curative'. The evaluation of novel therapeutic strategies for induction of remission and prevention of CD recurrence therefore remains a major area of investigation. Notably, in recent years, trials of pre- and pro-biotics, nutritional supplements such as omega-3 fatty acids (16), and immune stimulants such as granulocyte macrophage-colony stimulating factor (GM-CSF) (17;18) have been conducted in CD, some of which have shown promise.

1.1.2 Ulcerative colitis and other inflammatory bowel diseases

'Inflammatory bowel disease' encompasses several other distinct clinical entities besides CD, including ulcerative colitis (UC), eosinophilic colitis, lymphocytic colitis and collagenous colitis. Each has distinctive pathological features and is thought to have a different underlying aetiology. Of these, by far the most common is UC, which affects approximately 100 individuals per 100,000 in the United Kingdom (UK). The cardinal clinical symptoms of UC include diarrhoea, typically with blood and mucus, tenesmus and lower abdominal cramps. Malaise and low grade fever may also be present. As in CD, UC is a systemic disorder, and a sizeable proportion of patients experience extra-intestinal manifestations. The most common include arthritis, ankylosing spondylitis, iritis and uveitis, primary sclerosing cholangitis, erythema nodosum and pyoderma gangrenosum. Although there is overlap in the clinical presentation of UC and CD, there are a number of features which distinguish the two conditions and suggest that they are in fact distinct disease entities, each with a different underlying aetiopathogenesis.

UC typically affects the rectum and large bowel only (although in a small proportion of patients a 'backwash ileitis' occurs), in contrast to CD where the rectum is usually spared. 55% of patients have rectal inflammation only (proctitis), 30% have 'left sided' ulcerative colitis that does not extend beyond the splenic flexure, and 15% have inflammation that extends throughout the entire large bowel (pancolitis). Secondly, the inflammation in UC is typically continuous and superficial in nature, being characteristically restricted to the mucosa, in contrast to the transmural inflammation of CD. Other histopathological features of UC include crypt abcess formation and goblet cell depletion, which is much less conspicuous in CD. Granulomata are also not observed in UC (19).

1.1.3 Inflammatory bowel disease is associated with genetic and environmental factors

Epidemiological studies have indicated roles for both genes and environment in the pathogenesis of CD. Environmental factors associated with CD include smoking tobacco, which confers both increased risk of developing CD and also influences the clinical phenotype (2); smokers being more likely to have ileal disease as opposed to colonic or ileocolonic involvement (20). Dietary factors may also be important in the development of CD – in particular, associations have been reported between CD and intake of dietary fat (21;22) and refined carbohydrates such as sucrose (23;24).

It is also well established that CD has a genetic component, which has been confirmed by twin and family studies. Up to one third of patients have a positive family history of CD, numerous multiplex pedigrees have been reported (25-27), and furthermore, twin studies have consistently demonstrated a greater concordance in monozygotic than dizygotic twins (28;29). In addition, genome-wide association studies (GWAS) have identified genetic loci associated with CD susceptibility, which are discussed in detail in section 1.4.

UC is also associated with genetic and environmental factors. However, in contrast to CD, smoking appears to confer protection against the development of UC (30-32). Genetic factors also play a less prominent role, with reported concordance rates in monozygotic twins significantly lower than for CD (28). This further indicates likely differences in the aetiopathogenesis of the two conditions.

1.2 Theories of Crohn's disease pathogenesis

Numerous hypotheses have been proposed as to the underlying cause of CD. These include an infectious aetiology, a dysfunctional mucosal barrier, and an aberrant immunological response to antigens – including autoimmunity and immunodeficiency. Whilst each theory has some supportive evidence, no single hypothesis has been unequivocally proven. It is likely that CD has a complex aetiology, where multiple factors interact to give rise to a CD phenotype. This is supported by the wide range of environmental and genetic susceptibility factors that are associated with the development of CD. Another important consideration is that CD is a heterogeneous syndrome, as illustrated by the variability in clinical presentation and phenotype discussed in section 1.1.1. It is therefore plausible that the underlying pathological mechanisms differ between different patients, but give rise to the same overall outcome in the development of CD.

A fundamental area of contention is whether the immune response is generally over- or under- active in CD, and the relative contribution of both the intestinal barrier and microbiota in disease initiation and progression. Intuitively, a chronic inflammatory disorder such as CD would be expected to arise from a hyper-active immune response; this however assumes that the chronic inflammatory response is in itself the primary defect. In investigating complex syndromes such as CD, a considerable challenge is the

distinction of ‘primary’ or ‘initiating’ factors from phenomena that could be secondary or compensatory to other defects.

Another problem in defining the aetiology of CD is a lack of a clear animal model that accurately reproduces all the features of human CD. Numerous animal models of inflammatory bowel disease have been described, including chemically induced colitides, inbred animal strains, knockout or transgenic animals and adoptive transfer models (33;34). These are very useful tools to investigate the physiology of bowel inflammation, and potential pathological influences. However, the direct applicability of most to human CD is often unclear. There are striking differences in pathological features, with relatively few models demonstrating granuloma formation, discontinuous transmural inflammation and extra-intestinal manifestations.

1.2.1 Abnormal mucosal barrier function in Crohn’s disease

The intestinal mucosa is made up of a number of different cellular components, including a layer of simple columnar epithelial cells, and an underlying lamina propria and muscularis mucosa. Within the epithelial layer, a number of other cell types are present, including intraepithelial lymphocytes and goblet cells. In the crypts of Lieberkühn within the small bowel, Paneth cells are also found, which may have a role in secretion and in host defense. The gastrointestinal epithelial cells normally form a relatively impermeable physical barrier to luminal contents, a function that is facilitated by tight junctions. Tight junctions are multi-protein complexes composed of transmembrane proteins (such as occludins, claudins and junctional adhesion molecules), scaffolding proteins and regulatory molecules such as kinases, and act to impede the flux of solutes along the paracellular pathway (35). Tight junction function is thought to be a key determinant of intestinal permeability.

Mucosal surfaces are also covered by a thick layer of mucus, largely composed of mucin glycoproteins. Mucus acts as a physical and chemical barrier to microbes, provides lubrication for the flow of luminal contents, and concentrates antimicrobial molecules close to the intestinal epithelium. Antimicrobial compounds contained within the mucus layer include defensins, lectins, immunoglobulins (such as sIgA, IgG and IgM) and protease inhibitors. In addition, the mucus mixture also contains trefoil peptides, which may confer structure to the mucus layer and influence wound healing and apoptosis, and phospholipids, the precise role of which remain to be fully elucidated (36).

Several studies have reported increased bowel permeability in CD patients (37-39). The underlying mechanism remains debated, as does whether it represents a primary phenomenon or a consequence of chronic inflammation. Although many studies investigated active patients, and pro-inflammatory cytokines such as tumour necrosis factor (TNF), interferon (IFN)-γ and interleukin (IL)-1β are known to influence tight junction permeability (40;41), abnormal intestinal permeability has also been reported in macroscopically normal small bowel of CD patients (42), which may be predictive of disease recurrence (43). Increased permeability has also been found in a proportion of healthy first-degree relatives (44) and spouses (45) of patients with CD. Taken together, these observations suggest that abnormal permeability may be a primary defect, with both genetic and environmental influences. Alterations in tight junction proteins, including dislocation of Rab13, vasodilator-stimulated phosphoprotein and zonula-occludin 1 has been reported in the mucosa of patients with inactive CD, which indicate a possible molecular basis for the abnormal intestinal permeability via the paracellular pathway (46).

Aberrant transcellular permeability may also occur in CD. The early lesions of CD are characteristically distributed overlying Peyer's patches, the site of the follicle-associated epithelium where microfold (M) cells are located (47). M cells are specialised epithelial cells that are capable of transporting macromolecules and microorganisms from the intestinal lumen to the underlying lymphoid tissues within the mucosa (48-50). Interestingly, a recent study demonstrated that *Escherichia coli (E. coli)* could be readily translocated across M cells, particularly strains of *E. coli* associated with the CD mucosa. This was found to be modified by a variety of substances found in foodstuffs, including the emulsifier polysorbate-80 (a common component of processed foods), which increased the bacterial translocation. This may indicate a mechanistic link between dietary factors and abnormal intestinal permeability, and could partly account for the increasing incidence of CD in Western populations (51).

A number of animal studies underscore the importance of mucosal barrier dysfunction in the induction of bowel inflammation. Transgenic mice manipulated to express a dominant negative N-cadherin, a junctional adhesion protein, develop spontaneous bowel inflammation (52). Furthermore, the introduction of mutations in the mucin encoding gene *MUC2* also results in spontaneous colitis, although the histopathological features are more reminiscent of UC (53). Relatively few animal models demonstrate isolated small bowel inflammation, although the few that have been described highlight a likely role for intestinal permeability in the development of ileitis. These include the inbred SAMP1/ Yit (Samp) mouse strain, which displays increased ileal permeability, preceeding the onset of a spontaneous ileitis. In this model, the inflammation described is not dissimilar to human CD, with discontinuous, transmural leukocytic infiltrates and coalescence of macrophages into aggregates (54;55). In addition, cyclooxygenase-2 deficient mice fed an atherogenic diet containing cholate (a

primary bile acid) develop transmural inflammation predominantely affecting the ileum and caecum (56). A plausible explanation for this observation is the strong emulsifying action of bile acids, which could be damaging to the mucosa. Indeed, bile acids have been shown to increase permeability in CaCo2 monolayers (57) and bacterial translocation in human intestinal biopsy samples (58). This process could also be influenced by species of intestinal bacteria, some of which can deconjugate and convert primary bile acids to secondary bile acids such as deoxycholate, which may represent the more toxic species (59).

The increased mucosal permeability observed in CD could contribute to the disease pathogenesis by directly facilitating increased translocation of bacteria and other antigenic material from the lumen to the bowel wall. Alternatively it may be an indicator of reduced mucosal resistance, which could increase its susceptibility to the effects of exogenous agents, bacterial infection, bile acids and other emulsifiers, intra-luminal pressure and non-steroidal anti-inflammatory drugs. It is apparent that mucosal barrier dysfunction may in itself not be sufficient to cause CD, as illustrated by the demonstration of abnormal permeability in a proportion of healthy relatives and spouses of patients with CD, and furthermore in patients with other gastrointestinal disorders, such as irritable bowel syndrome (60;61) and coeliac disease (62). However, clearly if ingress of luminal contents into the underlying tissues is followed by an abnormal immune response to this material, the development of a chronic inflammatory state could be promoted.

1.2.2. Infectious aetiology, 'Dysbiosis' and Crohn's disease pathogenesis

The human gastrointestinal tract is in continuous contact with prokaryotes from birth onwards; it is estimated that over 10^{14} microorganisms are present in the human gut, including 1,000-1,150 different species (63). The number of prokaryotes varies

significantly with anatomical location. The highest concentrations of bacteria are found in the colon, which contains approximately 10^{11} cells/g, whereas the terminal ileum contains in the region of 10^{7}-10^{8} bacteria/g, and the proximal small bowel 10^{2}-10^{3}/g (64). Although the human 'microbiome' varies greatly between individuals (65), it is thought to be relatively stable over time in health. It has long been postulated that the intestinal microbiota could have a key role in the pathogenesis of inflammatory bowel disease. There is a clear requirement for bowel luminal contents in the pathogenesis of CD, as demonstrated by experiments where the faecal stream was diverted in CD patients, which resulted in resolution of CD lesions (66;67). Re-introduction of small bowel effluent into the excluded ileum resulted in disease recurrence.

Over the years, numerous specific microorganisms have been postulated as causative of CD (Figure 1.2). The diversity and lack of unifying characteristics between these organisms is noteworthy – the list includes bacteria, yeasts and viruses, Gram positive and Gram negative bacteria, and aerobic and anaerobic species. Polymerase chain reaction (PCR) and immunocytochemistry reveal a multitude of organisms can be detected in CD tissue samples, including *Mycobacterium avium* subspecies *paratuberculosis* (MAP) (68), *E. coli*, *Listeria*, *Streptococcus* (69), *Helicobacter pylori* (70), *Klebsiella*, *Pseudomonas aeruginosa* (71), and viruses such as Epstein Barr virus (72). In recent years, considerable research has been directed towards several particular species, including MAP, *Candida albicans (C. albicans)* and *E. coli.*

MAP has been suspected to play a causative role in CD since Dalziel initially recognised similarities between CD and Johne's disease, a granulomatous ileitis in cattle caused by *Mycobacterium paratuberculosis*. In support of a role for MAP in CD pathogenesis, mycobacterial 'spheroplasts' (cells which contain mycobacterial DNA but lack a complete cell wall) have been isolated from intestinal resection tissue (68;73).

Organism	Illustrative references
Bacteria	
Gram positive	
Streptococcus sp	Liu et al. (1995) Gastroenterology 108(5):1396–1404
Listeria monocytogenes	Liu et al. (1995) Gastroenterology 108(5):1396–1404
Clostridium sp	Bolton (1980) Lancet 1(8165):383–384
Gram negative	
Escherichia coli	Darfeuille-Michaud et al. (1998) Gastroenterology 115(6):1405–1413
Yersinia enterocolitica	Lamps et al. (2003) Am J Surg Pathol 27(2):220–227
Helicobacter sp	Puspok et al. (1999) Am J Gastroenterol 94(11):3239–3244
Bacteroides fragilis	Prindiville et al. (2000) Emerg Infect Dis 6(2):171–174
Pseudomonas sp	Wei et al. (2002) Infect Immum 70(12):6567–6576
Chlamydia trachomatis	Schuller et al. (1979) Lancet 1(8106):19–20
Coxiella burnetti	Kangro et al. (1990) Gastroenterology 98(3):549–553
Klebsiella pneumoniae	Tiwana (2001) Rheumatology 40(1):15–23
Mycobacteria	
Mycobacterium paratuberculosis	Chiodini et al. (1984) J Clin Microbiol 20(5):966–971
Mycobacterium kansasii	
Atypical	
Mycoplasma pneumoniae	Kangro et al. (1990) Gastroenterology 98(3):549–553
L-forms	Belsheim et al. (1983) Gastroenterology 85(2):364–369
Viruses	
Paramyxomavirus	
Measles	Wakefield et al. (1993) J Med Virol 39(4):345–354
Herpersvirus	
Epstein-Barr	Yanai et al.(1999) Am J Gastroenterol 94(6):1582–1586
Cytomegalovirus	Dimitroulia et al. (2006) Inflamm Bowel Dis 12(9):879–884
Yeasts	
Candida albicans	Standaert-Vitse et al. (2006) Gastroenterology 130(6):1764–1775
Saccharomyces cerevisiae	Main et al. (1988) BMJ 297(6656):1105–110

Figure 1.2 Micro-organisms postulated to have a role in CD pathogenesis. Adapted from (80).

These can transform into acid-fast mycobacterial species after long term culture. A subsequent study reported that MAP could also be cultured from blood samples of patients with CD (74). Furthermore, serological studies have demonstrated the presence of antibodies against MAP-specific proteins in a proportion of individuals with CD (75), and PCR (76) and Fluorescent in situ hybridisation-based methods (77) have confirmed the presence of MAP DNA in granuloma isolated from CD patients. A recent meta-analysis of 28 studies revealed that MAP positivity, as detected by PCR based techniques, occurred at a significantly higher frequency in CD than controls (odds ratio 7.01, 95% confidence interval 3.95-12.4) (78).

However, the hypothesis that MAP infection causes CD remains controversial. Firstly, there are a number of epidemiological, clinical and pathological differences between Johne's disease and CD (79;80). Secondly, a number of studies have not replicated a convincing association between MAP and CD. In one study of 130 CD patients, all blood cultures were found to be negative for MAP (81). Furthermore, other studies have failed to detect MAP in intestinal biopsy samples (82) and microdissected granulomas (83) from CD patients, raising the possibility that the reported MAP detection could be an artefact of contamination, or due to underlying geographic differences between CD patients. A recent trial of MAP eradication therapy in CD revealed equivalent relapse rates between control and placebo arms after 2 years therapy, in spite of demonstrating some short term benefit (84). Finally, the efficacy of anti-TNF agents in CD, which can trigger reactivation of mycobacterial infections as a side effect (15), seems incompatible with the notion that MAP infection is the underlying cause of CD. However a contributory role of MAP, in combination with other genetic and immunological factors cannot be excluded.

E. coli is a Gram negative coliform that is an important component of the normal intestinal flora. Many subspecies of *E. coli* exist without causing human disease, and indeed some strains may be beneficial, providing that the mucosa is not damaged. However, many strains have acquired specific virulence factors that give them an advantage in certain environments; these have been implicated in a number of different diseases (85). *E. coli* antigens were initially demonstrated in a proportion of CD tissues by immunocytochemistry (69). Furthermore, *E. coli* have been cultured from lymph nodes of patients with CD (86), and a small-scale study revealed that the majority of granulomas from CD patients have detectable *E. coli* DNA by PCR (87), suggesting a possible pathogenic role.

In the past decade, adherent, invasive strains of *E. coli* (AIEC) have been the focus of considerable attention. These subspecies, reported to be increased in faeces (88) and ileal mucosa (89;90) of patients with CD, display increased adherence to epithelial cell layers *in vitro*. These interactions may be facilitated by interaction of type I pili with carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) expressed on the apical surface of epithelial cells (91). Transgenic mice expressing human CEACAM6 develop a severe colitis after infection with a virulent strain of AIEC (92), supporting a potential role for these interactions in the development of bowel inflammation. However, CEACAMs are known to be upregulated by pro-inflammatory cytokines (91), and the presence of AIEC in epithelial cells is not readily demonstrable *in vivo*. As discussed in section 1.2.1, interactions with other specialised cell types such as M cells (51) could be a critical determinant in determining whether these species penetrate from the lumen into the bowel wall, which is likely be modified by exogenous agents and/or inherent mucosal barrier defects.

Once AIEC reach the underlying tissues of the bowel wall, they interact with cells of the immune system. In particular, they are understood to be capable of infecting macrophages, where they can survive and replicate without causing death of the host cell (93). High levels of the pro-inflammatory cytokine TNF are released by macrophage cell lines on infection with the bacteria. Furthermore, interactions of AIEC with macrophages may trigger granuloma formation, as demonstrated by studies in human peripheral blood mononuclear cells (PBMCs) (94). These findings point towards a potential role for *E. coli* and AIEC in CD pathogenesis, most likely via interactions with the mucosal barrier and the innate immune system.

C. albicans is a fungal organism present in the gastrointestinal tract commensal flora of a substantial proportion of the human population (95); the degree and frequency of colonisation has been reported to be increased in CD patients and their healthy relatives compared to controls (96). *C. albicans* typically colonises mucosal surfaces without causing disease. However, in immunocompromised individuals, the organism can cause opportunistic infections, some of which can be life threatening. The organism has a multilayered cell wall, consisting of an outer layer largely composed of mannans and mannoproteins and an inner 'skeleton' of β-glucans and chitin (97). The cell wall of *C. albicans* contains a mannan with a specific mannose α1-3 mannose terminal disaccharide. This is also present in other microbes, including *Saccharomyces cerevisiae* (*S. cerevisiae*) and some mycobacterial subspecies (98), and has been identified as an epitope for the generation of anti-*S. cerevisiae* antibodies (ASCA) (99;100). ASCA have been demonstrated in 50-60% of CD patients (101;102), are stable over time and do not correlate with clinical disease activity (103), and they may predict the development of inflammatory bowel disease in control individuals (104). The presence of ASCA may also be of diagnostic importance, as the prevalence is significantly greater in CD patients than in those with UC (102;105). ASCA are

detectable in 20-25% of healthy relatives of CD patients (106). ASCA titres correlate with *C. albicans* colonisation in healthy relatives of CD patients, although surprisingly, not in CD patients themselves (96). Together these observations may be indicative of a primary role for aberrant *C. albicans*-immune system interactions in the development of CD, which could be determined by a combination of genetic and environmental factors.

Recently, the concept of 'dysbiosis' has been a major subject of investigation in CD. This refers to 'qualitative or quantitative changes in the intestinal microflora profile, their metabolic activity and local distribution' (107). CD is characterised by a reduced diversity of faecal microbiota (108). Reduced numbers have been reported for the Firmicutes phylum, *Clostridium* cluster IV, *Clostridium* cluster XI and subcluster XIVa and higher numbers for *Bacteroides* species (108-110). Whilst interesting, caution should be taken when inferring whether these alterations represent primary phenomena in CD pathogenesis. CD is characterised by persistent diarrhoea, and many patients require immunosuppressant medication, which may have effects on the profile of intestinal microbiota (111). Some of the changes, particularly in the Firmicutes phylum, have also been observed in patients with infectious colitis (112;113). Dysbiosis has been reported in healthy relatives of patients with CD, although the alterations were not the same as those observed in the patients (114). This indicates that some of the changes that occur in CD could be secondary to the disease state, although further studies should be conducted to determine whether the alterations observed in the healthy relatives represent primary abnormalities.

Whilst numerous studies have demonstrated alterations in the intestinal microbiota in CD, and suggested roles for these organisms in the disease pathogenesis, to date no single infectious agent has unequivocally been shown to be sufficient to cause CD. MAP, AIEC and *C. albicans* are all detectable in a proportion of unaffected

individuals (78;90;96). Furthermore, CD is not regarded as a contagious disease. The disorder has a clear hereditary component, as demonstrated by recent genetic studies (115;116). Its pathogenesis also clearly involves an abnormal immune response, given the observation of leukocytic infiltrates and granuloma in CD lesions, and the efficacy of immunosuppressive medication in CD. In the light of this, it seems unlikely that the pathogenesis of CD can be wholly explained by an infectious agent.

However, given the clear role of luminal contents in the development of CD lesions, it is very likely that CD arises from abnormal host interactions with the intestinal microflora in susceptible individuals. In addition, alterations of the microflora could influence disease pathogenesis via effects on intestinal barrier integrity and immune system function. Indeed, there are indications that such effects could be diverse, and in part dependent on the species of microbe. On one hand, *Faecalibacterium prausnitzii*, a member of the Firmicutes phylum, is reduced in CD, and low levels within ileal mucosa are associated with endoscopic recurrence. This bacterium exhibits anti-inflammatory effects both *in vitro* and *in vivo* (117). On the other hand, there is evidence that bacteria such as bifidobacteria (which are also reduced in patients with CD (118)) are important for stimulation of immune function, including enhancement of phagocytic activity, as well as macrophage TNF and IL-6 production (119;120). Furthermore, the probiotic mixture VSL#3 was shown to prevent the onset of inflammation in Samp mice, which was related to stimulation of TNF release by epithelial cells (121). These apparently paradoxical observations may support the notion that the pathogenesis CD involves temporally distinct phases, each with different molecular mediators.

1.2.3 Autoimmunity

The case for CD arising from an autoimmune aetiology or hyper-reactive adaptive immune response stems from the observation of pathological leukocytic infiltrates and elevated levels of pro-inflammatory cytokines in CD lesions, when compared to normal healthy tissue. The infiltrating CD4+ lymphocytes in CD lesions appear to be characterised by high production of IL-2 and IFN-γ, giving rise to a Th1 type immunological response (122). Furthermore, immunosuppressive regulatory T cells are decreased in actively inflamed CD tissue in comparison to normal mucosa (123), and Th17 cells (pro-inflammatory T lymphocytes with a potential role in autoimmunity) are present (124). In support of the autoimmunity hypothesis, a number of autoantibodies have been detected in subsets of CD patients, mostly against antigens produced in exocrine pancreas, neutrophils and intestinal goblet cells (125;126). Furthermore, immunosuppressive drugs such as corticosteroids are effective in inducing remission. Finally, GWAS have highlighted potential associations with genes involved in the IL-23 axis, which may relate to abnormal Th17 cell regulation, although these are not specific for CD (116).

However, a number of observations argue against autoimmunity being a primary aetiological factor in CD. Firstly, CD does not fulfil Witebsky's postulates for an autoimmune condition and there is no animal model that reproduces all of the clinicopathological features of the human disease (127). The mechanistic significance of the auto-reactive lymphocytes and autoantibodies to the disease pathogenesis remains unproven. In addition, many genetic studies have failed to show significant associations between CD and a particular human leukocyte antigen (HLA) haplotype, with the possible exception of HLA DRB1*0103, which has been associated with colonic CD in several studies (128;129). The lack of strong HLA association is in contrast to well-

established autoimmune diseases such as type 1 diabetes, where HLA associations can be readily identified by linkage and GWAS analysis, and are understood to account for a significant portion of the genetic risk (130;131). Indeed, recent genetic variation 'profiling' using data collected in the Wellcome Trust Case Control Consortium (WTCCC) study revealed minimal clustering of CD with five autoimmune conditions, including multiple sclerosis, ankylosing spondylitis, autoimmune thyroid disease, rheumatoid arthritis and type 1 diabetes (132). Furthermore, whilst there are clearly associations with CD to the IL-23 axis, there are indications that the functions of this cytokine axis are diverse and not restricted to Th17 regulation. Indeed, studies in mice have revealed an important role for IL-23 in protection against infection, including ensuring optimal neutrophil recruitment and bacterial clearance in response to infection with *Listeria monocytogenes* (133).

1.2.4 Immunodeficiency

It was first recognised in the 1970s that CD may arise from an immune deficiency state, characterised by an impaired acute inflammatory response. Early studies demonstrated this phenomenon using 'skin windows': small dermal abrasions that permit *in vivo* assessment of acute inflammation. Neutrophil accumulation into skin windows was found to be dramatically impaired in CD patients (134). More recently, the impaired neutrophil recruitment was also shown to occur in the bowel, using a novel 'serial biopsy' technique, where paired samples were taken from same area of normal rectal or ileal mucosa at baseline (to initiate an acute inflammatory response) and 6 hours subsequently (135). Although a deficient immune response may at first seem incompatible with the chronic inflammatory state that characterises CD, it was postulated that a sub-optimal acute inflammatory response would lead to impaired clearance of bacteria from the bowel wall. The persistence of this material could trigger

an adaptive immunological response, which would ultimately result in the development of chronic inflammation, and constitutional symptoms.

Recently, this hypothesis was investigated experimentally by subcutaneous injection of killed *E. coli* into the forearms of patients with CD and controls. In healthy control subjects, a dramatic increase in local blood flow at inoculation sites was observed, as determined using laser Doppler scanning. This response was grossly attenuated in CD patients, indicating an underlying weak acute inflammatory response. Accumulation of ^{111}indium labelled neutrophils to these sites was also impaired in CD, in concordance with the previous findings in skin windows. To determine whether this affected bacterial clearance, an additional set of experiments were performed, in which the bacteria were labelled with ^{32}P, and the rate of disappearance of radioactivity from the injection sites determined. Clearance was found to be dramatically slower in CD patients compared to controls. Extrapolation of clearance curves to a point where 99% of the inoculated material would be cleared predicted total clearance times of 44.3 days in CD patients, compared to 10.2 days in healthy control individuals (136).

Importantly, the defects in neutrophil accumulation and bacterial clearance were demonstrated in patients with inactive CD, and were not apparent in patients with other chronic inflammatory conditions, such as inactive UC, indicating that the phenomenon is not simply a consequence of a chronic inflammatory state (134-136). Although one study demonstrated the presence of serum chemotaxis inhibitors in CD that were purported to explain the observation of impaired neutrophil recruitment (137), active patients were investigated in this study, and inhibitors were also found in patients with UC, suggesting that this is not likely to be the underlying mechanism, at least in patients with quiescent CD.

In support of the immunodeficiency hypothesis, patients with congenital, monogenic disorders of phagocyte function frequently develop non-infectious bowel inflammation that is markedly similar to CD. Chronic granulomatous disease (CGD) is one such disorder, which is characterised by defective killing and digestion of bacteria by neutrophils, as a result of mutations in NADPH oxidase (138). Approximately half of these patients develop bowel inflammation in the absence of a demonstrable microbial infection, which is clinically and histopathologically indistinguishable from the inflammation that occurs in CD (139). In addition, strong associations between other monogenic disorders of neutrophil function and CD-like enteritis have been highlighted in recent reviews. Mutations in single genes can result in failure of neutrophil production and accumulation (congenital neutropenias and leukocyte adhesion deficiency), impaired digestion (glycogen storage disease 1b) and aberrant vesicle trafficking and phagolysosomal fusion (Chediak-Higashi and Hermansky-Pudlak syndrome), all of which result in a grossly impaired innate immune response to bacteria. The observation of non-infectious bowel inflammation in a substantial proportion of these patients bolsters support for a model of CD pathogenesis in which innate immunodeficiency and defective clearance of bacteria plays a critical role (140). In contrast, primary and secondary adaptive immunodeficiency disorders do not appear to be strongly associated with CD, with the possible exceptions of common variable immunodeficiency syndrome and Bruton's agammaglobulinaemia, where B cell and antibody dysfunction predominate (141).

The concept that CD involves an attenuated initial innate immune response is in also in keeping with the histopathological features of CD, especially the observation of granulomatous inflammation. Granulomata are frequently observed in CD lesions; a recent meta-analysis of 21 studies reported that 47.0% of CD patients have demonstrable granulomata (142). In fact, the true proportion of patients may well be

even greater than this, as illustrated by a comprehensive step sectioning study of surgically resected intestines from CD patients, which demonstrated the presence of granulomata in all individuals (143).

Granulomatous inflammation is a characteristic immunological response following exposure to antigens that are inadequately cleared by innate immune cells, such as the bacterium *Mycobacterium tuberculosis*, and functions to contain the material and prevent further dissemination (144). Whilst certain stimuli may inevitably trigger granulomata formation in a host, in other cases defective phagocyte function may be the critical precipitant, such as in patients with CGD. These individuals develop granulomatous inflammation in response to relatively non-pathogenic stimuli such as coagulase negative staphylococci (145;146) It is conceivable that in CD, inadequate neutrophil influx to sites of bacterial ingress in the bowel, and defective clearance of this material, could be followed by granuloma formation as a subsequent compensatory response.

Although there are few animal models that convincingly describe colitis occurring in conjunction with innate immunodeficiency, a number of mouse models support a role for defective phagocyte function in the generation of intestinal inflammation and merit discussion here. Mice with targeted deletions of *STAT3* are one such example, where impaired innate immune function, including reduced NADPH oxidase activity, was demonstrated. Interestingly, these mice had histopathological features reminiscent of CD, with transmural inflammation and granuloma formation (147). Additionally, mice with certain innate immune defects may have a more severe phenotype in dextran sodium sulphate (DSS) induced models of colitis. Of particular note, mice deficient in Toll-like receptor 4 (TLR4), a key pathogen pattern recognition receptor (PRR) of the innate immune system, have earlier and more pronounced

gastrointestinal bleeding than wild type mice after administration of DSS. Interestingly, this coincides with increased bacterial translocation and impaired neutrophil recruitment (148).

Recent GWAS have highlighted associations with variants in genes with roles in innate immune function, including *NOD2*, *ATG16L1* and *IRGM* (116). These associations add credence to the concept that CD involves impaired innate immunity, and are discussed in more detail in section 1.4.

If CD arises from a systemic defect in innate immunity, one might expect patients to manifest increased susceptibility to infection. This may well be the case, and several studies have reported higher incidences of acute gastroenteritis (149) and urinary tract infection (150) in patients with CD. However, larger scale studies are required to confirm this finding, which must take into account important confounding factors such as surgery, malnutrition and use of immunosuppressant medication (80;136). Another important consideration is bacterial load – most acute infections arise from the multiplication of a small number of initial inoculating organisms, which even the partially attenuated immune response in CD may be able to control. In contrast, the terminal ileum and colon contain a large number of bacteria (approximately 10^8/g and 10^{11}/g respectively, which could potentially 'overwhelm' the impaired clearance mechanisms. Interestingly, subcutaneous injection studies revealed that the defective clearance of ^{32}P labelled *E. coli* was dose-dependent. At lower doses of bacteria (10^6 or less), clearance of bacteria was normal in CD, whereas at larger doses, grossly delayed clearance was observed (136).

1.2.5 Integrated models of Crohn's disease pathogenesis

Given the various strands of supportive evidence for each theory, it is possible that mucosal barrier dysfunction, the balance of the intestinal microflora, innate

immunodeficiency and chronic inflammation act together in CD pathogenesis to generate lesions and constitutional symptoms. Our laboratory has recently proposed a '3 stage model' of the disease pathogenesis, whereby CD occurs in three temporally distinct stages. The first of these is ingress of bacteria and/or antigenic material into the bowel wall, potentially enhanced by an underlying abnormality in the mucosal barrier. In stage 2, innate immunodeficiency results in impaired clearance of this material. Chronic granulomatous inflammation and adaptive immune responses are subsequently provoked in stage 3, culminating in the development of CD (Figure 1.3) (151).

1.3 Impaired acute inflammation in Crohn's disease

1.3.1 Acute inflammation and the role of macrophages

Acute inflammation is a generic mechanism by which the body responds to infection, trauma and other potentially harmful stimuli, and is associated with the cardinal signs of heat, redness, swelling, pain and loss of function. The acute inflammatory process is associated with various cellular events, the most striking of which is recruitment and infiltration of neutrophils and monocytes to the inflammatory site, which enables killing and clearance of microorganisms and removal of harmful material. Initially, neutrophils are recruited to sites of inflammation, which peak between 4 and 6 hours after an insult. Subsequently, the number of infiltrating mononuclear cells rises, reaching a peak at 18 to 24 hours (152).

Acute inflammation may be initiated by a number of mechanisms, including triggering of the complement cascade by microbial components or antibody, and by recognition of stimuli by resident cells such as macrophages. Subsequently various important mediators of inflammation, including pro-inflammatory cytokines, chemokines, nitric oxide, histamine and lipids such as leukotrienes and prostaglandins act to promote and propagate the acute inflammatory response (153). A number of these

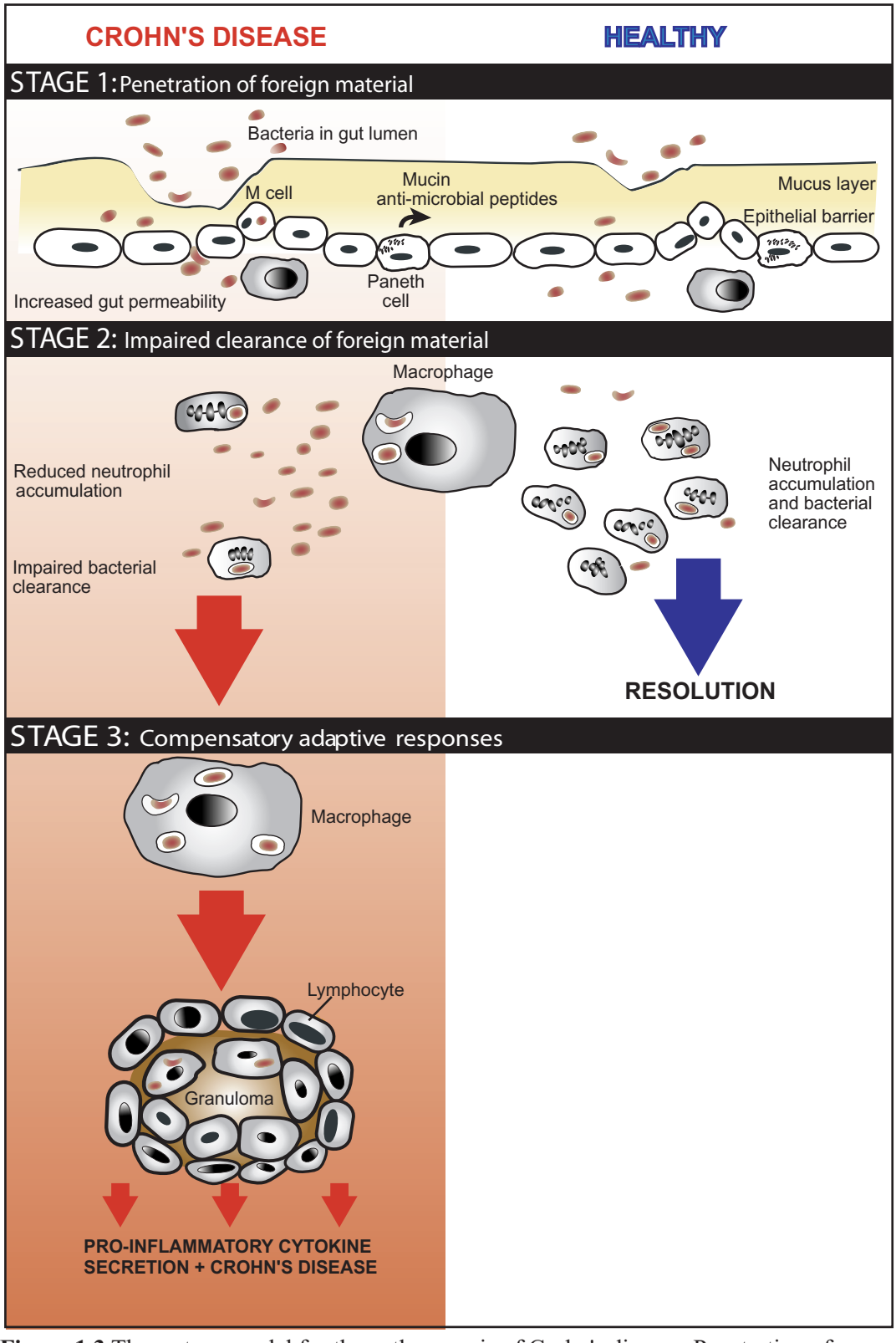


Figure 1.3 Three stage model for the pathogenesis of Crohn's disease. Penetration of luminal contents occurs in stage 1, via paracellular and transcellular pathways, facilitated by an abnormal mucosal barrier. In stage 2, healthy indviduals mount a strong acute inflammatory response, characterised by neutrophil accumulation and clearance of material. In CD patients, neutrophil influx is defective as a consequence of impaired macrophage function, resulting in impaired bacterial clearance. This acts as a trigger for stage 3 (compensatory adaptive responses), which leads to chronic pro-inflammatory cytokine secretion and the development of the CD lesion.
Adapted from (151).

trigger changes in the vasculature, including vasodilatation, alterations in permeability and upregulation of adhesion molecules, which facilitate the recruitment of leukocytes to the inflammatory site. Recruitment of leukocytes such as neutrophils is a tightly regulated, multistep process that involves successive interactions between leukocytes and endothelium (154). Initially, neutrophils interact loosely with endothelial cells via selectins (155). Subsequently, activation of neutrophils enables integrin-dependent, stable interactions to occur with the endothelium, allowing extravastation through the endothelium to sites of acute inflammation, down a chemoattractant gradient (155;156).

In the subsequent days and weeks, the inflammation may resolve in a co-ordinated fashion, in a process that involves apoptosis of neutrophils and their clearance by macrophages (157;158). This may trigger an alteration in macrophage cytokine production, whereby release of IL-1β, IL-8 and IL-10 is inhibited and production of transforming growth factor-β (TGF-β), a critical cytokine in wound healing, is increased (159;160). Various lipid mediators such as lipoxins have a pivotal role at this stage of inflammation (161). Alternatively, the inflammation may not resolve and progress to a chronic inflammatory state. Clearly, whether or not inflammation persists could well be dependent on both the adequacy of the acute inflammatory response to clear material, as illustrated by granulomatous conditions such as tuberculosis, CGD and CD, and on the resolving mechanisms, as illustrated by UC, where bacterial clearance is normal but persistent, non-resolving inflammation is observed (162;163).

Phagocytic cells, including neutrophils and macrophages, play a pivotal role in acute inflammation. Macrophages differentiate from circulating peripheral blood mononuclear cells (PBMCs), which migrate into tissues in both the steady state and in response to inflammatory stimuli (164;165). Their key functions include sensing of microbial stimuli, phagocytosis, antigen presentation and pro-inflammatory cytokine

secretion. In later stages, macrophages also have a critical role in resolution of the inflammatory response, maintenance of tissue homeostasis and promotion of wound healing.

Macrophages express a rich array of receptors that enable microbe detection. These receptors are located in the plasma membrane, cytosol and vacuolar compartments, thereby facilitating recognition of both extracellular and intracellular pathogens. Toll-like receptors (TLRs), Nod-like receptors (NLRs) and C-type lectin receptors, termed pattern-recognition receptors (PRRs) because of their ability to sense pathogen-associated molecular patterns (PAMPs), are critically important in the sensing of microbial components and induction of innate immunity (166). TLRs are type I transmembrane receptors that contain an extracellular leucine-rich repeat domain and an intracellular Toll / IL-1 receptor (TIR) domain (167;168). Their role in innate immunity was originally discovered in *Drosophila* (169), and following this human homologs of *Drosophila* Toll were identified (170), which have similarly important immunological functions.

Mammalian species have at least 10 different TLRs; each recognising specific microbial components. Key TLRs involved in the recognition of bacterial stimuli include TLR2, TLR4, TLR5 and TLR9. TLR2 recognises a range of ligands including lipoteichoic acid, peptidoglycan and a synthetic tripalmitoylated lipopeptide (*N*-palmitoyl-*S*-[2,3-bis(palmitoyloxy)-propyl]-(*R*)-cysteinyl-(lysyl)3-lysine (Pam_3CSK_4)). This broadness in specificity may be partly enabled by the ability of TLR2 to form heterodimeric complexes with both TLR1 and TLR6 (166;171). TLR4 functions as the receptor for lipopolysaccharide (LPS) (172), a component of Gram negative bacteria, and TLR5 is involved in recognition of flagellin, a protein found in bacterial flagella (173). In contrast, TLR9 recognises unmethylated CpG motifs in bacterial DNA (174).

Engagement of ligand with TLR results in activation of downstream signalling transduction pathways. Depending on the specific TLR, these may involve the myeloid differentiation primary response protein 88 (MyD88) adaptor (MyD88-dependent signalling), or other adaptors such as TIR domain-containing adaptor inducing IFN-β (TRIF) (175). Activation of downstream signalling cascades leads to induction of pro-inflammatory cytokines and chemokines, which are critical in the initiation of an acute inflammatory response and leukocyte recruitment (170).

Whilst macrophages clearly have a generalised role in orchestration of acute inflammatory responses, it is well recognised that the phenotype and function of both monocytes and macrophages is heterogeneous and dependent on the stimuli received from the surrounding environment. Treatment of macrophages with IFN-γ increases their microbicidal activity, and the levels of secreted pro-inflammatory cytokines such as IL-12, TNF and IL-23 (176;177). On the other hand, exposure of macrophages to cytokines such as IL-4 and IL-13 results in 'alternative activation'. Alternatively activated macrophages are characterised by high release of IL-10 and IL-1 receptor antagonist, and expression of distinctive markers such as CD163 and scavenger receptors; they may have roles in tuning inflammatory responses, wound healing and angiogenesis (177;178). They may also be involved in the response to helminths. This distinction has led to the concept of M1 (classically activated) and M2 (alternatively activated) macrophages, although it is increasingly recognised that the macrophage phenotype may be more of a 'continuum', with M1 and M2 representing extremes of phenotype and opposite ends of a spectrum (178).

1.3.2 Mechanism of impaired neutrophil recruitment in Crohn's disease

Although neutrophil accumulation to sites of acute inflammation is defective in CD, in the majority of patients, various parameters of neutrophil function appear normal. No

differences were observed in the chemotactic response of CD neutrophils *ex vivo* (179), and random motility of CD neutrophils was found to be normal in another study (180).

Investigations of the neutrophil respiratory burst in CD yielded somewhat conflicting results, with some studies demonstrating increased or normal respiratory burst while others showed a reduction (181-184). A more recent study conducted in our laboratory using a large cohort (n=100) of quiescent patients demonstrated a modest (but statistically significant) reduction in superoxide generation in response to stimulation with phorbol-12-myristate-13-acetate (PMA) in the CD cohort compared to controls. However, as digestion of ^{35}S-methionine-labelled *E. coli* was shown to be normal in the majority of patients, the biological or pathogenic significance of the decreased respiratory burst may be minimal. In spite of this, the study did highlight three patients with congenital disorders of neutrophil function and concurrent CD, in whom both the respiratory burst and bacterial digestion were shown to be defective (185). In one of these patients, a mutation in glucose-6-phosphatase catabolic-3 (G6PC3) was demonstrated, with concurrent effects on N- and O- linked glycan synthesis and glycosylation of gp91 phox, a component of NADPH oxidase (186).

If the majority of patients with CD do not have an inherent defect in neutrophil function, why is neutrophil recruitment to sites of inflammation so dramatically impaired? It was postulated that an underlying defect in macrophages could be responsible for the impairment in acute inflammation. Macrophages cultured from quiescent CD patients showed decreased production of interleukin-8 (IL-8) in response to stimulation with C5a, wound fluid and TNF (135). Furthermore, in response to stimulation with heat-killed *E. coli* (HkEc), CD macrophages released deficient levels of a range of pro-inflammatory cytokines in comparison to healthy control (HC) cells, including TNF, IL-4, IL-5, IL-13, IL-15 and IFN-γ. In response to HkEc, no difference

was observed in the secreted levels of the chemokines IL-8, IP-10 and RANTES, or in the release of the anti-inflammatory cytokines IL-10 and IL-1Ra (136). The release of TNF from CD macrophages was compared with HC macrophages at 4 hours, 6 hours and 24 hours after stimulation with HkEc. TNF reached maximal secretion within 6 hours in HC and CD cells. Depressed levels of TNF release from CD macrophages were observed all time points, suggesting that the impairment in pro-inflammatory cytokine release is absolute and not the result of delayed kinetics. The finding of impaired TNF release from CD macrophages was also replicated in a recent study that used live *E. coli* and MAP as stimuli. However, diminished IL-10 release and increased IL-23 secretion were also found. It is possible that this could be an effect of disease activity, as no reference was made as to whether patients included in this study were in remission or had active disease (187).

1.3.3 Mechanism of defective macrophage cytokine secretion in Crohn's disease

Interestingly, the defect in pro-inflammatory cytokine release did not appear to arise from abnormal control of cytokine gene transcription. Microarray analysis of the gene transcription profiles of HC and CD macrophages revealed equivalent levels of pro-inflammatory cytokine mRNA after stimulation with HkEc; a finding that was confirmed by quantitative PCR. Furthermore, in spite of the impaired release, no difference in TNF mRNA stability was observed between HC and CD macrophages. The defect also did not appear to relate to a difference in M1/ M2 macrophage populations. Initial M1 ($CD14^+CD16^-$) and M2 ($CD14^+CD16^+$) monocyte populations were similar between HC, CD and UC patients, and mRNA levels of maturation markers F4/80, L-selectin and ICAM-1 were similar after *in vitro* differentiation (136).

However, the impaired release in pro-inflammatory molecules from CD macrophages appeared to be due to an underlying defect in vesicle trafficking. The

amounts of intracellular cytokines, including TNF were investigated by western blotting. Reduced amounts of intracellular cytokines were found in CD macrophages compared to HC; however incubation with brefeldin-A, an inhibitor of protein translocation from the endoplasmic reticulum (ER) to the Golgi apparatus, resulted in normalisation to equivalent levels between HC and CD cells. This suggested that there was no abnormality in cytokine gene translation; rather the defect must lie in the post-translational trafficking of cytokines for secretion (136).

The role of the lysosome in intracellular cytokine trafficking was investigated by inclusion of monensin and chloroquine during macrophage HkEc stimulation. Chloroquine is a weak base that partitions into acidic compartments and elevates the pH, whereas monensin acts as a sodium-proton ionophore; both however have the consequence of inhibiting lysosomal function. In CD macrophages, incubation with the lysosomal inhibitors significantly increased the levels of intracellular cytokines, which did not occur in HC cells. Overall, these observations suggest that in CD macrophages, a trafficking defect results in mistargeting of pro-inflammatory cytokines to lysosomal compartments, where they are degraded rather than released through the normal secretory pathway. Interestingly, lysosomal, rather than proteasomal degradation of proteins appeared to be the central to the impairment in cytokine secretion, as incubation of cells with MG132 (a proteasomal inhibitor) was without effect (136). However, the molecular basis of this abnormality remains uncharacterised.

1.3.4 Pathways of cytokine secretion and relevance to CD

The process of cytokine secretion is highly regulated and is thought to proceed through a number of distinct pathways. The pathways utilised differ for individual cytokines and vary between cell types. Certain cytokines are thought to be secreted primarily via the classical ‘canonical’ secretory pathway, whereas others (such as IL-1β) are secreted by

alternative, unconventional pathways. In some situations, cytokines are also packaged into granules following processing in the Golgi complex, where they are stored until appropriate signals for release are received (188;189).

The canonical secretion pathway commences with protein synthesis in the ER, where proteins are appropriately folded and quality checked. Proteins are subsequently loaded onto vesicles for transport to the Golgi apparatus. In the Golgi apparatus, proteins are further modified and glycosylated before reaching the Trans-Golgi network (TGN), the last station in the Golgi complex. After the TGN, a diverse range of vesicles, carrier proteins and organelles transport proteins to the cell surface membrane, where they are released from the cell by exocytosis (188;189). In specialised cells such as macrophages, components of these pathways can be upregulated upon cell activation to increase the trafficking of proteins such as cytokines, thereby promoting their release.

Many studies directed towards characterising the molecular mechanisms of cytokine secretion have focused on the control of TNF secretion from cell lines such as RAW cells (a murine macrophage cell line). TNF is synthesised as a 26kDa precursor molecule in the endoplasmic reticulum and in most situations is released via the canonical pathway. TNF is delivered via recycling endosomes to the cell surface, where the transmembrane precursor is cleaved by TNF converting enzyme (TACE), to release from the cell a 17 kDa protein, which acts in a paracrine manner (190). It is generally accepted that the majority of cells are not able to pre-store TNF in granules to any significant degree, with the exception of mast cells, which are able to direct TNF towards granules in a mannose-6-phosphate receptor dependent (MPR) pathway, and release it upon mast cell degranulation (191;192).

TNF trafficking from the TGN to the plasma membrane in macrophages is dependent on a number of molecules. At the TGN, p230, a trans-Golgi network golgin

that labels a subset of tubulovesicular structures, is required in the first steps of TNF secretion (193). TNF is transported from the TGN to recycling endosomes through a complex of Soluble NSF Attachment protein receptor (SNARE) proteins, which includes syntaxin 6 and Vti1b (194) . Recycling endosomes appear to have a critical role for subsequent delivery of TNF to the plasma membrane, as demonstrated by recent studies. TNF was shown to colocalise with the transferrin receptor and the SNARE protein vesicle-associated membrane protein 3 (VAMP3), both of which are markers of the recycling endosome, and expression of a dominant negative Rab11 protein (another recycling endosomal marker) resulted in impaired delivery of TNF to the cell surface (194). TNF is subsequently delivered to cholesterol-dependent lipid rafts on the plasma membrane, especially concentrated around phagocytic cups (195).

In addition to the vesicle trafficking proteins, a number of other intracellular components are essential for efficient post-Golgi trafficking of TNF. The first is an intact cytoskeleton, as agents such as nocodazole and cytochalasin D, which disrupt microtubules and F-actin filaments respectively, inhibit TNF secretion in RAW macrophages (196). The second is appropriate membrane lipid composition, which will be governed by the rate of synthesis of lipid species, relative to how rapidly they are broken down. The balance of sphingolipids, phospholipids and cholesterol within a membrane can affect its ability to fuse (‘fusogenicity’), as well as membrane budding and fission, thereby exerting a strong influence on intracellular vesicle trafficking. This is discussed in more detail in chapter 4. Of particular note, one study recently demonstrated the importance of phosphatidylcholine, an abundant phospholipid in eukaryotic membranes, in the secretion of TNF. Cells that are deficient in CTP: phosphocholine cytidylyltransferase (CCT), an enzyme that catalyses the rate limiting step in phosphatidylcholine biosynthesis, show an interesting parallel to CD macrophages in that they secrete deficient levels of TNF compared to wild type cells

upon activation. Although the synthesis of TNF appeared to be unaffected in these cells, confocal microscopy revealed retention of TNF within the Golgi apparatus, explaining the impaired release into the medium. This phenomenon was found to be relevant in vivo, as CCT knockout mice developed a more diffuse pneumonia after inoculation with *Streptococcus pneumoniae*, and a higher mortality rate compared to wild type mice (197).

The intracellular trafficking of other cytokines is generally less well characterised than for TNF, however it is appreciated that other cytokines may be secreted by distinct mechanisms. IL-6, another important pro-inflammatory cytokine, also requires recycling endosomes for its secretion, but distinct subcompartments from those utilised by TNF (198). Other cytokines such as IL-1β are thought to be released through alternative, non-canonical pathways. IL-1β is released independently of the ER and the Golgi apparatus, in a process that may depend on membrane-derived microvesicles. At some point between the synthesis of IL-1β on free ribosomes and its release, cleavage of pro-IL-1β by caspase-1 occurs, enabling the mature form of IL-1β to be released (188). This processing is in turn dependent on inflammasome assembly and activation. Inflammasomes are multiprotein complexes, activated upon infection or cellular stress, that mediate caspase-1 dependent processing of pro-inflammatory cytokines (199). Genetic variation in NLR family, pyrin domain-containing 3 (*NLRP3*), a component of the NLRP3 inflammasome, has been associated with CD (200), suggesting a possible relevance to the disease pathogenesis. However, the associations have not been universally replicated in other cohorts (201).

Certain cytokines may be released by multiple mechanisms, for example in the case of IL-15, which is primarily understood to traffic via the canonical pathway in complex with its high affinity receptor subunit, IL-15Rα (202). However, monocytes

express IL-15 at the cell surface independently of the IL-15Rα, suggesting contributions from non-canonical pathways in IL-15 release in these cells (203).

An unanswered question is the degree to which these pathways overlap between different immune cell types, for example macrophages and lymphocytes. In addition, as many studies of cytokine trafficking have utilised cell lines and animal models, the relevance to primary human macrophages is unclear. Nevertheless, given the prominent defect in pro-inflammatory cytokine secretion observed in CD macrophages, which appears to relate to an underlying defect in vesicle trafficking, such studies may point to interesting candidate molecules that warrant further investigation. The contribution of any of these molecules to the impairment in cytokine secretion observed in CD is as yet undetermined. Of note, a number of lines of evidence suggest abnormal fatty acid and lipid compositions in CD, which could exert strong effects on vesicle trafficking in macrophages, as discussed in chapter 4. In addition, GWAS have indicated associations with a number of genes that have roles in autophagy and intracellular trafficking, including *ATG16L1* and *IRGM* (discussed in detail in section 1.4). It is also possible that polymorphisms in these genes influence disease pathogenesis by interfering with intracellular trafficking and secretion of pro-inflammatory cytokines in macrophages.

1.3.5 Other macrophage defects in Crohn's disease

The macrophage defects in CD may not be restricted to abnormal pro-inflammatory cytokine secretion alone. A recent study conducted in our laboratory demonstrated defective apoptosis of macrophages in CD after stimulation with the phorbol ester PMA. This was shown to relate to impaired mitochondrial depolarisation and cytochrome c release on stimulation with PMA. Given the prominent role for both neutrophil and macrophage apoptosis in the resolution of inflammation (204), it is plausible that the increased resistance of CD macrophages to apoptosis leads to their

persistence in inflamed bowel tissue. This would prolong cytokine secretion and result in persistent, non-resolving inflammation and thus promote the development of CD lesions. The same study also demonstrated diminished generation of H_2O_2 in macrophages upon activation with PMA, although this did not appear to mechanistically relate to the impairment in apoptosis (205). It is likely that defective apoptosis could represent a more general phenomenon in CD, as neutrophils (206) and lamina propria T cells (207) also display abnormalities in this process.

Interestingly, the monoclonal antibody infliximab, a widely utilised drug in the management of CD, has been proposed to exert its beneficial effects by cross-linking of membrane bound forms of TNF and stimulation of leukocyte apoptosis within the lamina propria (208). This substantiates a possible role for abnormal apoptosis in CD pathogenesis, and furthermore indicates that the mechanism of action of the anti-TNF agents may be indirect, rather than inhibition of TNF *per se*.

1.3.6 Macrophage function in ulcerative colitis

In contrast to CD, monocyte-derived macrophages from UC patients were found to release normal levels of TNF and IFN-γ after stimulation with HkEc. After stimulation with HkEc and LPS, UC macrophages were shown to release increased amounts of CXCL10, RANTES and IL-12p70. After TLR4 stimulation, secreted levels of IFN-β were also found to be significantly increased, relating to overactivation of the TLR4-TRIF signalling pathway (163).

The elevation in pro-inflammatory cytokine release may account for the impaired resolution of inflammation in UC patients observed after subcutaneous injection of HkEc (135;162;163). These findings further highlight the fact that UC and CD are distinct disease entities, with different underlying immunological abnormalities and pathogenetic mechanisms. Interestingly, specific ‘probiotic’ bacteria (*Lactobacillus*

casei) have been shown to inhibit CXCL10 secretion by inhibiting vesicular pathways involved in its release, which may explain the protective effect of VSL#3 in murine models of bowel inflammation (209). This could indicate that certain probiotics may be of clinical benefit in patients with UC.

1.4 Genetic factors in Crohn's disease and their relationship to impaired acute inflammation

If CD arises from a systemic defect in acute inflammation, one would predict certain sequence variants in genes with roles in innate immune function to confer susceptibility to CD. Recently, considerable advances have been made in understanding genetic loci that are associated with CD, and a number of these contain genes with known or predicted roles in innate immunity. Initially, linkage analysis in familial cases of CD and positional cloning strategies identified a number of important susceptibility loci. More recently, the advent of the Human Genome and International HapMap projects, coupled with recent technological advances have enabled GWAS to be conducted in CD and other complex disorders. In such studies, genotyping of up to one million selected SNPs is performed in thousands of cases and controls in a high throughput manner using whole genome microarrays. For each SNP, the allele frequency in cases and controls is compared to identify disease-associated polymorphisms. Whilst GWAS are regarded as 'hypothesis-generating', it should be noted that they are conducted under a 'common disease, common variant' hypothesis, in which much of the genetic variation of a common complex disease is assumed to be due to relatively few common variants (210).

1.4.1 NOD2

NOD2 was the first gene to be discovered that confers susceptibility to CD. The IBD1 susceptibility locus, containing several genes including *NOD2*, was first discovered in

1996 by two point sib-pair linkage analysis in families affected with CD (25). It was not until five years later that *NOD2* was identified as the relevant gene, using positional cloning strategies, DNA sequencing and case-control analysis. Three CD-associated single nucleotide polymorphisms (SNPs) were identified in *NOD2* (SNP8, SNP12 and SNP13), two of which result in substitutions of single amino acids (R702W and G908R) in the leucine-rich repeat domain of the protein. The other polymorphism (SNP13) is a frameshift alteration (1007fs) that generates a premature stop codon, predicted to result in a truncated protein (211;212). Individuals that are heterozygous for one of the polymorphisms have a 2-4 fold increased risk of developing CD, and those that are homozygous or compound heterozygous a 20-40 fold elevated risk (213).

The NOD2 protein is a PRR that is expressed in mononuclear phagocytes, epithelial cells and Paneth cells. Although LPS was originally thought to be the ligand for NOD2, it was subsequently shown that muramyl dipeptide (MDP), a component of bacterial peptidoglycan, is the specific agonist (214). Sensing of MDP by NOD2 results in activation of downstream signalling pathways, including NF-κB activation and ultimately pro-inflammatory cytokine gene transcription. The three CD-associated SNPs result in loss of function and impaired induction of pro-inflammatory cytokines (including IL-8, TNF and IL-1β) in response to MDP (135;215). Such a finding is consistent with an immunodeficiency model of CD, where defective macrophage function results in delayed neutrophil recruitment and impaired clearance of material. Although the deficit in acute inflammation in CD did not appear to relate to the presence of *NOD2* polymorphisms, addition of exogenous MDP to skin windows increased levels of pro-inflammatory mediators. This did not occur in patients homozygous for polymorphisms in *NOD2*, in whom MDP was without effect. It was therefore postulated that NOD2 may play a compensatory role in boosting the acute

inflammatory response to bacteria *in vivo*, an ability that is abolished in a subset of CD patients in whom *NOD2* polymorphisms are present (135).

In spite of this, the overall impact of *NOD2* polymorphisms on inflammation remains an area of contention. NOD2 has been suggested to downregulate TLR2 responses in mice, thereby exerting anti-inflammatory effects (216). Defective release of the anti-inflammatory cytokine IL-10 has been demonstrated in CD patients with *NOD2* variants, and a recent study suggested that the frameshift mutation may in fact exert a 'gain of function', inhibiting phosphorylation of the nuclear riboprotein hnRNP-A1 and actively suppressing IL-10 transcription. This effect was not observed for wild type NOD2 (217). There is also evidence to suggest that NOD2 could have a role in maintenance of mucosal barrier function. *NOD2* variants have been associated with increased ileal permeability (218). Furthermore, abnormalities in the *NOD2* pathway may also result in to abnormal expression of human β-defensin, an important antimicrobial component of the mucosal barrier (219). NOD2 may also have a role in autophagy, as discussed in section 1.4.3. Therefore, the precise role of *NOD2* in the pathogenesis of CD may well be complex.

1.4.2 IBD5

The IBD5 locus was originally discovered in a genome-wide scan of 158 Canadian sib-pair families with early-onset disease (220). It is located in the chromosome 5q31-q33 region, and it appears that the presence of a specific haplotype confers susceptibility to CD. The underlying pathogenic variant in this region remains much debated, and strong linkage disequilibrium exists with a cytokine cluster (221). However, a putative candidate is the *OCTN* gene, which is involved in carnitine transport (222). L-carnitine in turn contributes to the transport of long chain fatty acids into mitochondria for β-

oxidation. *OCTN* variants associated with CD may act to impair L-carnitine transport (222), and thus potentially β-oxidation of fatty acids.

1.4.3 ATG16L1, IRGM and autophagy

In 2007, three independent GWAS identified a polymorphism in the autophagy-related 16-like 1 (*ATG16L1*) gene that is strongly associated with CD (116;223;224). The associated variant in *ATG16L1* was reported to be a nonsynonymous SNP, resulting in a threonine to alanine substitution (T300A) in the ATG16L1 protein. Individuals homozygous for the risk allele have an approximately 1.65 fold increased risk of CD, and 2.2 fold increased risk of ileal disease (225). The WTCCC study also reported two other CD-associated SNPs in the chromosomal region 5q33.1, located in close proximity to the immunity-related GTPase family, M (*IRGM*) gene (130). The association was subsequently replicated in a number of independent cohorts (226;227). The polymorphisms identified were not located within the coding region of the *IRGM* gene itself, and resequencing of *IRGM* in 248 individuals revealed only three coding sequence variants, one of which was a synonymous SNP and the remaining two were not associated with CD (226). It was therefore postulated that the SNPs might influence the expression of IRGM. In a subsequent study, a deletion haplotype was discovered to be in perfect linkage disequilibrium (r^2=1.0) with rs13361189, one of the original SNPs identified, which was shown to give rise to differential IRGM expression (228). More recently, a potential effect of the synonymous SNP on gene expression was also demonstrated. This exonic variant is a target for a family of microRNAs (miR-196), which are capable of downregulating IRGM expression. Under inflammatory conditions, downregulation of risk variant IRGM by miR-196 was reported to be impaired in comparison to wild type IRGM (229).

ATG16L1 and IRGM are understood to have functional roles in autophagy. Autophagy refers to any intracellular degradative pathway that involves delivery of cytoplasmic cargo to lysosomal compartments. Three forms of autophagy are recognised – macroautophagy, microautophagy and chaperone-mediated autophagy (230). Macroautophagy has a role in the degradation of intracellular organelles and cytosolic proteins, involving the formation of a double membrane structure ('the autophagosome') to initially sequester this material. Subsequently, the autophagosome fuses with lysosomes which results in degradation of material. Microautophagy, in contrast, does not require autophagosome formation; instead material is initially engulfed and sequestered by the lysosomal membrane itself. Chaperone-mediated autophagy is involved in selective degradation of cytosolic proteins and occurs independently of vesicle trafficking (231). Autophagy is a tightly regulated process, and upregulation occurs upon starvation, ER stress and cellular activation.

Of potentially greater relevance to CD, autophagy may also have a role in bacterial clearance and the immune response to bacteria (232). Activation of PRRs such as TLRs and NLRs and their downstream signalling cascades induces autophagy. Furthermore, induction of autophagy pathways via TLR signalling during phagocytosis results in enhanced phagolysosomal fusion and destruction of intracellular microbes in macrophages (233). Autophagy has also been shown to be important for antigen processing and major histocompatibility complex (MHC) class II presentation in dendritic cells, thereby influencing $CD4^+$ T lymphocyte responses (234). In addition, autophagy may have roles in autophagosome-independent destruction of parasite-derived membranes (such as from *Toxoplasma gondii*), (235;236) and in pro-inflammatory cytokine production by PBMCs (237).

At a molecular level, ATG16L1 forms an oligomeric complex with ATG5 and ATG12, which is necessary for autophagosome formation. The complex is understood to function in elongation of the isolation membrane (a small 'crescent like' membrane precursor of the autophagosome), possibly by conjugation of microtubule-associated protein 1 light chain 3 (LC3) to the phospholipid phosphatidylethanolamine (238). Cells deficient in ATG16L1 demonstrate defective autophagy induction in response to a range of stimuli, including serum starvation, rapamycin treatment and intracellular *Salmonella typhimurium (S. typhimurium)* infection (223). In cell lines expressing the CD-associated variant ATG16L1 protein, basal autophagy is equivalent compared to wild type cells; however mutant cells were reported to have a reduced efficiency of anti-bacterial autophagy, as demonstrated by decreased capture of *S. typhimurium* within autophagosomes (239). These findings are in keeping with the concept that clearance of foreign material is important in the development of bowel inflammation.

Chimaeric mice with a targeted deletion of ATG16L1 in haematopoietic cells display defective autophagy, and increased macrophage production of IL-1β in response to LPS and *E. coli*, which was proposed to explain the more severe phenotype in the mutant mice after DSS induced colitis. This may result from impaired regulation of inflammasome activity (240). The relevance of ATG16L1 and autophagy to CD pathogenesis may also extend beyond phagocytes – a recent study investigating mice hypomorphic for ATG16L1 expression identified abnormalities in the granule exocytosis pathway in Paneth cells – an interesting parallel with the finding of defective post-translational trafficking of pro-inflammatory molecules in CD. This study also raises the possibility that the product of the *ATG16L1* gene could have a more generalised role in vesicle trafficking. Transcriptional profiling of ATG16L1 deficient Paneth cells revealed abnormal gene expression in a number of pathways that could underlie the observed defects in granule exocytosis, including PPAR signalling and

lipid metabolism, which may be consequential or compensatory changes in response to alterations in ATG16L1 expression or vesicle trafficking (241).

Recent investigations have suggested that NOD2 and ATG16L1 may function in overlapping pathways. Firstly, MDP has been shown to induce autophagy in a NOD2 dependent manner in innate immune cells. The MDP-induced autophagy was shown be important for bacterial handling and NOD2-mediated antigen presentation, by influencing intracellular trafficking and surface expression of MHC class II molecules. CD patients with *NOD2* or *ATG16L1* polymorphisms were shown to have defective autophagy in response to MDP, and impaired antigen presentation and CD4+ T cell responses after exposure to *Salmonella enterica* (242). Secondly, ATG16L1 has been shown to modulate pro-inflammatory cytokine secretion in response to MDP. Although abnormal IL-1β release in response to MDP was not originally observed in ATG16L1 deficient mice (240), PBMCs from healthy individuals and CD patients carrying the T300A polymorphism were shown to secrete increased levels of the pro-inflammatory cytokine IL-1β after stimulation with MDP (243). Although interesting, the effect of autophagy, and thus possibly *ATG16L1* polymorphisms, on cytokine release is likely to be complex. Contrasting effects may be observed for different stimuli, cytokines and cell types. A recent study revealed that autophagy inhibition in PBMCs using 3-methyladenine was associated with enhanced IL-1β release, but impaired TNF secretion downstream of TLR stimulation (237). It is unknown whether impaired autophagy or the presence of *ATG16L1* variants could contribute to the defective secretion of pro-inflammatory cytokines observed in response to HkEc in CD macrophages.

1.4.4 Other CD-susceptibility loci

In addition to *NOD2*, IBD5, *ATG16L1* and *IRGM*, numerous other important susceptibility loci have been identified in CD. In 2006, a relatively uncommon coding

variant was identified in the IL-23 receptor (*IL-23R*) gene, which confers strong protection against CD (244). Furthermore, a meta-analysis of three GWAS published in 2008 demonstrated over 30 different CD-susceptibility loci. As well as verifying the associations with *ATG16L1*, *IRGM* and *IL-23R*, various novel loci were also identified. Some of these contain genes involved in immune system function, and especially the Th17 pathway (116). For example, *IL-12B, JAK2* and *STAT3*, all appear to be involved in the IL-23 axis, and the regulation of CD4+ T cells that produce high levels of IL-17 (Th17 cells). Of note, polymorphisms in these genes have also been associated with UC (245;246), suggesting possible roles as modifiers of a chronic inflammatory state. A more recent meta-analysis has increased the number of CD-susceptibility loci to 71 (115), again highlighting associations with genes implicated in innate and adaptive immunity.

In addition to the susceptibility loci identified in the GWAS meta-analyses, a number of other variants have been associated with CD in smaller scale studies. These include a non-synonymous SNP in *TLR4* (247), and promoter polymorphisms in the *TLR9* (248) and *CD14* (249) genes, further suggesting that innate immune dysfunction could be an important aspect of CD pathogenesis. Additionally, associations have been identified with polymorphisms in mucin-encoding genes such as *MUC3A* (250), and the HLA haplotype DRB1*0103 (128;129). The reasons why some of these associations have not been convincingly replicated in the GWAS meta-analysis, and various other studies, are unclear. It is very possible that some represent false positive discoveries. However, another important consideration is the heterogeneity in disease expression between patients. Notably, both the *TLR4* polymorphism and HLA DRB1*0103 haplotype have been most strongly associated with colonic disease in some studies (251;252). It is possible that the stringent statistical thresholds applied in GWAS could preclude identification of subtle differences in the frequencies of variants in certain

disease subtypes. Therefore, whilst some of these associations should be interpreted with caution, their potential relevance to CD cannot be disregarded. Interestingly, a recent GWAS that specifically investigated patients with early-onset IBD identified associated variants in loci containing the *ZMIZ1*, *IL27* and *HORMAD2* genes (253).

1.4.5 Limitations of GWAS and 'Missing heritability'

It is incontestable that GWAS have shed considerable light on the genetic architecture associated with CD, and have provided some important insights into the mechanisms of CD pathogenesis. However, the functional relevance of many of the associated variants remains relatively uncharacterised. In addition, it is unclear whether the some of the polymorphisms identified indeed represent the true pathogenic variants, or whether they are simply 'proxy markers' in linkage disequilibrium with a pathogenic variant. As discussed, the *IRGM* variant originally identified was shown to be in linkage disequilibrium with an upstream deletion polymorphism, which more likely represents the causal variant in this case. Further fine mapping and resequencing of these regions may help clarify this issue.

It should also be emphasised that the associated variants have a low penetrance and a comparatively high frequency in the general population, even in the case of *NOD2*. Considering a random sample of 100,000 individuals, approximately 15,000 would be heterozygous for one of the three CD-associated polymorphisms in *NOD2*, with around 500 compound heterozygous or homozygous (254). Approximately 100 individuals in the sample would be predicted to develop CD; 28 of whom would carry at least one *NOD2* variant. Only 8 of the 100 patients would be predicted to be compound heterozygous or homozygous for one of the three variants (255). A similar situation exists for *ATG16L1*, but with even weaker effects.

It is increasingly clear that the individual and cumulative effects of polymorphisms identified by GWAS are small, and explain only a proportion of the disease heritability (the portion of phenotypic variance attributable to additive genetic factors) for complex disorders. This has led to the concept of 'missing heritability' - that much of the variation underlying genetic risk is yet to be discovered (256). Considering CD specifically, the 32 susceptibility loci identified in the original GWAS meta-analysis were estimated to account for less than 20% of the heritability (116). Adding the 39 new loci identified in the more recent meta-analysis increased the proportion of heritability explained to only 23.2%, in spite of the greatly increased study population size (6,333 and 15,056 cases and controls respectively compared to 3,230 and 4,829 in the original) (115). This suggests that the majority of the unexplained heritability is unlikely to be discovered simply by conducting larger scale GWAS in their current form.

1.4.6 The missing heritability of CD remains unexplained

The nature of the undiscovered 'missing heritability', and the strategies for finding it, are at present contentious. It has been postulated that rare or low frequency variants (where minor allele frequency is less than 5% of the population) could be important contributors, especially as these are poorly detected by current GWA genotyping arrays (257). Although common SNPs can, in some cases facilitate the discovery of rarer, pathogenic variants by association, the overall capacity of common variants to tag rare variants may well be limited (258). Rare mutations or variants can clearly be critical in the development of bowel inflammation. Up to 50% of patients with certain rare, monogenic disorders of phagocyte function develop bowel inflammation that is histologically identical to that of CD (139;140), as discussed in section 1.2.4. Furthermore, a recent study of two families with early-onset inflammatory bowel

disease revealed the presence of point mutations in the IL-10 receptor, which resulted in impaired IL-10 signalling. Although the phenotype observed in these patients was somewhat different from that of sporadic CD patients, and the mutations were not found in 90 patients with adult onset CD, the cases did highlight how rare mutations can be important determinants of susceptibility to bowel inflammation (259).

Structural variation, including copy number variants such as duplications and deletions, inversions and translocations are also incompletely assessed by GWAS and could further account for some of the unexplained heritability (257). Interestingly, it has been estimated that 8% of the population carry deletions or duplications greater than 500kb with an allele frequency of less than 5% (260). It is unlikely that a disease association of these large structural alterations would have been detected by GWAS. Finally, epigenetic effects such as DNA methylation and histone modification may warrant further investigation (261), given the role of these modifications in the regulation of gene expression. Clearly, a combination of strategies could well be required to identify the missing heritability. Whilst additional data from GWAS and large scale sequencing projects could be powerful, the results must be interpreted in the light of differences in gene expression and functional investigations.

1.5 Outline of Thesis

1.5.1 Summary of background information

The aetiopathogenesis of CD remains unproven, although it is understood to be highly complex and heterogeneous. It is likely that a combination of genetic and environmental factors result in abnormal immune system-microbiota interactions, which are central to the development of CD lesions. GWAS have identified polymorphisms in over 70 genes that are associated with CD, which include the pathogen pattern recognition receptor *NOD2*, two genes involved in autophagy (*ATG16L1* and *IRGM*), and various others

including *IL-23R.* Whilst providing important clues into the underlying genetic susceptibility and pathogenesis of CD, the polymorphisms have a comparatively high frequency in the general population; the penetrance of CD in individuals carrying any single polymorphism is generally very low. In addition, the functional mechanisms by which both the genes and CD-associated polymorphisms influence disease pathogenesis are likely to be subtle and in many cases remain incompletely understood. Finally, it is clear that only a small proportion of the total genetic risk has been explained by GWAS, which has led to the concept of 'missing heritability'.

Mounting evidence suggests a role for defective acute inflammation in the pathogenesis of CD. Acute inflammation is the mechanism by which the body responds to noxious stimuli such as bacteria; cells of the innate immune system such as macrophages and neutrophils have a vital role in this process. Patients with CD have a grossly impaired acute inflammatory response to bacteria, as demonstrated by skin window studies and subcutaneous injection of killed *E. coli*. Neutrophil accumulation at sites of acute inflammation is impaired in CD, which results in defective clearance of bacteria. The persistence of microbial material may act as a trigger for the development of a chronic inflammatory state in the bowel wall. Although neutrophil function is normal in the majority of CD patients, a number of immunological abnormalities in macrophages have been identified that could underlie the impotent acute inflammatory response. Monocyte-derived macrophages from CD patients release sub-optimal levels of pro-inflammatory cytokines such as TNF in response to HkEc, due to abnormal post-translational targeting of these molecules to lysosomal compartments. However, the molecular mechanisms responsible for this defect remain uncharacterised.

1.5.2 Summary of investigations conducted and hypotheses

In this thesis, the macrophage response to microbial stimuli is further characterised. Furthermore, molecular defects underlying the impaired pro-inflammatory cytokine release in CD are investigated using several different approaches. Firstly, a 'hypothesis based' approach is employed. Molecular defects previously associated with CD, and molecules with prominent roles in vesicle trafficking, are investigated as candidates for the impaired secretion of pro-inflammatory cytokines. Specifically, polymorphisms identified as associated with CD by GWAS, and lipid species (including sphingolipids and phospholipids) are investigated.

Secondly, a 'hypothesis-generating' approach is taken, using transcriptomic data from cultured macrophages. Alterations in gene expression in CD macrophages are analysed by classical methods and a novel 'outlier analysis' strategy, enabling detection of gross defects in gene expression in individual CD patients. The functional relevance of a candidate molecule identified by this strategy is subsequently investigated *in vitro*.

The key hypotheses investigated in this thesis include:

1. Macrophage pro-inflammatory cytokine secretion, especially TNF, is impaired in response to a range of microbial stimuli in CD (chapter 3).

2. CD-susceptibility polymorphisms identified by GWAS influence TNF release from CD macrophages (chapter 3).

3. Alterations in macrophage lipids such as sphingolipids and phospholipids underlie the defective pro-inflammatory cytokine release and disordered macrophage function in CD (chapter 4).

4. Abnormalities in gene expression identified by transcriptomic profiling strategies indicate heterogeneous molecular defects of relevance to the impaired acute inflammatory response in CD (chapter 5).

Chapter 2: Materials and Methods

2.1 Subject recruitment and selection

Patients with CD and UC were recruited from the Gastroenterology outpatient clinic at University College London Hospitals Foundation NHS Trust (UCLH). Patients included in the study fulfilled internationally accepted criteria for the diagnosis of either CD or UC (262). Patients were excluded from the study if only non-specific features of inflammatory bowel disease were present, if the diagnosis had been made less than one year previously, or if the histological and clinical diagnoses were inconsistent. All patients were between 18 and 75 years of age and had quiescent disease, as determined by the Harvey-Bradshaw disease activity index (263) or partial Mayo activity index (264) for CD and UC respectively. Only CD patients with a Harvey-Bradshaw score of less than three, or UC patients with a partial Mayo score of two or less were included in the study (with the exception of shotgun lipidomics analysis of bowel biopsies). Unless indicated, all patients were receiving either no treatment or a stable dose (for the preceding 3 months) of 5-aminosalicylates alone. None of the patients included in the study had infections with either Human Immunodeficiency virus (HIV), Hepatitis B or Hepatitis C.

Healthy volunteers were recruited from the Department of Medicine, UCL. All healthy volunteers were between 18 and 75 years of age, had no previous or family history of inflammatory bowel disease, and were not receiving immunosuppressant medication. For shotgun lipidomics analysis of bowel biopsies, control individuals were recruited that were undergoing colonoscopy for diagnostic or screening purposes. None of these individuals had any evidence or family history of inflammatory bowel disease.

Ethical approval for all studies was obtained from the Joint UCL/UCLH Committees on the Ethics of Human Research (project number 02/0324). Written informed consent was obtained from all patients and healthy volunteers. No subject was studied more than once in each of the different sets of experiments.

Clinical information of patients and volunteers was recorded in an encrypted and password-protected database, registered and covered by the Data Protection Act 1998. Clinical details that were recorded included date of birth, current medication, previous medication, previous surgery, Montreal classification (site of disease, age at diagnosis, disease behaviour), Harvey-Bradshaw or partial Mayo score, smoking status, family history, SNP genotype information and co-morbidities.

2.2 Cell culture and assays

2.2.1 Preparation of stimuli for cell culture assays

Heat-killed E. coli

A fully antibiotic sensitive, laboratory adapted strain of *E. coli* (NCTC 10418) was obtained and grown overnight at 37^{o}C in Luria-Bertani (LB) medium. The following day, *E. coli* were harvested and washed repeatedly with phosphate buffered saline (PBS) (GIBCO, Paisley, UK). Bacteria were killed by heat treatment at 60^{o}C for 1 hour. Bacterial concentrations were determined by optical density (OD_{600} = 0.365 equates to 10^{8} *E. coli*/ ml (265)). Sterility of the final sample was confirmed by multiple cultures on LB agar plates.

Heat-killed C. albicans

A standard strain of *Candida albicans* (ATCC 10231) was cultured overnight at 30°C in Yeast peptone dextrose (YPD) medium. The following day, *C. albicans* were harvested

and washed three times with PBS. The concentration of cells was determined by optical density (OD_{520}=0.38 is equivalent to 10^7 *C. albicans* /ml (266)) and resuspended in PBS to a dilution of $5x10^6$. *C. albicans* were killed by heat treatment at 60°C for 1 hour. Sterility of the final sample was confirmed by multiple cultures on YPD agar plates.

2.1.2 Primary macrophage isolation, culture and stimulation

Peripheral venous blood samples were collected from HC, CD and UC patients into 50 ml syringes (Terumo Medical) containing 5 U/ml heparin (LEO laboratories, Princes Risborough, UK.). PBMCs were isolated by differential centrifugation (2,000 rpm, 30 minutes, 20°C) over Lymphoprep (Axis-Shield, Oslo, Norway). Subsequently, cells were washed twice in sterile PBS (GIBCO, Paisley, UK), first at 1,400 rpm (5 minutes, 20°C) and subsequently at 1,200 rpm (5 minutes, 20°C). Monocytic cells were prepared from PBMCs by adherence (136). To achieve this, mononuclear cells were resuspended in serum free RPMI-1640 medium (Invitrogen, Paisley, UK), supplemented with 100 U/ml penicillin (GIBCO), 100 µg/ml streptomycin (GIBCO) and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.4 (Sigma-Aldrich), and plated at approximately $5x10^6$ cells onto 20 cm^2 Nunclon™ Surface tissue culture dishes (Nunc, Roskilde, Denmark). Cells were incubated at 37°C in an atmosphere containing 5% CO_2, to allow monocytic cells to adhere. After 2 hours, the serum free medium containing non-adherent cells was removed and cultures were washed once with 10 ml sterile PBS. 10 ml of RPMI-1640 medium supplemented with 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich), 100 U/ml penicillin (GIBCO), 100 µg/ml streptomycin (GIBCO) and 20 mM HEPES buffer pH 7.4 (Sigma-Aldrich) was then added. Cells were then incubated for 5 days at 37°C, 5% CO_2, enabling maturation of monocytic cells into macrophages. On day 2, cultures were supplemented with a further 10 ml of RPMI-1640 with 10% FBS.

After 5 days of culture, cells were harvested by scraping into 10 ml PBS. A pellet of cells was obtained by centrifugation at 1,200 rpm (5 minutes, 20°C). Cells were resuspended in X-Vivo-15 medium (Cambrex, MD, USA) at a density of 10^6 cells/ml. Cells were then plated into BD Falcon™ 96 well tissue culture plates at a density of 10^5 cells/well or 8 cm^2 Nunclon™ Surface tissue culture dishes at 10^6 cells/dish, depending on the experiment. After overnight incubation, 10^5 cells were stimulated for up to 24 hours with 2 µg/ml Pam_3CSK_4 (Alexis Biochemicals, San Diego, USA), 200 ng/ml lipopolysaccharide (LPS) (Alexis), 500 ng/ml Flagellin (Alexis), 2.5 HkEc/ macrophage or 5 heat-killed *C. albicans*/ macrophage, depending on the experiment.

2.1.3 THP-1 cell culture and stimulation

THP-1 cells (a human pro-monocytic cell line) were cultured in RPMI-1640 medium (Invitrogen), supplemented with 10% FBS (Sigma-Aldrich), 20 mM HEPES (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO) and 50 µM 2-mercaptoethanol (Invitrogen). For cytokine assays, 10^5 cells were resuspended in 100 µl medium and stimulated for 6 hours with 2 µg/ml Pam_3CSK_4 (Alexis) or 2.5 HkEc/ cell.

2.1.4 THP-1 cell transfection and siRNA knockdown

Optineurin expression in THP-1 cells was depleted by transfection of small interfering RNAs (siRNAs) targeted against optineurin, using the Amaxa Cell line Nucleofector® kit V (Amaxa/Lonza, Basel, Switzerland). THP-1 cells were harvested by centrifugation and washed with PBS to completely remove medium. Cells were resuspended in Nucleofector® solution V, to obtain a concentration of 10^6 cells/ 100 µl. For each transfection, 100 µl of cell suspension was mixed with 60 pmol of appropriate siRNAs. Silencer® pre-designed and validated siRNA directed against optineurin was used to

deplete expression (ID # 4392420, Ambion, Austin, TX, USA). Non-targeting Accell^{TM} siRNA was also used as a negative control for off-target effects (catalog # D-001910-01-05, Dharmacon, Thermo Scientific). Transfection was performed by means of electroporation using an Amaxa Nucleofector® device, using programme U-01. Following transfection, cells were resuspended in RPMI-1640 medium (Invitrogen), supplemented with 10% FBS (Sigma-Aldrich), 20 mM HEPES (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (GIBCO) and 50 µM 2-mercaptoethanol (Invitrogen) and cultured at 37°C in an atmosphere of 5% CO_2. Subsequent assays were performed 24 hours after transfection.

2.1.5 TNF Bioassay

Release of TNF was determined using the L929 cytotoxicity bioassay, a sensitive technique for the detection of biologically active TNF in tissue culture supernatants (267). A TNF sensitive clone of murine L929 fibroblast cells was kindly provided by Professor B. Beutler (The Scripps Institute, La Jolla, CA, USA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% FBS (Sigma-Aldrich), 100 U/ml pencillin-streptomycin (Invitrogen) and 100 µg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2. A confluent monolayer of cells was trypsinised and resuspended in DMEM at 2 X 10^5 cells/ml in DMEM. L929 cells were plated into 96-well flat bottomed tissue culture plates at a density of 2 X 10^4 cells/ well, and incubated overnight at 37°C in 5% CO_2. After overnight culture, the medium was removed and replaced with 50 µl DMEM containing 0.04 mg/ml cycloheximide (Sigma-Aldrich), and incubated at 37°C in 5% CO_2. After 30 minutes incubation, 50 µl of cell free supernatant (collected from primary macrophages or THP-1 cells as previously described) was added to individual wells, at a dilution of 1:10-1:250,

depending on the experiment. Serially diluted recombinant human TNF (100–0 pg/ml; R&D Systems) was used to determine the standard curve for the assay.

After 18 hours, 25 µl of 2.5 ng/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, tetrazolium salt) was added to each well and incubated for 4 hours at 37°C in 5% CO_2. Supernatants were then discarded and 100 µl/well of lysis solution (90% isopropanol, 0.5% SDS, 0.04 M HCl, and 10% H_2O) added to each well. After 1 hour incubation at room temperature, absorbance in each well at 570 nm was determined using a FLUOstar OMEGA plate reader and software (BMG LABTECH Ltd., Aylesbury, UK). Data were exported into Microsoft Excel for subsequent analysis. TNF and multiplex cytokine measurements were normalised to correct for cell number using the MTT assay (primary macrophages) or propidium iodide DNA fragmentation assay (THP-1 cells).

2.1.6 Multiplex cytokine measurement

Cultured macrophages from HC individuals, CD and UC patients were stimulated for 24 hours with HkEc (2.5/ macrophage). After 24 hours, supernatants were removed and stored at -70°C until the cytokine assay was performed. Supernatants were replaced with X-Vivo-15 medium containing MTT, and an MTT assay was performed (see below) to correct for cell number. GM-CSF, IFN-γ, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70 and TNF concentrations in the supernatants were determined using the Meso Scale Discovery™ (MSD) Human Pro-inflammatory 9-plex cytokine assay (Meso Scale discovery, Gaithersburg, MD, USA). This 96 well multi-spot assay permits simultaneous detection of cytokines by electrochemoluminescence, enabling profiling of multiple cytokines in a single sample of supernatant. Each well in this assay has 10 carbon electrodes, 9 of which are pre-coated with appropriate anti-cytokine antibodies;

the 10^{th} is coated with BSA and gives a blank reading. Cytokine measurements were determined as per manufacturer's instructions.

In brief, supernatants were diluted 1:8 in tissue culture medium supplied with the assay (diluent 1). Calibrators were serially diluted in diluent 1, to obtain standards ranging from 10000 to 2.4 pg/ml. 200 µl of blocking solution containing 1% Blocker B in PBS was added to each well, and the plate shaken (500 rpm) for 1 hour at room temperature. Subsequently, plates were washed once using 200 µl/ well PBS + 0.05% Tween-20. 25 µl of diluted standards and samples were then added to the appropriate wells, and the plate was incubated for 1.5 hours at room temperature with shaking (500 rpm). The plate was washed three times with 200 µl PBS + 0.05% Tween-20, and 25 µl of detection antibody solution (prepared in diluent 100) was added. After shaking the plate (500 rpm) for 1 hour at room temperature, the detection antibody solution was removed and the plate washed three times with 200 µl PBS + 0.05% Tween-20. 150 µl 2X Read buffer was added to each well and electrochemoluminescence measured using a MSD Sector Imager 2400 plate reader. Raw electrochemoluminescence data were automatically converted into cytokine concentrations with reference to the standards, using Discovery Workbench 3.0 software (MSD). This calculated data were exported into Microsoft Excel for further data analysis.

2.1.7 MTT cell viability assay to estimate cell number

Cell viability of primary macrophages was ascertained using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, tetrazolium salt) assay. 25 µl of 2.5 ng/ml MTT was added to each well and incubated at $37^{o}C$, 5% CO_2. After 24 hours, supernatants were removed and 100 µl/well of lysis solution (90% isopropanol, 0.5% sodium dodecyl sulphate, 0.05 M NH_4Cl, 9.5% H_2O) was added to each well. After 1 hour incubation at room temperature, the absorbance was determined at 570 nm

using a FLUOstar OMEGA microplate reader and software (BMG LABTECH Ltd, Aylesbury, UK).

2.1.8 Propidium iodide staining and flow cytometry

Cell viability of THP-1 cells after transfection was assessed by propidium iodide incorporation and flow cytometry. 24 hours after siRNA transfection, THP-1 cells were permeabilised in 0.1% Triton X-100/ PBS with 2 µM propidium iodide (Sigma Aldrich) for 1 hour in the dark. DNA fragmentation was assessed by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences, NJ, USA), and analysis performed using Cellquest^{TM} software. The proportion of DNA giving fluorescence below the G_{1-0} gated peak (gated as M1) was used as a measured of apoptosis.

2.1.9 SDS-PAGE and Western blotting

Western blotting was used to determine intracellular optineurin levels in THP-1 cells after siRNA knockdown, and levels of optineurin levels in primary macrophages. In the case of THP-1 cells, $2x10^5$ cells were harvested by centrifugation, washed once with PBS and lysed in Laemmli sample buffer (0.06 M Tris-HCl pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% β-mercaptoethanol (VWR), 0.04% (w/v) bromophenol blue (VWR, prepared as 3X concentrate). 1X sample buffer was freshly prepared containing a dissolved Complete protease inhibitor cocktail tablet (1 tablet/10ml) (Roche Diagnostics GmbH, Germany), and phosphatase inhibitor cocktails 2 and 3 (1:100) (Sigma Aldrich). In the case of primary macrophages, $2x10^5$ cells were cultured in 24 well tissue culture plates (Nunc), washed once with PBS and lysed in Laemmli sample buffer, prepared as detailed previously. Prior to electrophoresis, samples were heated to 95°C for 5 minutes, and subsequently centrifuged for 2 minutes at 15,000 g at 4°C.

Proteins in the lysates were resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using the method of Laemmli (268). 10% resolving gels were cast by mixing 1.65 ml of ProtoGel 30% (w/v) acrylamide/ 0.8% bisacrylamide (National Diagnostics, Atlanta, USA) with 1.25 ml of 1.5 M Tris pH 8.8, 50 µl 10% SDS (Sigma-Aldrich), 2 ml distilled water, 50 µl of 10% freshly prepared ammonium persulphate (Sigma-Aldrich), and 4 µl tetramethylethylenediamine (TEMED) (Sigma-Aldrich). Stacking gels were created by mixing 500 µl ProtoGel 30% (w/v) acrylamide/ methylene bisacrylamide (National Diagnostics) with 380 µl of 1 M Tris pH 6.8, 2.1 ml distilled water, 40 µl of 10% SDS (Sigma-Aldrich), 40 µl ammonium persulphate and 4 µl TEMED. Electrophoresis was conducted using Hoefer Mighty Small II SE 250 systems units (Hoefer, CA, USA) at 30 mA per gel.

Proteins were subsequently transferred onto Hybond P nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK) using a semi dry transfer system (Trans-Blot SD semi-dry transfer cell, Bio-Rad) in transfer buffer (200 mM glycine, 0.1% SDS (w/v), 10% methanol (v/v), 25 mM Tris-HCl pH 8.8). In initial experiments, transfer was confirmed using reversible staining with Ponceau Red (Sigma-Aldrich). Membanes were subsequently blocked for 1 hour with 5% non-fat milk (w/v) in TBS/0.05% Tween-20 (v/v). Following blocking, membranes were probed overnight at 4°C with primary antibodies directed against optineurin (Sigma-Aldrich, 1:1000 in TBS/0.05% Tween-20 (v/v) containing 5% bovine serum albumin (w/v)) or β-actin (Sigma-Aldrich; 1:1000 in TBS/0.05% Tween-20 containing 5% non-fat milk (w/v)). After overnight incubation, membranes were washed three times in TBS/0.05% Tween-20 for 5 minutes. Subsequently, membranes were probed with HRP-conjugated anti-rabbit IgG antibody (Amersham Biosciences, 1:2000 in TBS/0.05% Tween-20 containing 5% non-fat milk) for 1 hour at room temperature. After three further 5 minute washes in TBS/0.05% Tween-20, blots were developed using the Enhanced Chemiluminescence

method (ECL PLUS; Amersham Biosciences), and the autoradiograph processed with an X-Omat film developer (Kodak, Hertfordshire, UK). Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD), with band densities normalised to β-actin loading controls.

2.3 Genomic DNA preparation and analysis

2.3.1 Genomic DNA extraction

For SNP genotyping and DNA sequencing, genomic DNA was extracted from peripheral blood samples. 1-2 ml of peripheral venous blood was collected from subjects into an ethylenediaminetetraacetic acid (EDTA) tube (BD Biosciences). Genomic DNA was extracted using the QIAamp DNA blood Mini Kit (Qiagen GmbH, Hilden, Germany), in accordance with the manufacturer's instructions. Optical density readings were determined for OD_{260}/OD_{280} and OD_{260}/OD_{230} using a NanoDrop ND-1000 spectrophotometer (Fisher Scientific, Loughborough, UK) to assess protein and solvent contamination respectively.

2.3.2 Genotyping of Crohn's disease associated polymorphisms

HC, CD and UC patients were genotyped for 34 single nucleotide polymorphisms that were previously identified as convincingly associated with CD by GWAS (116;253). Details of the polymorphisms are shown (Table 2.1). Genotyping was performed by the Sanger Centre using the iPLEX™ Gold Assay (Sequenom® Inc., San Diego, CA, USA). Assays for all SNPs were designed using the eXTEND suite and MassARRAY Assay Design software version 3.1 (Sequenom® Inc.). Amplification was performed in a total volume of 5 µL containing ~0.06-0.4 ng genomic DNA, 100 nM of each PCR primer, 500 µM of each dNTP, 1.25 x PCR buffer (Qiagen), 1.625 mM $MgCl_2$ and 1 U HotStar Taq® (Qiagen). Reactions were heated to 94°C for 15 minutes followed by 45

dbSNP ID	Chromosome	Potential candidate genes	Risk Allele	HC Frequency	CD Frequency	Odds ratio
rs11209026	1p31	*IL23R*	G	0.932	0.973	2.66
rs9286879	1q24	*TNFSF18, TNFSF4, FASLG*	G	0.246	0.285	1.22
rs11584383	1q32	*C1orf106, KIF21B*	T	0.710	0.733	1.12
rs3024505	1q32	*IL10, IL19*	A	0.156	0.179	1.18
rs10210302	2q37	*ATG16L1*	T	0.525	0.597	1.34
rs4613763	5p13	*PTGER4*	C	0.120	0.163	1.43
rs10067603	5q3	*SLC22A4, SLC22A5, IRF1, IL3*	A	0.789	0.821	1.23
rs13361189	5q33	*IRGM*	C	0.086	0.114	1.37
rs10045431	5q33	*IL12B*	C	0.757	0.792	1.22
rs6908425	6p22	*CDKAL1*	C	0.784	0.805	1.14
rs7746082	6q21	*PRDM1*	C	0.299	0.325	1.13
rs2301436	6q27	*CCR6*	T	0.467	0.504	1.16
rs1456893	7p12	*IKZF1, ZPBP, FIGNL1*	A	0.697	0.724	1.14
rs1551398	8q24		A	0.610	0.647	1.17
rs10758669	9p24	*JAK2*	C	0.349	0.387	1.18
rs4263839	9q32	*TNFSF15, TNFSF8*	G	0.681	0.721	1.21
rs17582416	10p11	*CREM*	G	0.344	0.374	1.14
rs10995271	10p21	*ZNF365*	G	0.392	0.442	1.23
rs1250550	10q22	*ZMIZ1*	C	0.678	0.710	1.16
rs7927894	11q13	*C11orf30*	T	0.389	0.427	1.17
rs11175593	12q12	*MUC19, LRRK2*	T	0.023	0.037	1.64
rs3764147	13q14	*C13orf31*	G	0.245	0.275	1.17
rs8049439	16p11	*IL27, SH2B1, EIF3C, LAT, CD19*	C	0.378	0.409	1.14
rs2066844	16q12	*NOD2* (R702W, SNP8)	T	0.036	0.081	2.36
rs2066845	16q12	*NOD2* (G908R, SNP12)	C	0.017	0.041	2.50
rs2066847	16q12	*NOD2* (L1007finsC, SNP13)	C	0.022	0.087	4.24
rs2872507	17q21	*GSMDL, ZPBP2, ORMDL3, IKZF3*	A	0.458	0.491	1.14
rs744166	17q21	*MLX, STAT3*	A	0.583	0.612	1.13
rs2542151	18p11	*PTPN2*	G	0.153	0.183	1.24
rs10500264	19q13		G	0.807	0.829	1.16
rs1736135	21q21		T	0.577	0.613	1.16
rs762421	21q22	*ICOSLG*	G	0.383	0.423	1.18
rs2412973	22q12	*MTMR3*	A	0.457	0.487	1.13
rs4821544	22q12	*NCF4*	C	0.330	0.351	1.10

Table 2.1 Details of the 34 SNPs genotyped and used to determine overall genetic risk score for CD. SNPs were selected based on the results of two GWAS (116;253). The potential candidate genes of interest for each locus are those reported in (115).

cycles at 94°C for 20 seconds, 56°C for 30 seconds and 72°C for 1 minute, then a final extension at 72°C for 3 minutes. Unincorporated dNTPs were digested with shrimp alkaline phosphatase (SAP) prior to iPLEX™ Gold allele specific extension with mass-modified ddNTPs using an iPLEX Gold reagent kit (Sequenom® Inc.). SAP digestion and extension were performed according to the manufacturer's instructions with reaction extension primer concentrations adjusted to between 0.7-1.8 µM, dependent upon primer mass. Extension products were desalted and dispensed onto a SpectroCHIP using a MassARRAY Nanodispenser prior to MALDI-TOF analysis with a MassARRAY Analyzer Compact mass spectrometer. Genotypes were automatically assigned and manually confirmed using MassARRAY TyperAnalyzer software version 4.0 (Sequenom® Inc.).

2.3.3 Calculation of genetic risk scores

Weighted genetic risk scores (GRS) for CD, given the 34-SNP genotype, relative to mean population risk were calculated for each individual using a logistic regression model, with allele counts as predictors of disease status as outcome, assuming additivity within and between loci. The model intercept was set based on the allele frequencies, and regression co-efficients were derived from odds ratio, both taken from a recent meta-analysis (115). For those SNPs that were not directly assessed in the meta-analysis, the best proxy SNP was used. The allele frequencies and odds ratio for the three *NOD2* SNPs were taken from a *NOD2* specific meta-analysis (269). Only those individuals where >95% of the SNPs could be reliably determined were included in the analysis. Potential genotyping errors were assessed via deviations from Hardy Weinberg equilibrium.

2.3.4 Sequencing of OPTN region

Sequencing of the *OPTN* region in outlier individuals, including all exons and flanking sequences, was conducted by PCR amplification and automated Sanger chemistry. The reference sequence of the *OPTN* gene was obtained from the University of California Santa Cruz (UCSC) genome browser. Forward and reverse primers were designed for specific amplification of *OPTN* exons, promoter and downstream reactions, using Primer3 software (270). Sequences of these primers are shown (Figure 2.1A).

PCR was carried out in 25 µl reactions containing 12.5 µl 2X Hotstar Taq master mix (Qiagen GmbH), 200 pmol of the appropriate forward and reverse primers (Eurofins MWG operon), the equivalent of 20 ng DNA per reaction, and made up to a total volume 25 µl using DNAse free water (Qiagen GmbH). PCR was conducted using a DNA engine Tetrad 2 Peltier Thermal cycler (Bio-Rad, Hercules, CA, USA). Reactions were initially heated at 95°C for 2 minutes. Subsequently, reactions were subjected to 35 cycles of denaturation at 95°C (30 seconds), annealing at 56-60°C, depending on the specific primer (30 seconds), and extension at 72°C (30 seconds). For products over 900 base pairs, an extension time of 1 minute was used. After 35 cycles, reactions were incubated at 72°C for 10 minutes. Negative control reactions containing no DNA template were performed in parallel to test for contamination.

Following PCR, 5 µl of the reactions were subjected to agarose gel electrophoresis, stained with ethidium bromide and visualised using a Chemi-Doc™ XRS system (Bio-Rad), to assess amplification of a specific PCR product of the appropriate size, and absence of contamination (Figure 2.1B). Prior to DNA sequencing, PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH), in accordance with the manufacturer's instructions. DNA was eluted in 30 µl DNAse free water, and the concentration measured using a NanoDrop ND-1000

A

Primer pair	Product size (base pairs)	Exon/ Region coverage		Primer sequence
1	940	Promoter	F	5'-CAGCCTCTAGCCTCATCTGC-3'
			R	5'-CACCCTGCCCATTCCATA-3'
2	390	Promoter, 1	F	5'-CTTGGTCGGGTGGGGTAT-3'
			R	5'-GCGGGTACCGTTTTCAGG-3'
3	1558	2,3,4	F	5'-TCACCAAGTGTGAAGGTGGA-3'
			R	5'-GCCTTGCCAAATGCTAAATC-3'
4	656	5	F	5'-GGGCAGGACCAGTATTTGAA-3'
			R	5'-CTTCCAAGACCAGGCAAAAC-3'
5	332	6	F	5'- GTGCCCAGCCTTAGTTTGAT-3'
			R	5'-CTTGGCTTGTGTTGACAAGAA-3'
6	343	7	F	5'- TGGGTTGCATGTCACAAAAA-3'
			R	5'-CCAGTTTGAGCTGCAACATTA-3'
7	593	8	F	5'-GCATTGTAAGCTGGCCTCTC-3'
			R	5'-CAGAAAGCACATTGCTTGGA-3'
8	268	9	F	5'- TTTTGAAAACCCCTGATCCTT-3'
			R	5'-GTGTGTGGGTGTGGTAGTGG-3'
9	371	10	F	5'-TGGTTCAGCCTGTTTTCTCC-3'
			R	5'-TCATGCTCACACATTAACTGGA-3'
10	734	11,12	F	5'-ACTGCGACGTAAAGGAGCAT-3'
			R	5'-AACGTTCAACAGTTTCTGTTCATT-3'
11	329	13	F	5'-CGGCCAGAGCTGATAATTAAA-3'
			R	5'-TGCTTTCCAATGCGAGAATA-3'
12	313	14	F	5'-AGCAGGATTGTGCATCTGTG-3'
			R	5'-GACAGGCACCTCTTCTACGG-3'
13	261	15	F	5'-TGAACCTTGGCAGTGTAGTTTG-3'
			R	5'-TGAAGTGGAATTTTTCTTCAAGC-3'
14	1762	16	F	5'-AACTGCCTGCAAAATGGAAC-3'
			R	5'-TGAGGTGTGGTAGGGGACTC-3'
15	749	Downstream	F	5'-TTGCCAAGAGTAAATAACACTGGA-3'
			R	5'-TCGTAAACCTGTTAAGAGTGCAA-3'
16	957	Downstream	F	5'-GAAGGCCATTTCACGTAGTCA-3'
			R	5'-AAGGGATTCCTCTCACACCA-3'
17	975	Downstream	F	5'-CAGGGACACCACAGGCTATT-3'
			R	5'-GGATCCCGACACAAAATCAC-3'
18	890	Downstream	F	5'-GCCTCTTTGGGCCATATTTT-3'
			R	5'-GAGCCTGAATCCAATTTCCA-3'

B

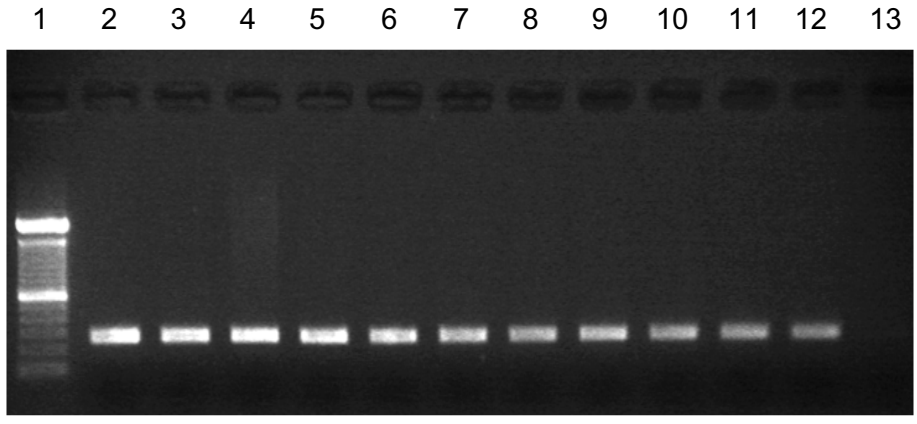


Figure 2.1 PCR Amplification of *OPTN* gene for sequencing. **(A)** Primer sets used for PCR amplification of *OPTN* gene, showing product sizes, region coverage and sequences for the forward (F) and reverse (R) reactions. **(B)** Representative gel showing PCR amplification of a fragment using primer pair 8, and subsequent agarose gel electrophoresis. In lane 1, 100 base pair ladder is shown. In lanes 2-12, specific amplification of a 268 base pair product is shown in outlier patients (lanes 3-9) and controls (lane 2 and 10-12). Lane 13 shows no DNA template control reaction, showing absence of contamination.

spectrophotometer (Fisher Scientific), and resuspended to obtain a concentration of 2-10 ng/µl, depending on the fragment length. Sequencing of the fragments was conducted at the Wolfson Institute for Biomedical Research, UCL by automated Sanger dideoxy sequencing reactions.

DNA sequence chromatograms were viewed using FinchTV (Geospiza Inc., Seattle, WA, USA). The chromatogram quality and presence of heterozygous changes were inspected by eye. Sequences of amplified fragments were aligned with reference sequences using the ClustalW algorithm on the SDSC Biology workbench 3.2 website (http://workbench.sdsc.edu/), with default parameter settings. Sequences were searched for previously recognised variants in the *OPTN* gene, as documented in the National Centre for Biotechnology Information (NCBI) SNP database (dbSNP).

Linkage disequilibrium (the non-random association of alleles at two or more loci) between polymorphisms indentified in the optineurin outlier patients was determined using SNP annotation and proxy search (SNAP) software (271). This software package calculates pair-wise linkage disequilibrium between variants, using phased genotype data from the International HapMap and 1000 genomes projects. In order to assess whether any of the identified SNPs were associated with gene expression, data from expression quantitative trait loci (eQTL) studies was interrogated using Genevar (GENe Expression VARiation) software (272).

2.4 Lipid extraction and analysis

2.4.1 Sphingolipid analysis of cultured macrophages

Monocyte-derived macrophages were cultured onto 8 cm^2 dishes (Nunc) as described in section 2.1.2 and stimulated for 4 hours with HkEc (2.5:1). Cells were harvested in PBS

and pellets obtained. Cell pellets were resuspended in 200 µl PBS and sonicated. 10 µl aliquots were obtained for protein determination.

The ceramide content of the solution remaining was determined using high performance liquid chromatography tandem mass spectrometry (HPLC-MS), as previously described by Bielawski *et al.* (273). Prior to extraction, cell lysates were spiked with appropriate internal standards synthesised by the Lipidomics Core at the Medical University of South Carolina. A 50 µl volume of 1 µM internal standard solution containing 17-carbon (C) analogues of sphingosine, which occur at trace amounts in nature (17C Sphingosine-1-phosphate, 17C24:1 ceramide and 18C17:0 ceramide), in methanol was added to all samples. Following addition of the internal standards, 2 ml of an iso-propanol:water:ethyl acetate mixture (30:10:60) was added, samples were vortexed and sonicated periodically for 30 seconds. Samples were centrifuged for 10 minutes at 4000 rpm, and supernatant transferred to a new vial. This process was repeated once. Lipid extracts were evaporated to dryness under liquid nitrogen. The dry residue was resuspended in 150 µl Mobile Phase A solution (1 mM ammonium formate dissolved in methanol containing 0.2% formic acid). 20 µl of the resulting solution were injected into the HPLC system.

HPLC-MS analysis was performed in the Lipidomics Core at the Medical University of South Carolina on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer operating in a Multiple Reaction Monitoring positive ionisation mode. Measurements of macrophage sphingolipids were normalised to total protein content.

2.4.2 BCA assay

The protein content of cell lysate aliquots was determined using the bicinchoninic acid (BCA) assay (Pierce/ Thermo Fisher Scientific Inc.). Appropriate dilutions of samples were prepared and transferred to a 96 well plate. BCA reagent (20:1 BCA reagent A:

BCA reagent B) was added to each well and heated at 60^{o}C. After 30 minutes, the plate was read using a FLUOstar OMEGA plate reader (BMG LABTECH Ltd.) at 562 nm and the protein concentration was determined by reference to known concentrations of bovine serum albumin (BSA) standards. Absolute measurements of sphingolipids obtained using mass spectrometric analysis were normalised to the total protein content of the lysate.

2.4.3 Preparation of samples for phospholipid analysis and stable isotope incubation

Monocyte-derived macrophages from HC and CD patients were cultured onto 8 cm^2 dishes (Nunc) at a density of 10^6 cells/ well. After overnight incubation, medium was removed and replaced with X-vivo-15 (Cambrex) supplemented with deuterated choline (*methyl*-d_9-choline, 100 µg/ml (Sigma Aldrich)), deuterated inositol (*myo*-d_6-inositol, 100 µg/ml, (C/D/N isotopes, Quebec)) and deuterated serine (serine-d_3, 100 µg/ml (C/D/N isotopes)), in the presence or absence of HkEc (2.5:1). Inclusion of deuterated ('stable') isotope labelled choline, inositol and serine in the culture medium enables mass-spectrometric distinction of newly synthesised phospholipid species from 'endogenous' species, and allows assessment of metabolic flux through phospholipid synthesis pathways.

Macrophages were incubated with the stable isotope labelled compounds for 3 hours at 37^{o}C in an atmosphere of 5% (v/v) CO_2. Subsequently, medium was removed and cells were washed once with Hanks Balanced Salt Solution (Invitrogen). Cells were then lysed in 1 ml ice cold methanol and cell fragments were stored at -20^{o}C until lipid extractions were performed.

2.4.4 Phospholipid extraction and analysis by electrospray ionisation mass spectrometry

Total lipid was extracted from macrophages using chloroform and methanol as described previously (274). Prior to lipid extraction, cell lysates were spiked with appropriate internal standards, including 2 nmol dimyristolphosphatidylcholine (PC 14:0/14:0), 0.2 nmol 17:0 lyso-phosphatidylcholine, 0.8 nmol dimyristoylphosphatidylethanolamine (PE 14:0/14:0), 0.4 nmol dimyristoylphosphatidylserine (PS 14:0/14:0), 0.4 nmol dimyristoylphosphatidylglycerol (PG 14:0/14:0), 0.2 nmol dimyristoylphosphatidic acid (PA 14:0/14:0) per sample. All of these species added as internal standards are present at trace amounts in nature. An 800 µl volume of 0.9% NaCl, 1 ml chloroform and 1 ml methanol was subsequently added, and samples were sonicated for 5 minutes. A further 1 ml of chloroform was added followed by 1 ml HPLC-grade water. Samples were thoroughly mixed by vortexing, and centrifuged at 1,000 g for 10 minutes at 20^{o}C. The lower phase was carefully aspirated into new borosilicate tubes using a glass Pasteur pipette. Samples were dried under nitrogen at 37^{o}C for 45 minutes. When dry, 2X 500 µl chloroform was added, the samples were transferred to 1 ml mass spectrometry vials (Waters, Milford MA), and dried under nitrogen again at 37^{o}C for 45 minutes.

For analysis, samples were reconstituted in 30 µl of a solution containing 20% butanol, 60% methanol, 16% water and 4% concentrated aqueous NH_3 and introduced by direct infusion into a triple quadrupole mass spectrometer (Quattro Ultima, Micromass, Manchester, UK) equipped with a nanoflow electrospray ionisation interface. Phospholipid and neutral lipid species, both endogenous and with incorporated stable isotope-labelled substrates, were selectively detected and quantified from a variety of precursor (P) and neutral loss (NL) scans. Phosphatidylcholine (PC)

was analysed in positive ionisation as P184+ and P193+ scans for endogenous and newly synthesised (D_9) PC. Phosphatidylinositol (PI) and phosphatidylserine (PS) were analysed in negative ionisation, as P241- and P247- scans for endogenous PI and newly synthesised (D_6) PI respectively, and NL87- and NL90- for endogenous PS and and newly synthesised (D_3) PS respectively. Data were processed using MassLynx software (Waters) and analysed using a macro developed at the University of Southampton, as employed in previous studies (275). The macro enabled spectra to be smoothed, background subtracted, converted into centroid format and exported into individual Excel sample files, which were imported into the analyser programme. Correction for the ^{13}C isotope was performed prior to calculation of percentage composition and incorporation of labelled phospholipid head groups. The fractional incorporations of *methyl*-d_9-choline, *myo*-d_6-inositol and serine-d_3 into PC, PI and PS species respectively were calculated relative to the total abundance. Only species of PC, PI and PS that made up >2% of the total molar percentage of PC, PI or PS respectively were considered detectable.

2.4.5 Shotgun lipidomics analysis of ileal biopsies

Shotgun lipidomics analysis was performed on ileal biopsies from CD patients and control individuals, in collaboration with Dr Xianlin Han (Washington University, St Louis). Shotgun lipidomics is a technique for analysing the global lipid profile ('lipidome') in biological extracts, whereby lithium adducts of lipid species are formed prior to extraction and mass spectrometric analysis (276). Ileal biopsies were obtained from non-inflamed ileum at colonoscopy. Tissue samples were snap frozen in liquid nitrogen and stored at -70^{o}C until sample processing. Analysis of the lipidome was performed as described previously (277). Briefly, samples were homogenised in 1 ml ice-cold LiCl aqueous solution (50 mmol/L) using a Potter-Elvehjem tissue grinder.

Aliquots of the homogenates were obtained and protein content determined using the BCA assay. Internal standards, including dimyristoylphosphocholine (15 nmol/mg protein), dimyristoylphosphatidylserine (1 nmol/mg protein), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoglycerol (4.2 nmol/mg protein), 1,2-dipentadecanoyl-*sn*-glycero-3-phosphoethanolamine (18.75 nmol/mg protein), 17C16 ceramide (40 pmol/mg protein) and triheptadecenoylglycerol (10 nmol/mg protein) were added at this stage. Lipid extraction was performed using a modified Bligh and Dyer procedure, and samples were dried under nitrogen gas. ESI-MS was performed as previously described using a triple-quadrupole mass spectrometer (ThermoElectron TSQ Quantum Ultra, San Jose, CA, USA) (277).

2.5 RNA preparation and analysis

2.5.1 RNA extraction from HC, CD and UC macrophages

Monocyte-derived macrophages were cultured from HC, CD and UC donors as described above, resuspended in X-vivo-15 medium (Cambrex) and cultured onto 8 cm^2 tissue culture dishes (Nunc) at a density of 10^6 cells/ dish. Total RNA was extracted from unstimulated and 4 hour-HkEc-stimulated macrophages using the RNeasy Mini kit with RNase free DNase treatment (Qiagen), in accordance with the manufacturer's instructions. The RNeasy Mini kit enables up to 100 µg RNA greater than 200 bases in length to be extracted from homogenised cell lysates, using an RNeasy silica membrane and a series of high salt buffer solutions in the presence of ethanol.

Optical density readings were determined for OD_{260}/OD_{280} and OD_{260}/OD_{230} using a NanoDrop ND-1000 spectrophotometer (Fisher Scientific, Loughborough, UK) to assess protein and solvent contamination respectively. Prior to microarray analysis, RNA integrity was analyzed by assessing ribosomal RNA band 28S/18S ratios using an

Agilent Technologies Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) high resolution electrophoresis system. All samples had an RNA integrity number above 8.

2.5.2 Microarray hybridisation

500 ng of total RNA for each sample was amplified and purified using the Illumina TotalPrep-96 RNA Amplification kit (Ambion, Huntingdon, UK), according to the manufacturer's instructions. Biotin-Labelled cRNA was then normalized to a 150 ng/µl concentration and 750 ng was hybridised to Illumina Human-WG6 v3.0 Expression BeadChips (Illumina) for 16 hours at 58°C. Following hybridisation, beadarrays were washed and stained with streptavidin-Cy3 (GE Healthcare, UK). Beadarrays were then scanned using the Beadarray reader and image data were then processed using Genome Studio software (Illumina).

2.5.3 Microarray data analysis

Expression data were exported from Genome Studio software (Illumina) after $\log_2$ transformation to stabilise the variance and cubic spline normalisation. Data were exported without background subtraction. Detection p-values associated with each probe expression value were also exported. Batch variation was minimised using ComBat, an established R algorithm for preventing batch effects in microarrays (278). Probes that reached a minimum detection p-value of $p<0.01$ in a single chip were included in the subsequent outlier and differential gene expression analysis, to enable detection of over-expressed outliers in individual CD patients and HC individuals. Probes that had associated detection p-values of $p>0.01$ in all chips were excluded from the analysis.

Differentially expressed genes between groups were computed using MultiExperiment viewer TM4 Microarray Software suite version 4.7 (279). Probes were

initially identified that reached an uncorrected p-value threshold of $p<0.01$ and a minimum raw fold change of 1.2 between groups. Correction for multiple testing was performed using an adjusted Bonferroni method. Analysis was also performed in R programming language using customised software developed by the Department of Computer Sciences, UCL, which enabled correction for multiple testing using the less stringent method of Benjamini and Hochberg (280). Principal component analysis (PCA) was conducted to determine whether global macrophage gene expression profiles could be separated on the basis of disease or batch of microarray analysis. PCA is a mathematical algorithm for reducing the dimensionality of datasets, by finding the principal components (directions) along which the variation is maximal. PCA was conducted using the PCA tool of MultiExperiment viewer TM4 Microarray Software suite, with default settings.

Gene expression outlier analysis was performed using customised software developed in collaboration with Daniel Roden and Anna Lobley (Department of Computer Sciences, UCL). In brief, this software enables identification and visualisation of abnormally expressed genes ('outliers') in individual patients compared to a comparison cohort of healthy individuals, and utilises both the R programming language with *Biobase* package from the bioconductor platform (281), and the Java programming language. A configurable Graphical User Interface is provided to run the analysis. Two adjustable thresholds are used to identify potential probe-set outliers: (i) the significance level (p-value) of the standardised deviation of the expression levels from the test sample, when compared to the mean expression levels of the comparison group; and (ii) the ($\log_2$) fold-change between the expression level from the test sample and the average of the comparison group.

To assess the significance of the standardised deviation of the test sample expression values from the comparison group average, a Z-score is calculated, using *equation 1*, for each probe.

$$Z = \frac{E_{sample} - \mu}{\sigma} \qquad \textit{(equation 1)}$$

Where: E_{sample} is the expression level of the test sample; and μ and σ are the mean and standard deviation of the expression levels of the samples in the comparison group respectively. From these Z-scores, p-values are calculated by determining the likelihood that the standardised expression value of the probe-set, in the test sample, would be selected at random from a normal distribution with a mean of 0 and standard deviation of 1. Fold changes between expression level in the test sample and mean expression in the HC comparison group were also calculated. For outlier analysis, the threshold p-value was set at $p<0.005$ and minimum fold change was set at 1.5 compared to mean expression in the HC cohort.

MetaCore software (GeneGo, St Joseph, MI, USA) was used to determine the significantly enriched Gene Ontology (GO) processes and pathway networks within the differentially expressed and outlier gene lists. MetaCore is an integrated software suite that enables identification of GO processes and pathway networks that are enriched within datasets in an unbiased manner, based on a manually curated database. Illumina probe IDs, with associated fold changes and p-values where appropriate, were uploaded onto the MetaCore pathway analysis package. Significantly enriched GO processes and MetaCore pathway networks within the gene lists were identified and ranked in order of p-value.

2.5.4 cDNA conversion and Quantitative PCR

Prior to performing quantitative PCR, 0.5 µg of RNA was converted to cDNA by oligo d(T) priming and reverse transcription using the Promega (Qiagen GmbH) reverse transcription kit, as per manufacturer's instructions. Real time quantitative PCR analysis of optineurin expression was performed using TaqMan® gene expression assay kits (Applied Biosystems, Warrington, UK), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous control. TaqMan gene expression assay identifiers were Hs01014552_m1 (optineurin) and Hs03929097_g1 (GAPDH). Efficiency of both primer sets was estimated to be above 95%, using serially diluted pooled cDNA templates.

All quantitative PCR reactions were conducted in ABgene 96 well PCR plates (Abgene, Epsom, UK), in 20 µl total volumes containing 1 µl of 20X TaqMan gene expression assay and 10 µl of 2X TaqMan Universal Master Mix (Applied Biosystems). A 4 µl volume of cDNA equivalent to 5 ng of total RNA was used for each quantitative PCR reaction. Real time quantitative PCR was conducted using an Eppendorf Mastercycler® ep realplex Thermal Cycler (Eppendorf, Hamburg, Germany). PCR cycling conditions utilised included an initial 10 minute hold at 95^{o}C, followed by 40 cycles of denaturation (15 seconds at 95^{o}C) and primer annealing and extension (1 minute at 60^{o}C). All reactions were performed in triplicate. The cycle at which fluorescence reached an arbitrary threshold of 100 relative units (cycle threshold, Ct) was determined for each reaction. Data were exported from realplex software into Microsoft Excel for further analysis.

Relative expression of optineurin in the CD patients of interest compared to HC was determined using the comparative Ct (ΔΔ Ct) method (282). Briefly, mean Ct values obtained for GAPDH amplification were subtracted from mean Ct values for

OPTN amplification for each sample. The average difference in these values between HC and patient samples was determined, and the exponent to base 2 calculated to determine the fold difference in expression.

2.6 Statistical analysis

Unless otherwise indicated, all statistical calculations were performed using GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA, USA). A p-value <0.05 was considered statistically significant. The Komolgorov-Smirnov test was used to determine whether distributions of datasets were Gaussian.

2.6.1 Analysis of cytokine secretion measurements, and relationships to phenotypes and genotypes (chapter 3)

Statistical significance was assessed using an unpaired t-test or Mann-Whitney U test as appropriate. For multiple comparisons between groups, statistical significance was assessed by one-way analysis of variance (ANOVA) with Tukey post test, or for non-normally distributed data, Kruskal-Wallis one-way ANOVA with Dunn's post test.

Correlation between cytokine measurements and GRS was ascertained using Spearman's rank correlation co-efficient. A correlation was considered significant when $p<0.05$.

2.6.2 Analysis of macrophage lipids in CD (chapter 4)

Statistical significance of differences between groups was determined using a paired or unpaired t-test. A p-value was considered significant when $p<0.05$.

2.6.3 Analysis of transcriptomic abnormalities in CD (chapter 5)

Microarray data were analysed using MultiExperiment viewer TM4 Microarray Software suite and customised outlier analysis software, as described in section 2.5.3.

For all other experiments, statistical significance was assessed using a paired or unpaired t-test. For multiple comparisons between groups, statistical significance was assessed by one way ANOVA with Tukey post-test.

Chapter 3: The macrophage response to microbial stimuli in Crohn's disease, and genotype-phenotype correlations

3.1 Introduction

Recent evidence strongly implicates a role for macrophages in the pathogenesis of CD. Macrophages are key orchestrators of an acute inflammatory response, involved in pro- and anti-inflammatory cytokine secretion, antigen presentation and phagocytosis. Our laboratory recently demonstrated that CD patients have a grossly impaired acute inflammatory response to killed *E. coli in vivo*, with associated defects in pro-inflammatory cytokine secretion by CD macrophages in response to HkEc. The latter was related to abnormal post-translational trafficking of cytokines within these cells, resulting in mistargeting and degradation of these proteins in lysosomal compartments (136).

A wide variety of microbes have been postulated as causative in CD. These include Gram positive and Gram negative species, mycobacteria, viruses, and yeasts such as *C. albicans* that are commonly found in the commensal microflora of patients and healthy individuals (85;96). Whilst direct causality has not been proven for any organism, there is a clear requirement for luminal contents in the development of CD lesions; abnormal interactions of intestinal microbiota with the innate immune system may be crucial in CD pathogenesis.

Intestinal flora provides a complex and diverse range of ligands for pathogen pattern recognition receptors (PRRs) expressed by macrophages, including Toll-like receptors (TLRs) and NOD-like receptors (NLRs). TLRs are transmembrane PRRs with critical roles in pathogen sensing by macrophages. Each TLR recognises specific bacterial or viral components; key TLRs in the response to bacteria include TLR2,

TLR4 and TLR5. Ligand-receptor engagement results in intracellular signalling cascades and the induction of effector responses important for the innate immune defence against microbes, as discussed previously in section 1.3.1 (166). The potential relevance of TLRs in the response to intestinal microbiota is demonstrated by TLR4 knockout mice (148). These mice have earlier and more pronounced gastrointestinal bleeding than wild type mice after administration of DSS, which coincides with increased bacterial translocation and impaired neutrophil recruitment. Differential expression of TLRs has been documented in biopsy samples from IBD patients (283;284). A number of studies have reported associations between TLR4 polymorphisms and CD (247), although these were not identified in the recent large meta-analysis.

Recently, GWAS have identified polymorphisms within susceptibility loci that are believed to account for approximately 23% of the total heritability of the disease (115;116). Some of the strongest associations include *NOD2*, which encodes a PRR, the IL-23 receptor *IL-23R*, and two genes with putative roles in autophagy (*ATG16L1* and *IRGM*). Many of the CD-associated genes identified to date appear to have important roles in sensing, clearance and propagation of the inflammatory response to commensal microflora. However, although many of the loci are suspected to influence the innate immune system, their precise functional roles remain to be elucidated, as well as the mechanisms by which the associated SNPs influence disease susceptibility.

Here the macrophage response to microbial stimuli in CD is further characterised. Pro-inflammatory cytokine secretion is investigated in response to stimulation with the Gram negative bacterium *E. coli*, TLR ligands and the yeast *C. albicans*. The relationship of the macrophage response to both patient phenotypic data and previously identified genetic susceptibility loci are interrogated.

3.2 Results

3.2.1 Optimisation of stimuli for cytokine secretion assays

The relationships of heat-killed *E. coli* (HkEc), heat-killed *C. albicans* (HkCa) and TLR stimulus dose to TNF release were investigated in HC macrophages, in order to determine the concentrations resulting in near maximal TNF release for use in subsequent experiments. Optimal TNF release occurred at a 5:1 ratio of HkCa: macrophage and a 2.5:1 ratio of HkEc: macrophage (Figure 3.1A, B). Dose-response relationships of TLR agonists were previously determined by Dr Farooq Rahman. The optimum doses of Pam_3CSK_4, LPS and flagellin were found to be 2 μg/ml, 200 ng/ml and 500 ng/ml respectively. TNF release reached maximum by 6 hours in response to HkEc and TLR agonists and was stable over a 24 hour period, whereas IL-8 and IL-10 reached a maximum by 24 hours.

3.2.2 Pro-inflammatory cytokine secretion from CD macrophages after HkEc stimulation is defective

In order to validate the previous finding of impaired pro-inflammatory cytokine release in CD, peripheral blood monocyte-derived macrophages were cultured from a new cohort of quiescent CD (n=43), UC (n=25) patients and HC individuals (n=39), which differed from the previous study (136). The demographics of these patients are shown (Appendix 1 Table IA). Eight CD patients were receiving immunosuppressive therapy (6-mercaptopurine, azathioprine or methotrexate), 21 were receiving 5-ASA and 14 were receiving no treatment. Macrophages were stimulated with HkEc (2.5:1) for 24 hours. Supernatants were collected and secretion of the pro-inflammatory cytokines TNF, IL-2, IL-6, IFN-γ, GM-CSF, IL-1β and IL-12p70 were determined using a multiplex cytokine assay. Secreted levels of the chemokine IL-8 and the anti-

inflammatory cytokine IL-10 were also determined. At the dilution used, levels of all cytokines were within the detectable range of the assay.

In the CD cohort, the secreted levels of TNF after HkEc stimulation were significantly reduced compared to both HC individuals ($p<0.05$) and UC patients ($p<0.05$) (Figure 3.2A, B). Median TNF secretion from CD macrophages was 12,276 pg/ml (interquartile range 7,645-23,871 pg/ml) compared to 21,991 pg/ml (interquartile range 11,080-35,059 pg/ml) in HC and 22,065 pg/ml (interquartile range 12,798-42,675 pg/ml) in UC. Release of IFN-γ was also significantly attenuated in CD compared to HC ($p<0.01$) (Figure 3.2C). A trend towards attenuated IL-6 secretion was also observed in CD (median 8,117 pg/ml, interquartile range 5,239-14,746 pg/ml) compared to HC (median 12,179 pg/ml, interquartile range 6,319-25,276 pg/ml), although this did not reach statistical significance.

Secretion of IL-2, GM-CSF, IL-1β, IL-12p70, IL-8 and IL-10 did not differ between HC and CD patients. These findings therefore provide confirmatory evidence of the selective defect in the release of a number of pro-inflammatory cytokines previously identified in CD macrophages.

No significant differences in the secreted levels of cytokines between UC and HC macrophages were found, with the exception of IL-10, the release of which was significantly elevated in UC compared to HC ($p<0.05$) macrophages. Taken together, the results suggest that the defective release of pro-inflammatory cytokines observed in CD is likely to be specific to CD, rather than consequential to a chronic inflammatory state in the bowel.

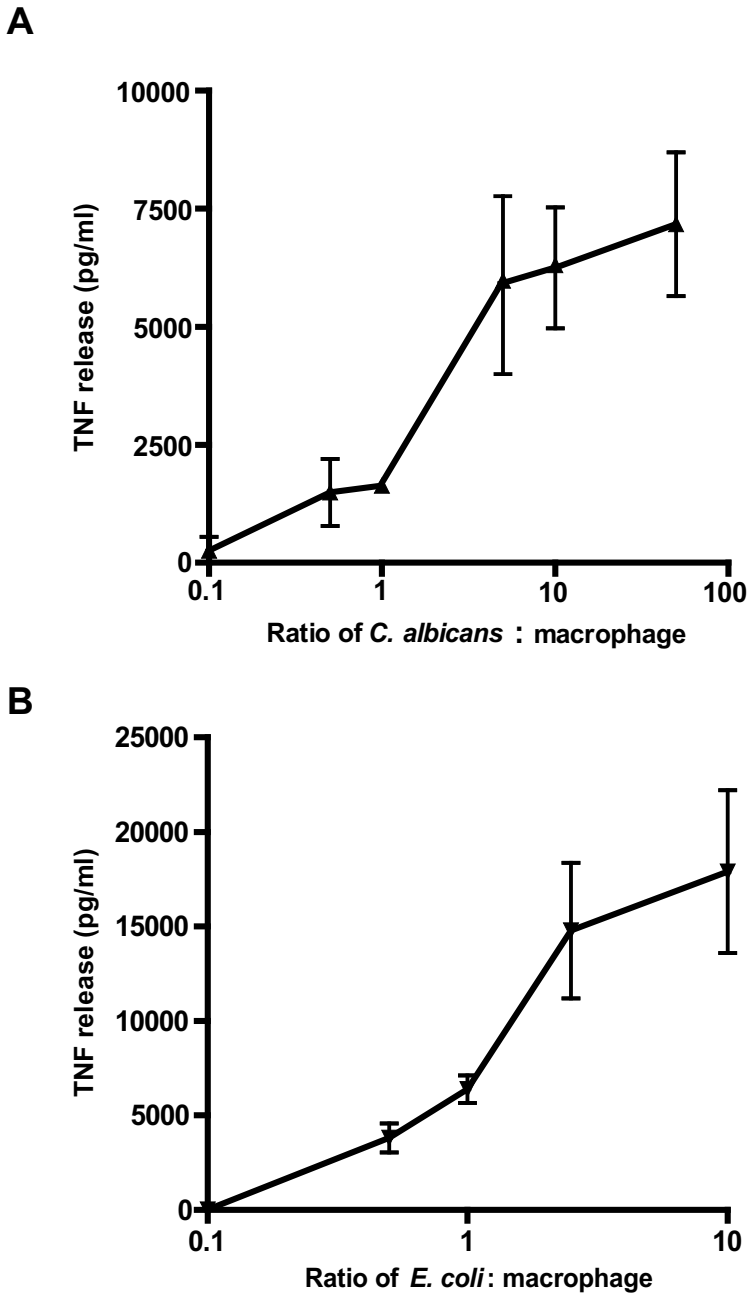


Figure 3.1 Dose response curves of TNF release with increasing doses of *C. albicans* and *E. coli*. Macrophages from HC donors (n=3) were cultured and stimulated with **(A)** heat killed *C. albicans* and **(B)** *E. coli* at differing ratios of micro-organism : macrophage. TNF release was determined using the TNF bioassay. Ratios of micro-organism are presented on a logarithmic scale. Data shown are mean of 3 experiments with error bars representing standard error of the mean (SEM).

A

		HC (n=39)	CD (n=43)	UC (n=25)
Cytokine (pg/ml)	TNF	**21,991** (11,080-35,059)	**12,276 *** (7,645-23,871)	**22,065** (12,798-42,675)
	IL-2	**244** (179-299)	**239** (203-320)	**223** (179-337)
	IL-6	**12,179** (6,319-25,276)	**8,117** (5,239-14,746)	**9,802** (4,936-18,762)
	IFN-γ	**2,501** (1,318-7,270)	**1,213 **** (866-2,145)	**1,375** (955-2,959)
	GM-CSF	**780** (429-1828)	**781** (529-1269)	**977** (535-1,384)
	IL-1β	**110** (77-154)	**101** (70-142)	**118** (74-162)
	IL-12p70	**185** (141-289)	**161** (134-218)	**203** (152-264)
	IL-8	**99,540** (83,332-122,714)	**109,502** (88,397-131,726)	**113,581** (87,746-139,834)
	IL-10	**6,889** (4,552-12,663)	**9,167** (6,372-13,097)	**11,869 *** (8,269-19,573)

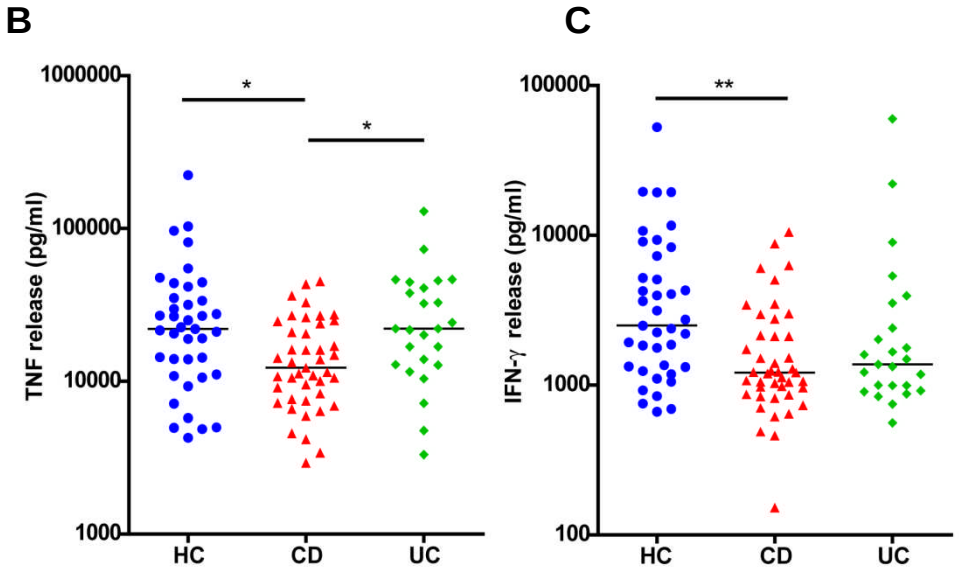


Figure 3.2 Pro-inflammatory cytokine release from CD macrophages after HkEc stimulation is deficient compared to HC and UC macrophages. Monocyte-derived macrophages were cultured from HC (n=39), CD (n=43) and UC patients (n=25) and stimulated with HkEc for 24 hours. Cytokine release was determined by the MSD pro-inflammatory 9-plex assay. **(A)** Cytokine secretion from HC, CD and UC macrophages. Data are expressed as **median** (interquartile range).* indicates $p<0.05$ compared to HC, **$p<0.01$ compared to HC. Dot plots show the impaired **(B)** TNF and **(C)** IFN-γ secretion from CD macrophages. Data are presented on a logarithmic scale. * indicates $p<0.05$, ** $p<0.01$

There was a significant positive correlation between the secreted levels of TNF and IFN-γ release in HC individuals (R_s=0.613, p<0.0001) and CD patients (R_s=0.302, p<0.05) (Figure 3.3A). Likewise, IL-6 secretion was significantly correlated with TNF in HC (R_s=0.617, p<0.0001) and CD (R_s=0.718, p<0.0001) (Figure 3.3B), indicating a relationship between the attenuated release of TNF, IFN-γ and IL-6 from CD macrophages. In contrast, there was no relationship between macrophage TNF release and IL-10 secretion in HC individuals (R_s= -0.261, p>0.05) or CD patients (R_s=0.14, p>0.05).

3.2.3 Release of TNF is impaired in response to other microbial stimuli

Having confirmed an impairment in macrophage pro-inflammatory cytokine release in response to HkEc stimulation, the investigation was extended to determine whether this was a phenomenon specific to *E. coli*, or whether the defect could also be seen in response to other microbial stimuli. Monocyte-derived macrophages were cultured from CD patients and HC individuals and stimulated with TLR ligands and heat-killed *C. albicans* (HkCa). Demographics of patients included in the TLR and HkCa studies (Appendix 1 Table IB, C) are shown. All patients had quiescent disease, and were receiving either no medication or 5-ASA, with the exception of two patients in the HkCa study who were receiving azathioprine.

Monocyte-derived macrophages were stimulated with Pam_3CSK_4 (a TLR1/2 agonist (166)), LPS (a TLR4 agonist (172)) and HkCa, for 6-24 hours and release of TNF quantified using the L929 bioassay, a sensitive assay for the detection of biologically active TNF. Data presented for Pam_3CSK_4 and LPS stimulation show combined results incorporating data obtained previously by Dr Farooq Rahman. Data for flagellin (TLR5 agonist (173)) were collated by Dr Farooq Rahman and are shown here for completeness. The previous experiments also employed the L929 bioassay, and

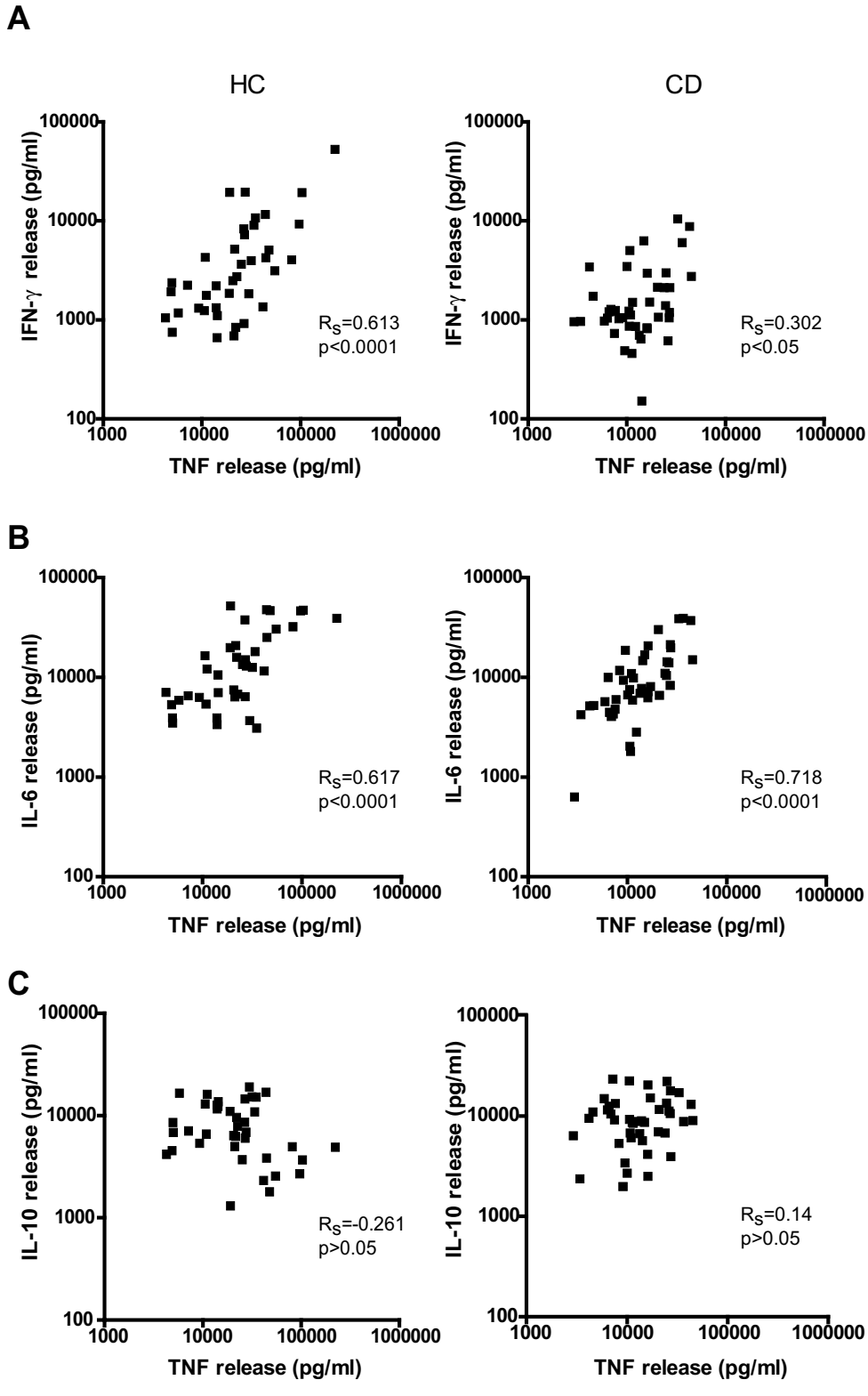


Figure 3.3 Correlation of TNF secretion with **(A)** IFN-γ, **(B)** IL-6 and **(C)** IL-10 release from HC and CD macrophages. Macrophages were cultured from HC (n=39) and CD (n=43) donors and stimulated for 24 hours with HkEc. Release of TNF, IFN-γ, IL-6 and IL-10 were compared, as determined using the MSD pro-inflammatory 9-plex assay. All data are presented on a logarithmic scale. R_S is Spearman's rank correlation co-efficient.

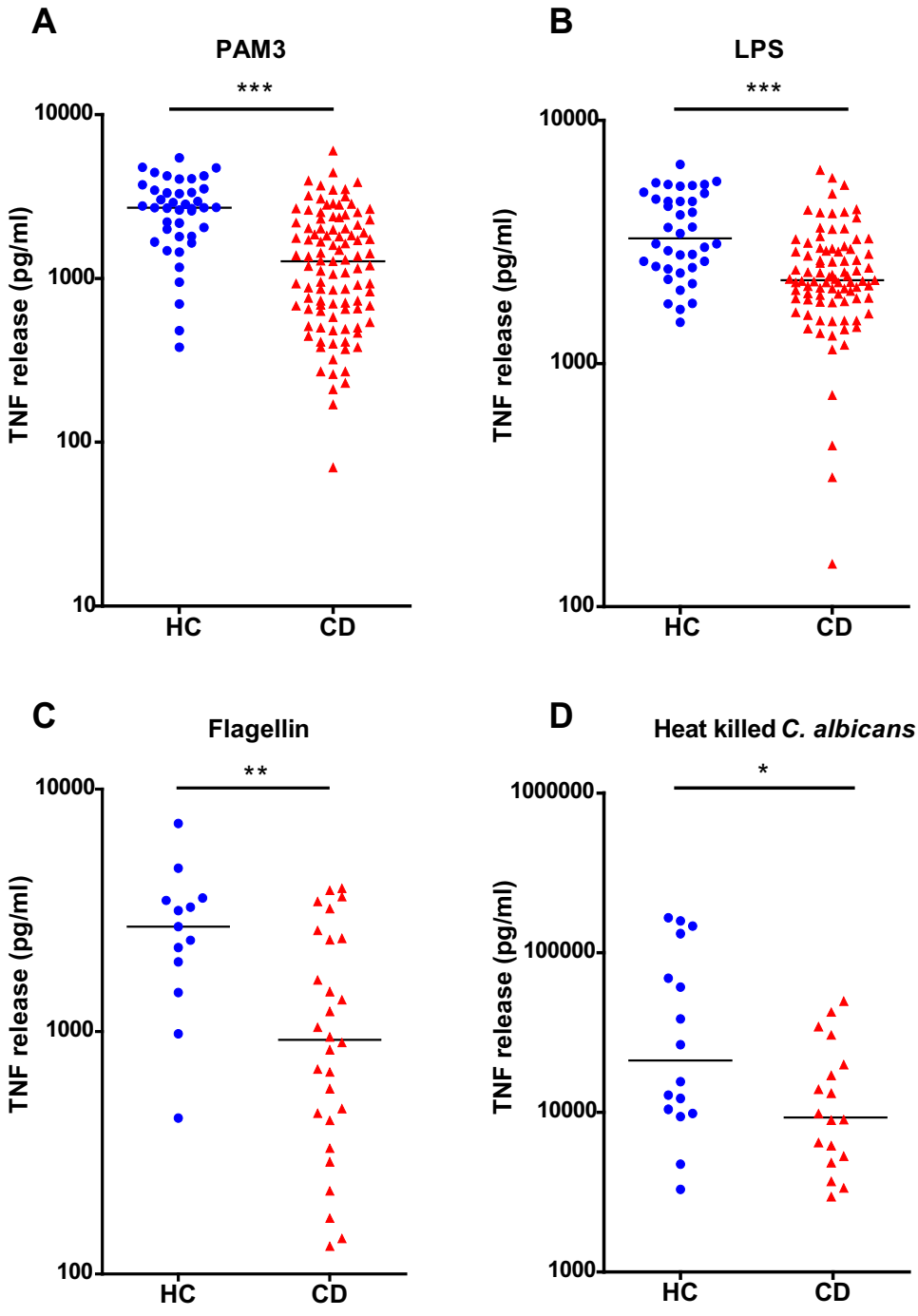


Figure 3.4 Macrophages from CD patients release attenuated levels TNF in response to multiple stimuli. Monocyte-derived macrophages were stimulated for 6-24 hours with the TLR agonists Pam_3CSK_4, LPS and flagellin, and release of TNF quantified using the TNF bioassay. Heat-killed *C. albicans* was also used as a stimulus. **(A)** TNF release from HC (n=41 donors) and CD (n=101) macrophages in response to TLR2 stimulation with Pam_3CSK_4 (PAM3). **(B)** TNF release from HC (n=38) and CD (n=87) macrophages in response to the TLR4 agonist LPS. **(C)** TNF release from HC (n=13) and CD (n=28) macrophages in response to TLR5 stimulation with flagellin. **(D)** TNF release from HC (n=16) and CD (n=18) macrophages after 24 hours stimulation with heat-killed *C. albicans*. All data are presented on a logarithmic scale. * represents $p<0.05$, ** $p<0.01$ and ***$p<0.001$.

no statistical difference was identified between the two batches of data. Secreted levels of TNF into tissue culture medium without stimuli were minimal over 24 hours and did not differ between HC and CD macrophages ($p>0.05$).

TNF release from CD (n=101) patient macrophages after TLR2 activation with Pam_3CSK_4 was significantly ($p<0.001$) reduced compared to HC (n=41) (Figure 3.4A). Median TNF release from CD macrophages in response to Pam_3CSK_4 was 1,270 pg/ml (interquartile range 650-2,155 pg/ml) compared to 2,700 pg/ml (interquartile range 1,805-3,495 pg/ml) from HC cells, representing a 53% reduction from the HC cohort.

Similarly, after TLR4 stimulation using LPS, TNF release from CD macrophages (n=87) was significantly ($p<0.001$) lower than TNF release from HC macrophages (n=38) (Figure 3.4B). Stimulation with flagellin, a TLR5 agonist, likewise revealed diminished TNF release from CD macrophages compared to HC (CD n=28 and HC n=13, $p<0.001$) (Figure 3.4C).

Differential levels of TNF release were also observed in response to HkCa (Figure 3.4D). HC macrophages released, on average 21,109 pg/ml TNF after 24 hours stimulation with HkCa (n=16). In contrast, CD macrophages released 9,304 pg/ml in response to HkCa (n=18); 56% less than that of the control group. The difference in TNF release was statistically significant ($p<0.05$).

Correlation analysis was used to determine whether there was a relationship between attenuated TNF release downstream of TLR2, TLR4 and HkEc stimulation in the CD cohort. There was a significant positive correlation between TLR2 induced TNF release and secreted levels of TNF after HkEc ($R_s=0.521$, $p<0.05$) (Figure 3.5A). Furthermore, there was a weak but significant correlation between TLR2 and TLR4 induced TNF release in the CD group ($R_s=0.281$, $p<0.01$) (Figure 3.5B). Insufficient

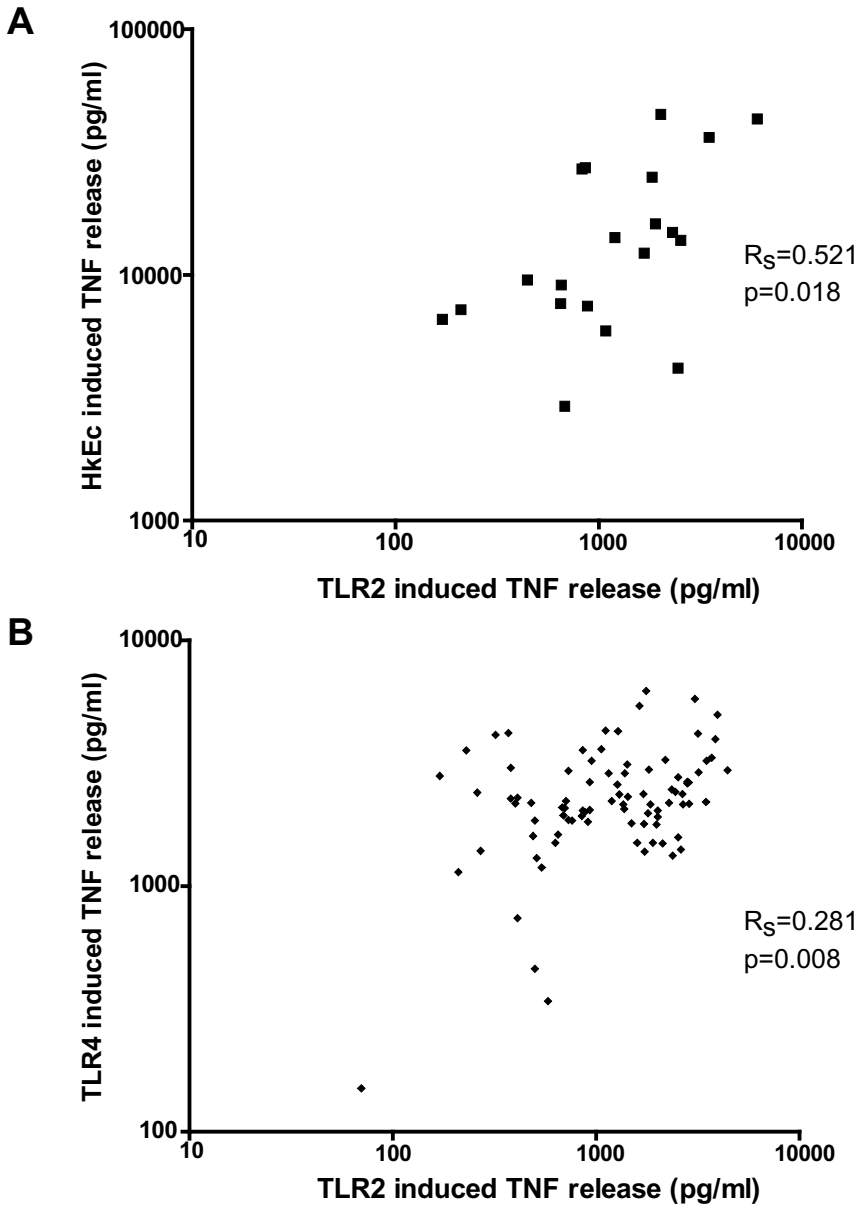


Figure 3.5 Correlation of attenuated TNF release in CD between microbial stimuli. **(A)** Correlation between secreted levels of TNF from CD macrophages (n=20) in response to TLR2 stimulation with Pam_3CSK_4 and HkEc. **(B)** Correlation between secreted levels of TNF from CD macrophages (n=87) in response to TLR2 stimulation with Pam_3CSK_4 and TLR4 stimulation with LPS. R_S is Spearman's rank correlation coefficient.

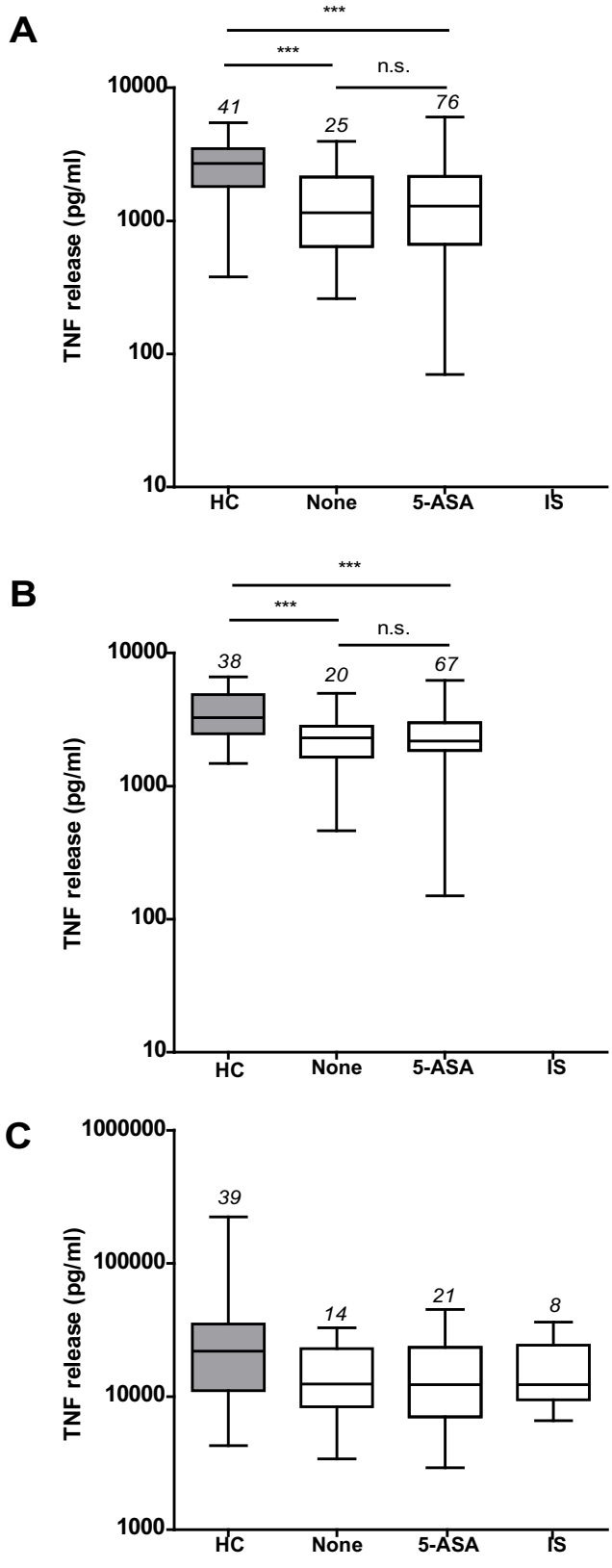


Figure 3.6 Impaired TNF secretion from CD macrophages is not related to treatment regimes. Macrophage TNF release from HC and CD patients after **(A)** Pam_3CSK_4 stimulation, **(B)** LPS stimulation and **(C)** HkEc stimulation, with CD patients subdivided on the basis of treatment regimes, including no treatment (None), 5-aminosalicylates (5-ASA) or immunosuppressant (IS, azathioprine or methotrexate). Data are presented as box and whisker plots on a logarithmic scale showing median, interquartile range and range. Numbers for each group are shown in italics. *** indicates $p<0.001$; no other differences were statistically significant.

overlap in the patients studied between the TLR4, HkEc and HkCa cohorts precluded assessment of correlation between these stimuli.

3.2.4 Impaired TNF release is not dependent on treatment regimes, age, gender and smoking status

In order to assess whether use of medication could have a confounding influence on TLR2 induced macrophage TNF release, the CD cohort was subdivided into those receiving either no medical treatment (n=25) or 5-ASA alone (n=76) at the time of venesection. No patients in this cohort were receiving immunosuppressants. Both groups displayed impaired TNF secretion after Pam_3CSK_4 exposure compared to HC ($p<0.001$). Furthermore, there was no significant difference in TNF release between patients receiving no treatment or those prescribed 5-ASA (Figure 3.6A). Similar results were obtained for TLR4 activation with LPS (Figure 3.6B).

Furthermore, levels of TNF secretion in response to HkEc were equivalent between patients receiving no treatment, 5-ASA or immunosuppressants (azathioprine or methotrexate) (Figure 3.6C), although none of the groups reached statistical significance against the HC cohort. Taken together, these observations suggest that the impaired TNF release from CD macrophages is independent of treatment regimes at the time of sample collection.

In addition, no associations were identified between TNF release and age, gender and smoking status after TLR2 stimulation (Figure 3.7A, B, C). Similar results were obtained for TLR4 and HkEc stimulation.

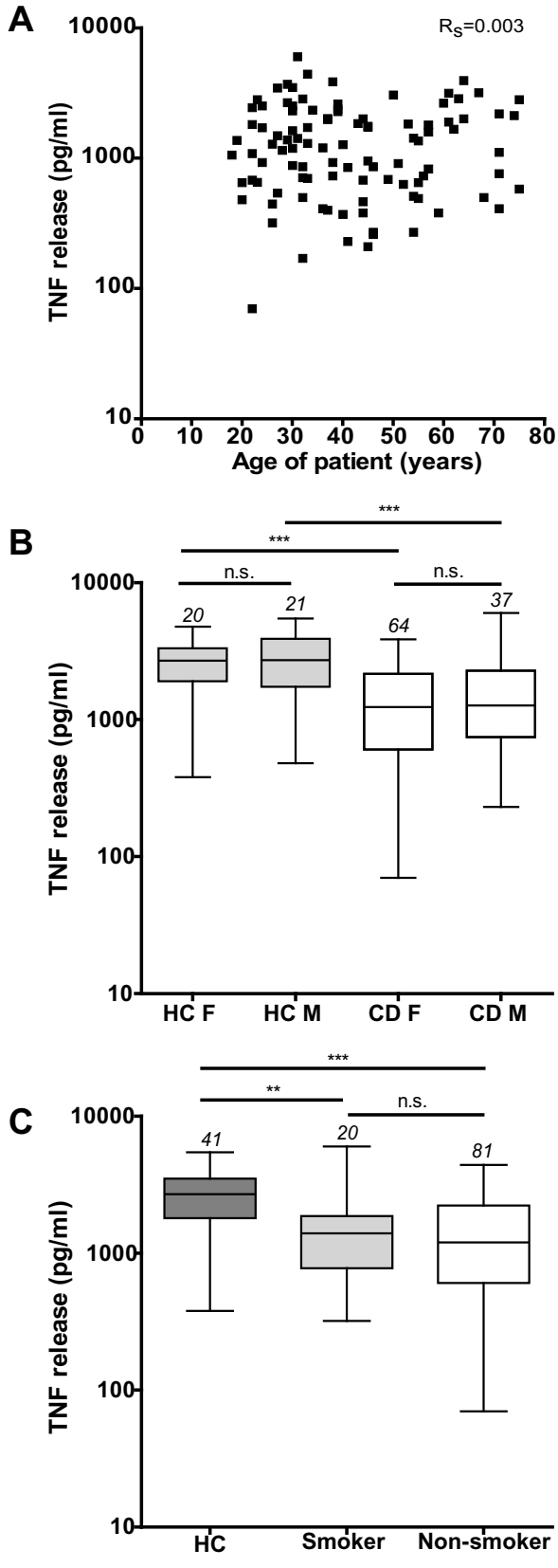


Figure 3.7 Defective release of TNF is not associated with age, gender or smoking status. CD patients and where applicable HC were divided by **(A)** age, **(B)** gender (M=male, F=female) and **(C)** current smoking status and TNF secretion in response to TLR2 stimulation compared between groups. Results for TNF aganist age are shown as a scatter plot with corresponding R_S value. All other results are expressed as box and whisker plots on a logarithmic scale showing median, interquartile range and range. Numbers in each group are shown in italics. *** represents $p<0.001$, **$p<0.01$. All other changes were not significant (n.s.)

3.2.5 Association between aberrant TNF release and disease phenotype

It is now recognised that CD is a heterogeneous syndrome and can be divided into phenotypic subtypes. Where information was available, CD patients were sub-divided into groups according to Montreal classification, a system that classifies patients based on age at diagnosis (A), anatomical location of disease (L), and disease behaviour (B) (4). After TLR2 stimulation, TNF release was comparable between patients diagnosed with CD under 16 years (A1, n=15), those diagnosed between 17 and 40 years (A2, n=65) and those diagnosed over 40 years of age (A3, n=21) (Figure 3.8A). Similarly, equivalent levels of TNF were released between patients with ileal (L1, n=32), colonic (L2, n=44) and ileocolonic (L3, n=24) disease (Figure 3.8B). Considering disease behaviour, patients with stricturing disease (B2, n=23), released significantly reduced levels of TNF compared to those with pure inflammatory disease (B1, n=63) and those with penetrating disease (B3, n=15), suggesting that an abnormality in TLR2 induced TNF could be particularly relevant in stricture pathogenesis.

Considering TLR4 stimulation with LPS, there were no significant differences in TNF release between patients subdivided by age of diagnosis (Figure 3.9A). Macrophages from patients with colonic involvement (n=41) released diminished levels of TNF downstream of LPS ($p<0.05$) compared to patients with ileal disease (n=25) (Figure 3.9B). Macrophages from one patient with isolated upper GI disease (L4) secreted TNF at levels that were ~5% of median HC levels. This patient had early-onset disease (A1) and stricturing behaviour (B2). In contrast to TLR2, there were no differences between patients when subdivided by disease behaviour (Figure 3.9C).

Considering HkEc stimulation, there was a trend for patients diagnosed with CD under 16 years of age (n=11) to release less TNF than patients diagnosed over 40 years

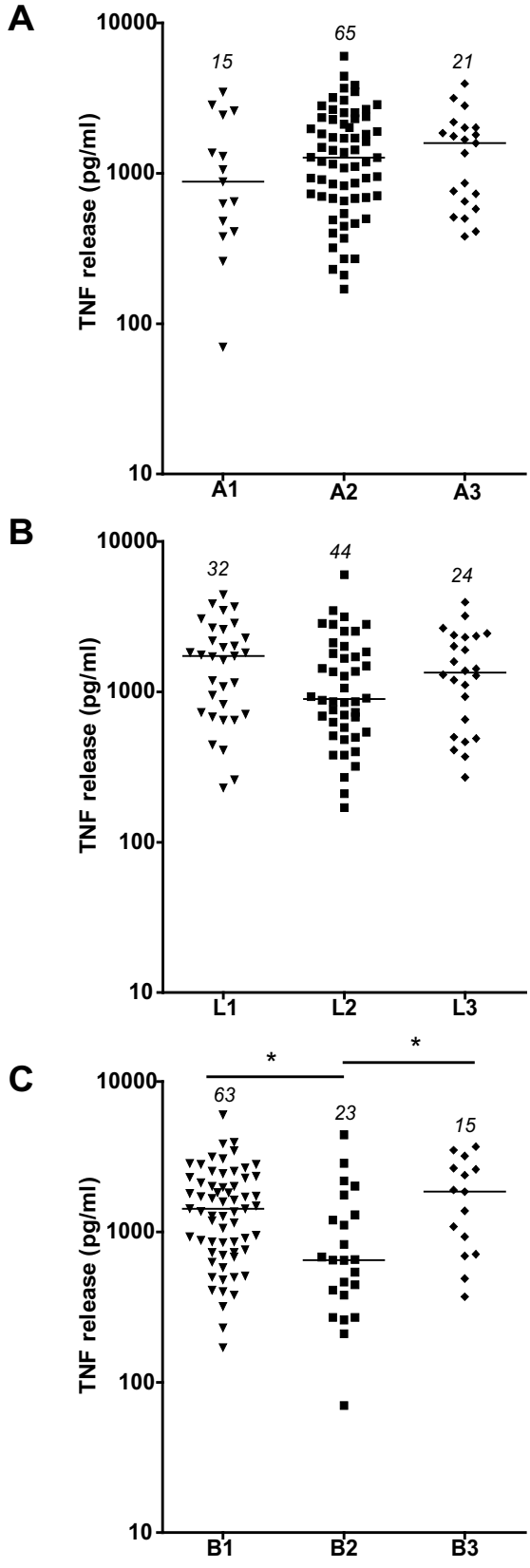


Figure 3.8 TNF release from CD macrophages in response to TLR2 stimulation, with CD patients subdivided by Montreal phenotypic classification. Monocyte-derived macrophages were cultured from CD patients and stimulated with Pam3CSK4. **(A)** Subdivision by age at diagnosis, where A1, A2 and A3 represent age below 16, 17-40 and above 40 years respectively. **(B)** Subdivision by disease location, where L1, L2 and L3 are ileal, colonic and ileocolonic disease. **(C)** Subdivision by phenotype, where B1, B2 and B3 represent inflammatory, stricturing and penetrating disease respectively. Results are presented on a logarithmic scale. Numbers in each group are shown in italics. * represents $p<0.05$. No other changes were statistically significant.

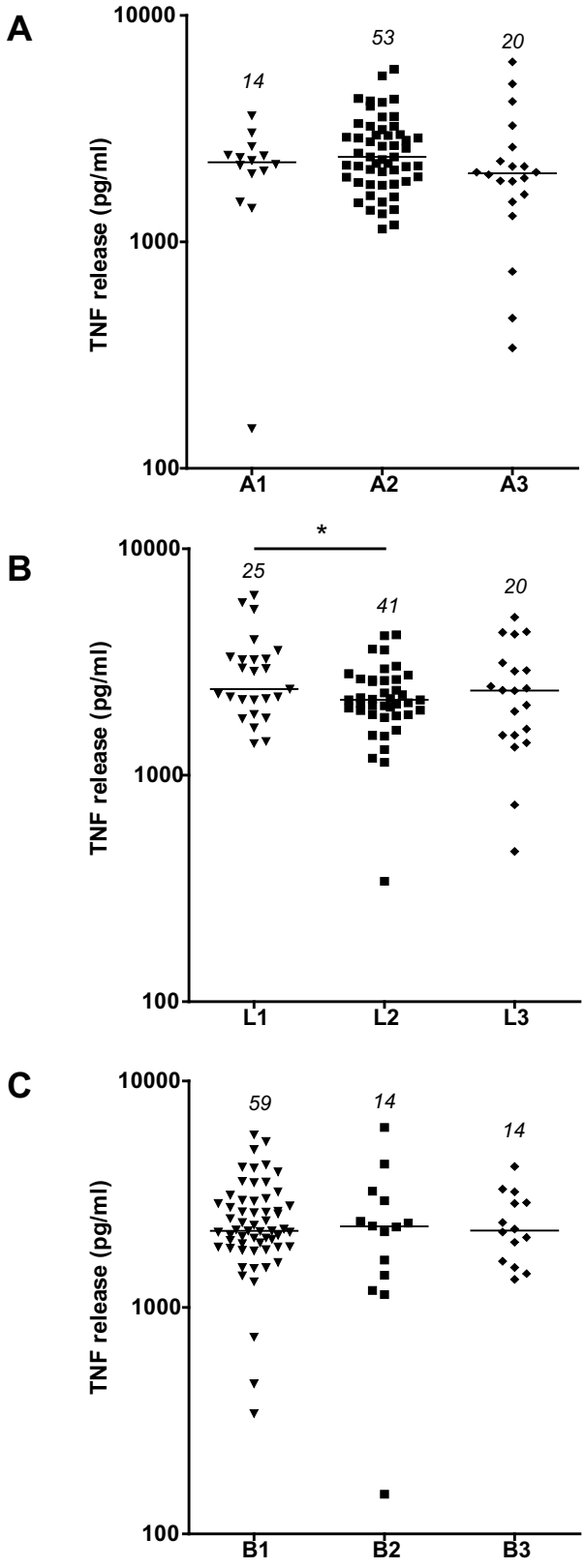


Figure 3.9 TNF release from CD macrophages in response to TLR4 stimulation, with CD patients subdivided by Montreal phenotypic classification. Monocyte-derived macrophages were cultured from CD patients and stimulated with LPS. **(A)** Subdivision by age at diagnosis, where A1, A2 and A3 represent age below 16, 17-40 and above 40 years respectively. **(B)** Subdivision by disease location, where L1, L2 and L3 are ileal, colonic and ileocolonic disease. **(C)** Subdivision by phenotype, where B1, B2 and B3 represent inflammatory, stricturing and penetrating disease respectively. Results are presented on a logarithmic scale. Numbers in each group are shown in italics. * represents $p<0.05$. No other changes were statistically significant.

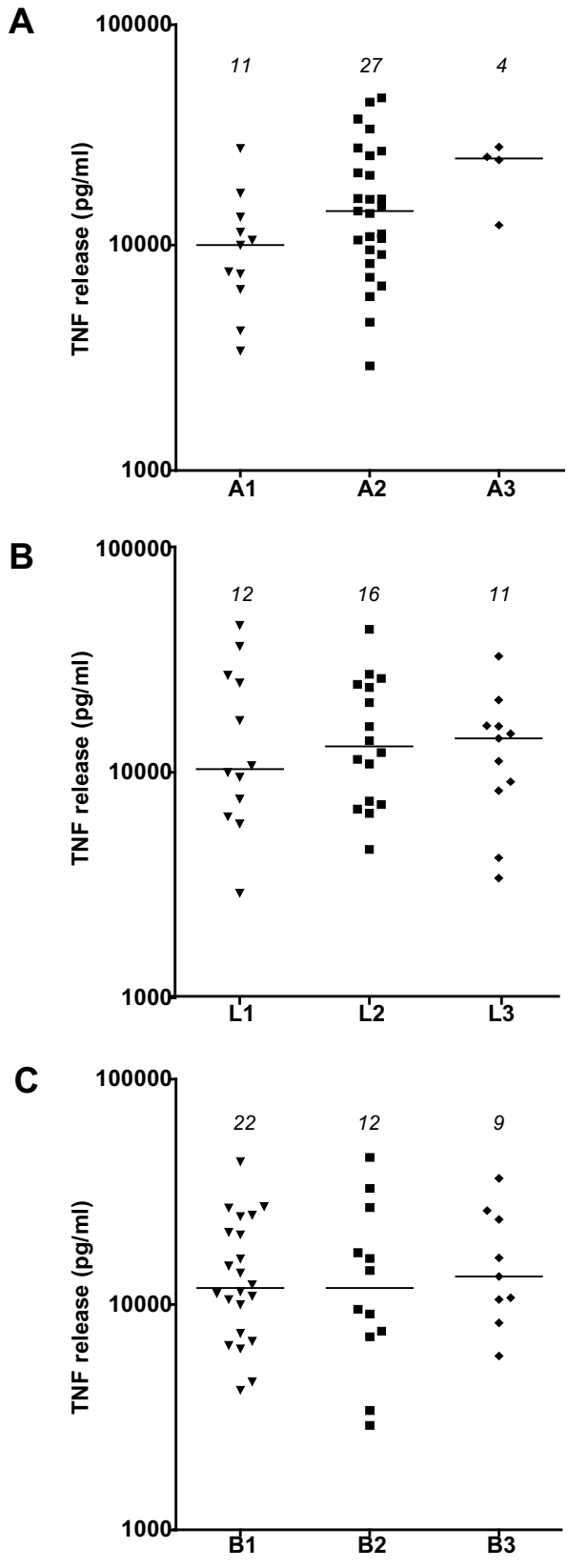


Figure 3.10 TNF release from CD macrophages in response to HkEc stimulation, with CD patients subdivided by Montreal phenotypic classification. Monocyte-derived macrophages were cultured from CD patients and stimulated with HkEc **(A)** Subdivision by age at diagnosis, where A1, A2 and A3 represent age below 16, 17-40 and above 40 years respectively. **(B)** Subdivision by disease location, where L1, L2 and L3 are ileal, colonic and ileocolonic disease. **(C)** Subdivision by phenotype, where B1, B2 and B3 represent inflammatory, stricturing and penetrating disease respectively. Results are presented on a logarithmic scale. Numbers in each group are shown in italics.

of age (n=4) ($p<0.05$) (Figure 3.10A), although this was not statistically significant ($p>0.05$) (Figure 3.10A). Secreted levels of TNF were not significantly different between patients with ileal (n=12), colonic (n=16) and ileocolonic (n=9) disease (Figure 3.10B), and levels were comparable between patients with inflammatory, stricturing and penetrating disease (Figure 3.10C).

3.2.6 Relationship of impaired TNF secretion to GWAS susceptibility loci

GWAS have identified numerous susceptibility loci associated with CD. A meta-analysis of three genome-wide association studies in 2008 identified 32 susceptibility loci that were convincingly associated with CD (116). More recently, a GWAS meta-analysis using a larger cohort of patients identified 39 additional associated variants, accounting for an additional 3% of the total heritability (115). A GWAS study of early-onset IBD patients, published in 2009, identified three additional associations in patients with CD (253). Of the polymorphisms identified to date, the strongest association is with *NOD2,* which encodes an intracellular PRR that recognises MDP, a component of peptidoglycan. The three CD-associated polymorphisms in *NOD2* have been shown to result in defective pro- and anti- inflammatory cytokine induction after MDP (215). The effect of *NOD2* polymorphisms on TNF release downstream of TLR2 stimulation was investigated (Figure 3.11A). Patients homozygous or compound heterozygous for polymorphisms in NOD2 (n=5) secreted, on average 710 pg/ml (interquartile range 546-1,505 pg/ml) TNF in response to TLR2, compared to 1,270 pg/ml (interquartile range 630-2,120 pg/ml) in patients wild type for *NOD2* polymorphisms (n=71), however this reduction was not statistically significant. There was also no detectable relationship between attenuated TNF release and polymorphisms in the autophagy-associated genes *ATG16L1* and *IRGM* (Figure 3.11B, C).

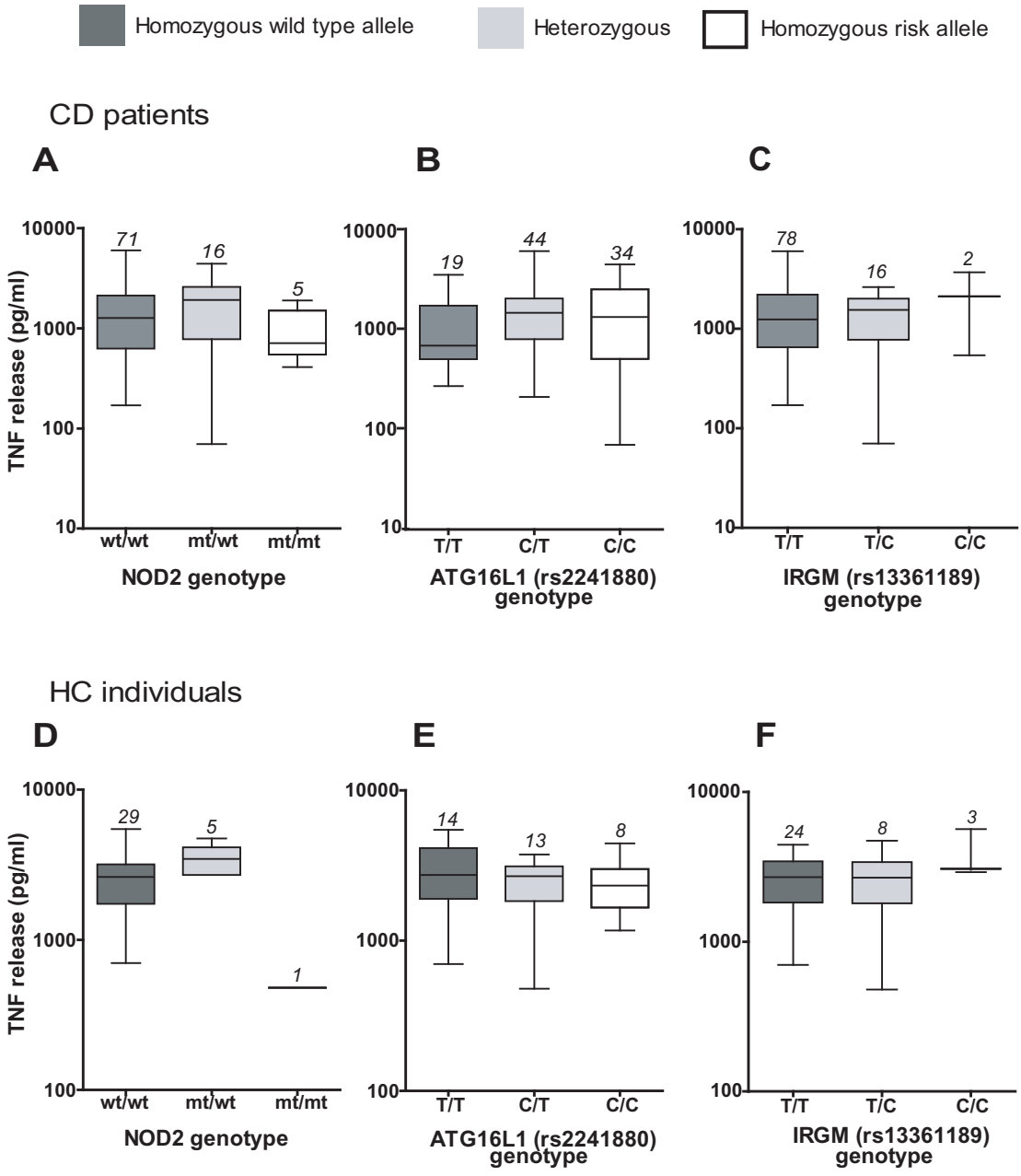


Figure 3.11 Relationship between TNF secretion after TLR2 stimulation and *NOD2*, *ATG16L1* and *IRGM* genotypes. CD patients were typed for polymorphisms associated with CD and separated into homozygous non-risk (dark grey), heterozygous (light grey) and homozygous risk (white) for **(A)** *NOD2* (rs2066844, rs2066845, rs2066847), showing wild type (wt/wt), heterozygous (mt/wt) and homozygous or compound heterozygous individuals (mt/mt) **(B)** *ATG16L1* and **(C)** *IRGM*, and the corresponding TNF levels released after TLR2 stimulation shown. Corresponding results for HC are shown in figures **(D), (E)** and **(F)**. Data are presented as box and whisker plots on a logarithmic scale showing median, interquartile range and range. No significant differences were observed between genotypes.

Similar findings were apparent in the HC cohort. HC carrying one *NOD2* risk allele (n=5) released TNF at equivalent levels to individuals with two wild type alleles (n=29). One healthy individual was identified who was homozygous for *NOD2* risk alleles. This 25 year old male had a history of atopic eczema but no other medical history of note, and no family history of IBD. Macrophage TNF release in response to TLR2 stimulation was attenuated from this individual (480 pg/ml, ~38% of median HC secretion) (Figure 3.11D). The release of TNF from HC macrophages in response to Pam_3CSK_4 did not differ significantly between wild type, heterozygous or homozygous individuals for *ATG16L1* and *IRGM* CD-associated polymorphisms (Figure 3.11E, F).

In addition to *NOD2*, *ATG16L1* and *IRGM*, none of the 28 other CD-associated polymorphisms tested (25 from the 2008 meta-analysis (116) and 3 from the 2009 study (253)) showed detectable associations with TNF release downstream of TLR2 activation. No CD patients or HC individuals in the cohort were homozygous for the minor *IL-23R* allele, precluding assessment of how TNF release in response to TLR2 activation could be influenced by this protective genotype.

The influence of GWAS susceptibility loci on HkEc-induced TNF release was also assessed. Although median TNF release from CD patients carrying two NOD2 risk alleles (n=3) was slightly reduced compared to CD patients with two wild type alleles (n=26), this difference was not statistically significant (Figure 3.12A). As for TLR2 stimulation, there was no significant difference in HkEc-induced TNF release between CD patients carrying no, one or two *ATG16L1* or *IRGM* risk alleles (Figure 3.12B, C). Similar results were obtained in the HC cohort (Figure 3.12D, E, F). TNF release in response to HkEc from HC individuals heterozygous for CD-associated polymorphisms in *NOD2* (n=5) did not differ significantly from the wild type group (n=26). Macrophage TNF release from the HC individual homozygous for CD-associated

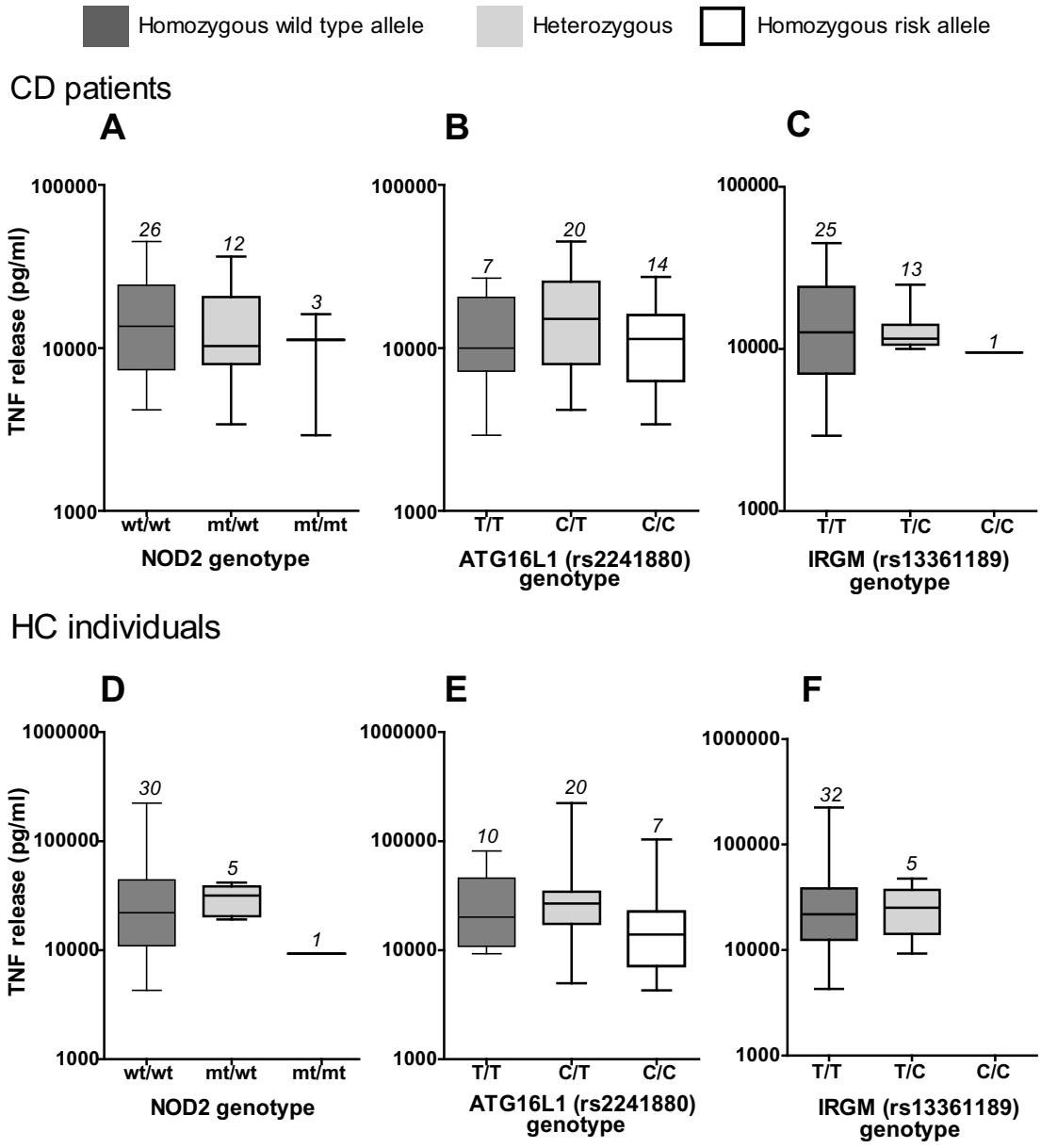


Figure 3.12 Relationship between TNF secretion after HkEc stimulation and *NOD2*, *ATG16L1* and *IRGM* genotypes. CD patients and HC were typed for CD-associated polymorphisms and separated into non-risk (dark grey), heterozygous (light grey) and homozygous risk (white) for **(A)** *NOD2* (rs2066844, rs2066845, rs2066847) where wt/wt= wild type, mt/wt = heterozygous and mt/mt = homozgyous or compound heterozygous, **(B)** *ATG16L1* and **(C)** *IRGM*, and the corresponding TNF levels released after stimulation shown. Corresponding results for HC are shown in figures **(D)**, **(E)** and **(F)**. Data are presented as box and whisker plots on a logarithmic scale showing median, interquartile range and range. No significant differences were observed between genotypes.

NOD2 polymorphisms in response to HkEc was in the lowest quartile for the HC cohort (9279 pg/ml). No HC individuals were homozygous for the *IRGM* risk allele.

Furthermore, none of the other 28 polymorphisms were significantly associated with TNF release in response to HkEc, in either CD or HC patients. As for TLR2, no patients were homozygous for the *IL-23R* minor allele, meaning that the contribution of this protective genotype to macrophage TNF release in response to HkEc could not be assessed.

3.2.7 Relationship of attenuated TNF release to overall genetic risk score

Polymorphisms in multiple genetic loci are associated with increased susceptibility to CD. Emerging evidence suggests that products encoded by these loci may map to common pathways and networks, giving rise to abnormal phenotypes relevant to CD pathogenesis. The overall contribution of 34 GWAS susceptibility loci (31 from the 2008 meta-analysis, and 3 from the more recent GWAS of early-onset disease) to impaired macrophage TNF release was assessed by calculation of a CD 'genetic risk score' (GRS) for each individual. This risk score was based on the genotype of the 34 CD-associated polymorphisms, weighted by odds ratio of each locus and assuming additive genetic effects. A risk score of 1 represents average risk for a control population. Only individuals where >95% of the SNPs could be reliably determined were included in this analysis.

Overall, our entire cohort of 356 CD patients displayed enrichment for the 34 CD susceptibility alleles, as shown by the increased GRS compared to 221 HC individuals ($p<0.001$). This cohort has been collated over a 10 year period by a number of individuals (M. Harbord, D. Marks, F. Rahman, B. Hayee, G. Sewell, A. Walker, A. Smith, A. Levine, P. Smith and A. Segal). Of note, a wide variation in GRS was observed in both groups, ranging between 0.07 and 9.07 in the HC cohort and 0.14 to

37.2 in the CD cohort. GRS for patients in the TLR2 and HkEc study did not differ from the overall cohort, indicating a representative sample of patients ($p>0.05$); (Figure 3.13A, B).

The HC and CD study cohort of patients was divided into two groups – those with an overall GRS of less than 1, and those with a GRS greater than 1. There was no difference in TLR2-induced TNF release between CD patients with a GRS less than 1 (n=47), and those with a GRS greater than 1 (n=43), suggesting that the impaired TNF secretion in response to TLR2 is unrelated to patients' overall genetic risk as identified by GWAS (Figure 3.14A). Intriguingly, macrophages from HC individuals with a GRS greater than 1 (n=16) released significantly ($p<0.05$) more TNF than those with a GRS less than 1 (n=18) (Figure 3.14B), suggesting that healthy individuals with a greater burden of GWAS risk alleles could be protected from developing CD by virtue of a strong, TLR2-mediated macrophage response to bacteria.

In contrast to the results for TLR2, after HkEc stimulation, CD patients with a GRS greater than 1 (n=20) released significantly ($p<0.05$) less TNF than patients with a GRS less than 1 (n=18) (Figure 3.14C). Furthermore, there was a weak but significant negative correlation between HkEc-induced TNF release and GRS in the CD cohort ($R_s=-0.38$, $p=0.018$) (Figure 3.15). This indicates a relationship between attenuated TNF release in response to HkEc and an increased burden of GWAS susceptibility alleles. In the HC cohort, patients with a GRS over 1 also secreted reduced TNF in response to HkEc than those with a GRS less than 1, although this did not reach statistical significance (Figure 3.14D).

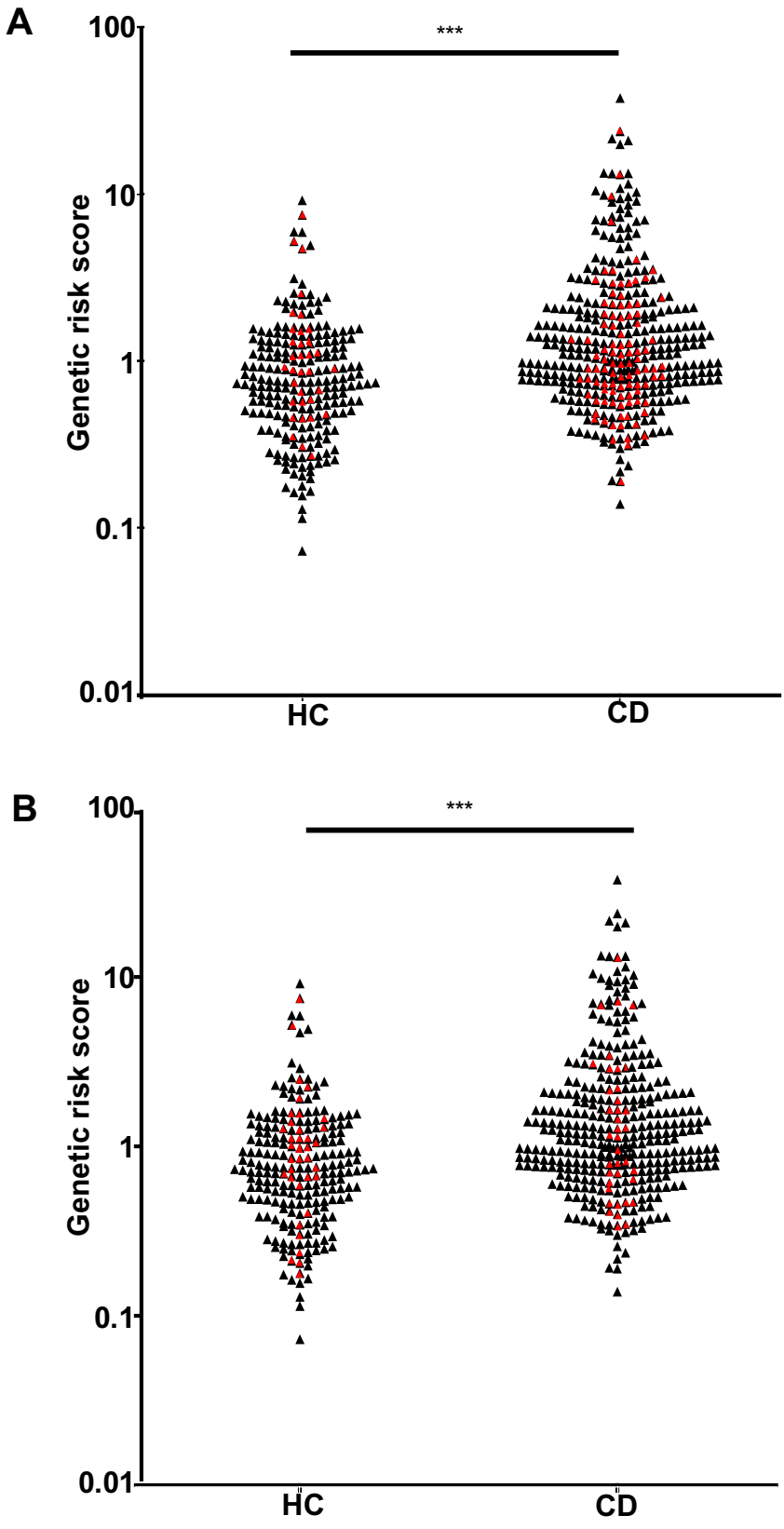


Figure 3.13 Genetic risk scores for developing CD in the total UCLH and study cohorts. The UCLH cohort consists of CD patients (n=356) and HC individuals (n=221). *** represents $p<0.001$ for difference in genetic risk score of the entire UCLH cohort of CD patients compared to HC individuals. **(A)** TLR2 study cohort patients are highlighted in red. **(B)** HkEc study cohort are highlighted in red. The genetic risk scores of the HC and CD studied in the TLR2 and HkEc cohorts did not differ from the total UCLH HC and CD patient cohorts.

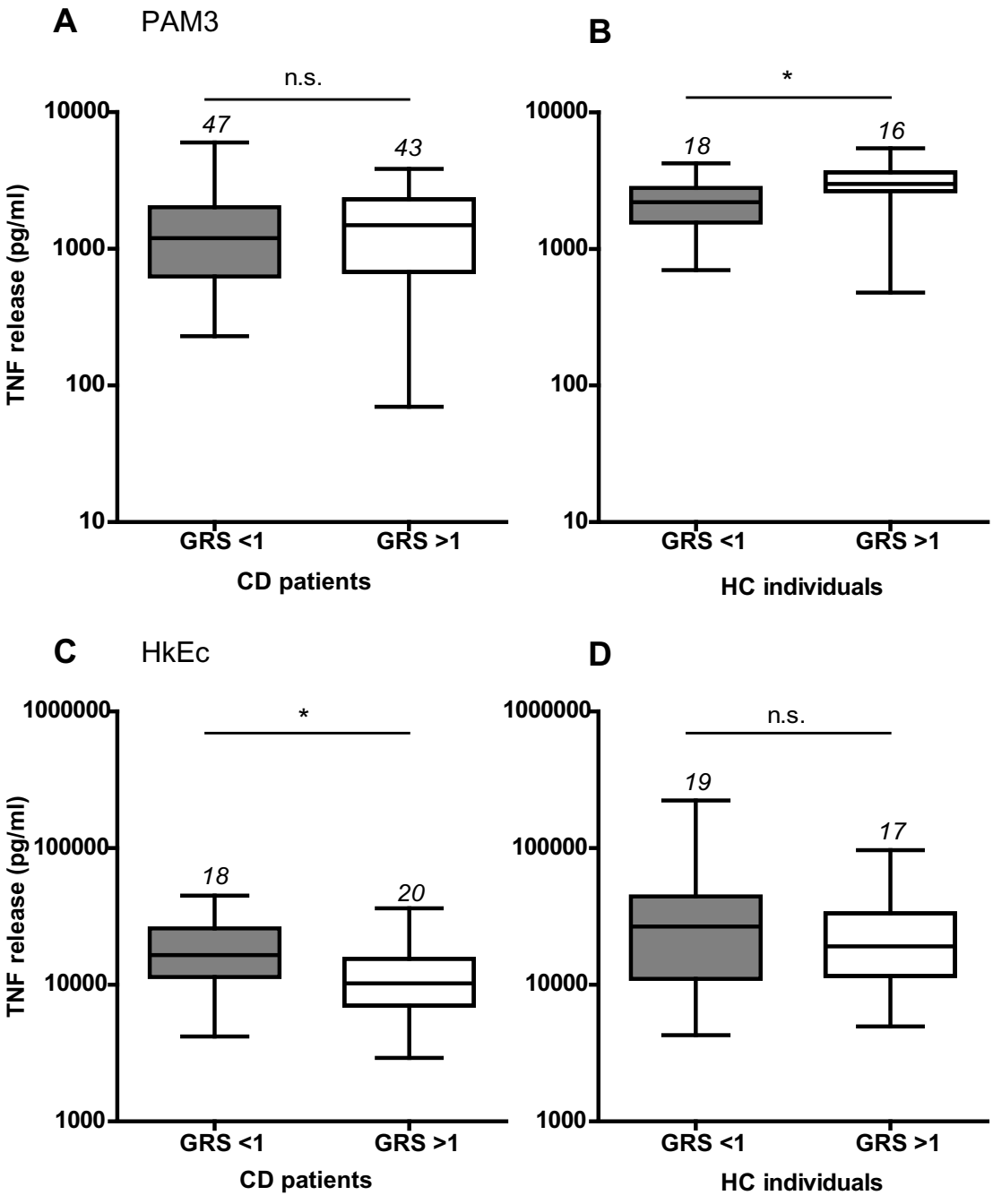


Figure 3.14 HC and CD individuals separated into risk of developing CD (Genetic risk score, GRS) based on a 34 SNP genotype, and relationship to the TLR2 and HkEc response. Susceptibility variants of SNPs associated with the development of CD are common in both HC and CD individuals. The **(A)** CD and **(B)** HC cohorts were divided into individuals with GRS <1 and GRS >1, and macrophage TNF response to TLR2 (PAM3) stimulation compared. **(C, D)** Corresponding results for HkEc stimulation. Data are presented as box and whisker plots on a logarithmic scale showing median, interquartile range and range. Numbers in each group are shown in italics. * indicates $p<0.05$.

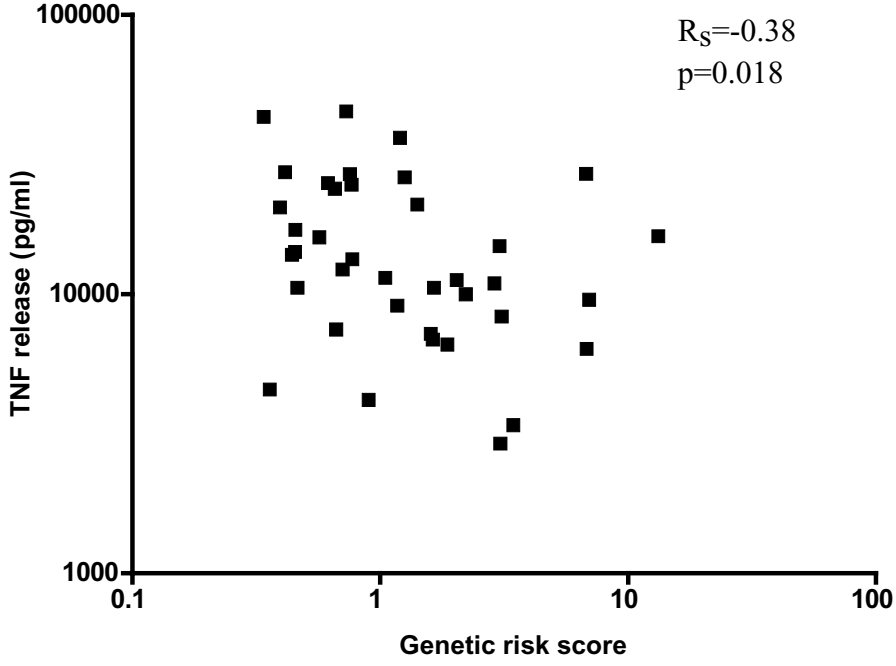


Figure 3.15 Correlation between TNF release from CD macrophages after HkEc stimulation and genetic risk score. Genetic risk score for CD patients (n=38) was calculated from the genotype of 34 risk-associated variants and correlated with TNF release after HkEc stimulation. Data are presented on logarithmic scale. R_S value shown is Spearman's rank correlation coefficient, with corresponding p-value.

3.3 Discussion

In vivo studies clearly demonstrate that the CD phenotype is characterised by an impaired acute inflammatory response and delayed bacterial clearance (135;136). Whilst neutrophil function in the majority of patients is normal, macrophages from CD patients were previously shown to release deficient levels of pro-inflammatory cytokines in response to HkEc, as a result of aberrant degradation of these molecules in lysosomal compartments (136). In this chapter, the abnormal release of pro-inflammatory cytokines in response to HkEc was verified in a second cohort of CD patients and HC individuals. Macrophages from CD patients demonstrate attenuated TNF release downstream of multiple microbial stimuli, including the TLR2, TLR4 and TLR5 agonists, and HkCa, which stimulates a variety of TLR and non-TLR receptors such as the C-type lectin mannose receptor and dectin 1 (97). In addition, whilst there was a generalised depression in TNF release to various stimuli in the overall CD cohort, a number of subtle differences in the levels of TNF release were apparent between patient phenotypic subgroups, which were stimulus specific.

CD is a complex syndrome, the pathogenesis of which is likely to occur in many temporally distinct phases. Our group recently proposed a ‘three stage’ model for CD pathogenesis, where mucosal damage and penetration of bacteria and other particulate matter into the bowel wall are followed by an inadequate acute inflammatory response and incomplete bacterial clearance. Subsequently, a compensatory adaptive immunological response develops, associated with chronic, granulomatous inflammation and an elevation in pro-inflammatory cytokines that is characteristic of the ‘active’ phase of CD (151). The finding of deficient macrophage secretion of pro-inflammatory cytokines such as TNF in response to microbial stimuli is consistent with this pathogenic schema. TNF is a pleiotropic cytokine with a prominent role in the

initiation of an acute inflammatory response, upregulating endothelial cell adhesion molecules such as ICAMs necessary to ensure neutrophil influx to sites of acute inflammation (285). Defective macrophage TNF release may therefore provide an explanation for the deficient neutrophil recruitment observed at acute inflammatory sites in CD.

Animal studies further support the concept that inadequate TNF production during an acute inflammatory response leads to impaired bacterial clearance, increasing susceptibility to bowel inflammation and driving chronic granulomatous pathology. TNF deficient mice display enhanced susceptibility to DSS-induced colitis, and a greater 7 day mortality in comparison to wild type mice (286). Furthermore, in zebrafish models infected with *Mycobacterium marinum*, loss of TNF signalling results in increased bacterial burden, which is associated with accelerated granuloma formation (287).

The secreted levels of the chemoattractant IL-8 after HkEc stimulation did not differ between CD and HC macrophages. This may be surprising, given the previous demonstrations of attenuated IL-8 levels in skin windows from CD patients, and defective macrophage release of IL-8 in response to stimuli such as C5a and wound fluid (135). There are two important considerations here. Firstly, TNF is an inducer of NF-κB activation (288), thereby triggering IL-8 gene transcription; abnormalities in IL-8 could therefore occur secondary to defective release of TNF, depending on the timing and nature of the stimulus investigated. Secondly, whilst macrophages are important producers of chemokines during acute inflammation, neutrophils are also recognised to secrete significant amounts of IL-8 upon activation (289). IL-8 levels were determined 24 hours after skin window creation, when neutrophil influx is dramatically impaired in CD. It is therefore possible that the observed reduction in IL-8 in skin windows is a

consequence of differential neutrophil recruitment between HC and CD patients. This may well still be of pathogenic significance and could act to further compound an initial defect in the acute inflammatory response in CD.

Attenuated TNF secretion in CD was observed in response to a variety of stimuli, including TLR agonists and HkCa, demonstrating that the abnormalities previously described are not purely a phenomenon specific to HkEc. The observation of impaired macrophage TNF release in response to HkCa is noteworthy, given the presence of ASCA in a substantial percentage of CD patients, and to a lesser extent in their healthy relatives, in whom ASCA titres correlate with *C. albicans* colonisation (101;290;291). Defective pro-inflammatory cytokine release from macrophages could lead to impaired clearance of *C. albicans in vivo*; the persistence of *C. albicans* or its components could then trigger the generation of ASCA. However, a primary role for *C. albicans* itself in CD remains an area of controversy, given the lack of clear microscopic evidence of invasive candidosis in CD, and expression of the ASCA epitope in other microbes, including MAP (98). Interestingly, MAP stimulation of CD macrophages may also be associated with attenuated TNF release (187). Therefore, the results cannot be extrapolated as proof of a causative role for any individual organism in the pathogenesis of CD. Rather, the results suggest an attenuated macrophage response to a variety of microbial stimuli; the nature of the critical macrophage-microbe interactions in the mucosa is however likely to be complex and heterogeneous between individuals.

The observation of attenuated TNF release in response to a variety of microbial stimuli is consistent with an abnormality in post-translational trafficking of pro-inflammatory cytokines in CD macrophages. Although post-translational handling of cytokines in response to TLR agonists and *C. albicans* has not been directly addressed here, surface expression of TLRs, levels of pro-inflammatory cytokine mRNA after

TLR agonist stimulation, as well as mRNA stability, were found to be unaltered in other studies conducted in our laboratory (Dr Farooq Rahman, unpublished findings). This indicates that there is no abnormality in the downstream signalling pathways of TLRs, and in the control of cytokine gene transcription after TLR stimulation, further suggesting a post-translational defect in pro-inflammatory cytokine handling in these cells. The correlations observed between secreted levels of TNF in response to TLR2 and HkEc stimulation, and between TNF release after TLR2 and TLR4 stimulation, also indicate a likely degree of overlap in the underlying cellular and molecular mechanisms responsible for the attenuated pro-inflammatory cytokine release downstream of each stimulus.

A strong correlation was observed between the secreted levels of TNF, IL-6 and IFN-γ, indicating that the impaired release of these pro-inflammatory cytokines in CD macrophages is related. One possible explanation is that impaired release of IL-6 and IFN-γ is secondary to an initial impairment in TNF secretion, given the ability of TNF to influence IL-6 gene transcription and release (292). However, the selective impairment in a number of pro-inflammatory cytokines could also relate to abnormalities in a specific vesicle subset. Studies conducted in RAW macrophage cell lines revealed that overlapping mechanisms are involved in the post-translational trafficking of TNF and IL-6, both of which require subcompartments of the recycling endosome for their release (198). It could therefore be hypothesised that abnormalities exist in the recycling endosomal compartment of CD macrophages, which prevent adequate pro-inflammatory cytokine release on exposure to microbial stimuli. This is currently being addressed in parallel microscopy studies on cellular vesicle trafficking being conducted in our laboratory. Interestingly, the release of the pro-inflammatory cytokine IL-1β was unaffected from CD macrophages in response to HkEc. The intracellular machinery necessary for IL-1β processing and trafficking is distinct from

that of TNF and IL-6, involving membrane-derived microvesicles and inflammasome activity (188). The finding of equivalent IL-1β release indicates that these alternative pathways of cytokine secretion are likely to function normally in CD macrophages in response to *E. coli*, and further argues in favour of specific cellular and molecular defects that give rise to a selective abnormality in cytokine secretion.

Classification of patients in these studies using the Montreal system revealed a number of subtle differences in the macrophage TNF response between phenotypes. The release of TNF after TLR2 stimulation was most attenuated in patients with stricturing disease. Stricture formation is characterised by abnormal wound healing and excessive fibrosis of the bowel wall. This may relate to abnormal myofibroblast migration and collagen deposition in the context of chronic or relapsing inflammation (293). It is possible that a defective TLR2 response predisposes CD patients to stricture formation, either by direct influences on myofibroblast function and collagen formation, or indirectly by predisposing to a more severe disease course. Interestingly, polymorphisms in *NOD2* have been associated with a stricturing phenotype in genetic studies (294;295), supporting the concept that a defective response to microbes downstream of PRRs could be an important prerequisite for the development of strictures.

In contrast, macrophage TNF release downstream of TLR4 stimulation could be particularly pertinent in the development of colonic CD. The large bowel contains larger numbers of bacteria than the ileum (64), and distinct species, including high numbers of anaerobic, Gram negative *Bacteroides* species (65). Some controversy exists as to whether these species primarily stimulate a TLR2 or TLR4 response (296;297). Nevertheless, in a context of a high, complex load of bacterial ligands in the underlying colonic mucosal tissues (that could occur after the mucosal barrier is breached),

adequacy of macrophage TNF release downstream of TLR4 may be a critical factor in ensuring adequate neutrophil recruitment and bacterial clearance in the colon. A small scale genetic study revealed an association of the D299G *TLR4* polymorphism with colonic CD (251), potentially adding credence to this hypothesis. This could exert a small contributory role in the impaired TNF release; most likely in concert with other, distinct molecular and cellular defects, however this has not yet been fully addressed.

The stimulus specific differences in TNF release between patient subphenotypes add to a growing body of evidence that CD is a highly heterogeneous syndrome, with various patterns of disease associated with distinct genetic and functional abnormalities. It is therefore likely that the underlying molecular mechanisms responsible for such functional defects in macrophages, including pro-inflammatory cytokine secretion, are also highly heterogeneous. A variety of abnormalities could give rise to subtly different patterns of macrophage cytokine release between patients, predisposing to a particular disease course.

In an attempt to identify molecular defects underlying the impaired pro-inflammatory cytokine secretion, patients were genotyped for 34 SNPs associated with CD, and TNF release in response to TLR2 and HkEc compared between genotypes. No associations could be detected between any specific CD-associated polymorphism and TNF release, either in response to HkEc or TLR2 stimulation. A possible exception was *NOD2*, as CD individuals and one HC individual that were homozygous for *NOD2* variants demonstrated a trend towards attenuated TNF release compared to wild type individuals. Although studies conducted on macrophages from NOD2 knockout mice revealed normal TNF release in response to TLR2 activation with peptidoglycan (298), further studies may be required to clarify the role of NOD2 in the human macrophage response to TLR2 and HkEc.

Polymorphisms associated with disease through GWAS are mainly markers of shared genetic regions which are present at increased frequency in individuals with diagnosed disease. It is clear that the effects of individual genetic polymorphisms associated with disease by GWAS may be relatively weak, as demonstrated by the high frequency of disease associated SNPs in healthy populations and low penetrance, even in the case of *NOD2*. However, it is likely that the cumulative effects of multiple susceptibility polymorphisms may be of greater relevance to disease pathogenesis. Gene products may interact together in particular functional pathways, giving rise to an abnormal phenotype; statistical approaches (299) and functional studies (242) have identified a number of such pathways where interactions could occur in CD. In order to account for the possibility of cumulative effects of GWAS polymorphisms on macrophage TNF release, overall genetic risk scores were calculated for each individual patient. In the CD group, patients with an increased burden of GWAS risk alleles had a more severe impairment in TNF release in response to HkEc.

Although correlation cannot be regarded as equating to causality, the observed relationship is nevertheless consistent with the notion that the products of GWAS susceptibility loci interact to depress the macrophage response to HkEc in CD. The precise mechanisms can only be speculated on at this stage, especially given that no single polymorphism could be identified as strongly associated with TNF release. Larger scale and functional studies will be required to delineate which polymorphisms and interactions are responsible for the relationship. It is possible that the products encoded by GWAS risk alleles interact to influence intracellular trafficking pathways relevant to pro-inflammatory cytokine release in response to HkEc. In support of this, the NOD2 protein may be targeted to membranes on activation (300), and its agonist MDP is localised to acidified vesicles upon uptake into primary macrophages; a process that occurs via a clathrin and dynamin-dependent endocytic pathway (301). In addition,

stimulation of the NOD2 receptor with MDP leads to induction of autophagy (242;302) and recruitment of ATG16L1 to the plasma membrane (302). Recent studies have implicated a role for autophagy in controlling the release of pro-inflammatory cytokines such as TNF and IL-1β from macrophages, and notably incubation of cells with the autophagy inhibitor 3-methyladenine reduces TNF release in response to TLR stimulation (237). Although this was proposed to relate to reduced transcription of the TNF gene, influences at the post-translational level cannot be ruled out.

Whilst tempting to speculate that the products of GWAS susceptibility loci could interact to influence vesicle trafficking pathways, there are a number of other possible explanations for the relationship. Distinct abnormalities could exist in the cytokine trafficking pathway in CD macrophages, but the effect could be compounded by an increased load of GWAS polymorphisms, acting to further impair the macrophage response to Gram negative bacteria such as *E. coli*. Furthermore, the association may also be explained by protective alleles interacting to increase TNF release in response to HkEc stimulation, partially compensating for an inadequate innate immune response to HkEc. Regions containing *IL-23R* and *TNFSF15* are both examples of loci where certain alleles can confer protection against CD (116;244), and interestingly IL-23 has been shown to induce inflammatory macrophages to produce TNF in murine models of autoimmune inflammation (303). Finally, the possibility of indirect associations between GRS and macrophage TNF release cannot be excluded.

There was no association between overall GRS and macrophage TLR2 response in CD patients, suggesting these risk alleles have no major role in the impaired release of TNF downstream of TLR2 stimulation. Somewhat paradoxically, macrophages from HC individuals with a GRS of more than 1 produced significantly more TNF than those with a GRS less than 1. This could indicate a protective role of a strong TLR2 response

in HC individuals with a higher burden of GWAS hits, which might be able to compensate to prevent the development of CD.

Overall, the lack of association of GRS with TNF release downstream of TLR2 activation, and weak relationship with HkEc stimulation, suggests that the molecular basis of impaired pro-inflammatory cytokine secretion by CD macrophages remains incompletely understood. A number of additional loci have recently been reported as associated with CD, several of which contain genes encoding proteins involved in vesicle and protein trafficking (*VAMP3, NDFIP1, SCAMP3*) (115), which could have a role in pro-inflammatory cytokine release by macrophages. It has been estimated that approximately 23% of the heritability of CD can be explained by the variants identified to date by GWAS (115). Furthermore, it has recently been postulated that rare variants and structural rearrangements could account for some of the 'missing heritability' (257). It is very possible that some of these mutations, which are likely to be highly heterogeneous between individual patients, could be important determinants of the macrophage response to bacterial stimuli.

Chapter 4: Investigation of macrophage lipids in Crohn's disease

4.1 Introduction

Mounting evidence, including data presented in the previous chapter, suggests the pathogenesis of CD involves a defective innate immune response to microbial material. Patients with CD have delayed clearance of bacteria *in vivo*, which is likely to relate to abnormal macrophage function. Cultured macrophages from CD patients release deficient levels of pro-inflammatory cytokines in response to HkEc. Defective TNF release is also observed in response to a range of stimuli, including TLR agonists and the yeast *C. albicans*, as demonstrated in chapter 3.

The abnormality in cytokine secretion was shown to relate to post-translational handling of pro-inflammatory molecules, as discussed in chapter 1. Whilst the level of cytokine gene transcription in response to HkEc was equivalent between CD and HC macrophages, studies using inhibitors of protein trafficking and lysosomal function revealed mistargeting of pro-inflammatory cytokines to lysosomes in CD macrophages, where they are degraded rather than released through the normal secretory pathway (136). However, the molecular mechanisms responsible for this abnormality remain uncharacterised.

In this chapter, sphingolipids and phospholipids are investigated as candidates for the defective macrophage function observed in CD, given the prominent roles of these lipids in membrane trafficking, autophagy and apoptosis.

4.1.1 Introduction to sphingolipids and their functions

The sphingolipids and phospholipids are major constituents of eukaryotic cell membranes. Whilst both have long been understood to have a critical role in the maintenance of membrane structure, the rich array of functions of each lipid class is

increasingly being uncovered (304). Sphingolipids were originally named by J.L.W Thudichum in 1884, as a testament to their enigmatic or "sphinx-like" nature (305). All sphingolipids contain a sphingoid base backbone. In humans, the sphingoid base is typically sphingosine (an 18 carbon length base containing one double bond) or sphinganine (a saturated 18 carbon length base). Ceramide, the simplest sphingolipid, is made up of sphingosine and a fatty acid, which are linked by an amide bond (Figure 4.1A). The fatty acid can vary in carbon chain length, ranging from 16 to 26 atoms, and in the number of double bonds (degree of unsaturation), giving rise to a multitude of different ceramide 'species'. The precise biological roles of each individual ceramide species remain somewhat of an enigma. Ceramide is considered a structural backbone for all other complex sphingolipids, including sphingomyelin and glycosphingolipids (306).

Ceramide may be generated from two distinct pathways, including 'de novo' synthesis from fatty acyl-CoA and serine, and by hydrolysis of sphingomyelin or other complex sphingolipids. The former involves condensation of serine and fatty acyl-CoA by serine palmitoyltransferase to form 3-ketosphinganine. Reduction of 3-ketosphinganine results in sphinganine generation, which can be N-acylated to form dihydroceramide, and further desaturated to form ceramide (307). Serine palmitoyltransferase catalyses the rate limiting step of this reaction, whereas sphingomyelinases catalyse the breakdown of sphingomyelin to release ceramide and phosphocholine. Ceramide may be phosphorylated to ceramide phosphate (308); used to synthesise glycosphingolipids, sphingomyelin or sulphatides, or broken down by ceramidase enzymes to release sphingosine and free fatty acids (306;309). The pathway of sphingolipid metabolism is illustrated diagrammatically (Figure 4.1B).

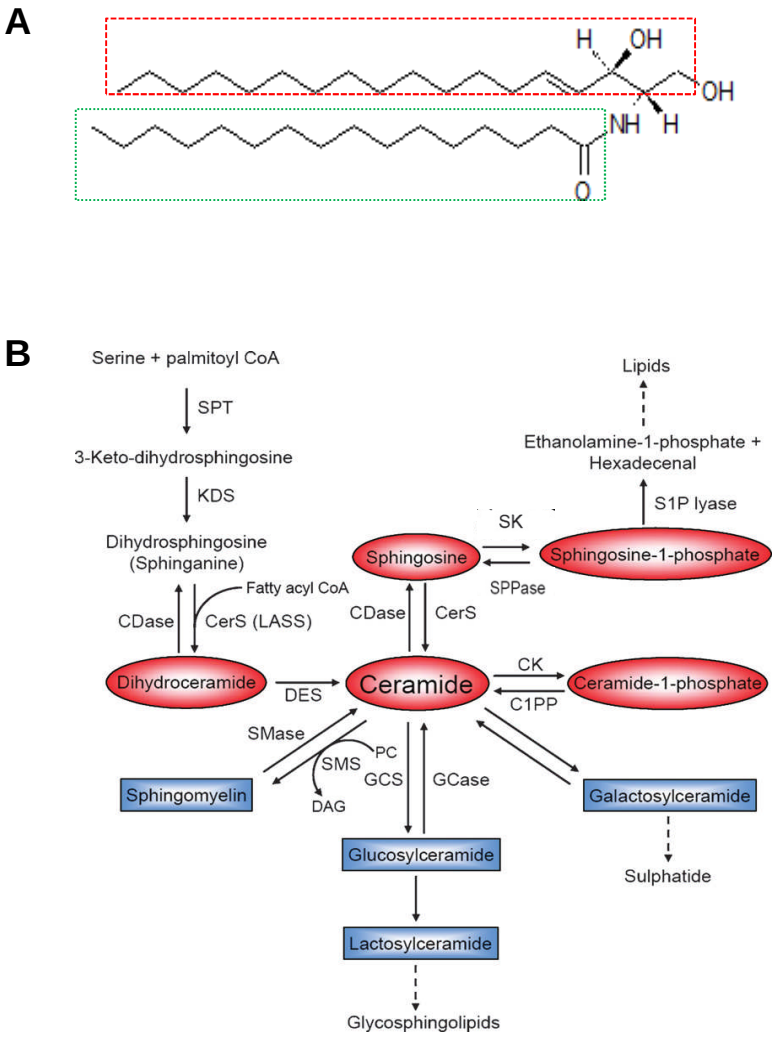


Figure 4.1 Diagrams of ceramide structure and sphingolipid metabolism pathway. **(A)** Structure of C16 ceramide, showing sphingosine (red box) attached to a C16 fatty acid (green box) via an amide bond. Adapted from http://lipidlibrary.aocs.org/lipids/ceramide/index.htm. **(B)** Sphingolipid metabolism pathway. Note the central role of ceramide. Adapted from (309).

Sphingolipids have diverse effects on cellular physiology. Firstly, the sphingolipid content has strong influences on the biophysical properties of cell membranes. Ceramide has been shown to facilitate fission and fusion of membranes, to increase the order of acyl chains, and permeabilise lipid bilayers (310). Importantly, slug-a-bed (*SLAB*) (ceramidase) mutant *Drosophilia* show impaired neurotransmitter release, which relates to alterations in presynaptic ceramide content and changes in synaptic vesicle fusion, exocytosis and trafficking (311). Furthermore, macrophages from acid sphingomyelinase (ASM) deficient mice have impaired phagolysosomal fusion, which results in grossly defective killing of *Listeria monocytogenes*, suggesting a role for sphingolipids in the regulation of 'fusogenicity' of membranes, with consequential biological effects on cell function (312). Some of the biological effects of ceramides may also depend on their tendency to self-associate, which can lead to the formation and stabilisation of ceramide-enriched microdomains ('lipid rafts') (313;314). Although their existence remains an area of controversy, a number of studies indicate that lipid rafts may serve to cluster receptors and signalling proteins such as CD95 and CD40 (315;316).

It is increasingly apparent that sphingolipid metabolism and inflammatory responses are intimately connected (317). On one hand, bacterial ligands such as LPS, as well as pro-inflammatory cytokines such as TNF and IL-1β, have been shown to stimulate an increase in intracellular ceramide levels (318;319) by various mechanisms, including serine palmitoyltransferase activation (320). Moreover, sphingolipids are involved in the induction of certain cytokines, such as IL-6 (321). Ceramide and ceramide-1-phosphate may also depress TNF release (322), most likely via effects at the post-translational level and influencing TACE activity (323).

Sphingolipids show another parallel to the acute inflammatory defect of CD, in that they may also influence inflammatory responses via effects on apoptosis of immune cells. Ceramide is a well-established apoptotic mediator, which may exert effects via signalling molecules such as protein kinase C zeta (324) and ceramide-activated protein phosphatases (325). It may also exert effects on mitochondria directly; increasing the permeability of the outer membrane via formation of 'ceramide channels' (326). This facilitates the release of small proteins such as cytochrome c from mitochondria, which can trigger caspase activation and thereby apoptosis.

Furthermore, ceramide has been shown to have important roles in the control of autophagy, a process strongly implicated in the pathogenesis of CD. Alterations in sphingolipid metabolism could therefore provide a mechanistic connection between the defective trafficking of cytokines and impaired autophagy in CD pathogenesis. Addition of exogenous ceramide has been shown to induce autophagy (327), which may relate to modulation of signalling networks or changes in the biophysical properties of membranes (306).

4.1.2 Phospholipids: structure, synthesis and biological functions

Glycerophospholipids (also known as phospholipids) are the predominant lipid components of eukaryotic cell membranes. Phospholipids are composed of two fatty acids attached to a glycerol backbone via ester, ether or vinylether bonds, and a headgroup comprised of phosphate and polar moieties. Various classes of phospholipids exist, which are defined by the structure of the headgroup. The principal phospholipid classes include phosphatidylcholines (PC), phosphatidylethanolamines (PE), phosphatidylinositols (PI) and phosphatidylserines (PS), where the headgroups are choline, ethanolamine, inositol and serine respectively (Figure 4.2A). As for sphingolipids, the nature of the two fatty acids may vary both in carbon chain length

(typically from 14 to 26 carbon atoms) and the number of double bonds (normally between 0 and 6), which gives rise to a complex repertoire of different possible molecular species within each phospholipid class (328;329). Fatty acids found in phospholipids typically include palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), arachidonic acid (C20:4), the essential fatty acid linoleic acid (C18:2), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6).

All glycerophospholipids are synthesised from the precursor phosphatidic acid (PA). PA may be dephosphorylated to yield diacylglycerol (DAG), which is used in the synthesis of PC, PE and PS. CDP-Diacylglycerol (CDP-DAG) may also be generated from PA by action of the CDP-diacylglycerol synthase. The CDP-DAG may be converted to PI, phosphoglycerol (PG) and cardiolipin (CL) in subsequent reactions (328) (Figure 4.2B).

PC is the most abundant phospholipid in eukaryotic cell membranes and functions as a major structural component. Synthesis of PC generally occurs via the CDP-choline (Kennedy) pathway (330), although certain cells such as hepatocytes are also capable of generating PC from PE in a series of reactions catalysed by the enzyme phosphatidylethanolamine N-methyltransferase (PEMT) (331). The first step in the Kennedy pathway is the phosphorylation of choline to produce phosphocholine, catalysed by choline kinase (332;333). The choline utilised in this reaction is obtained from the diet or from the breakdown of lipids and acetylcholine (334). Subsequently, the phosphocholine is converted to CDP-choline in a condensation reaction utilising cytidine triphosphate (CTP) and catalysed by the CTP: phosphocholine cytidylyltransferase enzyme (CCT). Distinct α and β isoforms of CCT exist, which are encoded by two separate genes. The transcripts encoded by these genes may undergo alternative splicing to generate multiple protein products. CCT-α isoforms predominate

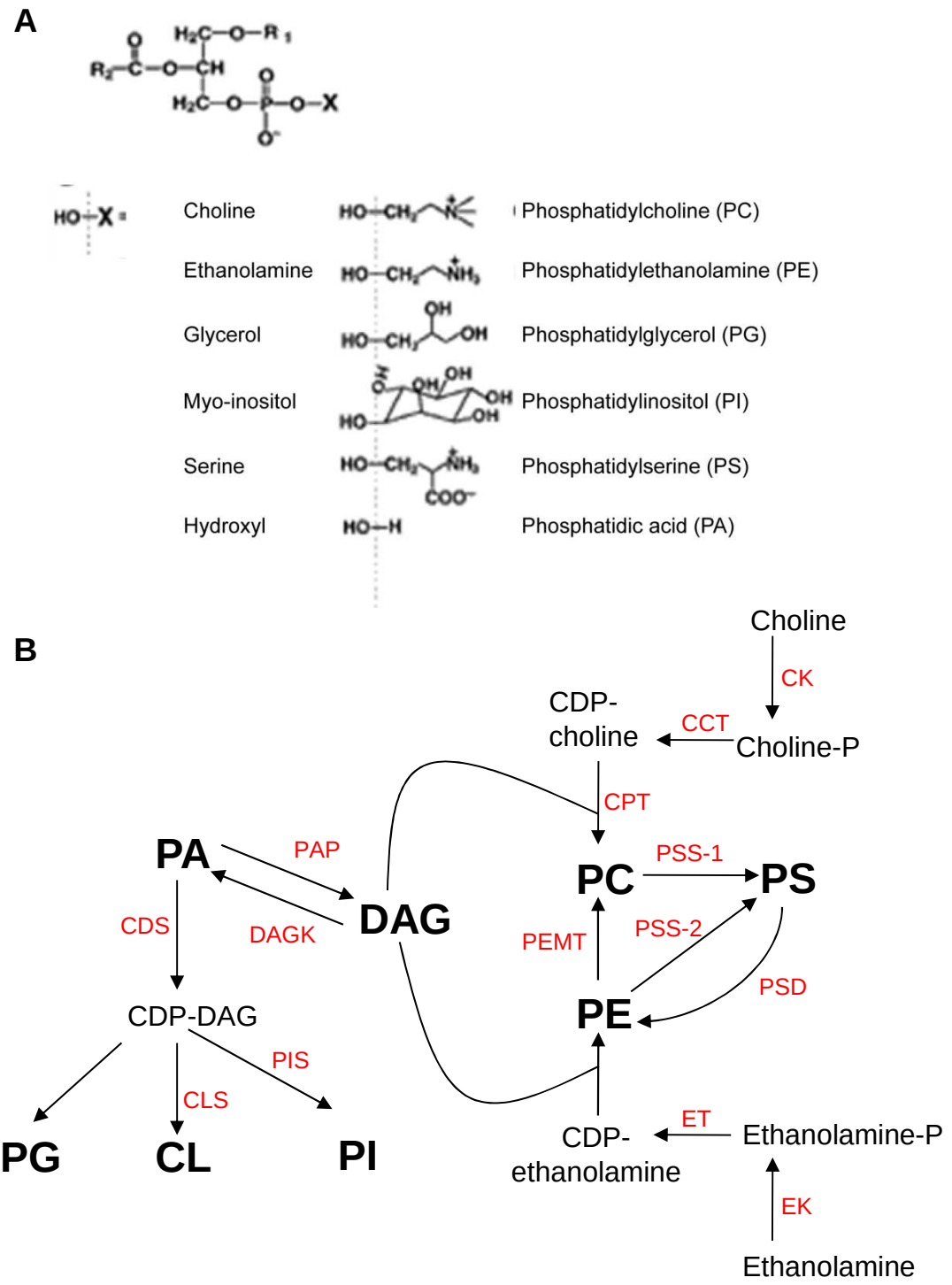


Figure 4.2 Structure and biosynthesis of phospholipids. **(A)** Generic phospholipid structure, showing a glycerol backbone to which two fatty acids (R1 and R2) are attached. A head-group (X) is also attached to glycerol via a phosphodiester bond. The nature of the head group determines the phospholipid class, as indicated. Adapted from (276). **(B)** Schematic diagram of the phospholipid biosynthetic pathway. Enzymes catalysing each reaction are shown in red. The major metabolites are highlighted in bold (PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI , phosphatidylinositol; CL, cardiolipin; PG, phosphatidylglycerol; DAG, diacylglycerol. Adapted from (328).

in most tissues, generally being expressed at levels 10 to 30 fold higher than CCT-β (335;336). The reaction catalysed by CCT is regarded as the rate limiting step in PC synthesis (337) and is tightly regulated. The final step in the Kennedy pathway is the transfer of phosphocholine from CDP-choline to DAG by a phosphotransferase enzyme, thereby generating PC.

PC is an important structural component of all cell membranes, including intracellular vesicles (338). A number of studies have highlighted the importance of PC and phospholipid composition in ensuring efficient intracellular membrane trafficking. In *Drosophila,* mutations in CCT give rise to an altered ratio of PC to PI and consequential alterations in endocytosis, resulting in aberrant localisation of the epidermal growth factor and Notch receptors (339). In yeast, the phospholipid transfer protein SEC14p is required for efficient export of proteins from the Golgi apparatus (340), and mutations in the CDP-choline pathway overcome this requirement (341). Together these observations indicate that SEC14p regulation of membrane phospholipid dynamics is important in maintaining the 'secretory competence' of Golgi membranes.

The generation of PC is understood to have a number of important roles in macrophage function. Differentiation of monocytes into macrophages is associated with an increase in PC content, and alterations in the degree of PC saturation (342). The same study reported that induction of fatty acid synthesis during differentiation influenced macrophage ultrastructure and organelle development, possibly by influencing the synthesis of PC and PE. Furthermore, PC biosynthesis has been shown to have a critical role in pro-inflammatory cytokine secretion in macrophages (197), as indicated in section 1.3.4. Murine macrophages deficient in CCT-α secrete reduced levels of TNF and IL-6 in response to LPS stimulation, whilst IL-1β and apolipoprotein E release are unaffected. Interestingly, although the synthesis of pro-inflammatory

cytokines in CCT-α deficient macrophages is normal, impaired release occurs due to abnormal post-translational handling and retention of these molecules in the Golgi apparatus. Given the parallels between the phenotype of CCT-α deficient macrophages and that of cultured macrophages from CD patients, it could be hypothesised that CD macrophages display abnormalities in PC synthesis that underlie the impairment in cytokine secretion.

In mammalian cells, PS is synthesised in a base exchange reaction from PC or PE, in which the headgroup is replaced by L-serine. Two distinct PS synthase enzymes are found in mammalian cells; PS synthase-1 (PSS-1) catalyses the synthesis of PS from PC (343), whereas PS synthase-2 (PSS-2) uses PE as the substrate (344). PS is understood to have a number of important biological functions, including as a cofactor for enzymes such as protein kinase C (345). PS is normally found at high concentrations at the plasma membrane, where it is asymmetrically distributed so that nearly all PS is located at the cytoplasmic face of the membrane bilayer. During apoptosis, loss of the phospholipid asymmetry occurs so that PS becomes distributed on the external membrane surface (346). PS externalisation by apoptotic cells is required for their uptake and clearance by macrophages (347), a process which is also dependent on the T cell immunoglobulin mucin (TIM) glycoproteins on the surface of macrophages and other phagocytic cells, which specifically bind PS (348).

PI is synthesised in a two step process from phosphatidic acid. Initially, CDP-DAG is synthesised from PA and CTP, catalysed by the enzyme CDP-DAG synthase. Subsequently, CDP-DAG is converted to PI in a condensation reaction with *myo*-inositol, which is catalysed by PI synthase (328). PI is an important structural lipid, and is also a substrate for various lipid kinases and phosphatases, which can generate phosphoinositide derivatives (phosphoinositides or PIPs). PIPs are important second

messenger molecules in signalling pathways involved in the control of cell growth, cytoskeletal re-organisation and membrane trafficking (349). Interestingly, PI 3-kinase, an enzyme that converts PI species to phosphatidylinositol-3,4,5-bisphosphate, was recently shown to be important for TNF trafficking from the Golgi apparatus to the plasma membrane in macrophages (350). The generation of phosphatidylinositol-3,4,5-bisphosphate via PI 3-kinase activity has also been shown to be important for autophagy (351).

4.1.3 Previous investigations of lipids in Crohn's disease and hypothesis

A number of studies have indicated that CD patients may have abnormalities in lipid metabolism, which could have a pivotal role in the disease pathogenesis. Case-control studies have shown positive correlations between dietary fat consumption and the development of CD (22), and in one small scale randomised controlled trial, a high fat content was found to attenuate the benefits of an elemental diet in patients with active disease (352). GWAS have also identified CD-associated variants in loci containing genes related to lipid metabolism. The recent GWAS meta-analysis identified a SNP on chromosome 11q12 in close proximity to *FADS1* (115), which encodes the fatty acid desaturase 1 enzyme. Genetic variation in this gene has been associated with alterations in the fatty acid composition in serum phospholipids (353). The WTCCC found moderate evidence of association with a SNP located in the *SPTLC2* gene encoding serine palmitoyltransferase, long chain base subunit 2 ($p=2.65x10^{-5}$) (130), although this association was not replicated in the recent GWAS meta-analysis (115). Furthermore, whilst the exact identity of the 'CD susceptibility gene' within the IBD5 locus remains contentious, a putative candidate is the organic cation transporter 1, which functions in the mitochondrial transport of L-carnitine (222).

Studies conducted on biological samples from CD patients have demonstrated decreased membrane fluidity in erythrocytes from CD patients, with concomitant increases in sphingomyelin and reductions in phosphatidylcholine and polyunsaturated acyl chains of phospholipid (354). Another study demonstrated alterations in glycosphingolipid metabolism in patients with CD (355). Increased concentrations of the glycosphingolipid lactosylceramide were reported in biopsies from CD patients, compared to controls and patients with UC. However, as inflamed tissue was investigated from patients with active CD, it is possible that these changes were secondary to a chronic inflammatory state in the bowel.

There is also considerable evidence describing fatty acid abnormalities in CD, although the results are somewhat conflicting. Plasma phospholipids of patients with CD were reported to have alterations in the fatty acid profile, with reductions in the percentage of arachidonate (20:4), and an increased saturation index (sum of saturated fatty acids in relation to unsaturated fatty acids) (356). Another investigation of peripheral blood mononuclear cells demonstrated an altered ratio of omega-3 (n-3) to omega-6 (n-6) polyunsaturated fatty acids (357). In contrast, two studies investigating serum fatty acids in patients with CD revealed an increase in unsaturated fatty acids, particularly arachidonate and docosahexaenoic acid (C22:6 n-3) (358;359). The changes were common to patients with active and quiescent disease, and patients with UC. Lipids from adipose and lymphoid tissues have been reported to contain more saturated but fewer polyunsaturated fatty acids, with preferential depletion of n-6 polyunsaturates in lymphoid cells and n-3 polyunsaturates in adipose tissue (360). Whilst the evidence for n-3 polyunsaturated fatty acids in the treatment of CD is not yet compelling (16), the efficacy of enteral nutrition in CD may depend on the relative content of monounsaturated compared to polyunsaturated fatty acids, suggesting that the balance of fatty acids could influence disease pathogenesis and course (361).

To date, no studies have been conducted investigating macrophage phospholipid and sphingolipid composition in CD. Given prominent roles of sphingolipids and phospholipids in the maintenance of membrane structure and vesicle trafficking, it was hypothesised that abnormalities in the macrophage sphingolipid or phospholipid composition could underlie the defective cytokine secretion observed in CD. Sphingolipids (including ceramides, dihydroceramides and sphingoid bases) in CD macrophages were therefore quantified using high performance liquid chromatography tandem mass spectrometry (HPLC-MS). Phospholipid composition and dynamics were investigated using stable isotope labelling and electrospray ionisation mass spectrometry (ESI-MS). Lipidomics analysis of ileal biopsies from CD patients was also performed to determine whether any alterations were also present in the mucosa.

4.2 Results

4.2.1 Sphingolipid composition of unstimulated HC and CD macrophages

The sphingolipid composition of HC and CD macrophages was quantified to determine any abnormalities that could explain the impairment in cytokine secretion and apoptosis. Macrophages were cultured, and the amounts of sphingoid bases, dihydroceramide and ceramide (in pmol/mg protein) were determined by HPLC-MS. Demographics of patients included in this study are shown (Appendix 1 Table IIA). Two batches of analysis were performed, the first consisting of unstimulated macrophages from HC (n=11) and CD (n=14) donors, and the second consisting of macrophages from HC (n=7) and CD (n=8) patients, with paired unstimulated and HkEc stimulated samples from these donors. Although similar patterns in the ceramide carbon chain length and degree of unsaturation were observed, the absolute values for the amounts of C16 and C24 ceramide differed significantly between batches ($p<0.001$).

Therefore, for simplicity only the results of the second batch are discussed below, to allow comparison of unstimulated and HkEc paired samples.

Ceramide species consisting of 14-26 carbon atom length fatty acids were detectable in HC and CD macrophages. C16 dihydroceramide, sphingosine and dihydrosphingosine (sphinganine), but not sphingosine-1-phosphate, were also detectable. The predominant species in both HC and CD macrophages were the C16:0, C24:0 and C24:1 ceramides (Figure 4.3A, B). In HC macrophages, C16:0, C24:0 and C24:1 ceramides constituted 22.4% $\pm$ 3.6%, 32.4 $\pm$ 2.1% and 37.6 $\pm$ 1.6% of the total ceramide content respectively. In CD macrophages, the C16:0, C24:0 and C24:1 ceramides constituted 18.4 $\pm$ 6.5%, 35.5 $\pm$ 12.5% and 38 $\pm$ 13.4% respectively. There were no statistically significant differences in the mean amounts of any ceramide species, dihydroceramide or sphingoid base between HC and CD macrophages in the unstimulated state (Figure 4.3C, D). The mean total cellular ceramide content was also not significantly different between HC (802.3 $\pm$ 82.7 pmol/mg) and CD (672.4 $\pm$ 56.5 pmol/mg) macrophages (Figure 4.3E).

Whilst ceramides are considered to have a central role in the sphingolipid pathway, they may be used to synthesise more complex species such as sphingomyelin and glycosphingolipids. A parallel study, conducted in our laboratory by Dr Andrew Smith and Professor Al Merrill using HPLC-MS, also revealed no significant differences in the sphingomyelin and hexosylceramide content of CD macrophages, further evidencing the lack of a gross defect in the sphingolipid pathway in CD macrophages.

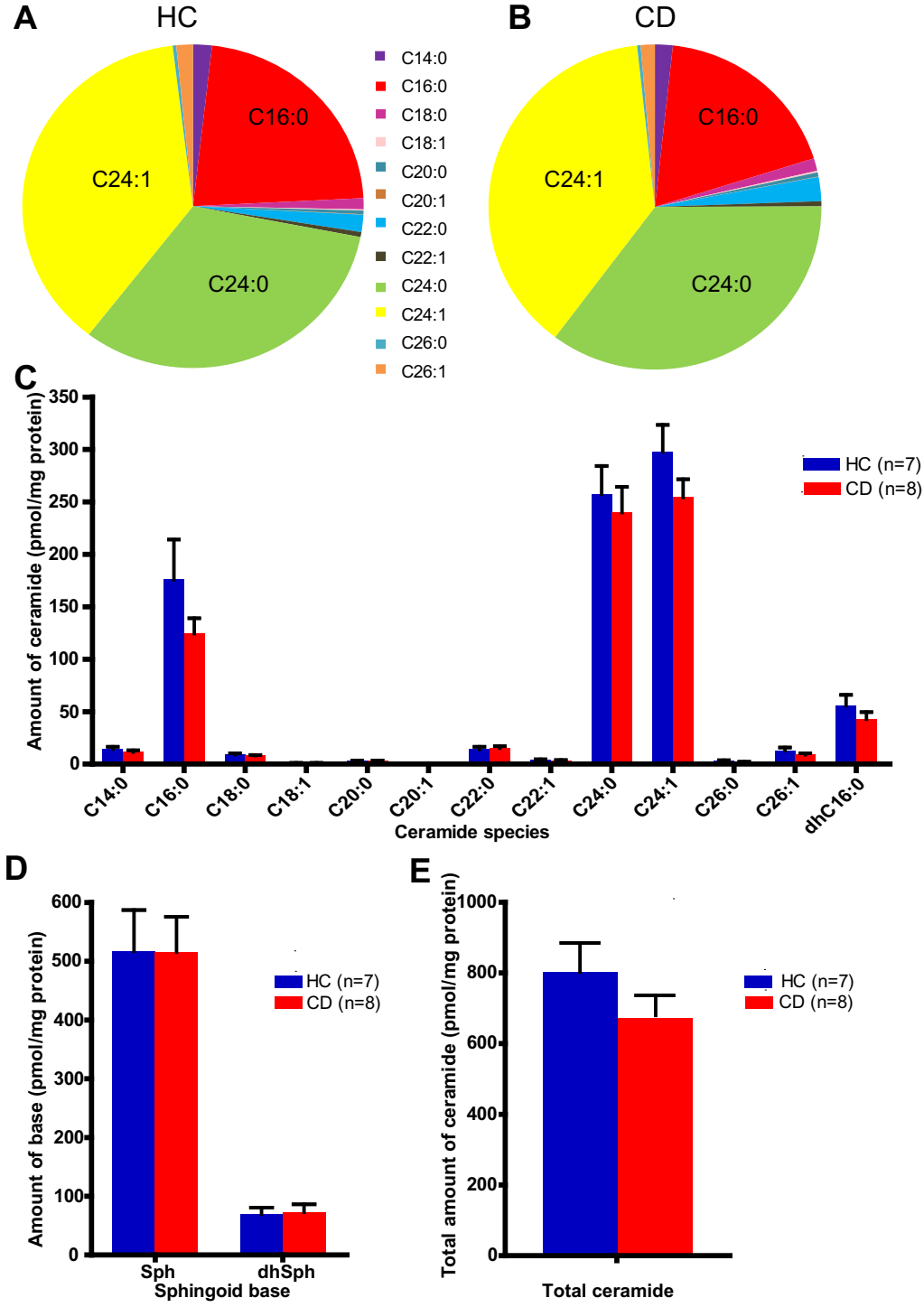


Figure 4.3 Ceramide and sphingoid base composition of unstimulated HC and CD macrophages. The amounts of individual ceramide species, consisting of between 14 and 26 carbon chain length fatty acids were quantified in HC (n=7) and CD (n=8) macrophages by high performance liquid chromatography tandem mass spectrometry. (**A, B**) Pie charts depicting the proportion of various ceramide species (C, fatty acid carbon chain length) in HC and CD macrophages respectively, expressed as a percentage of the total amount of ceramide detected. (**C**) The amounts of individual ceramide species (in pmol/mg protein) in unstimulated HC and CD macrophages. The amount of C16:0 dihydroceramide (dhC16:0) is also shown. No significant differences were identified between HC and CD cells. Results are expressed as mean + SEM. (**D**) The amounts of sphingosine (Sph) and dihydrosphingosine (dhSph) in HC and CD macrophages, expressed as mean + SEM. **(E)** Total amounts of ceramide in HC and CD macrophages, expressed as mean + SEM.

4.2.2 Sphingolipid composition of HkEc stimulated macrophages

In the absence of an obvious difference in the sphingolipid composition of resting HC and CD macrophages, it was subsequently determined whether HkEc stimulated CD macrophages had abnormalities in sphingolipid composition. The amounts of ceramide, dihydroceramide and sphingoid base species in HC (n=7) and CD (n=12) macrophages after 4 hours stimulation with HkEc was therefore determined. 4 hours after stimulation was chosen as this was the point where maximal rate of secretion of TNF occured in HC macrophages in response to heat-killed *E. coli* (136), and when targeting of TNF to lysosomal compartments occurred in CD macrophages.

In HC macrophages, stimulation with HkEc resulted in a significant reduction in C16:0 (42.3 $\pm$ 29.2% decrease, $p<0.05$), C24:0 (18.1 $\pm$ 6.3%, $p<0.05$) and C24:1 (24.1 $\pm$ 5.4%, $p<0.01$) ceramide species, and a significant increase in dihydrosphingosine (40 $\pm$ 13.5% increase, $p<0.001$) (Figure 4.4A, B, C, D, left panel). No other differences in ceramide or sphingoid base species between unstimulated and stimulated macrophages were statistically significant. Similarly, in CD macrophages, stimulation with HkEc resulted in a significant reduction in C24:0 (12 $\pm$ 4.3%, $p<0.05$) and C24:1 (21.4 $\pm$ 3.1%, $p<0.01$) ceramide species, with a concomitant increase in dihydrosphingosine content (25.8 $\pm$ 14.1%, $p<0.05$) (Figure 4.4A, B, C, D, right panel). Although the amount of C16:0 species also decreased in CD macrophages after stimulation, the reduction did not reach statistical significance. Comparison of mean amount (in pmol/mg of protein) of individual ceramide and dihydroceramide species (Figure 4.5A) and sphingoid bases (Figure 4.5B) did not reveal any significant differences between HC and CD macrophages in the HkEc stimulated state. The mean total ceramide content was 601.6 $\pm$ 79.4 pmol/mg in HC macrophages and 565.5 $\pm$ 42.1 pmol/mg in CD macrophages, which also did not represent a significant difference (Figure 4.5C).

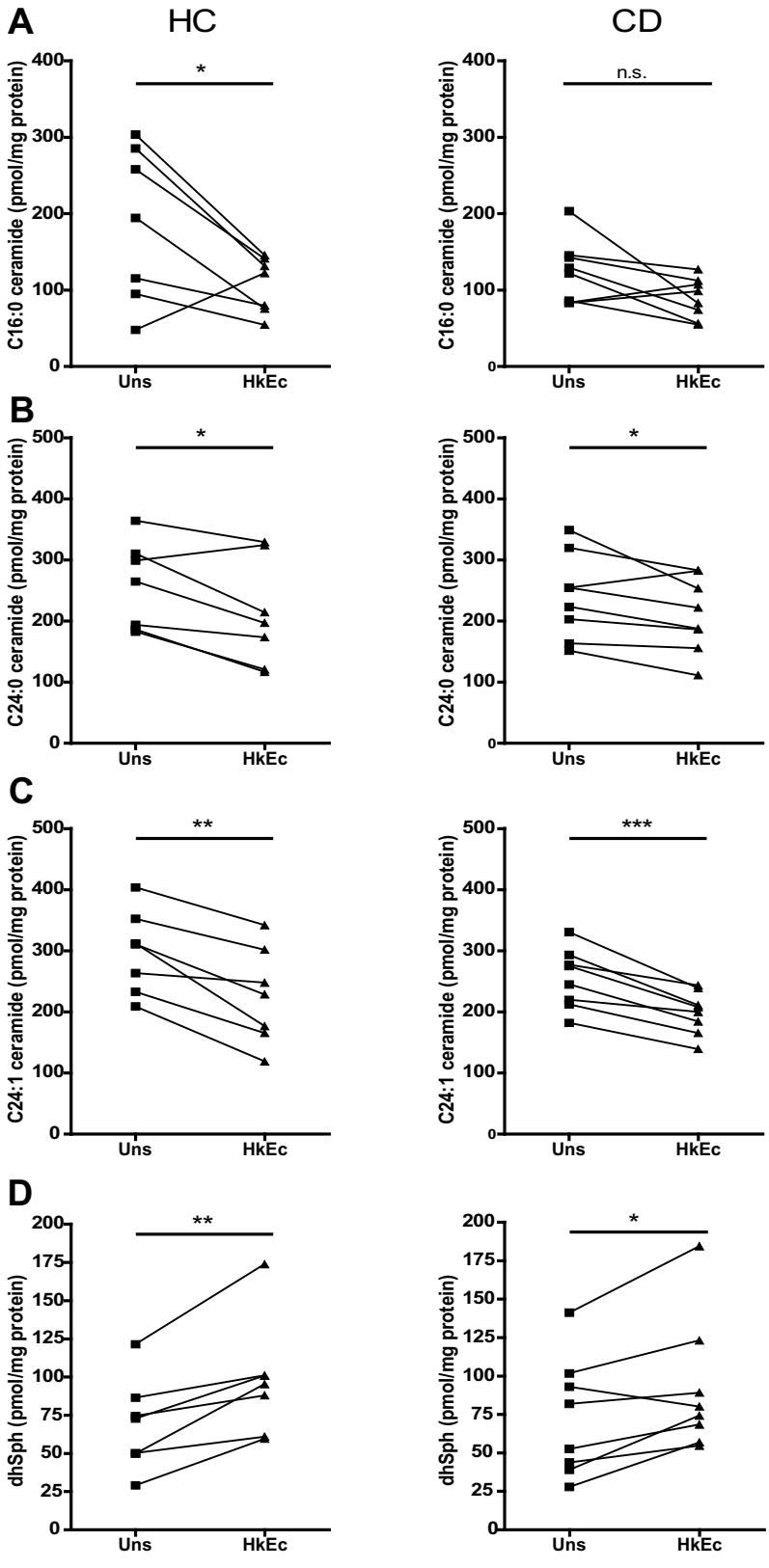


Figure 4.4 Alterations in amounts of ceramide and sphingoid bases in HC and CD macrophages after HkEc stimulation. Macrophages from HC (n=7) and CD (n=8) donors were cultured and stimulated with HkEc for 4 hours. Ceramide and sphingoid bases were quantified by HPLC-MS. Alterations in the amount of **(A)** C16:0, **(B)** C24:0, **(C)** C24:1 ceramides and **(D)** dihydrosphingosine (dhSph) are shown for HC (left panel) and CD (right panel) macrophages. * represents $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

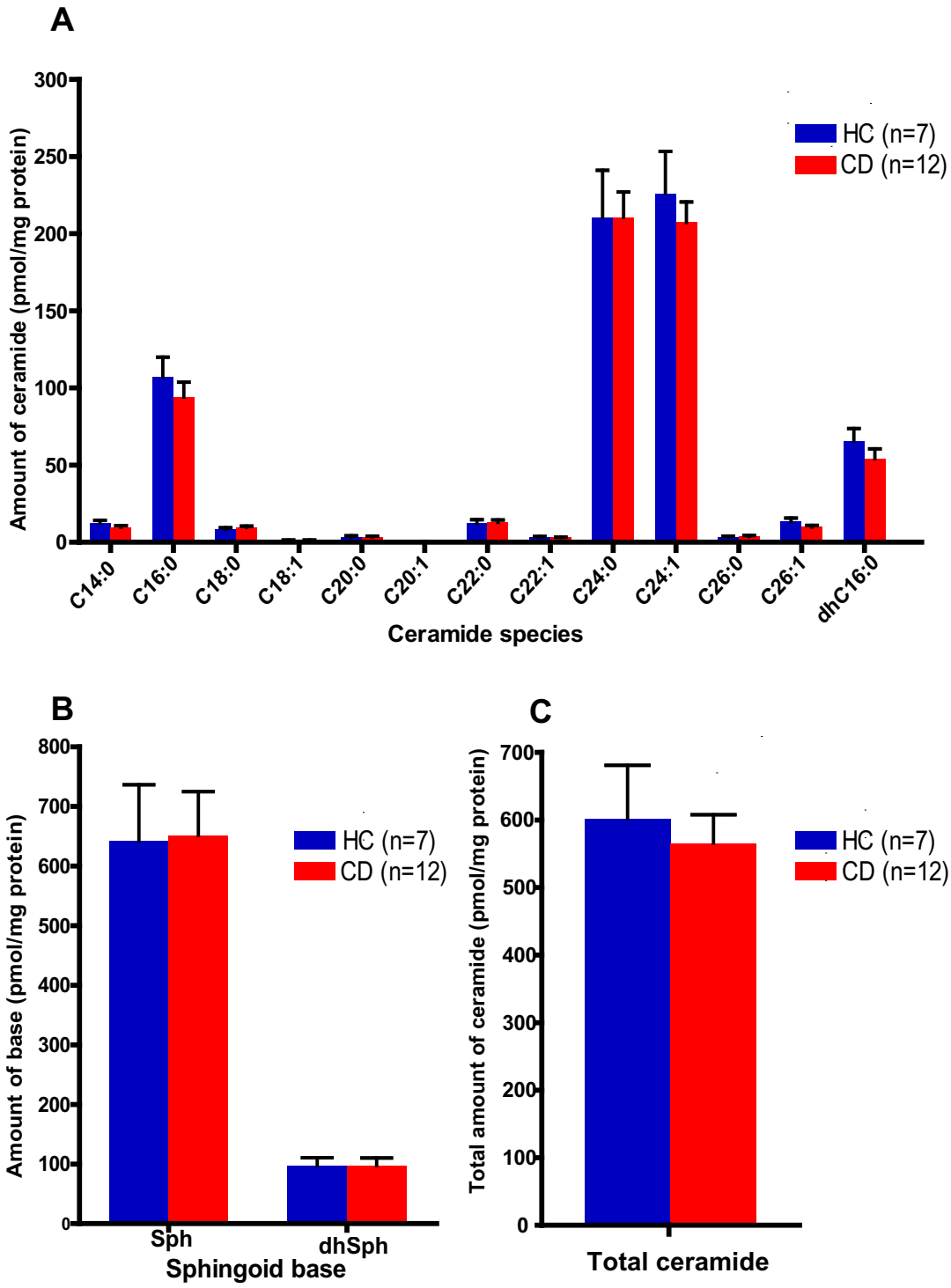


Figure 4.5 Ceramide and sphingoid base composition of HkEc stimulated HC and CD macrophages. Macrophages from HC (n=7) and CD (n=12) individuals were stimulated with HkEc for 4 hours and ceramides and sphingoid bases quantified by high performance liquid chromatography tandem mass spectrometry. (**A**) Amounts of individual ceramide species (C, fatty acid carbon chain length), expressed as pmol/mg protein. Results for C16:0 dihydroceramide (dhC16:0) are also shown. (**B**) Amounts of sphingosine (Sph) and dihydrosphingosine (dhSph). **(C)** Total amount of ceramide. No significant differences were found between HC and CD macrophages. Results are presented as mean + SEM.

4.2.3 Investigation of phospholipid dynamics in CD and HC macrophages

Relative quantification of the phospholipid species PC, PI and PS in CD and HC macrophages was subsequently performed by ESI-MS, as a collaborative project with the University of Southampton. Depending on the lipid class to be detected, ESI-MS quantification requires use of a 'triple quadrupole' mass spectrometry based system and a variety of 'precursor' and 'neutral loss' scans. Electrospray ionisation enables ionisation of neutral species and transfer of ions from solution into gaseous phase. The ions are then subjected to an electrical field, established by an assembly of three sets of four parallel metal rods ('quadrupoles'), which enables resolution of ions based on their mass to charge (*m/z*) ratio. Subsequently, the ions selected ('precursor ions') in the first quadrupole are subjected to further fragmentation in the second quadrupole, in a process known as 'collision induced dissociation'. The resulting fragments ('products') are then separated again in the third quadrupole, based on *m/z* ratio. There are several possible modes of data acquisition, including a 'precursor scan', where a unique product ion is selected in the third quadrupole, and the first quadrupole scans to detect a range of possible precursor ions (362).

In the case of endogenous PC, the species contain the choline headgroup of *m/z* 184+. The MS is programmed to perform a "precursor analysis" to detect positively charged species that undergo collision induced dissociation to give rise to the 184+ choline fragment. A representative spectrum, resulting from all lipid components in the sample containing the 184+ choline species is shown (Figure 4.6A). Similarly for endogenous PI species, the MS is programmed, in negative ionisation mode, to perform a precursor scan of fragments that degenerate to produce the 241- inositol species. In the case of PS, a 'neutral loss' scan is performed, where both quadrupoles 1 and 3 scan at a

constant difference in *m/z* ratio. This is used to monitor species that undergo loss of the 87 neutral fragment during collision induced dissociation.

Macrophages were incubated with stable isotope labelled *methyl*-d_9-choline, *myo*-d_6-inositol and serine-d_3 for 3 hours, in the presence or absence of HkEc. 3 hours was chosen as the appropriate time period based on the results of previous studies investigating phospholipid turnover in leukocytes (363) and macrophages (342). Lipids were subsequently extracted and the molar percentage of PC, PI and PS species quantified by ESI-MS. Inclusion of stable isotope labelled *methyl*-d_9-choline, *myo*-d_6-inositol and serine-d_3 permits distinction of 'newly synthesised' phospholipids from pre-existing or 'endogenous' species, thus enabling determination of metabolic flux rates through the phospholipid synthesis pathways (364). Demographics of individuals included in this study are shown (Appendix 1 Table IIB). Six patients in this study were receiving no treatment and eight were receiving 5-ASA. The mean body mass index (BMI) of the CD patients in this study was 23.3 $\pm$ 3.0 kg/m^2 (mean $\pm$ standard deviation). One of the patients was receiving vitamin B12 replacement therapy; none of the other patients had any evidence of impaired nutritional status. None of the patients were receiving enteral nutrition.

Endogenous and newly synthesised PC were determined in all samples, including unstimulated (HC n=10, CD n=13) and HkEc stimulated (HC n=7, CD n=8) macrophages. Endogenous and newly synthesised PS and PI were quantified in a subset of unstimulated (HC n=7, CD n=9) and HkEc stimulated samples (HC n=6, CD n=5).

4.2.4 Analysis of PC in HC and CD macrophages

Precursor scans of *m/z* 184+ and *m/z* 193+ were used to determine endogenous and newly synthesised PC species respectively in CD and HC macrophages. Representative PC spectra from one HC individual, generated as precursor scans of *m/z* 184+ and 193+ are shown (Figure 4.6A, B). The predominant peaks at *m/z* 760.8 and 786.8 correspond to endogenous PC 16:0/18:1 and PC 18:0/18:2 species respectively (Figure 4.6A). Newly synthesised PC species can clearly be distinguished from endogenous PC, generated from the *m/z* 193+ precursor scan (Figure 4.6B). The peaks at *m/z* 769.8 and *m/z* 795.8 correspond to newly synthesised PC 16:0/18:1 and PC 18:0/18:2 species respectively (9 *m/z* units greater than for the endogenous species).

Relative quantification of PC species was performed from the generated spectra using macro software developed at the University of Southampton. Only PC species that made up >2 % of the total molar percentage of PC were considered detectable. In unstimulated HC macrophages (n=10 donors), the predominant endogenous PC species were PC 16:0/16:0, PC 16:0/18:1 and PC 18:0/18:2, which made up 11.7 $\pm$ 0.64%, 22.0 $\pm$ 0.73% and 13.2 $\pm$ 0.66% of the total endogenous PC respectively. Similarly in unstimulated CD macrophages (n=13 donors), PC16:0/16:0, PC 16:0/18:1 and PC18:0/18:2 were the major species present, which made up 11.9 $\pm$ 0.75%, 22.2 $\pm$ 0.62% and 12.3 $\pm$ 0.84% of the total endogenous PC respectively. There were no significant differences in the molar percentage of any endogenous PC species between HC and CD macrophages. Stimulation with HkEc resulted in a significant reduction in the molar percentage of endogenous PC containing arachidonate (16:0/20:4) in both HC and CD macrophages. No other alterations were observed after stimulation and there were no significant differences between HC and CD macrophages (Figure 4.7A).

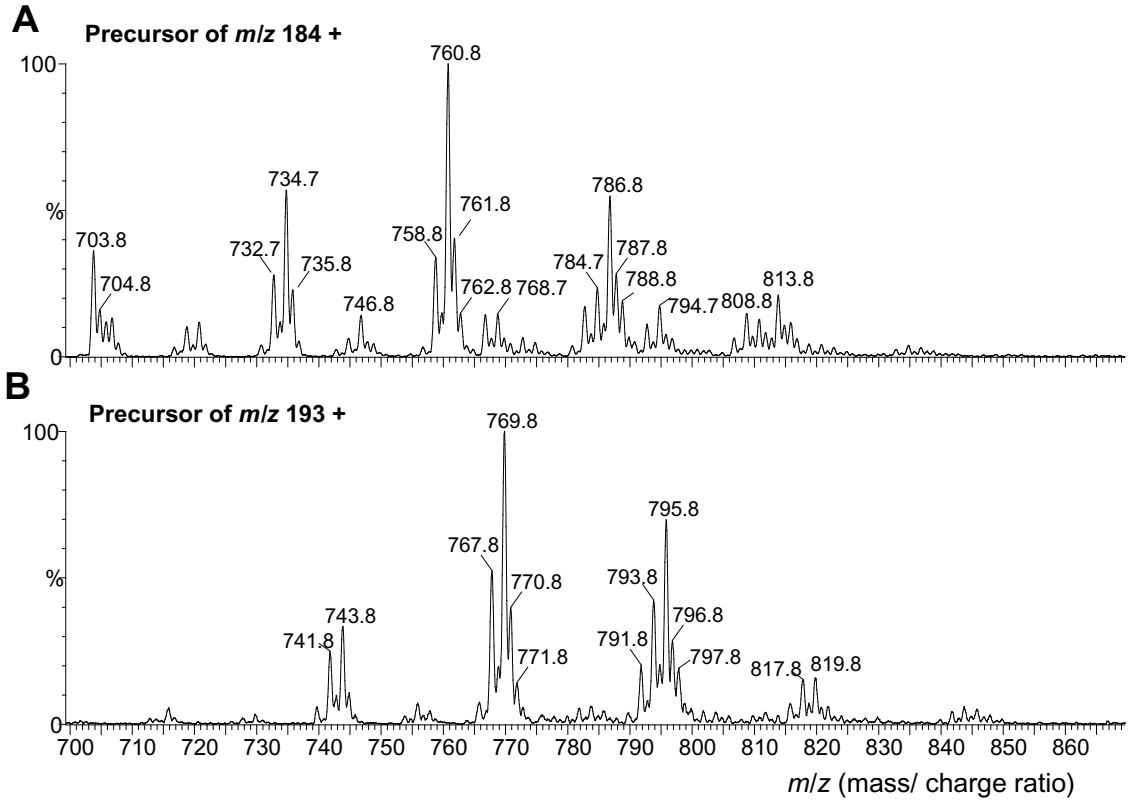


Figure 4.6 Electrospray ionisation mass spectrometric analysis of endogenous and newly synthesised PC. **(A)** Precursor scan of *m/z* 184+, indicating endogenous PC species. Note the peak at *m/z* 760.8, which corresponds to PC 16:0/18:1. **(B)** Precursor scan of *m/z* 193+ showing newly synthesised PC species 9 *m/z* units higher than the endogenous species, indicating incorporation of the *methyl*-d_9-choline stable isotope. The peak at *m/z* 769.8 corresponds to newly synthesised PC 16:0/18:1.

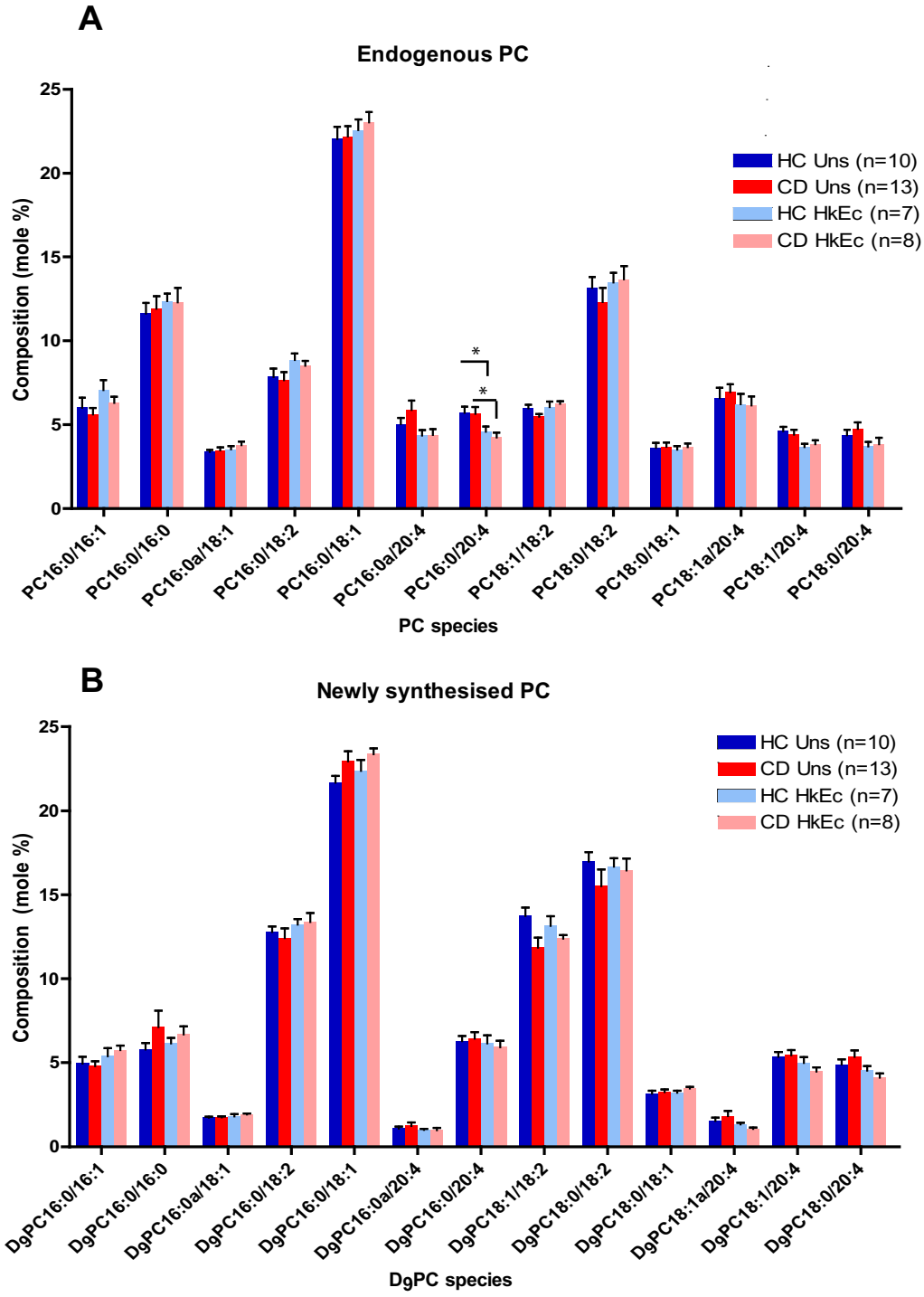


Figure 4.7 Composition of endogenous and newly synthesised PC species in HC and CD macrophages. Macrophages were cultured from HC individuals and CD patients, incubated with *methyl*-d_9-choline for 3 hours in the presence (HC n=7, CD n=8) or absence (HC n=10, CD n=13) of HkEc. Lipids were extracted and PC species quantified by electrospray ionisation mass spectrometry. (**A**) Molar percentage composition of endogenous PC species, consisting of various carbon chain length fatty acids, in unstimulated (Uns) and HkEc stimulated HC and CD macrophages. (**B**) Molar percentage composition of newly synthesised (D_9) PC species, in unstimulated and HkEc stimulated HC and CD macrophages. Results are expressed as mean + SEM. * indicates $p<0.05$.

The profile of newly synthesised PC species was similar to that of the endogenous PC profile of HC and CD macrophages. PC 16:0/18:1 and PC16:0/18:2 were the predominant newly synthesised species in HC and CD macrophages (Figure 4.7B). Stimulation with HkEc increased the proportion of PC 16:0/18:1 synthesised in HC macrophages, and increased the fraction of synthesised PC 16:0/16:1 in CD cells. There were no significant differences in the molar percentage of any species between HC and CD macrophages, either in the unstimulated state or after stimulation with HkEc.

The incorporation of *methyl*-d_9-choline into PC was also determined over 3 hours in HC and CD macrophages, as a measure of the global rate of synthesis of all PC species (Figure 4.8). The mean fractional incorporation of *methyl*-d_9-choline into PC over the 3 hour period was 10.3 $\pm$ 1.3% in unstimulated HC macrophages and 10.5 $\pm$ 1.5% in CD macrophages, indicating equivalent rates of PC synthesis in HC and CD cells. Stimulation with HkEc increased the mean fractional incorporation of *methyl*-d_9-choline into PC in HC macrophages (17.0 $\pm$ 1.1%, $p<0.01$), and CD macrophages (16.9 $\pm$ 1.1%, $p<0.01$) compared to unstimulated cells. Stimulation with HkEc was therefore associated with an increased rate of PC synthesis; however there were no significant differences between HC and CD macrophages.

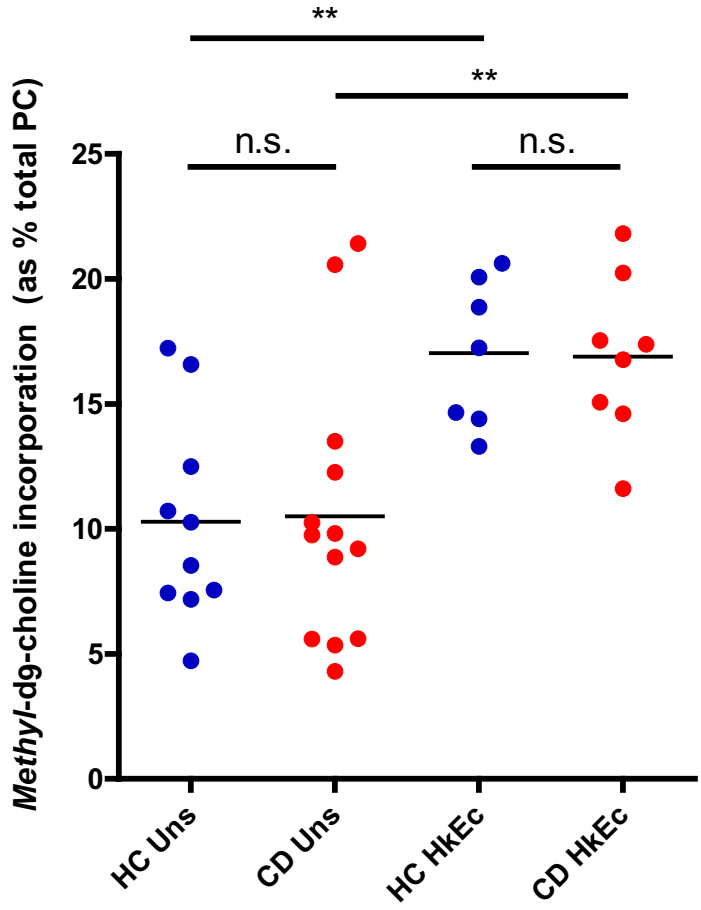


Figure 4.8 Incorporation of *methyl*-d_9-choline into HC and CD macrophage PC. Macrophages were cultured from HC and CD donors and incubated with *methyl*-d_9-choline for 3 hours. Results are shown for unstimulated (Uns) macrophages (HC n=10, CD n=13) and HkEc stimulated cells (HC n=7, CD n=8). Total lipid was extracted and analysed by ESI-MS as precursor scans of *m/z* 184+ and *m/z* 193+. The fractional incorporation of *methyl*-d_9-choline as a % of the total PC was determined.

4.2.5 Analysis of PS in HC and CD macrophages

Neutral loss scans of *m/z* 87 and *m/z* 90 were used to determine the profile of endogenous and newly synthesised PS species respectively in HC and CD macrophages. Representative PS spectra from one HC individual are shown (Figure 4.9). A representative neutral loss scan of *m/z* 87- is shown (Figure 4.9A); the peak at *m/z* 788.9 corresponding to endogenous PS 18:0/18:1. A representative neutral loss scan of *m/z* 90- is also shown (Figure 4.9B); peaks at *m/z* 763.8, 789.9, 791.9 and 813.9 correspond to the newly synthesised species PS 16:0/18:1, PS 18:0/18:2, PS 18:0/18:1 and PS 18:0/20:4. Spectra were converted to centroid format and used to determine the molar percentage composition of endogenous and newly synthesised PS species, together with the overall rate of PS synthesis.

The predominant endogenous PS species was a monounsaturated species containing 18 length carbon fatty acids (PS 18:0/18:1) (Figure 4.10A). This species made up 44.0 $\pm$ 1.9% of the endogenous PS in HC macrophages and 42.9 $\pm$ 1.1% in CD macrophages. Nine other endogenous PS species were detectable, each of which made up less than 10% of the total PS. There were no significant differences in the molar percentage of any PS species between unstimulated HC and CD macrophages. There were no alterations in any endogenous PS species after stimulation with HkEc, and no significant differences between HC and CD macrophages in the stimulated state.

The predominant newly synthesised PS species over the 3 hours in unstimulated HC and CD macrophages contained 18 carbon length fatty acids, including PS 16:0/18:1, PS 18:0/18:1, PS 18:0/18:2 and PS 18:0/20:4 species (Figure 4.10B). Although PS 18:0/18:1 made up over 40% of the endogenous PS, this species accounted for only 18.3 $\pm$ 1.7% of the total newly synthesised PS in HC and 16.9 $\pm$ 1.2% in CD macrophages, indicating possible differential turnover. No significant differences were

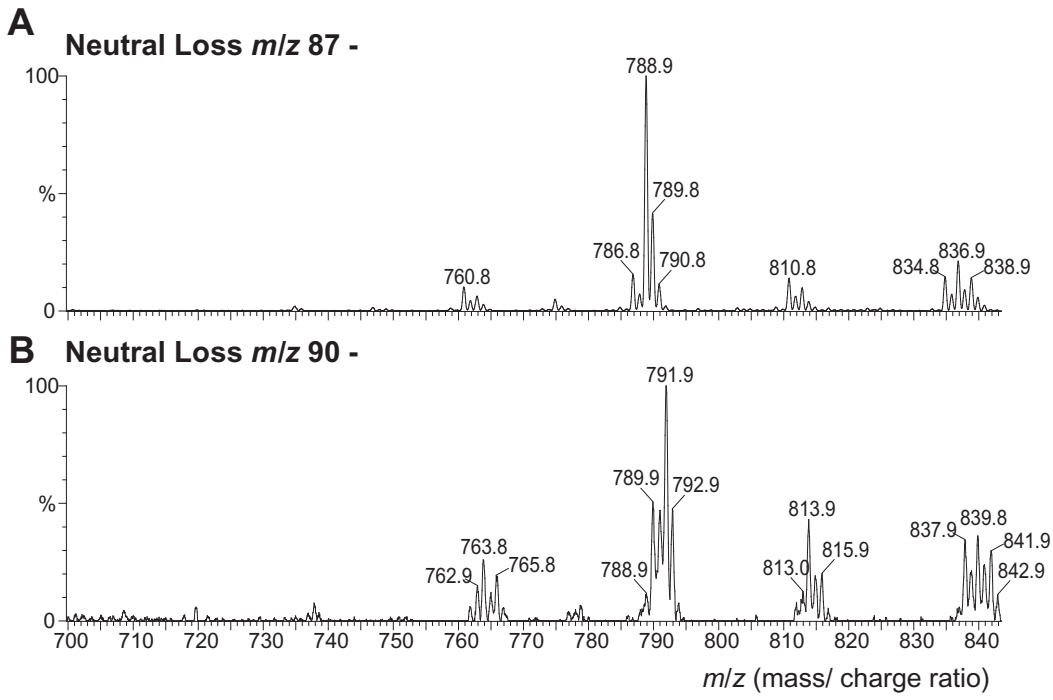


Figure 4.9 Electrospray ionisation mass spectrometric analysis of endogenous and newly synthesised PS. **(A)** Neutral loss scan of *m/z* 87 endogenous PS species. The peak at *m/z* 788.9 corresponds to PS 18:0/18:1. **(B)** Neutral loss scan of *m/z* 90, showing newly synthesised PS species 3 *m/z* units higher than the endogenous species, indicating incorporation of the serine-d_3 stable isotope. The peak at *m/z* 791.9 corresponds to newly synthesised PS 18:0/18:1.

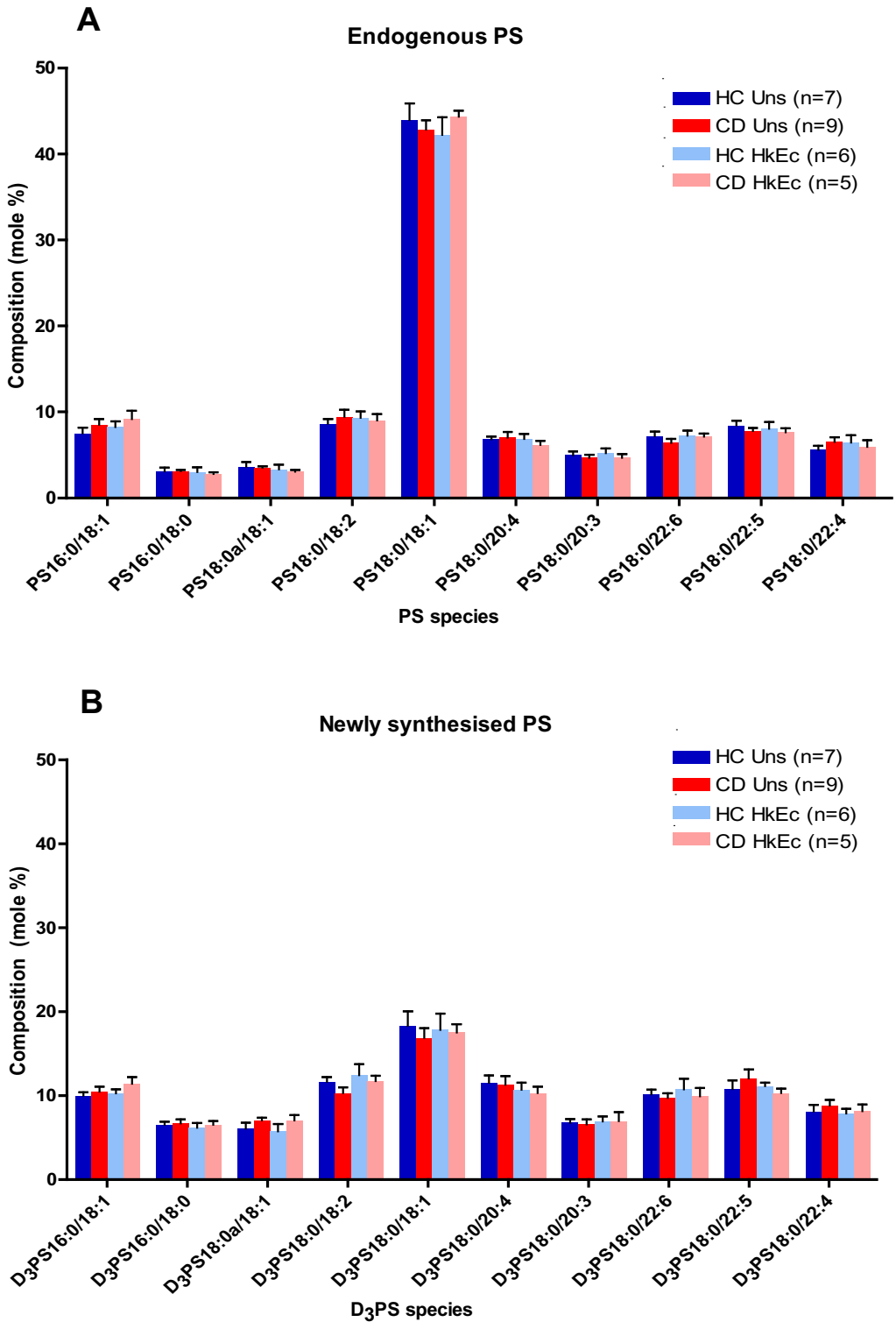


Figure 4.10 Composition of endogenous and newly synthesised PS species in HC and CD macrophages. Macrophages were cultured from HC individuals and CD patients, incubated with serine-d_3 for 3 hours in the presence (HC n=6, CD n=5) or absence (HC n=7, CD n=9) of HkEc. PS species were quantified by electrospray ionisation mass spectrometry. **(A)** Molar percentage composition of endogenous PS species, consisting of various carbon chain length fatty acids, in unstimulated (Uns) and HkEc stimulated HC and CD macrophages. **(B)** Molar percentage composition of newly synthesised (D_3) PS species, in unstimulated (Uns) and HkEc stimulated macrophages. Results are expressed as mean + SEM. No statistically significant differences were identified between HC and CD.

observed in the molar percentage composition of any PS species between unstimulated HC and CD macrophages. The profiles of the newly synthesised PS species were comparable between unstimulated and HkEc stimulated macrophages, and similarly there were no differences in the molar percentage of any PS species between HC and CD macrophages.

The rates of PS synthesis over 3 hours were inferred from the fractional incorporation of serine-d_3 within the total PS (Figure 4.11). The mean fractional incorporation of serine-d_3 into PS was 14.9 $\pm$ 3.0% in unstimulated HC macrophages and 16.0 $\pm$ 1.4% in unstimulated CD cells. This difference was not statistically significant, indicating equivalent rates of PS synthesis in unstimulated HC and CD macrophages. After stimulation with HkEc, there was no significant change in the fractional incorporation of serine-d_3 compared with unstimulated macrophages, and the value for HC cells (18.1 $\pm$ 4.5%) was not significantly different from CD macrophages (14.7 $\pm$ 0.8%), indicating equivalent rates of PS synthesis after HkEc stimulation.

4.2.6 Analysis of PI in HC and CD macrophages

The composition of endogenous and newly synthesised PI species was determined by precursor scans of *m/z* 241- and *m/z* 247- respectively in HC and CD macrophages. Representative PI spectra from one HC individual are shown (Figure 4.12). The major peak at *m/z* 885.8 corresponds to PI 18:0/20:4 (Figure 4.12A), the predominant native PI species detected in HC and CD macrophages. Peaks at *m/z* 841.8, 865.8, 867.8 and 891.8 correspond to the newly synthesised PI species 16:0/18:1, 18:1/18:2, 18:0/18:2 and 18:0/20:4 (Figure 4.12B). As for PC, the spectra were used to quantify the relative concentrations of native and newly synthesised PI species and to estimate the rate of PI synthesis in HC and CD macrophages.

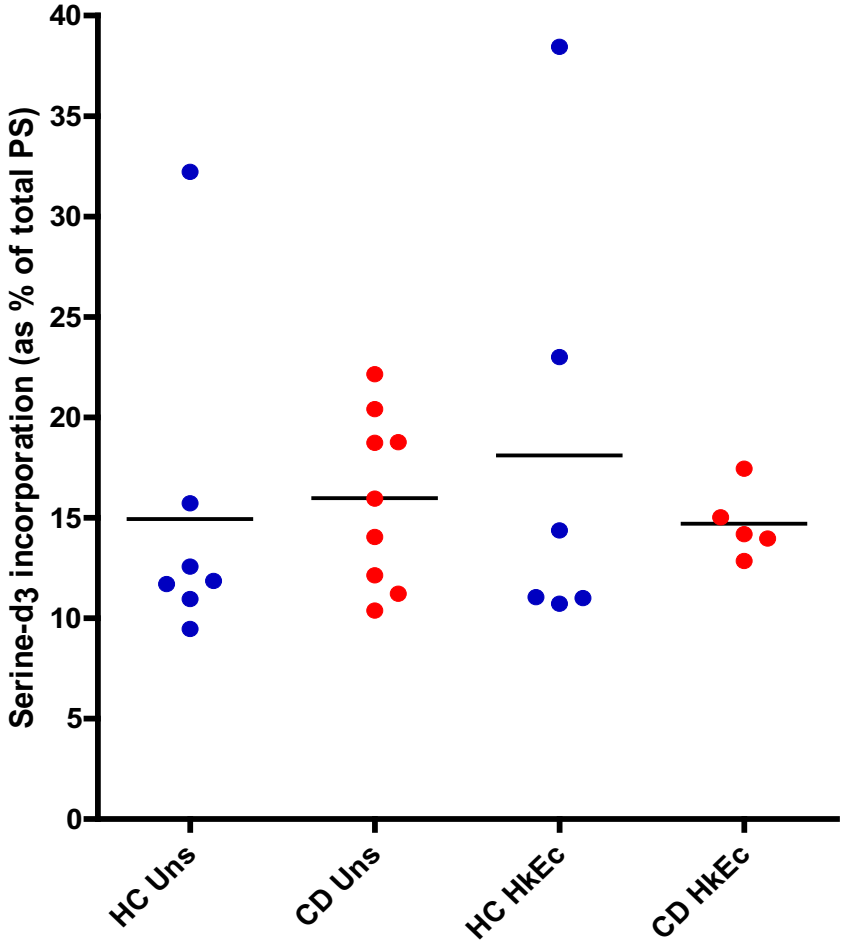


Figure 4.11 Incorporation of serine-d3 into HC and CD macrophage PS. Macrophages were cultured from HC and CD individuals, and incubated with serine-d3 for 3 hours. Results are shown for unstimulated (Uns) (HC n=7, CD n=9) and HkEc stimulated (HC n=6, CD n=5) macrophages. Total lipid was extracted and analysed by ESI-MS as neutral loss scans of *m/z* 87- and *m/z* 90-. The fractional incorporation of serine-d3 as a % of the total PS was determined.

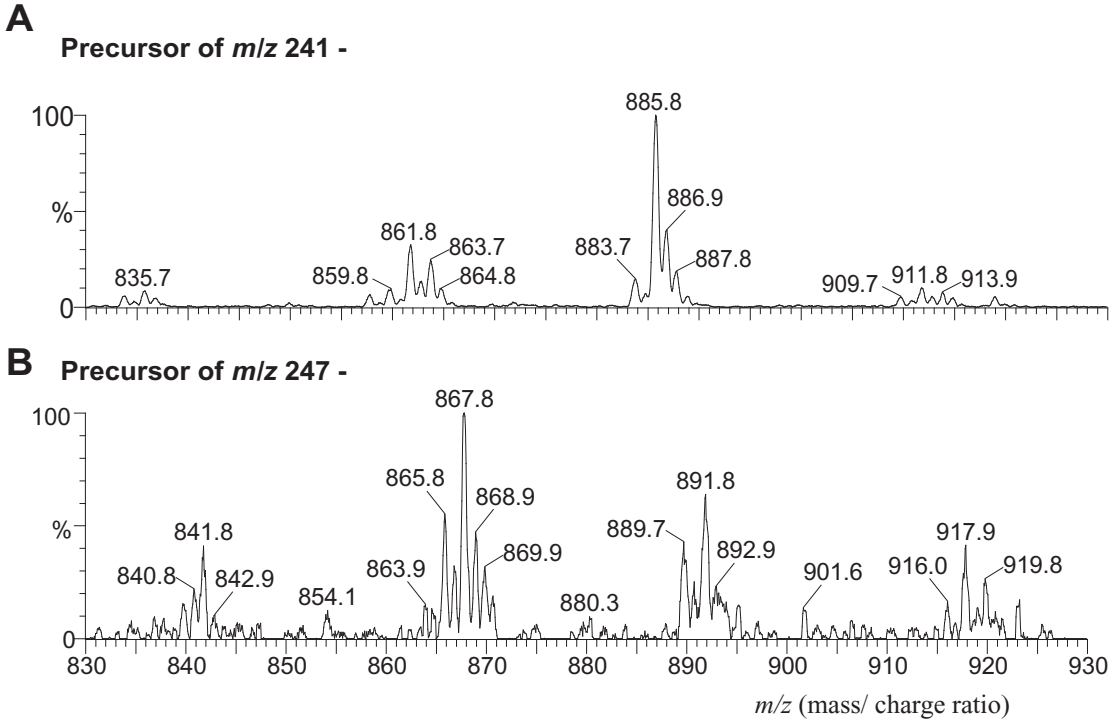


Figure 4.12 Electrospray ionisation mass spectrometric analysis of endogenous and newly synthesised PI species in macrophages. **(A)** Precursor scan of *m/z* 241-, indicating endogenous PI species. Note the peak at *m/z* 885.8, indicating endogenous PI 18:0/20:4. **(B)** Precursor scan of *m/z* 247-, indicating newly synthesised PI species. The peaks at *m/z* 867.8 and 891.8 correspond to newly synthesised PI 18:0/18:2 and 18:0/20:4 respectively.

The predominant endogenous PI species detected was PI 18:0/20:4, making up 42.9 ± 2.7% and 44.0 ± 1.8% of the total native PI in HC and CD macrophages respectively. With the exception of PI 18:0/18:2, all other species detectable contributed to less than 10% of the total endogenous PI. The endogenous PI profiles were equivalent between HC and CD macrophages, and the composition was unaltered after HkEc stimulation (Figure 4.13A).

The profile of newly synthesised PI species was strikingly different to that of endogenous PI, in both HC and CD macrophages (Figure 4.13B). In contrast to endogenous PI, no single species predominated; PI 18:0/20:4 accounted for only 12.3 ± 1.1% of the newly synthesised PI in HC macrophages and 12.9 ± 1.8% in CD macrophages. PI 18:0/18:2 made up an additional 12.6 ± 1.0% of the newly synthesised PI in HC and 13.0 ± 1.3% in CD macrophages. 10 additional species accounted for the remaining newly synthesised PI, each contributing between 5-10% of the total in HC and CD cells. The difference from the endogenous PI profiles could indicate rapid turnover of certain PI species or a relatively stable pool of arachidonate containing-PI species within macrophages. The proportion of newly synthesised PI 16:0/18:1 was significantly reduced in CD macrophages compared to HC in the unstimulated state ($p<0.05$) (Figure 4.13B, Figure 4.14). This did not appear to be related to use of 5-ASA medication (Figure 4.14C).

The fractional incorporation of *myo*-d_6-inositol into total cellular PI was calculated as a measure for the global rate of PI synthesis (Figure 4.15). The mean fractional incorporation of *myo*-d_6-inositol as a percentage of the total PI was 16.1 ± 2.3% in HC and 15.5 ± 1.5% in CD macrophages over 3 hours, suggesting that the overall rate of PI synthesis is unaltered in CD macrophages. In contrast to PC, there was

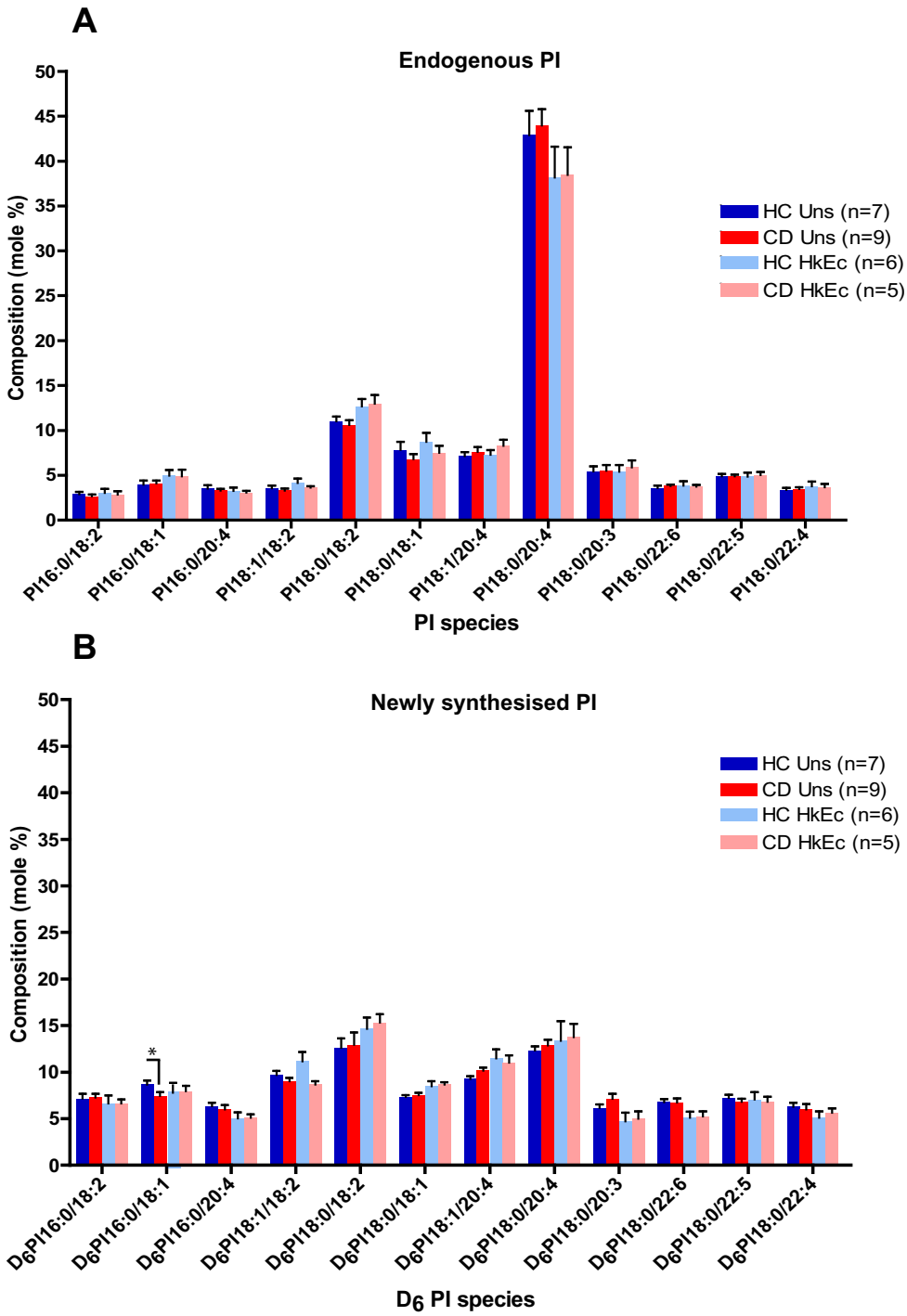


Figure 4.13 Composition of endogenous and newly synthesised PI species in HC and CD macrophages. Macrophages were cultured from HC individuals and CD patients, incubated with *myo*-d_6-inositol for 3 hours in the presence (HC n=6, CD n=5) or absence (HC n=7, CD n=9) of HkEc, and PI species quantified by electrospray ionisation mass spectrometry. (**A**) Molar percentage composition of endogenous PI species, consisting of various carbon chain length fatty acids, in unstimulated (Uns) and HkEc stimulated HC and CD macrophages. (**B**) Molar percentage composition of newly synthesised (D6) PI species, in unstimulated and HkEc stimulated HC and CD macrophages. Results are expressed as mean + SEM. * indicates $p<0.05$.

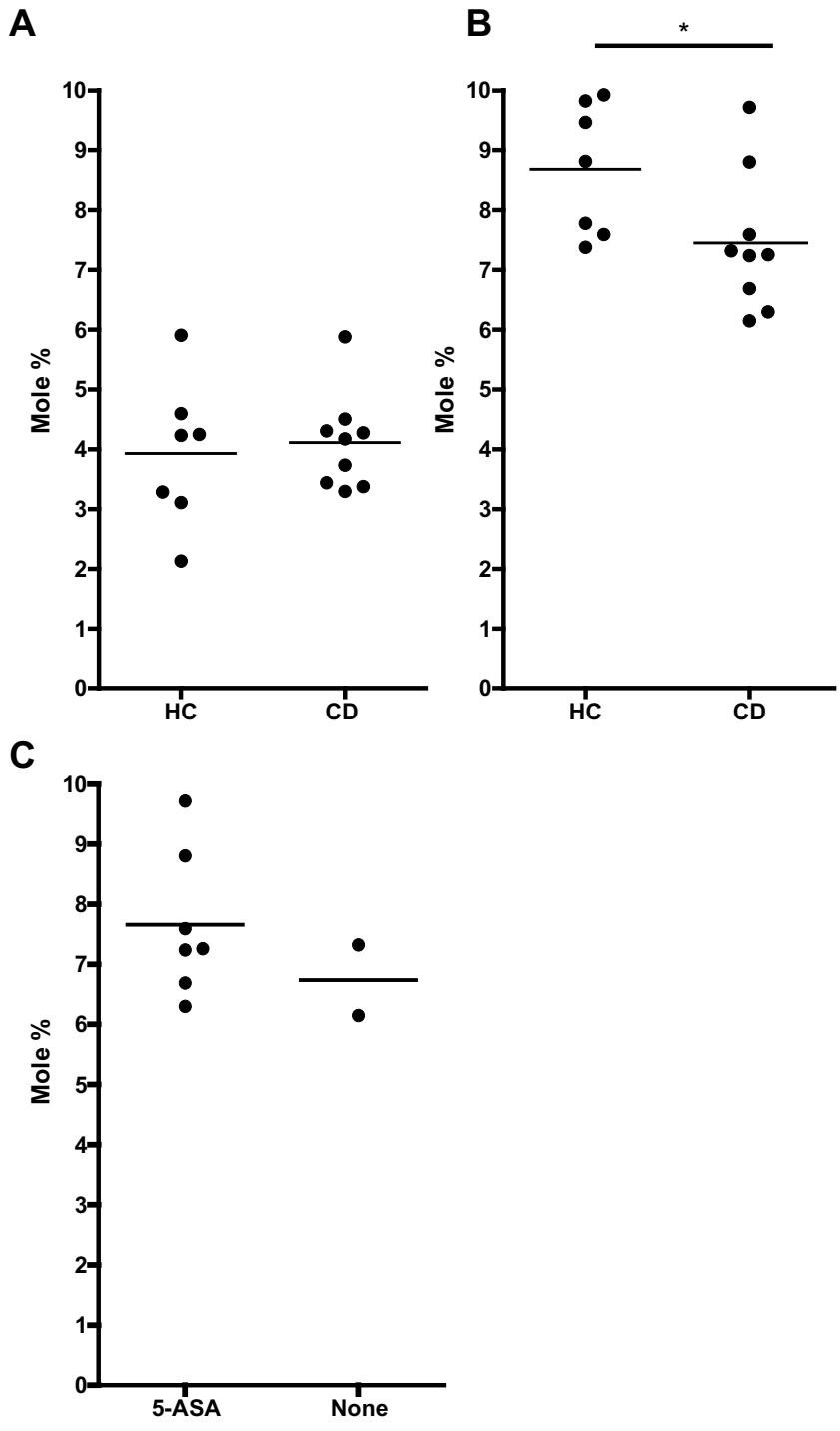


Figure 4.14 Alterations in the molar percentage of PI 16:0/18:1 in CD macrophages. **(A)** The molar perecentage of endogenous PI 16:0/18:1 was not significantly altered in CD (n=9) compared to HC (n=7). **(B)** The proportion of newly synthesised PI 16:0/18:1 over 3 hours, as determined using stable isotope labelled *myo*-d_6 inositol and ESI-MS, was reduced in CD macrophages compared to HC. **(C)** There was no difference in the proportion of newly synthesised PI 16:0/18:1 in patients receiving 5-ASA and those receiving no treatment (None). * Represents $p<0.05$.

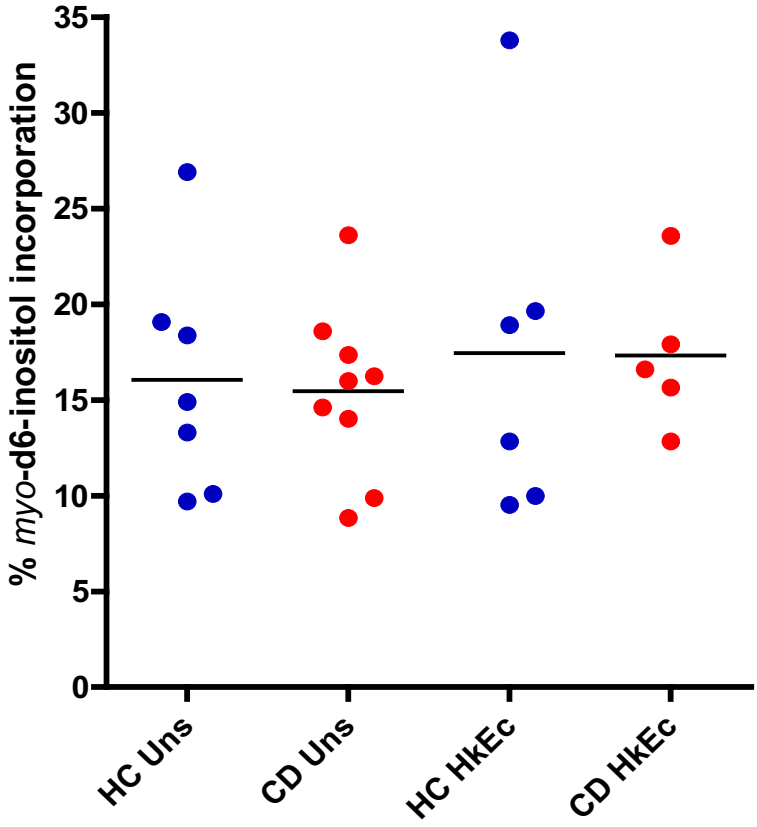


Figure 4.15 Incorporation of *myo*-d_6-inositol into HC and CD macrophage PI. Macrophages were cultured from HC and CD donors and incubated with *myo*-d_6-inositol for 3 hours. Total lipid was extracted and analysed by ESI-MS as neutral loss scans of *m/z* 241- and *m/z* 247-. Results are shown for unstimulated (Uns) (HC n=7, CD n=9) and and HkEc stimulated macrophages (HC n=6, CD n=5). No significant differences were identified.

no alteration in the percentage incorporation after stimulation with HkEc, indicating comparable rates of PI synthesis in unstimulated and HkEc stimulated macrophages.

4.2.7 Shotgun lipidomics analysis of ileal biopsies

CD is a complex, heterogeneous condition, and may involve both a defect in macrophages and perturbations in the mucosal barrier (151). Therefore, in addition to investigations on macrophages, 'shotgun lipidomics' was also performed on non-inflamed ileal biopsies from CD patients (n=5) and control individuals (n=5) to assess for possible defects in mucosal lipid composition. Biopsies from non-inflamed ileum were obtained from CD and control individuals at colonoscopy by experienced gastroenterologists (Dr Roser Vega, Dr Farooq Rahman). Two of the patients had macroscopic and microscopic evidence of active disease; the remaining patients had macroscopic and histologic features consistent with quiescent disease. Three patients were receiving no treatment, one was receiving 5-ASA and one was receiving methotrexate. Two patients had previous ileal resections (<20 cm); in these cases biopsies from the neoterminal ileum were obtained. Control individuals were undergoing colonoscopy for other purposes and had no evidence of inflammatory bowel disease.

This work was performed as a collaborative project with Dr Xianlin Han, Washington University School of Medicine. Shotgun lipidomics is a 2-dimensional mass spectrometry based technique that permits quantitation of a vast array of lipid species in biological samples (276;365). Whilst the overall amounts of PC, PS, CL, PG, PA, sphingolipids and cholesterol in the biopsy samples did not differ between CD and HC (Figure 4.16A), interestingly, a significant reduction PI 16:0/18:1 (as a % of total PI) was observed in CD compared to HC ($p<0.01$) (Figure 4.16B, C). This is the same species that had reduced synthesis in CD macrophages compared to HC.

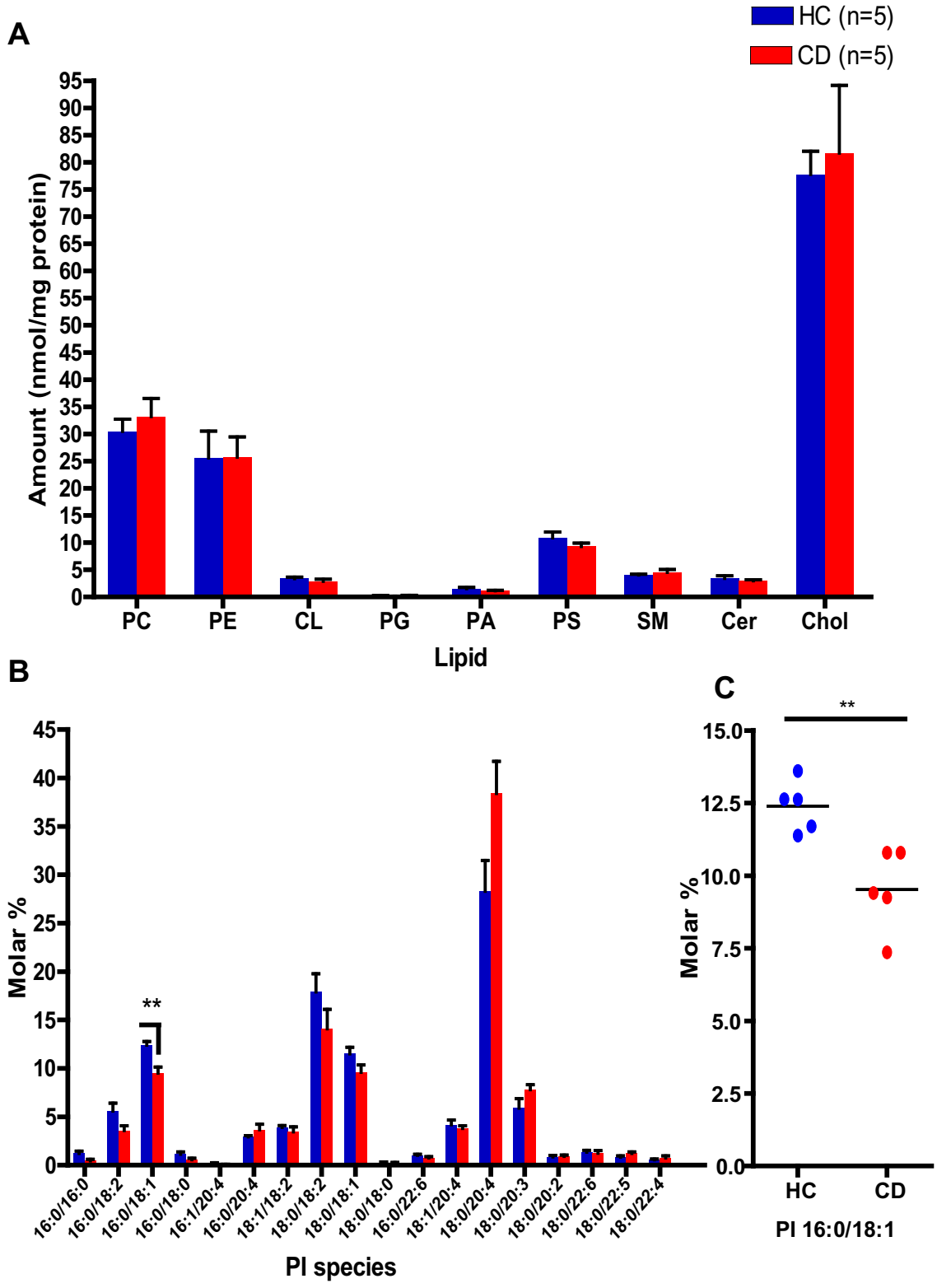


Figure 4.16 Shotgun lipidomics analysis of ileal biopsies. Ileal biopsies were obtained from non-inflamed ileum, and amounts of lipids were quantified by mass spectrometry. **(A)** Total phospholipid (PC, PE, CL, PG, PA, PS), sphingolipid (sphingomyelin, SM and ceramide, Cer) and cholesterol (Chol) content did not differ between HC and CD patients. **(B)** Molar percentage composition of phosphatidylinositol (PI) species. **(C)** Reduced molar percentage of PI 16:0/18:1 in CD biopsies compared to HC. ** represents $p<0.01$.

4.3 Discussion

In this chapter, sphingolipids and phospholipids were investigated as candidate molecular defects in CD macrophages. No gross abnormalities were identified in the ceramide, dihydroceramide or sphingoid base composition of CD macrophages, both in the unstimulated state and after stimulation with HkEc. In addition, the macrophage PC and PS composition, and rates of PC and PS synthesis, were shown to be normal in CD macrophages. The profile of endogenous PI species in CD macrophages also did not differ from HC. The newly synthesised PI species determined over a 3 hour time period displayed a reduced molar percentage of the monounsaturated species PI 16:0/18:1 in CD macrophages, although the molar percentage of other PI species, and the overall rates of PI synthesis were unaltered. The same species was present at reduced levels in ileal biopsies from CD patients.

Previous demonstrations of impaired pro-inflammatory cytokine release and apoptosis led to the hypothesis that macrophage sphingolipid or phospholipid composition may be an underlying abnormality in CD. Macrophages from CD patients release deficient levels of TNF in response to multiple stimuli, which (in the case of HkEc) resulted from abnormal post-translational trafficking of TNF to lysosomes (136). Sphingolipids and phospholipids are important components of vesicular membranes, and generation of PC has been shown to be critical for pro-inflammatory cytokine release from the Golgi apparatus in murine macrophages (197). However, given the data presented in this chapter, it is unlikely that gross defects in sphingolipid, PC, PI or PS composition underlie the impaired cytokine release observed in the majority of patients. Furthermore, impaired release of TNF does not appear to relate to alterations in the overall metabolic flux through the PC, PI or PS synthesis pathways, indicating that other mechanisms are likely to be responsible.

There were no detectable abnormalities in the fatty acid composition of endogenous sphingolipids, PC, PI or PS in CD macrophages, in terms of the carbon chain length and degree of saturation. This could be considered surprising, given the results of previous studies indicating fatty acid aberrations in CD patients. In particular, studies have demonstrated differences in the fatty acid profiles of plasma phospholipids (356) and PBMCs (357) in CD patients. There are several possible explanations for this discrepancy. Firstly, the study by Geerling et al. (356) included patients with active disease in their investigation, raising the possibility the alterations observed could be secondary to chronic inflammation; indeed studies in quiescent patients have yielded conflicting results (358). Secondly the profiles of ceramides, PC, PS and PI specifically were determined in this study, whereas others have addressed fatty acid composition in terms of the total percentage of fatty acids or phospholipid species. In addition, it is well established that differentiation of cells is often associated with significant alterations to the fatty acid profile. For example, differentiation of U937 promonocytic cells with dimethyl sulfoxide is associated with an increase in PC species containing arachidonate (366). Furthermore, fatty acid synthesis and desaturation is upregulated in primary monocytes upon M-CSF induced differentiation, which is associated with a shift in the phospholipid species profile towards those containing shorter, monounsaturated fatty acids (342). It is therefore likely that the phospholipid profiles of CD macrophages do not strongly resemble those of monocytes or PBMCs, hence concurrent abnormalities would not necessarily be expected.

In addition, the technique employed in this study, whilst able to distinguish lipids containing fatty acids of different chain lengths and degree of saturation, does not allow distinction between species containing omega-3 and omega-6 fatty acids with the same *m/z* ratio. Alterations in the omega-3 and omega-6 fatty acid balance have been described in PBMCs from CD patients (357); differences in CD macrophages could

therefore exist and be investigated in further studies. Techniques enabling distinction of individual phospholipid molecular species with the same *m/z* but containing different omega-3 or omega-6 fatty acids are currently in their infancy. However, an important consideration is the fact that the balance of omega-3 and omega-6 fatty acids in cultured cells may be largely governed by the culture medium. For example, FBS contains very low levels of linoleic acid, which can give rise to unphysiological cellular levels of omega-6 and omega-3 fatty acids, as demonstrated by studies in cystic fibrosis cell lines (367). A more relevant investigation may therefore be the interrogation of the omega-6 and omega-3 fatty acid balance in mucosal biopsies or intestinal macrophages from CD patients.

CD macrophages synthesised a reduced relative percentage of PI 16:0/18:1 over a 3 hour time period when compared to HC, although this was not associated with a alteration in either the overall rates of PI synthesis or the molar percentage of endogenous PI 16:0/18:1. Interestingly, shotgun lipidomic analysis of ileal biopsy samples also revealed abnormalities in this PI species in CD. Taken together, the two studies suggest that the observed reduction in PI 16:0/18:1 represents a genuine abnormality in CD.

Given that the overall rate of PI synthesis is normal in CD macrophages, this difference could relate to an alteration in the substrate preference of PI synthase in CD. Alternatively, abnormalities could exist in the fatty acid metabolism pathway, leading to altered fatty acid availability for phospholipid synthesis. The control of fatty acid synthesis and metabolism is a key determinant of membrane lipid composition, as illustrated by a recent study conducted in macrophages and dendritic cells. GM-CSF and IL-4 treated PBMCs were found to have reduced monounsaturated fatty acid species within certain phospholipid classes. This difference was attributed to decreased

stearoyl-CoA desaturase activity (368). Stearoyl-CoA desaturase is an enzyme involved in desaturation of palmitate (C16:0) and stearate (C18:0) to monounsaturated fatty acids. It could therefore be hypothesised that CD patients have differences in fatty acid synthesis and desaturation that give rise to the observed alteration in PI 16:0/18:1. Notably, the recent GWAS meta-analysis identified a CD-associated SNP in a region containing the *FADS1* gene (Fatty acid desaturase 1) (115). Whilst fatty acid desaturase 1 is distinct from stearoyl-CoA desaturase in its activity (being primarily involved in the conversion of essential fatty acids to highly unsaturated fatty acids), the association does add credence to the hypothesis that fatty acid desaturation may be relevant in the pathogenesis of CD.

It is plausible that abnormalities in the mucosal PI profile could be important in triggering barrier dysfunction in CD patients, facilitating ingress of luminal contents into the bowel wall. Phospholipids are present in mucus as well as cellular components of the mucosa, and are understood to play a pivotal role in enhancing barrier resistance. Administration of PC and PI is protective in rodent acetic acid-induced colitis models (369), and PC is known to protect against intestinal injury induced by bile acids (370). Alterations in the PC composition of intestinal mucus have also been identified in patients with UC (371). In contrast, no striking differences were reported in ileal or colonic mucus from CD patients compared to controls, with the possible exception of sphingomyelin and PC 16:0/16:0 content, which were both moderately increased in CD. Other phospholipid species, including PI, were not quantified. It is therefore possible that the phospholipid profile of mucus from CD patients is subtly altered. Alternatively, the cellular components of the mucosa could have a subtly different membrane lipid composition. This could alter the fluidity and facilitate bacterial translocation directly, or enhance susceptibility to damaging agents such as emulsifiers. Whilst endogenous profiles of PI appeared unaltered in macrophages, it is possible that subtle alterations in

the species of synthesised PI may have some functional effect on cytokine release, given the role of phosphatidylinositol phosphate derivatives in ensuring efficient secretion of TNF (350). Further functional studies are required to test this.

During this study, quantitation of total macrophage cellular sphingolipid, PC, PS and PI species was performed. It is however recognised that there exists a significant 'comparmentalisation' of lipid metabolism within cells, with particular enzymes in the pathway acting in specific subcellular compartments (309). The site of 'de novo' ceramide synthesis is the endoplasmic reticulum (372), whereas complex glycosphingolipid and sphingomyelin synthesis occurs in the Golgi apparatus. Breakdown of complex sphingolipids and ceramide can occur at the plasma membrane, or by the action of acid sphingomyelinase and acid ceramidase in the lysosomal compartment. Transport of sphingolipids between various compartments depends on vesicular transport or the action of transporter proteins such as CERT (373). Furthermore, analysis of subcellular membrane fractions has also revealed distinctive profiles of phospholipid classes and species within each fraction (364). It could be hypothesised that subcellular fractions of CD macrophages have abnormalities in the phospholipid or sphingolipid composition. However the limited yield of macrophages obtainable from peripheral blood samples makes such studies infeasible.

During the course of this investigation, HkEc stimulation of macrophages was shown to be associated with a number of alterations in ceramides and phospholipids, further evidencing roles for these lipids in inflammation and the innate immune response to microbes. LPS, TNF and IL-1β have previously been shown to cause a rapid increase in the levels of ceramide in macrophage cell lines (319). Unexpectedly, in this study, HkEc stimulation was associated with a reduction in the C16:0, C24:0 and C24:1 ceramides, and a concomitant increase in dihydrosphingosine content. This difference

could relate to differential effects of TLR and HkEc stimulation on the sphingolipid pathway, or alternatively the time frame investigated. Studies by MacKichan *et al*. investigated alterations in the ceramide content over a 30 minute period, whereas this study compared unstimulated with 4 hour HkEc stimulated macrophages. An altered ratio of ceramide to sphingoid bases in later stages of macrophage activation could be functionally important, given the diverse roles of ceramides in regulation of membrane structure, apoptosis and intracellular signalling, and dihydrosphingosine, which has a role in SNARE protein complex assembly and vesicle exocytosis (374).

Alterations in the phospholipid profile of macrophages were also observed after stimulation with HkEc. These included a reduction in the amount of endogenous PC 16:0/20:4. This is likely to relate to an increased activity of cytosolic phospholipase A_2 enzyme-α (cPLA$_2$-α), an enzyme involved in the generation of fatty acids for prostanoid synthesis. cPLA$_2$-α specifically hydrolyses arachidonate-containing phospholipids to release arachidonate (375), and preferentially binds PC (376). LPS stimulation is known to result in increased phosphorylation and activation of this enzyme (377), consistent with the observed reduction in PC 16:0/20:4 after stimulation. The findings are also in concordance with the effect of zymosan stimulation in human monocytes, which results in a reduction in PC species containing arachidonic acid. This has been attributed to increased PC hydrolysis by cPLA$_2$-α (378).

In addition, an increased metabolic flux down the PC synthesis pathway was observed after HkEc stimulation. The rate limiting step in this pathway is catalysed by CCT (337), and activity is tightly regulated. Activity of CCT is dramatically increased on binding to membranes, which induces a conformational change in the enzyme and increases its affinity for CTP (379). The presence of degraded phospholipids (380) and membrane curvature stress (381) are known to be key modulators of enzyme activity

which could foreseeably increase after HkEc stimulation, thereby increasing the rate of metabolic flux down the PC synthesis pathway.

In summary, in this chapter, the macrophage sphingolipid and phospholipid composition has been explored in CD, given the prominent roles of these molecules in vesicle trafficking and their relevance in cytokine secretion. Mass spectrometric analysis revealed no gross differences in the sphingolipid or phospholipid profiles in CD, although a subtle difference in the proportion of newly synthesised PI 16:0/18:1 was identified. The same species was also present at reduced levels in ileal biopsies from CD patients. A number of potential mechanisms could be responsible for this abnormality. Given the lack of gross abnormalities in phospholipid and sphingolipid composition in CD macrophages, an alternative 'hypothesis-generating' approach was subsequently employed to identify molecular mechanisms of the impaired cytokine release. This is explored in the subsequent chapter.

Chapter 5: Analysis of macrophage transcriptomic abnormalities in Crohn's disease

5.1 Introduction

CD is associated with attenuated monocyte-derived macrophage TNF release in response to a range of microbial agonists, as demonstrated in chapter 3. Whilst defective secretion is likely to relate to aberrant post-translational trafficking of pro-inflammatory cytokines (136), the molecular mechanisms responsible are incompletely understood. In chapter 3, no associations could be identified between any specific CD-susceptibility polymorphism and TNF release in response to HkEc and TLR2 stimulation. Whilst TNF release after HkEc stimulation was weakly correlated with overall genetic risk score, this was not the case for TNF release downstream of TLR agonists. Subsequently, a defective macrophage sphingolipid and phospholipid composition was postulated, given the roles of these lipids in vesicle trafficking and membrane structure. However, no profound abnormalities could be identified in the majority of the lipid species investigated. In this chapter, a 'hypothesis-generating' approach is taken to identify a molecular basis of impaired cytokine secretion, utilising macrophage whole genome expression data. Transcriptomic data were analysed using both conventional group comparisons and by 'outlier' analysis; the iterative comparison of individual patients with a reference group. This approach, whilst accepted in the field of oncology, has not previously been applied to CD and other multifactorial disorders.

CD is a complex and highly heterogeneous syndrome. Clinically, individual CD patients display variable age of onset, anatomical sites of involvement, disease course and response to treatment. In addition, genetic studies have revealed considerable genetic heterogeneity between CD patients. For example, considering the case of *NOD2*; only 8% of patients will be homozygous or compound heterozygous for disease-associated polymorphisms, with approximately one quarter of patients heterozygous for

one *NOD2* risk allele (255). Furthermore, the genetic risk score (GRS) in our cohort of patients, as calculated from a 34 SNP genotype, ranged from 0.14 to 37.2 (where 1 represents mean population risk). Given the heterogeneity of CD, it is likely that the precise molecular lesions underlying impaired macrophage function and defective acute inflammation will be highly variable between individual patients. Furthermore, given the underlying 'common disease, common variant hypothesis' assumed by most GWAS (210), it is doubtful that such heterogeneous lesions would have been discovered in these studies. Indeed, only 23% of the total heritability of CD is thought to be explained by the 71 CD-associated variants identified in the recently published GWAS meta-analysis (115). Some of the 'missing heritability', whilst controversial, may be explained by rare variants present in a subset of individuals with disease, possibly with large biological effects (256;257).

Recently, studies in the field of oncology have utilised transcriptomic data to identify molecular abnormalities in heterogeneous tumour sets. Cancers show a potential parallel to multifactorial disorders such as CD, in that each individual tumour possesses a distinctive genetic and transcriptomic profile that results in abnormal protein expression and the development of a neoplastic phenotype. For this reason, some of the studies have focused on microarray expression profiling of individual tumours, as opposed to investigating tumour groups as homogeneous entities. Specifically, cancer outlier profile analysis (COPA) interrogates abnormally expressed genes in subsets of cancer samples, by identifying the probes in individual samples that display expression outside a set threshold percentile from the median (outliers) (382). Pairs of probes are identified that are most frequently detected as mutually exclusive outliers in the disease group, to facilitate the identification of potential chromosomal translocations. Application of the COPA strategy to prostate cancer led to the discovery of translocations between the transmembrane protease gene *TMPRSS2* and the ETS

transcription factor genes *ERG* or *ETV1* in subsets of samples (383). The translocations placed androgen-responsive promoter sequences of *TMPRSS2* upstream of the transcription factor gene, causing over-expression of the gene family in prostate cancer. Hence, the chromosomal rearrangement was detected by its effects on gene expression.

Several microarray gene expression studies have been previously performed in CD and UC. The majority of these focused on bowel biopsies, in which a wide spectrum of cells are present, although several investigated PBMCs (384;385). More recently, our laboratory investigated the global transcription profiles of macrophages using Affymetrix gene arrays. Levels of pro-inflammatory cytokine mRNA were found to be equivalent between HC and CD macrophages. However, 27 differentially expressed probe sets were identified that were common to both ileal and colonic CD, corresponding to nine genes which had functions in vesicle trafficking and cytoskeletal organisation (136). This was consistent with a failure of cytokine secretion (a vesicle-mediated process) in CD macrophages. All of these studies used conventional group comparison methods for microarray data analysis. In this chapter, the results of a larger scale study of macrophage gene expression profiling, conducted using the Illumina microarray platform, are discussed. Gene expression data are interrogated in a number of ways, including by standard group comparisons of differential gene expression, and by using an 'outlier analysis' strategy to identify grossly under- and over-expressed genes in individual CD patients. The abnormal expression of one such gene is validated, and the functional relevance of this to the acute inflammatory deficit of CD is investigated.

5.2 Results

5.2.1 Differentially expressed genes between CD, HC and UC macrophages

In order to identify differences in gene expression between HC, CD and UC macrophages, microarray analysis of genome-wide gene expression was performed. Macrophages were cultured from CD patients (n=61), HC individuals (n=43) and UC patients (n=45). RNA was extracted from unstimulated macrophages, and macrophages stimulated for 4 hours with HkEc (CD n=30, HC n=14 and UC n=7). Microarray analysis was performed using Illumina Human-WG6 v3.0 Expression BeadChips. Demographics of patients included in the study are shown (Appendix 1 Table III). Eight CD patients in this study were receiving immunosuppressants (6-mercaptopurine, methotrexate or azathioprine), one patient was receiving low dose steroid therapy and four patients were receiving anti-TNF agents. The remainder were receiving either no treatment (n=16), or 5-ASA only (n=32). Four of the CD patients were related (from two distinct families). None of the other individuals included in the study were related.

Expression data were $\log_2$ transformed with cubic spline normalisation between arrays. Data from unstimulated samples were subsequently normalised across three batches of experiments using an established algorithm (278). All HkEc stimulated samples (CD n=30, HC n=14 and UC n=7) were analysed in one single batch with paired unstimulated samples from all donors. Therefore, the HkEc stimulated samples were excluded from the batch normalisation procedure, and for comparisons of HkEc with unstimulated samples, non-batch normalised data were used.

20,023 out of the total 48,802 probes (41.0%) were found to be expressed at detectable levels in macrophages, as determined using the detection p-values associated with each probe (see Materials and Methods section 2.5.3 for details). Differentially expressed genes were identified using the MultiExperiment viewer TM4 software suite,

an established software package for statistical analysis of microarray datasets (279). Probes were identified that were significantly different between CD and HC macrophages, using a threshold uncorrected p-value of $p<0.01$ and a minimum fold change of 1.2 compared to mean expression in either the HC or the UC cohort.

Comparing unstimulated CD with HC macrophages, 81 probes were identified that differed in expression at an uncorrected p-value of $p<0.01$ (Table 5.1). These probes represented 16 over-expressed genes and 55 under-expressed genes (Table 5.1A). A number of these genes have important functions in innate immunity; notably *NCF1*, and the transcription factor *STAT1*, were downregulated in CD macrophages. Furthermore, a number of the under-expressed genes in CD macrophages are located in loci implicated in CD susceptibility from GWAS, including *ICAM3* (115) and *LST1* (116). Comparison of CD with UC macrophages identified only 3 genes that were differentially expressed (Table 5.1B); *CST7*, *RPS6KA2* and *GSTM1*, indicating strong similarity between CD and UC profiles in this analysis. In both comparisons, none of the genes reached statistical significance after correction for multiple testing, either using an adjusted Bonferroni correction or the less stringent Benjamini and Hochberg False Discovery rate (FDR) correction that is commonly used in microarray analysis (280). Therefore, the results should be interpreted with the caveat that some or all of the differentially expressed genes could represent false positive discoveries, and may indicate that the transcriptomic profile of CD macrophages is not grossly abnormal.

Considering HkEc stimulated macrophages, 134 probes were differentially expressed between HC and CD macrophages at the applied thresholds. These probes corresponded to 120 unique genes, 44 of which were over-expressed and 76 were under-expressed in CD macrophages (Table 5.2, Table 5.3). Again, many of these genes have well-established roles in innate immune function. Consistent with the findings in

A

Over-expressed genes (p<0.01, >1.2 Fold change)			
Probe ID	**Gene ID**	**Fold change**	**P-value**
ILMN_1801077	ADFP*	1.42	3.40E-03
ILMN_1712475	HS3ST2	1.38	1.92E-03
ILMN_1669831	C6ORF192	1.35	5.77E-03
ILMN_2332964	LGMN*	1.35	6.39E-03
ILMN_1680738	C5ORF13	1.31	5.74E-03
ILMN_1745963	FOLR2	1.29	6.12E-03
ILMN_1784207	C1ORF128	1.25	5.33E-03
ILMN_1685625	UCP2	1.25	9.73E-03
ILMN_1787186	NOV	1.25	3.16E-03
ILMN_1704753	EPAS1	1.23	5.19E-03
ILMN_1772224	KCNJ5	1.23	4.90E-03
ILMN_1706531	ABCC5	1.22	4.86E-03
ILMN_1795342	MLPH	1.22	4.64E-03
ILMN_1709634	CMBL	1.22	5.58E-03
ILMN_1811616	EEPD1	1.22	3.62E-03
ILMN_1710124	CMTM8	1.20	5.52E-03
Under-expressed genes (p<0.01, >1.2 Fold change)			
ILMN_2160476	CCL22	-1.68	2.80E-04
ILMN_1691364	STAT1*	-1.64	9.14E-03
ILMN_1697309	NCF1	-1.62	8.50E-03
ILMN_1701114	GBP1	-1.48	9.62E-03
ILMN_1729801	S100A8	-1.48	3.46E-03
ILMN_1748473	GIMAP4	-1.47	1.27E-03
ILMN_1723035	OLR1	-1.44	3.54E-03
ILMN_1740418	CYP27B1	-1.44	7.10E-03
ILMN_2376108	PSMB9	-1.43	8.43E-03
ILMN_1680996	ALOX5	-1.42	2.01E-03
ILMN_1741727	QPCT	-1.42	9.29E-04
ILMN_1778321	SLC2A6	-1.42	6.22E-03
ILMN_1654696	C15ORF48*	-1.40	2.33E-03
ILMN_2366212	CD79B*	-1.40	6.37E-03
ILMN_1803945	HCP5	-1.39	3.35E-03
ILMN_1691341	IL7R*	-1.39	2.45E-03
ILMN_1751079	TAP1	-1.38	9.23E-03
ILMN_1797875	ALOX5AP	-1.38	3.40E-03
ILMN_1686664	MT2A	-1.37	8.35E-03
ILMN_1713751	ADAM19	-1.36	4.93E-03
ILMN_2307903	VCAM1	-1.36	4.94E-03
ILMN_1780465	CLEC5A	-1.35	4.16E-03
ILMN_2404154	SERPINA1*	-1.34	2.10E-03
ILMN_1774077	GBP2	-1.33	8.81E-03
ILMN_1769388	GJB2	-1.32	4.69E-03
ILMN_1779257	CD40	-1.32	7.15E-03
ILMN_1691071	FCRLA	-1.32	6.50E-03
ILMN_1655549	SIGLEC10	-1.31	5.05E-04
ILMN_1687538	ETS1	-1.31	6.84E-03
ILMN_1769383	GIMAP5	-1.31	8.70E-03
ILMN_2376204	LTB*	-1.30	7.18E-03
ILMN_2239754	IFIT3	-1.30	5.81E-03
ILMN_2329429	GIMAP6	-1.29	3.64E-03
ILMN_1747305	GIMAP8	-1.29	4.60E-03
ILMN_2325506	BCAS4	-1.28	8.89E-03
ILMN_1723412	ASCL2	-1.28	9.28E-03
ILMN_1663390	CDC20	-1.27	6.16E-03
ILMN_1674063	OAS2	-1.27	9.62E-03
ILMN_2212763	ICAM3	-1.26	6.12E-04
ILMN_1671054	HLA-A	-1.26	5.48E-04
ILMN_1714433	MARCKSL1	-1.25	9.14E-04
ILMN_1786065	UHRF1	-1.25	3.40E-03
ILMN_1758418	TNFSF13B	-1.24	6.33E-03
ILMN_1718936	LST1	-1.24	3.80E-03
ILMN_1663195	MCM7	-1.23	4.81E-03
ILMN_2049766	NFE2L3	-1.23	3.60E-03
ILMN_1725170	LOC650557	-1.23	5.98E-03
ILMN_1682799	STAMBPL1	-1.22	5.01E-03
ILMN_2176063	FCGR1A	-1.22	8.23E-03
ILMN_2391051	FCGR1B	-1.22	5.00E-03
ILMN_2373062	RHBDF2*	-1.22	4.64E-03
ILMN_2109708	ECGF1	-1.21	8.33E-03
ILMN_2337928	CXCR5	-1.21	2.69E-03
ILMN_2042771	PTTG1	-1.21	6.11E-03
ILMN_1769911	SLC38A1	-1.20	8.62E-03

B

Probe ID	Gene ID	Fold change	P-value
ILMN_1679826	CST7	1.21	6.28E-03
ILMN_1716218	RPS6KA2	1.23	2.97E-03
ILMN_1762255	GSTM1	1.28	8.02E-03

Table 5.1 Differentially expressed genes in unstimulated CD macrophages. Whole genome microarray analysis of gene expression was performed using Illumina Human-WG v3.0 expression arrays. Differentially expressed genes were identified using a minimum fold change of 1.2 and p<0.01 (uncorrected for multiple testing). **(A)** Differentially expressed genes between CD and HC macrophage groups. **(B)** Differentially expressed genes between CD and UC macrophage groups. Mean fold changes in CD compared to the comparator group are shown. * indicates genes where multiple probes were identified as differentially expressed, the most significant of which are shown. None of the genes remained statistically significant after correction for multiple testing.

Over-expressed genes (p<0.01, >1.2 Fold change)			
Probe ID	Gene ID	Fold change	P-value
ILMN_1713638	ICHTHYIN	1.81	3.03E-03
ILMN_1776967	LRRC50	1.66	4.16E-03
ILMN_1784863	CD36	1.59	5.48E-03
ILMN_1705570	H2AFY2	1.53	7.97E-03
ILMN_1671478	CKB	1.52	9.25E-03
ILMN_1689088	COLEC12	1.50	6.91E-03
ILMN_1680110	C10ORF116	1.48	2.59E-03
ILMN_2370091	NGFRAP1*	1.47	2.74E-03
ILMN_1687751	BAALC*	1.42	1.91E-03
ILMN_1725311	GCGR	1.40	3.96E-03
ILMN_1793615	ME3	1.40	7.88E-03
ILMN_1753370	ABTB2	1.39	5.14E-03
ILMN_1669831	C6ORF192	1.38	7.75E-03
ILMN_1789361	PKD2L1	1.37	2.81E-03
ILMN_1797293	SIGLEC15	1.30	5.93E-03
ILMN_1704753	EPAS1	1.28	9.55E-03
ILMN_2386008	MPZL1*	1.28	6.73E-05
ILMN_1815023	PIM1	1.28	6.07E-03
ILMN_2336130	SULT1A4	1.28	6.56E-03
ILMN_1815283	SULT1A3	1.27	2.77E-03
ILMN_2374036	CTSL1*	1.26	6.02E-03
ILMN_1781536	FAH	1.26	2.16E-03
ILMN_1703946	ADORA2B	1.26	7.34E-03
ILMN_2393149	ALOX15B	1.25	8.17E-03
ILMN_1872457	HS.547277	1.25	4.50E-03
ILMN_1718607	TSPAN4	1.25	2.09E-03
ILMN_1695962	SLC12A9	1.24	4.14E-03
ILMN_1677305	PVR	1.24	5.81E-03
ILMN_1791569	PLXNA1	1.24	1.86E-03
ILMN_1772224	KCNJ5	1.24	6.27E-03
ILMN_1751898	C12ORF4	1.23	3.22E-05
ILMN_1658333	ECM1	1.22	9.05E-03
ILMN_2282641	TBXAS1	1.22	1.81E-03
ILMN_1656521	CGI-96	1.22	9.92E-03
ILMN_1795839	SCCPDH	1.22	4.94E-04
ILMN_1812062	SGPP2	1.22	8.25E-03
ILMN_1652549	DTNA*	1.21	7.27E-04
ILMN_1695763	PDIA5	1.21	8.03E-04
ILMN_1745116	ABHD12	1.21	3.00E-03
ILMN_1651296	LOC143666	1.21	7.20E-03
ILMN_1706598	ACPL2*	1.21	1.62E-03
ILMN_1672605	C7ORF41	1.21	7.00E-03
ILMN_2285817	FAM89A	1.20	7.54E-03
ILMN_2355462	CYFIP1	1.20	3.72E-03

Table 5.2 Over-expressed genes in HkEc stimulated CD macrophages in comparison to HC. Whole genome microarray analysis of gene expression was performed using Illumina Human-WG6 v3.0 arrays, and differentially expressed genes were identified using a threshold fold change of 1.2 and $p<0.01$ (uncorrected for multiple testing). Mean fold changes in CD compared to HC macrophage groups are shown. * indicates genes where multiple probes were identified as differentially expressed, the most significant of which are shown. None of the genes remained statistically significant after correction for multiple testing.

Under-expressed genes (p<0.01, 1.2 Fold change)			
Probe ID	**Gene ID**	**Fold change**	**P-value**
ILMN_1697309	NCF1	-1.85	6.23E-03
ILMN_1693009	FGL2	-1.77	5.44E-03
ILMN_1782704	CD19	-1.59	4.20E-03
ILMN_2109416	NAPSB	-1.56	7.65E-03
ILMN_1741727	QPCT	-1.54	3.04E-03
ILMN_1729801	S100A8	-1.53	3.97E-03
ILMN_1677441	C20ORF123	-1.51	3.81E-03
ILMN_1667081	CCND2*	-1.50	1.60E-03
ILMN_1661646	BANK1	-1.47	5.65E-03
ILMN_1810214	JUND	-1.47	4.49E-03
ILMN_1670134	FADS1	-1.46	4.77E-03
ILMN_1790689	CRISPLD2	-1.42	3.73E-03
ILMN_1738675	PTPN6	-1.42	9.68E-03
ILMN_1680996	ALOX5	-1.41	7.12E-03
ILMN_2325506	BCAS4	-1.41	8.22E-03
ILMN_1661109	ATF3	-1.41	2.92E-03
ILMN_1677038	FLJ21986	-1.40	1.30E-03
ILMN_2338452	SERPINA1	-1.40	3.78E-03
ILMN_1655549	SIGLEC10	-1.40	2.94E-03
ILMN_1710962	TMEM97	-1.37	3.14E-03
ILMN_1727098	PPP1R16B	-1.37	6.94E-03
ILMN_1740875	FPR2	-1.37	3.98E-03
ILMN_1699669	BLR1	-1.35	8.93E-04
ILMN_1800540	CD55	-1.34	1.83E-03
ILMN_1723035	OLR1	-1.34	1.21E-03
ILMN_1671054	HLA-A	-1.34	5.24E-03
ILMN_1756501	ST6GAL1	-1.34	8.26E-04
ILMN_1691930	CBX6	-1.34	1.52E-03
ILMN_1685580	CBLB	-1.33	1.94E-03
ILMN_2217935	RFC1	-1.32	6.21E-03
ILMN_1682312	CYBB	-1.32	6.34E-03
ILMN_1663390	CDC20	-1.31	6.13E-03
ILMN_1683450	CDCA5	-1.31	2.08E-03
ILMN_2345353	LST1*	-1.31	1.06E-03
ILMN_1657395	HMGCR	-1.30	2.67E-03
ILMN_1786065	UHRF1	-1.30	3.75E-03
ILMN_1756928	RTN1	-1.30	5.33E-04
ILMN_2144088	FDFT1	-1.30	1.68E-03
ILMN_1677607	SC5DL	-1.30	5.38E-03
ILMN_2319952	VDR	-1.29	2.95E-03
ILMN_1738058	CPNE6	-1.28	5.97E-03
ILMN_1718766	MT1F	-1.28	1.62E-03
ILMN_2224143	MCM3	-1.27	3.55E-03
ILMN_2078697	ALPK1	-1.27	4.26E-03
ILMN_1686097	TOP2A	-1.27	6.04E-03
ILMN_2072296	CKS2	-1.27	3.69E-03
ILMN_2073604	EBP	-1.26	5.52E-03
ILMN_1809590	GINS2	-1.26	5.44E-03
ILMN_1712452	KIF20B	-1.26	4.27E-04
ILMN_2336595	ACSS2	-1.26	9.38E-03
ILMN_1737205	MCM4	-1.25	1.56E-04
ILMN_1732516	KNTC1	-1.25	1.66E-04
ILMN_2075334	HIST1H4C	-1.25	1.38E-03
ILMN_1793859	ALDH2	-1.24	9.59E-03
ILMN_1773125	ENTPD1	-1.24	7.00E-03
ILMN_1714730	UBE2C	-1.24	5.21E-03
ILMN_2370365	RFC4	-1.24	8.03E-04
ILMN_1722811	CDKN1B	-1.24	8.49E-03
ILMN_1732296	ID3	-1.23	1.62E-03
ILMN_1798654	MCM6	-1.23	6.34E-03
ILMN_1751143	C7ORF23	-1.23	7.04E-03
ILMN_1671554	LPIN1	-1.22	6.14E-03
ILMN_1781942	HMMR	-1.21	5.54E-03
ILMN_1775708	SLC2A3	-1.21	8.87E-03
ILMN_1663068	MGC33556	-1.21	2.99E-03
ILMN_2216582	LYL1	-1.21	2.65E-03
ILMN_1767470	SCPEP1	-1.21	9.82E-03
ILMN_1716895	RPA3	-1.21	1.74E-04
ILMN_1814985	PDLIM7	-1.21	8.77E-03
ILMN_1755974	ALDOC	-1.21	5.31E-03
ILMN_1802160	XIRP1	-1.21	8.30E-03
ILMN_2118472	C10ORF58*	-1.21	6.97E-03
ILMN_1771593	RRM1	-1.20	4.06E-03
ILMN_2366703	SGK3	-1.20	1.13E-03
ILMN_2216918	SHPK	-1.20	8.09E-03
ILMN_1668865	SLC2A14	-1.20	6.86E-03

Table 5.3 Under-expressed genes in CD macrophages after HkEc stimulation. Differentially expressed genes were identified compared to HC using a threshold fold change of 1.2 and p<0.01 (uncorrected for multiple testing). Mean fold changes in CD compared to HC macrophage groups are shown. * indicates genes where multiple probes were identified as differentially expressed, the most significant of which is shown. None of these genes remained statistically significant after correction for multiple testing.

unstimulated macrophages, *NCF1* was found to be under-expressed in CD macrophages after HkEc stimulation. Furthermore, *CYBB*, a gene encoding the cytochrome b-245 β chain of NADPH oxidase (386) was also downregulated in CD macrophages compared to HC. A number of the differentially expressed genes may also function in cytoskeletal control and vesicle trafficking. For example, *LRRC50*, a gene implicated in dynein arm assembly and regulation of actin-based brush border microvilli (387), was over-expressed in CD macrophages. In concordance with previous findings using the Affymetrix platform, no difference in the expression of pro-inflammatory cytokines such as TNF, IL-6, IFN-γ could be observed. However, there was surprisingly little overlap between the genes identified as differentially expressed in the previous Affymetrix dataset. None of the 18 differentially regulated genes identified as common between ileal and colonic CD (136) were significantly altered in this study. As for unstimulated macrophages, none of the genes reached statistical significance after correction for multiple testing. Comparison of CD with UC macrophages after HkEc stimulation revealed four over-expressed and three under-expressed genes (Table 5.4), suggesting minimal differences in expression between these two groups.

The lack of significance after correction for multiple testing may partly reflect the presumed heterogeneity of genetic and environmental factors contributing to CD. Therefore, the grouped microarray expression data were analysed for common Gene Ontology (GO) processes (388), and for the most significant pathway networks using GeneGo software, because these approaches were considered more likely to detect the effects of many heterogeneous risk factors on a small number of processes and pathways.

MetaCore pathway analysis software was used to determine the GO and GeneGo pathway networks significantly enriched within the datasets (taking an uncorrected p-

Over-expressed genes			
Probe ID	**Gene ID**	**Fold change**	**P-value**
ILMN_1790692	GNLY*	2.34	1.76E-03
ILMN_1682993	NKG7	1.63	5.76E-03
ILMN_1805449	TAPBPL	1.28	6.15E-03
ILMN_1737818	C12ORF43	1.26	7.86E-03
Under-expressed genes			
Probe ID	**Gene ID**	**Fold change**	**P-value**
ILMN_1797594	NFAT5	-1.23	9.72E-03
ILMN_1801616	EMP1	-1.24	6.53E-03
ILMN_2218935	GPR37	-1.30	6.53E-03

Table 5.4 Differentially expressed genes in CD compared to UC macrophage groups after HkEc stimulation. A threshold fold change of 1.2 and $p<0.01$ (uncorrected for multiple testing) was used to determine genes that were differentially expressed. Fold change in the CD group compared to UC is shown. * Represents genes where more than one probe was identified as significantly different. None of the genes were significant after multiple testing correction was performed.

value $p<0.01$ and 1.2 fold change as the threshold). Within the unstimulated differentially expressed gene list (comparing CD with HC), the most significantly enriched GO terms within the biological processes category included immune system processes, response to interferon-γ and defence response (Table 5.5A). In comparison, within the HkEc stimulated differential gene list, the most significantly enriched GO processes included small molecule metabolic processes, DNA strand elongation, immune effector processes and processes related to adenylate cyclase G protein signalling pathways (Table 5.5B). Amongst the unstimulated differential gene list, significantly enriched pathway networks included 'antiviral actions of interferons', and various pathways relating to immune response, cell cycle and apoptosis (Table 5.5C). In contrast, differentially expressed genes between HkEc stimulated HC and CD macrophages showed enrichment for the 'AP-1 in regulation of cellular metabolism' pathway, and various signalling pathways involved in the immune response and development (Table 5.5D). Furthermore, cytoskeletal remodelling pathways were significantly enriched amongst this dataset, consistent with the hypothesis that the cytoskeleton could play a role in the dysfunctional macrophage response to HkEc in CD.

5.2.2 Principal component analysis of CD, HC and UC macrophage gene expression

Principal component analysis (PCA) was also conducted to determine whether global macrophage gene expression profiles could be separated on the basis of disease or batch of microarray analysis. PCA is a mathematical algorithm for reducing the dimensionality of datasets, by finding the principal components (directions) along which the variation is maximal. PCA plots of samples, where each data point represents expression values of all the expressed probes in the array, can be used to visually assess similarities and differences between samples and whether they can be grouped (389).

A

GO processes: Unstimulated CD versus HC	P-value	Dataset genes	Total genes
immune system process	1.06E-18	40	1729
response to interferon-gamma	6.70E-17	15	136
interferon-gamma-mediated signaling pathway	1.78E-16	13	88
response to cytokine stimulus	5.03E-16	23	548
defense response	1.26E-15	31	1197
immune response	1.45E-15	29	1028
cytokine-mediated signaling pathway	5.03E-15	18	313
cellular response to interferon-gamma	5.87E-15	13	114
cellular response to cytokine stimulus	1.42E-13	18	380
response to stress	4.94E-13	45	3204

B

GO processes: HkEc stimulated CD versus HC	P-value	Dataset genes	Total genes
small molecule metabolic process	4.67E-11	47	2570
DNA strand elongation involved in DNA replication	2.94E-09	7	35
DNA strand elongation	4.47E-09	7	37
regulation of immune effector process	6.76E-09	14	282
DNA-dependent DNA replication	8.34E-09	9	89
alcohol metabolic process	2.51E-08	19	608
second-messenger-mediated signaling	2.64E-08	16	424
activation of adenylate cyclase activity by G-protein signaling pathway	3.78E-08	8	75
positive regulation of adenylate cyclase activity by G-protein signaling pathway	3.78E-08	8	75
regulation of adenylate cyclase activity involved in G-protein signaling pathway	3.78E-08	8	75

C

GeneGO Pathway Networks: Unstimulated CD versus HC	P-value	Dataset genes	Total genes
Immune response_Antiviral actions of Interferons	6.95E-07	5	52
Immune response_MIF-mediated glucocorticoid regulation	2.43E-03	2	22
Development_Thrombopoetin signaling via JAK-STAT pathway	2.43E-03	2	22
Immune response_Antigen presentation by MHC class I	3.92E-03	2	28
Cell cycle_Role of APC in cell cycle regulation	5.10E-03	2	32
Cell cycle_Spindle assembly and chromosome separation	5.42E-03	2	33
Immune response_Role of integrins in NK cells cytotoxicity	7.14E-03	2	38
Immune response_Th1 and Th2 cell differentiation	7.89E-03	2	40
Apoptosis and survival_Anti-apoptotic TNFs/NF-kB/Bcl-2 pathway	8.28E-03	2	41
Apoptosis and survival_Lymphotoxin-beta receptor signaling	8.68E-03	2	42

D

GeneGO Pathway Networks: HkEc stimulated CD versus HC	P-value	Dataset genes	Total genes
Transcription_Role of AP-1 in regulation of cellular metabolism	1.43E-07	13	38
Cell cycle_ESR1 regulation of G1/S transition	2.01E-07	12	33
Immune response_Human NKG2D signaling	1.16E-06	12	38
Development_Thrombopoietin-regulated cell processes	1.31E-06	13	45
Development_HGF signaling pathway	2.25E-06	13	47
Immune response_IL-2 activation and signaling pathway	3.77E-06	13	49
Cytoskeleton remodeling_TGF, WNT and cytoskeletal remodeling	7.42E-06	20	111
Cytoskeleton remodeling_Cytoskeleton remodeling	7.59E-06	19	102
Development_HGF-dependent inhibition of TGF-beta-induced EMT	1.03E-05	10	32

Table 5.5 Functional annotation of differentially expressed genes between CD and HC macrophage groups. Analysis was conducted using MetaCore Pathway analysis software. The most significantly enriched Gene Ontology (GO) processes amongst **(A)** unstimulated and **(B)** HkEc stimulated differential gene lists are shown. The most highly significantly enriched GeneGo pathway networks in the **(C)** unstimulated and **(D)** HkEc stimulated differential gene lists are also shown. The enrichment p-values and number of genes in the dataset (dataset genes) in comparison to the total GO database genes (total genes) are displayed in all tables.

Considering the unstimulated macrophage dataset, principal components 1 and 2 accounted for 25.8% and 10.2% of the data variability respectively. A further 5.7% of the variance was explained by principal component 3. Unstimulated samples from batches 1, 2 and 3 could not be separated by PCA (Figure 5.1A), suggesting that after the normalisation procedure, gross batch effects were not present. Unstimulated macrophage gene expression profiles from CD and UC patients and HC individuals could not be distinguished by disease group using PCA analysis (Figure 5.1B).

Similar results were obtained for HkEc stimulated samples. As expected, PCA was able to separate unstimulated samples from HkEc stimulated samples from all donors (Figure 5.2A), consistent with clear transcriptomic differences that occur on macrophage activation. However, PCA analysis of HkEc stimulated samples was not able to separate samples on the basis of disease grouping (Figure 5.2B). Therefore CD macrophages do not appear to have a distinct global expression signature or gross transcriptomic abnormality, when compared to either HC individuals or to patients with UC.

5.2.3 Identification of outlier probes in CD, HC and UC subjects and their biological function

Given the heterogeneity of CD, and the lack of striking differentially expressed genes between the HC, CD and UC groups, a customised outlier analysis was subsequently used to detect gross abnormalities in gene expression (outliers) in individual subjects, in comparison to the HC cohort. Probes were identified that were significantly over- or under- expressed in unstimulated macrophages from individual CD and UC patients compared to the HC cohort (see Materials and Methods section 2.5.3 for full details). Probes that reached a threshold p-value of $p<0.005$ (calculated using Z-scores, a Z score of ~2.5 corresponding to a $p<0.005$) and a minimum fold change of 1.5 compared to

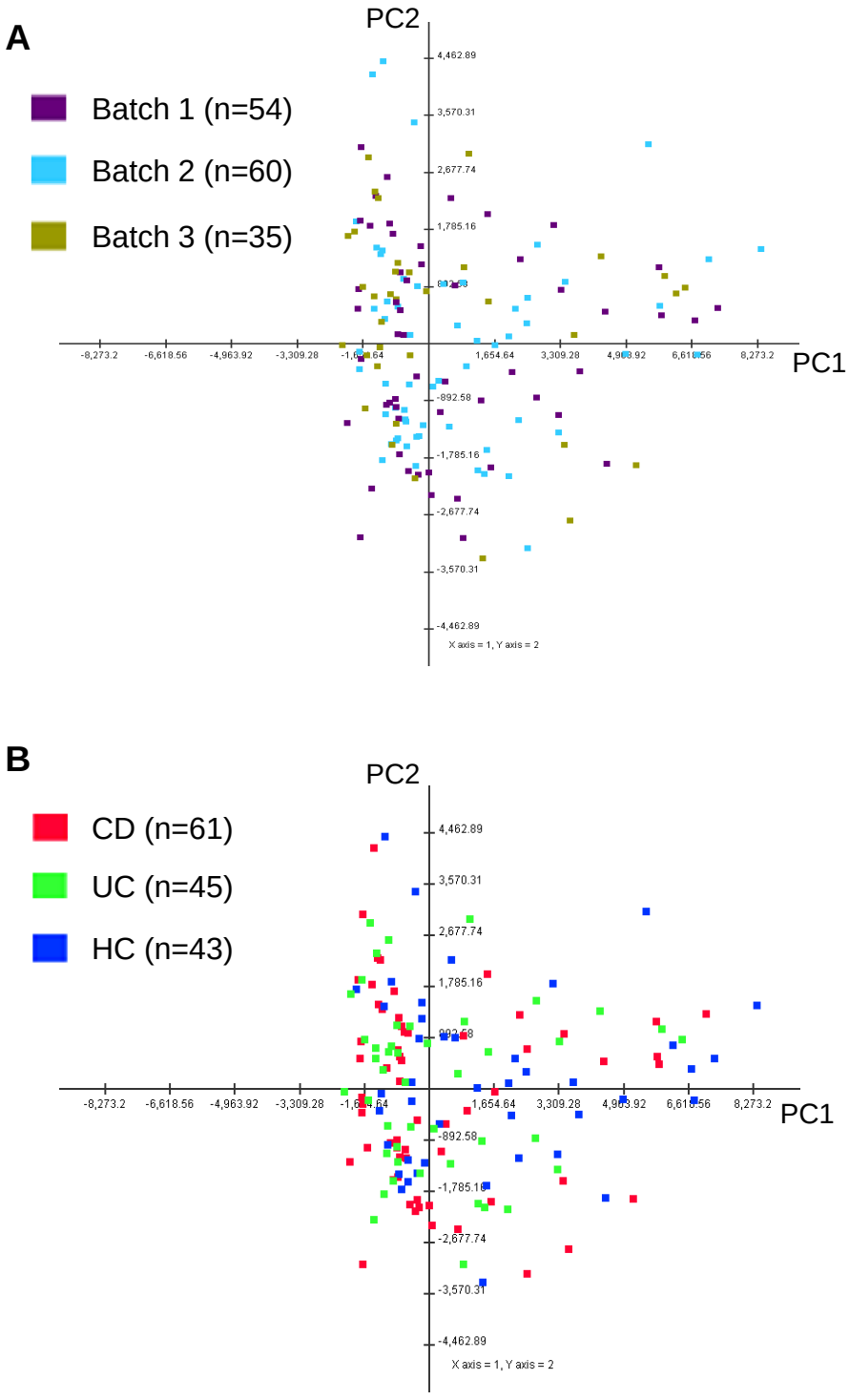


Figure 5.1 Principal component analysis of unstimulated macrophage gene expression profiles from CD, UC and HC patients. Array expression data were $\log_2$ transformed and PCA analysis was performed using MultiExperiment viewer TM4 Microarray software suite. Scatterplots for principal components (PC) 1 and 2 are shown, with the microarray results for each sample represented as a single point. **(A)** Array samples colour coded by batch. **(B)** Samples colour coded by disease grouping.

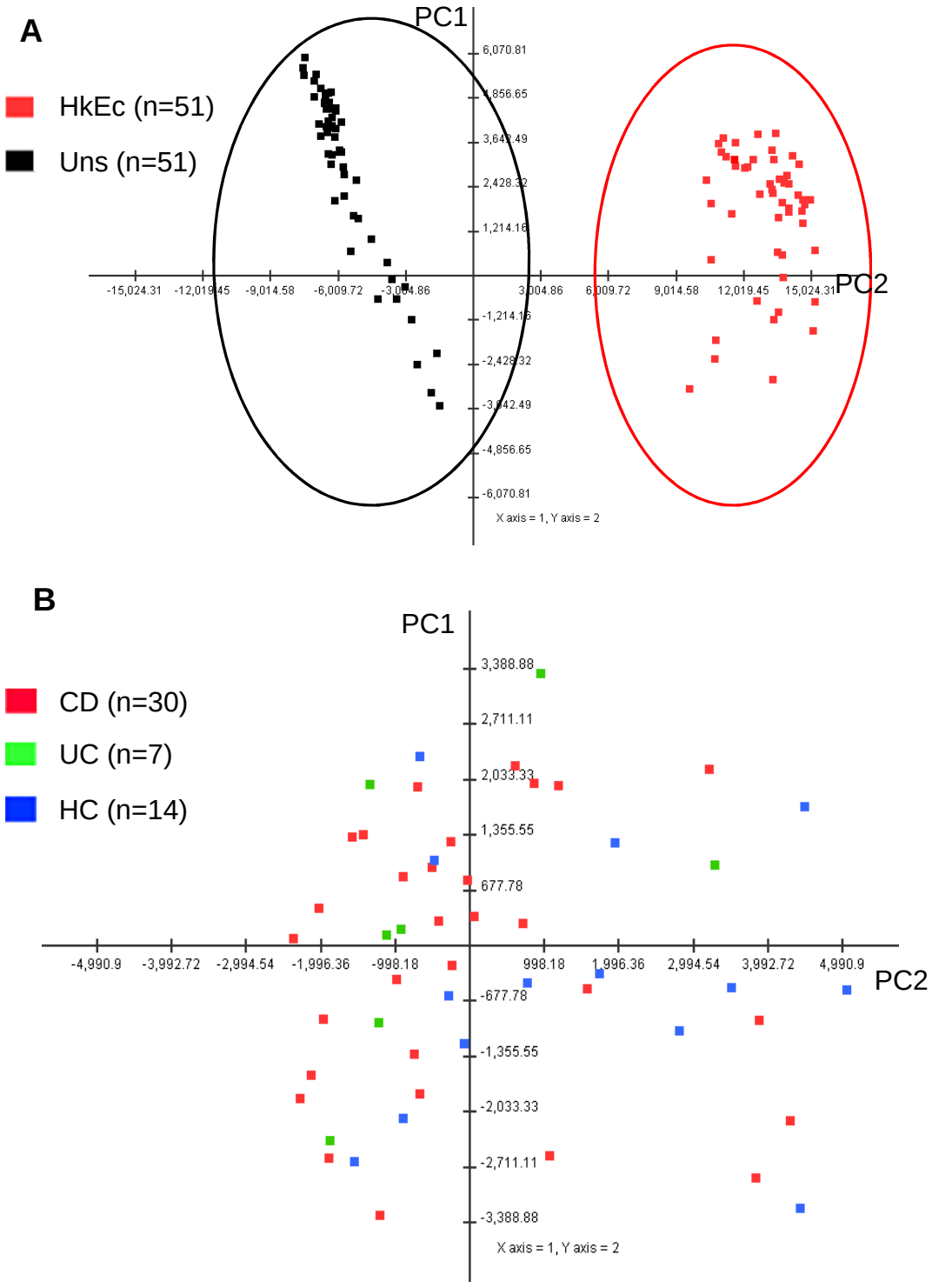


Figure 5.2 Principal component analysis of unstimulated and HkEc stimulated macrophages. Microarray expression data were $\log_2$ transformed and PCA analysis performed using MultiExperiment viewer TM4 software suite. Scatterplots for principal components (PC) 1 and 2 are shown, with the microarray results for each sample represented as a single point. (**A)** Principal component analysis of paired unstimulated and HkEc stimulated samples from CD and UC and HC macrophages, showing separation into HkEc (red) and unstimulated (black) groups. **(B)** Principal component analysis of HkEc stimulated macrophages from CD, HC and UC donors, colour coded by disease grouping.

mean expression in the HC cohort were considered to be significant outliers in an individual subject. Nineteen probes with expression that was significantly different between male and female individuals at FDR-corrected $p<0.001$ were excluded from the outlier analysis (Table 5.6).

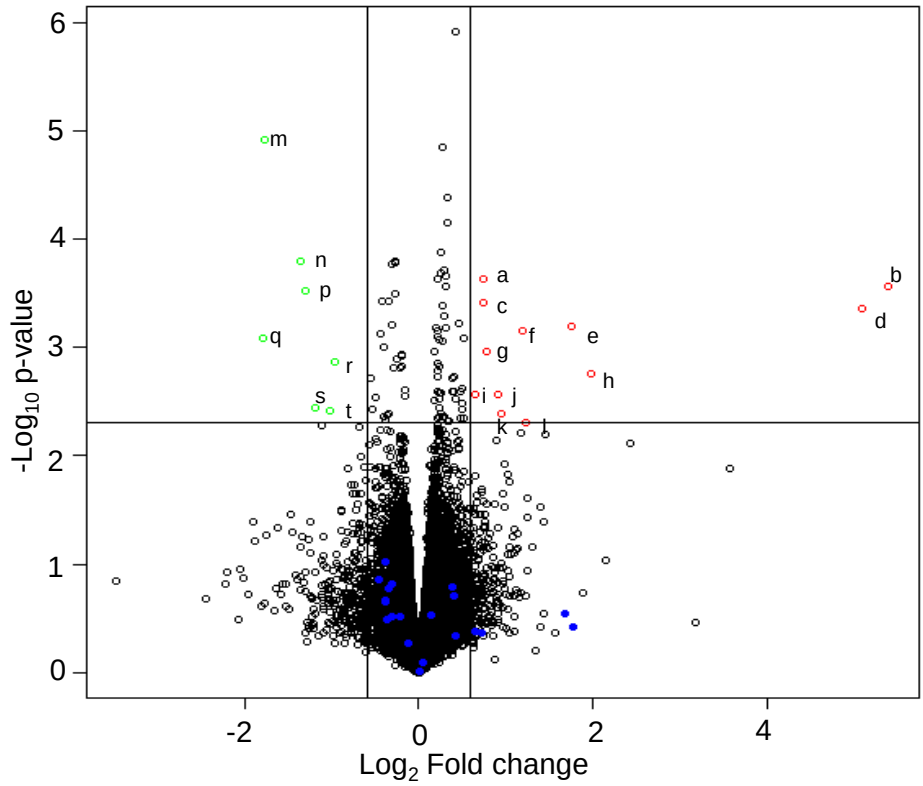
Customised software for outlier analysis was developed in collaboration with Daniel Roden and Anna Lobley, Department of Computer Sciences, UCL. This software permitted identification of outlier probes in individual patients and their visualisation by volcano plots (Figure 5.3A). The software also generated tables detailing the outlying probes in each individual patient (Figure 5.3B), and in the overall cohort. Outlier probes in HC subjects were also determined, by comparing expression of all probes in each individual subject with the remainder of the HC population.

The median number of outlier probes per individual was 27 (interquartile range 16-45) in the CD group, compared to 22 (interquartile range 10-76) and 25 (interquartile range 13-38) per individual in the HC and UC cohorts respectively (Figure 5.4A). None of these differences were statistically significant. Of the total 1,858 outlier probes identified in the CD cohort, 712 were under-expressed and 1,146 were over-expressed in one or more individual CD patients compared to mean HC expression. 20.7% of the under-expressed and 41.5% of the over-expressed outlier probes identified in the CD population were also abnormally expressed in one or more HC individuals. A further 18.0% of the under-expressed and 32.1% of the over-expressed outliers in the CD cohort were also identified as abnormal in one or more UC patients, leaving 478 over-expressed and 585 under-expressed outlier probes that were unique to the CD cohort (Figure 5.4B, C).

Probe ID	Gene ID	Chromosome	Raw fold change	Log_2 Fold change	Uncorrected P-value
ILMN_1783142	RPS4Y1	Yp11.3	15.45	3.9499	9.59E-71
ILMN_1670821	CYORF15A	Yq11.2	3.30	1.7224	2.14E-68
ILMN_1755537	EIF1AY	Yq11.2	7.89	2.9804	4.83E-67
ILMN_1685690	JARID1D	Yq11.2	2.92	1.5461	2.17E-63
ILMN_2228976	EIF1AY	Yq11.2	2.15	1.1055	1.26E-56
ILMN_2090059	ZFY	Yp11.3	1.49	0.5705	6.01E-42
ILMN_1764573	XIST	Xq13.2	-1.56	-0.6446	1.10E-34
ILMN_1710136	HDHD1A	Xp22.3	-1.33	-0.413	8.06E-34
ILMN_1776195	TMSB4Y	Yq11.2	1.34	0.4245	1.37E-33
ILMN_1810577	RPS4X	Xq13.1	-1.46	-0.5505	5.77E-31
ILMN_2166831	RPS4X	Xq13.1	-1.47	-0.5522	2.45E-28
ILMN_1687484	ZFX	Xp22.1	-1.21	-0.2799	9.72E-24
ILMN_1654488	UTX	Xp11.3	-1.29	-0.3689	1.05E-23
ILMN_1756506	CYORF15B	Yq11.2	1.19	0.2481	6.34E-19
ILMN_1873540	HS.412918	Yq11.2	1.19	0.2469	1.34E-18
ILMN_1786834	PRKX	Xp22.3	-1.33	-0.412	3.25E-16
ILMN_1794392	DDX3X	Xp11.4	-1.21	-0.2705	5.66E-15
ILMN_2205050	PRKX	Xp22.3	-1.31	-0.3889	1.18E-14
ILMN_1684873	ARSD	Xp22.3	-1.62	-0.6935	2.83E-10

Table 5.6 Differentially expressed probes between male and female individuals. Probes that were differentially expressed between male and female individuals (FDR $p<0.001$) were excluded from subsequent outlier analysis. Illumina probe identities (ID), corresponding gene identities, raw fold change (in male group compared to female group), log_2 transformed fold change and uncorrected p-values are shown. All probes were significant after FDR correction at $p<0.001$

A



B

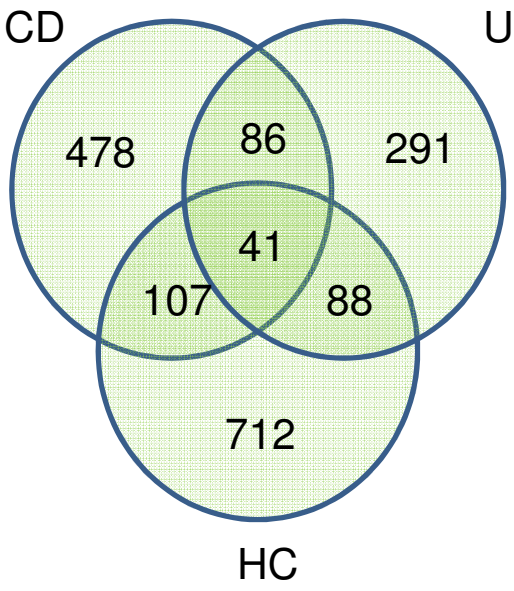
Over-expressed probes						
Probe ID	**Gene ID**	**Raw fold change**	**Log_2 Fold change**	**P-value**	**$-Log_{10}$ p-value**	**Volcano plot identity**
ILMN_2137625	LOC150763	1.67	0.74	2.34E-04	3.63	a
ILMN_2169801	TPSAB1	42.22	5.4	2.75E-04	3.56	b
ILMN_1660599	LOC653186	1.67	0.74	3.80E-04	3.42	c
ILMN_1676256	TPSAB1	33.82	5.08	4.37E-04	3.36	d
ILMN_1668134	GSTM1	3.39	1.76	6.31E-04	3.2	e
ILMN_1761312	CRHBP	2.3	1.2	7.08E-04	3.15	f
ILMN_1756469	GAMT	1.72	0.78	1.07E-03	2.97	g
ILMN_1762255	GSTM1	3.94	1.98	1.74E-03	2.76	h
ILMN_2346997	RAB23	1.89	0.92	2.69E-03	2.57	i
ILMN_2150851	SERPINB2	1.57	0.65	2.75E-03	2.56	j
ILMN_1668039	GYPC	1.93	0.95	4.17E-03	2.38	k
ILMN_1764754	RAMP1	2.35	1.23	4.90E-03	2.31	l
Under-expressed probes						
Probe ID	**Gene ID**	**Fold change**	**Log_2 Fold change**	**P-value**	**$-Log_{10}$ p-value**	**Volcano plot identity**
ILMN_1761450	DHRS4L2	-3.41	-1.77	1.23E-05	4.91	m
ILMN_1762666	DHRS4	-2.57	-1.36	1.58E-04	3.8	n
ILMN_2381899	OPTN	-2.46	-1.3	3.02E-04	3.52	p
ILMN_1660923	HCG4	-3.46	-1.79	8.32E-04	3.08	q
ILMN_1717477	PSD3	-1.93	-0.95	1.38E-03	2.86	r
ILMN_2262288	EEF1G	-2.27	-1.18	3.63E-03	2.44	s
ILMN_1676014	LOC728635	-2.03	-1.02	3.89E-03	2.41	t

Figure 5.3 Macrophage gene expression outliers in a representative CD patient (CD1). Gene expression outliers in unstimulated macrophages were identified by calculation of Z-scores and associated p-values for all expressed probes in each individual CD patient (n=63), relative to mean expression in the HC cohort (n=43). Probes that reached a minimum threshold of $p<0.005$ and 1.5 fold change were considered as outliers. **(A)** Volcano plot showing fold change of all expressed probes (on a $\log_2$ scale) in one CD patient (CD1) compared to mean HC expression, and corresponding p-values (on a $-\log_{10}$ scale). Under-expressed probes identified as outliers are shown in green and over-expressed probes in red. Probes with expression that was significantly different between male and female individuals were excluded from the analysis (blue). **(B)** Identities (ID) of outlier probes and corresponding genes in this individual, showing raw and log transformed fold changes and p-values, compared to expression in the HC cohort.

A

	Number of outlier probes per individual (median)	Interquartile range
CD (n=61)	27	16-45
UC (n=45)	25	13-38
HC (n=43)	22	10-76

B



C

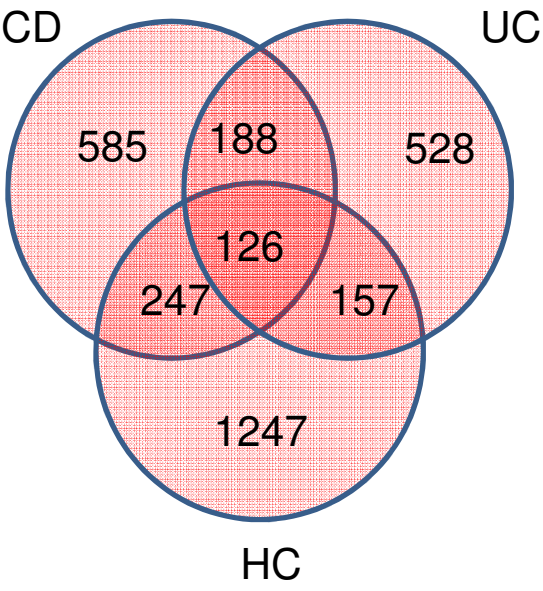


Figure 5.4 Numbers of outlier probes in unstimulated macrophages from CD, UC and HC individuals. **(A)** Median number of outlier probes per individual in CD, UC and HC patient macrophages with interquartile range. **(B)** Venn diagram showing the total number of under-expressed outlier probes in the CD, UC and HC cohorts, and number of overlapping outliers between the groups. **(C)** Corresponding results showing total number of over-expressed outliers detected in the CD, UC and HC cohorts.

MetaCore pathway analysis software was used to determine GO processes that were enriched within the total CD, UC and HC outlier probe list. The most highly significantly enriched GO processes within the CD outlier probes included cellular and metabolic processes, response to stress, response to wounding, immune system processes, cell death and establishment of localisation (Table 5.7A). The GO processes significantly enriched in the HC outlier probes were similar to CD (Table 5.7B), suggesting that overall, outliers in HC and CD macrophages have similar functional ontologies. Notably establishment of localisation and transport were amongst the top 20 most significant GO processes in the CD outliers but not in the HC group.

5.2.4 Overlap between macrophage gene expression outliers and GWAS susceptibility loci

To date, GWAS have identified 71 genetic polymorphisms associated with CD (115;116). In many cases the identified polymorphism may not represent the true pathogenic variant, and is likely to exist in linkage disequilibrium with other SNPs, giving rise to a 'susceptibility locus' containing several candidate genes. The unstimulated outlier gene list obtained in this study was screened to identify individual CD and UC patients with abnormal expression of genes linked to IBD by GWAS (115;116;390). Using the gene expression outlier analysis strategy, ten genes located in susceptibility loci were found to be abnormally expressed in our CD cohort, of which urotensin 2 (*UTS2*), caspase recruitment domain family, member 9 (*CARD9*) and chemokine (C-C motif) ligand 7 (*CCL7*) were identified as outliers in macrophages from two or more CD patients (Figure 5.5A, B). These may represent important targets for future investigation, and in particular, sequencing of the relevant genomic interval in these individuals could be conducted to search for rare variants or novel mutations.

A

GO processes: CD outliers	P-value	Dataset genes	Total genes
Cellular process	2.03E-26	1280	14811
Metabolic process	2.20E-23	931	10001
Cellular metabolic process	3.45E-22	805	8403
Small molecule metabolic process	1.58E-21	317	2570
Response to stress	2.57E-21	374	3204
Primary metabolic process	4.56E-20	797	8428
Response to wounding	2.95E-16	173	1251
Positive regulation of molecular function	6.80E-16	178	1312
Positive regulation of catalytic activity	1.43E-15	158	1123
Regulation of catalytic activity	2.90E-15	238	1959
Cell death	2.91E-15	158	1132
Positive regulation of biological process	4.52E-15	384	3611
Death	5.02E-15	158	1139
Regulation of molecular function	5.03E-15	271	2328
Immune system process	7.19E-15	215	1729
Negative regulation of biological process	7.22E-15	344	3158
Transport	2.86E-14	379	3596
Establishment of localization	4.16E-14	383	3652
Negative regulation of cellular process	4.98E-14	317	2891
Regulation of biological quality	5.49E-14	319	2916

B

GO processes: HC outliers	P-value	Dataset genes	Total genes
Response to stress	8.59E-40	576	3204
Immune system process	9.66E-39	365	1729
Positive regulation of biological process	6.58E-31	599	3611
Regulation of programmed cell death	1.28E-29	296	1430
Regulation of apoptosis	6.35E-29	292	1416
Regulation of cell death	1.01E-28	299	1467
Response to wounding	1.76E-28	266	1251
Response to organic substance	1.36E-27	398	2187
Immune response	1.88E-27	229	1028
Regulation of immune system process	3.34E-27	229	1032
Positive regulation of cellular process	5.23E-27	539	3261
Response to stimulus	1.92E-26	1130	8211
Cellular process	3.63E-26	1828	14811
Defense response	6.15E-26	251	1197
Multi-organism process	6.96E-24	274	1392
Small molecule metabolic process	1.01E-23	436	2570
Negative regulation of biological process	1.51E-23	512	3158
Metabolic process	2.23E-23	1313	10001
Cellular response to chemical stimulus	3.41E-22	288	1526
Negative regulation of cellular process	3.47E-22	472	2891

Table 5.7 Functional analysis of CD and HC gene expression outliers by gene ontology (GO) annotation. Analysis of functional ontology of all outlier probes detected in unstimulated CD and HC macrophages was performed using MetaCore Pathway analysis software. The 20 most significantly enriched GO processes within **(A)** the CD outlier dataset and **(B)** the HC outlier datasets are shown, with associated p-values. The number of genes in each GO process present in the dataset (dataset genes) in comparison to the total GO database (total genes) are shown.

A

dbSNP ID	Chromosome	Genes of Interest	Number of outlier individuals (p<0.005)					
			CD (61)		UC (45)		HC (43)	
			Down	Up	Down	Up	Down	Up
rs35675666	1p36	TNFRSF9, ERFFI1, **UTS2**		5		2		2
rs4077515	9q34	**CARD9**	2		2			
rs3091315	17q12	CCL2, **CCL7**		2				
rs1456896	7p12	**IKZF1**	1	1			2	
rs102275	11q12	**FADS1**	1					
rs694739	11q13	**PRDX5**, ESRRA	1				1	
rs11742570	5p13	**PTGER4**	1					
rs740495	19p13	**GPX4**, SBNO2	1					
rs3180018	1q22	SCAMP3, **MUC1**		1				1
rs4656940	1q23	**ITLN1**		1			1	

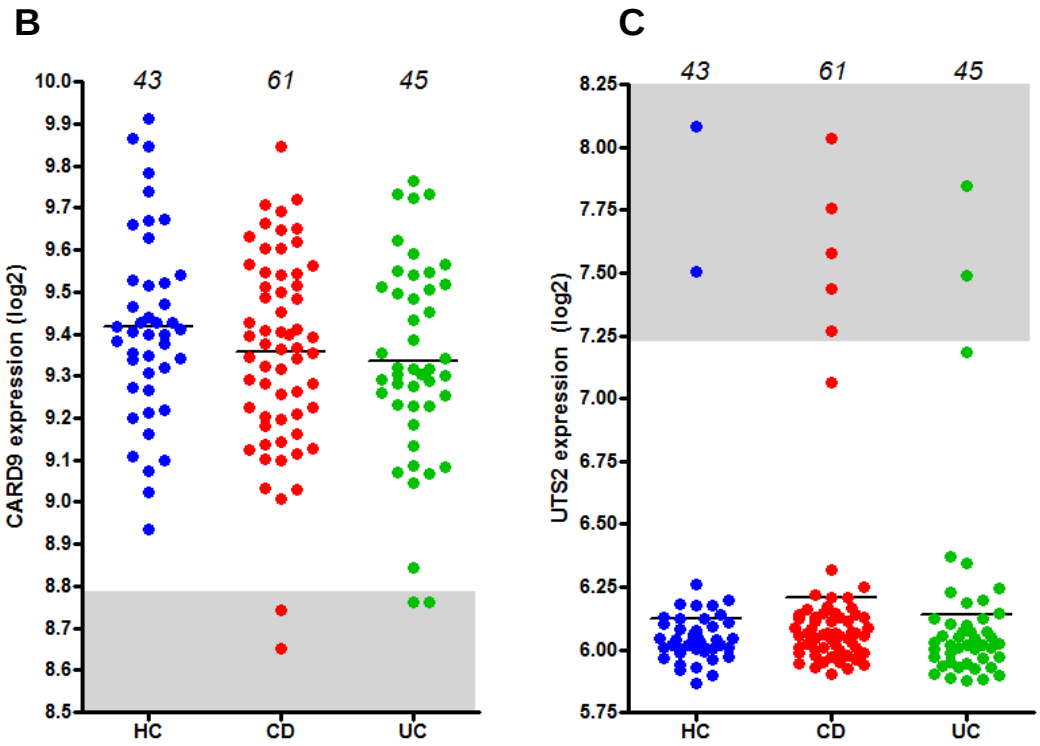


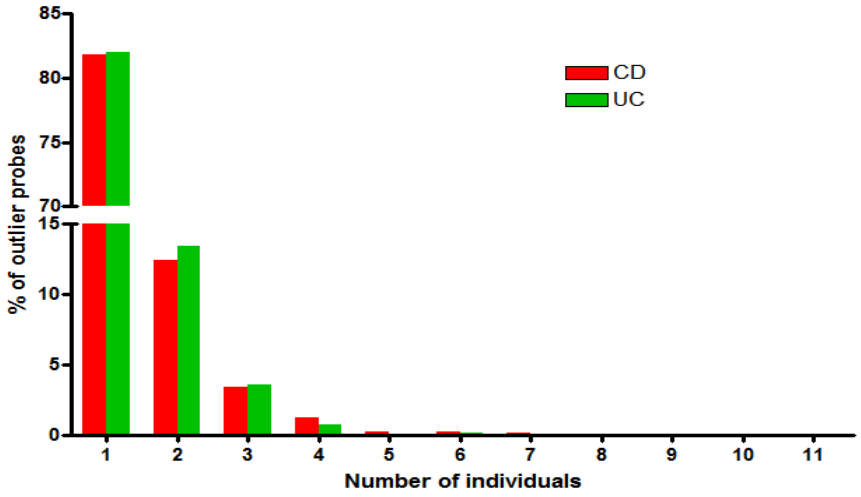
Figure 5.5 Abnormal macrophage expression of genes located in CD GWAS susceptibility loci in CD, UC and HC individuals. **(A)** SNPs associated with CD by GWAS, with their associated chromosomal location and genes located in the region of interest. The number of outlier individuals identified in the CD, UC and HC cohorts are shown. Genes that were identified as outliers are highlighted in bold. **(B)** Dot plot showing under-expression of *CARD9* in two patients with CD and two patients with UC (shaded area). **(C)** Dot plot showing over-expression of *UTS2* in five CD patients, two UC patients and two HC individuals (shaded area). Two additional patients with apparent over-expression marginally fall outside the significance threshold. Dot plots are shown on a logarithmic (to base 2) scale. All data shown are for unstimulated macrophages.

5.2.5 Outlier probes common between CD patients

The majority of abnormally expressed probes were specific to individual CD patients, with only 19.8% of under- and 25.5% of over-expressed probes detected as outliers in two or more subjects. The most frequent under-expressed outlier was present in seven CD patients, and the most frequently detected over-expressed outlier probe was found in 11 individuals (Figure 5.6A). Probes that were identified as abnormal in five or more CD patients are shown with the corresponding frequency with the HC and UC cohorts (Figure 5.6B). All of these outlier probes were detected as outliers in two or fewer HC individuals; the frequency was more variable in the UC cohort. A number of probes were not identified as outliers in any of the UC patients tested, such as ILMN_2103107 and ILMN_2105441, which correspond to ADAM-like, decysin 1 (*ADAMDEC1*) and immunoglobulin J polypeptide (*IGJ*) respectively, whereas others (such as ILMN_1664641, mediator subunit complex 4, *MED4*) were abnormally expressed in over 10% of UC patients. Interestingly, several of the frequently identified gene expression outliers have functional roles in apoptosis, which is impaired in CD macrophages in response to phorbol ester stimulation (205).

The most commonly under-expressed outlier probe in the CD cohort was ILMN_2381899, corresponding to the optineurin (*OPTN)* gene. Optineurin was under-expressed in seven CD patients (11.5% of the CD cohort investigated) and one individual with UC (2.2% of UC patients) (Figures 5.6B and Figure 5.7). No HC individuals were identified with deficient expression in this study. The optineurin predicted protein consists of 577 amino acids and has some predicted structural homology to NF-κB essential modulator (NEMO), a regulator of NF-κB activation (391). Recent functional studies have identified roles for optineurin in vesicle trafficking and maintenance of the Golgi apparatus (392), as well as in autophagy and

A

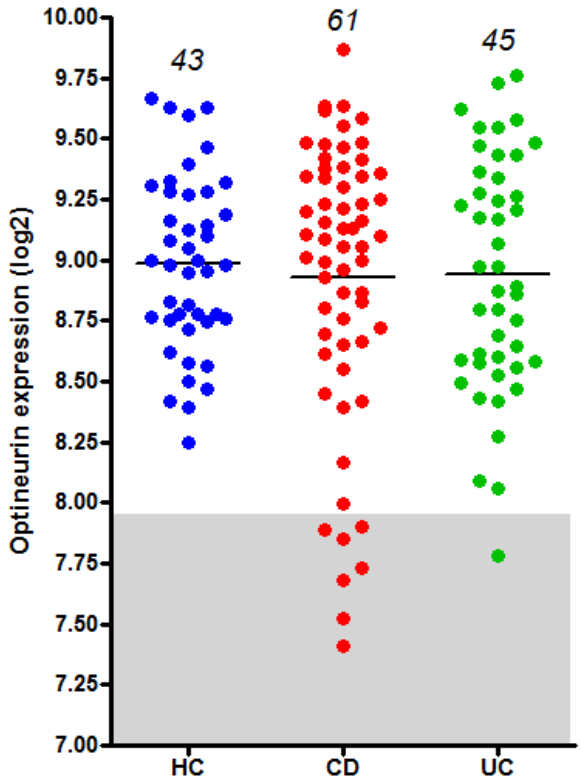


B

Under-expressed outlier probes (p<0.005, -1.5 fold change)						
		Number of Individuals				
Probe ID	Gene ID	CD (61)	UC (45)	HC (43)	Role/Function	Illustrative reference
ILMN_2381899	OPTN	7	1	0	Vesicle trafficking, autophagy	Wild P et al. 2011; *Science* 333(6039):228-33
ILMN_1772459	RPS23	7	4	1	Protein synthesis	Vladimirov SN et al. 1996; *Eur J Biochem.*239(1):144-9
ILMN_2103107	ADAMDEC1	6	0	1	Disintegrin and metalloproteinase	Bates EE et al. 2002; *Immunogenetics* 54(2):96-105.
Over-expressed outlier probes (p<0.005, 1.5 fold change)						
		Number of Individuals				
Probe ID	Gene ID	CD (61)	UC (45)	HC (43)	Role/Function	Illustrative reference
ILMN_2409384	SIGLEC7	11	1	1	Inhibitor of cell signaling	Avril T et al. 2004; *J Immunol* 173(11):6841-9.
ILMN_1710124	CMTM8	6	1	0	Apoptosis, EGFR signaling	Li D et al. 2007. *Int J Biochem Cell Biol* 39(11):2107-19.
ILMN_1875123	HS.572219	6	2	1	*Unknown*	
ILMN_1905324	HS.145134	6	4	2	*Unknown*	
ILMN_1662038	LARGE	5	3	0	Glycosyltransferase	Peyrard M et al. 1999.; *PNAS* 96(2):598-603
ILMN_1664641	MED4	5	5	1	Gene transcription	
ILMN_1671818	UTS2	5	2	2	Vasoconstriction, apoptosis	Ames R et al. 1999; *Nature* 401(6750):282-6.
ILMN_1732198	UTS2	5	3	2	Vasoconstriction, apoptosis	Ames R et al. 1999; *Nature* 401(6750):282-6.
ILMN_1691860	SPRY1	5	2	0	MAP kinase activator, Intracellular signaling	Lee JS et al. 2009; *J Immunol* 183(11):7178-86.
ILMN_1695290	FERMT2	5	2	0	Integrin signaling and Activation	Lai-Cheong JE et al. 2010; *Int J Biochem Cell Biol.* ;42(5):595-603.
ILMN_1707717	BAALC	5	3	0	*Unknown*	.
ILMN_1808566	TMEM180	5	0	0	*Unknown*	
ILMN_1760412	SHISA2	5	4	2	Development, cell signaling	Nagano T et al. 2006. *Development.* 133(23):4643-54.
ILMN_2105441	IGJ	5	0	0	Immunoglobulin linker molecule	Koshland ME. et al 1985; *Annu Rev Immunol* 3:425-53

Figure 5.6 Outlier probes common between CD patients. **(A)** Percentage of outlier probes detected in 1, 2 or more individuals, for the CD and UC cohorts. The majority of outlier probes were private to one individual only. **(B)** Under- and over-expressed outlier probes detected in 5 or more CD patients, showing corresponding number of CD, UC and HC patients detected as outliers, and functional role of the identified genes.

A



B

Probe ID	Gene ID	Chromosome	Fold change	P-value
ILMN_2381899*	OPTN	10p13	-2.42	1.69E-12*
ILMN_1781656	SCGB1D1	11q12	1.16	6.46E-05
ILMN_1731134	LOC730686	2q34	1.10	1.19E-04
ILMN_1773427	KANK1	9p24	1.53	1.37E-04
ILMN_1716869	GPM6A	4q34	1.10	1.72E-04
ILMN_1682399	CLOCK	4q12	1.25	2.00E-04
ILMN_1829389	HS.540247	15q22	1.09	2.01E-04
ILMN_1706511	TEF	22q13	1.13	2.13E-04
ILMN_1757370	SMPD1	11p15	1.20	2.19E-04
ILMN_2364357	RPS6KB2	11q13	1.21	2.35E-04

Figure 5.7 Abnormal optineurin (ILMN_2381899) expression in unstimulated CD macrophages. Microarray expression data were interrogated to identify gene expression abnormalities in individual CD and UC patients, using a threshold p-value of $p<0.005$ and a minimum fold change of 1.5 compared to HC. **(A)** $\log_2$-transformed expression data for optineurin, the most commonly underexpressed gene identified in the outlier analysis. Seven CD patients and one UC patient were identified with deficient optineurin expression (shaded area). Numbers in each group are shown in italics. **(B)** Differentially expressed probes between CD optineurin outlier patients (n=7) and HC (n=43), using classical group comparison of differential gene expression (uncorrected for multiple testing). No probes were significant after correction for multiple testing, with the exception of ILMN_2381899, OPTN (*).

clearance of intracellular bacteria (393). Abnormal optineurin expression is therefore a strong candidate molecular defect in a subset of CD macrophages, which could influence pro-inflammatory cytokine release via effects on vesicle trafficking. Genetic variation and mutations in the *OPTN* gene have previously been associated with primary open angle glaucoma (394), amyotrophic lateral sclerosis (ALS) (395) and Paget's disease of bone (396), however no studies have linked abnormalities in the *OPTN* gene to CD.

It is recognised that the presence of different SNPs between Illumina array probes and target transcripts can lead to mismatching and defective hybridisation, giving rise to 'artifactual' differences in gene expression (397). Therefore, before selecting candidates for further validation and functional study, a publically accessible database (397) of Illumina probe annotations was searched to determine whether any of the down- or up-regulated outlier probes common to five or more patients contained recognised variants. Only ILMN_1772459 (corresponding to RPS23) and ILMN_2409384 (corresponding to SIGLEC7) contained recognised SNPs within the probe sequence (rs3738 and rs273662 respectively). None of the other outlier probes common to five or more patients contained recognised SNPs, including ILMN_2381899, corresponding to optineurin. Sequencing of the *OPTN* probe region (50 nucleotides) in the seven CD outlier patients furthermore revealed perfect alignment with ILMN_2381899, indicating that the abnormal expression of optineurin is not due to defective probe hybridisation.

Transcriptomic abnormalities that occur concurrently with optineurin deficiency could be important mechanistically, for example by indicating contiguous gene deletions or duplications, defects in a common transcription factor, or secondary effects. Therefore, gene lists generated by the customised software were searched for the

presence of common outliers between individuals, and classical comparison of group differential gene expression was performed between the optineurin outlier CD patients (n=7) and HC individuals (n=43). No other outlier probes were common to three or more optineurin outlier individuals. Furthermore, classical group comparison of optineurin outlier patients with HC revealed no differentially expressed probes between the two groups after FDR correction (with the exception of ILMN_2381899, *OPTN*, as expected), suggesting the absence of other common gross transcriptomic abnormalities in these patients. The ten most significantly different probes (before multiple testing correction) are shown (Figure 5.7B).

5.2.6 Identification of optineurin gene expression outlier patients in other array datasets

The HkEc stimulated macrophage gene expression dataset obtained using the Illumina BeadChip array was subsequently interrogated for the presence of optineurin outlier patients. Using the same thresholds as previously, one CD patient was discovered to be a gene expression outlier for optineurin (Figure 5.8A). This patient was previously identified as having abnormal expression of optineurin in unstimulated macrophages.

Furthermore, the previously published Affymetrix gene expression dataset (136) was also investigated to identify additional CD patients with abnormal optineurin expression. UC patients were not included in this analysis due to batch differences in the level of expression between datasets that could not be resolved using conventional normalisation methods. One patient was identified with reduced expression of optineurin in unstimulated macrophages compared to HC (fold change compared to HC -3.24, p=0.009) (Figure 5.8B). This patient was not included in the Illumina study cohort. No outliers were identified in the HkEc stimulated dataset using the Affymetrix platform, although data were not available from the patient with abnormal expression

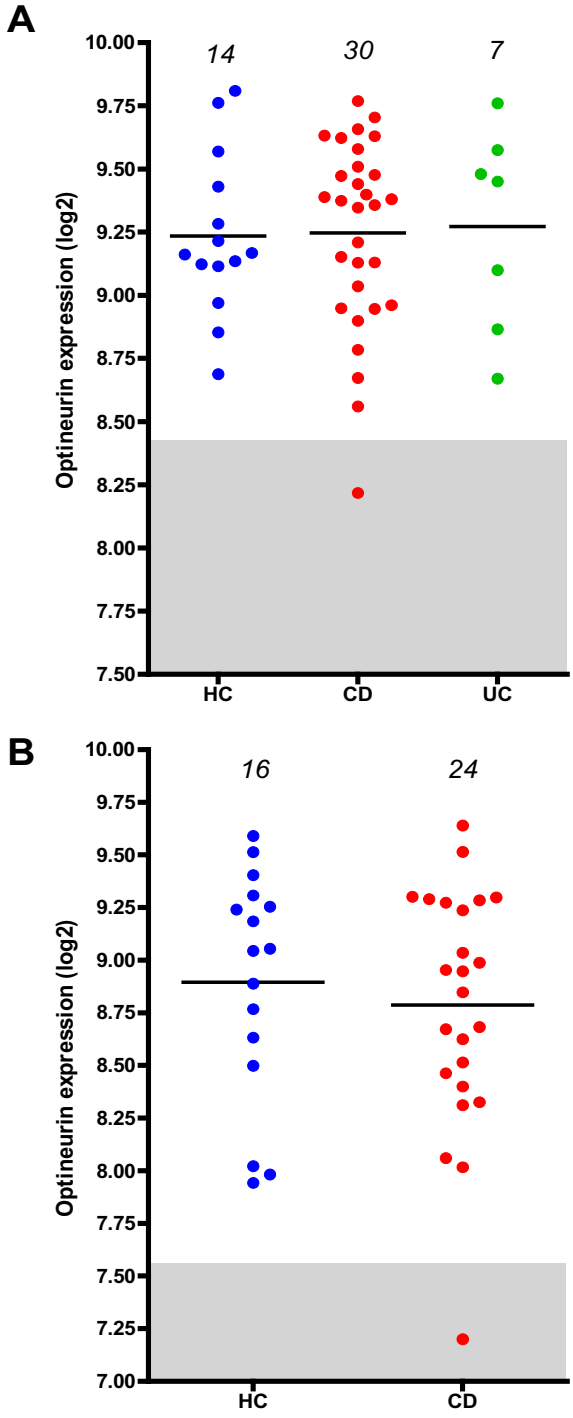


Figure 5.8 Abnormal optineurin expression in individual CD patients in two distinct array datasets. Patients were identified that were outliers for macrophage optineurin expression, using a threshold p-value of $p<0.005$ and a minimum fold change of 1.5 compared to mean HC expression. **(A)** Illumina microarray expression data of optineurin in cultured macrophages after stimulation for 4 hours with HkEc, showing one outlier CD patient (shaded area). **(B)** Affymetrix expression data of optineurin in unstimulated macrophages, showing one outlier individual (shaded area). Expression data are presented on a $\log_2$ scale. Numbers in each group are shown in italics.

identified in the unstimulated dataset. Overall, the results support the concept that a subgroup of CD patients have abnormal macrophage expression of optineurin, which can be detected under multiple conditions and across different microarray platforms.

5.2.7 Validation of abnormal optineurin expression in a subset of CD patients

In order to validate the abnormal expression of optineurin in the subset of patients identified in the microarray outlier analysis, TaqMan® real time quantitative PCR was performed. The relative expression of optineurin in the six of the outlier individuals was quantified in comparison to six HC individuals selected at random from the microarray study cohort. Results were normalised to expression levels of the housekeeper gene GAPDH. The outlier patients investigated had decreased optineurin expression compared to the HC individuals tested (mean reduction ~2.7 fold). This reduction was highly statistically significant ($p<0.001$) (Figure 5.9A), confirming the abnormality in macrophage optineurin expression in these CD patients at the mRNA level.

Western blotting was also performed in preliminary studies to investigate whether macrophage optineurin expression was reduced at the protein level. Samples from the one available outlier patient were compared with one HC lysate, in the unstimulated state, and after 4 and 24 hours stimulation with HkEc. A band was detectable at ~74kDa corresponding to the optineurin protein, in concordance with previously published studies (398). Macrophage expression of optineurin was reduced in all conditions compared to the controls tested (Figure 5.9B). Unfortunately cell lysates were not obtained from the majority of optineurin outlier individuals, preventing a more extensive assessment of optineurin protein expression.

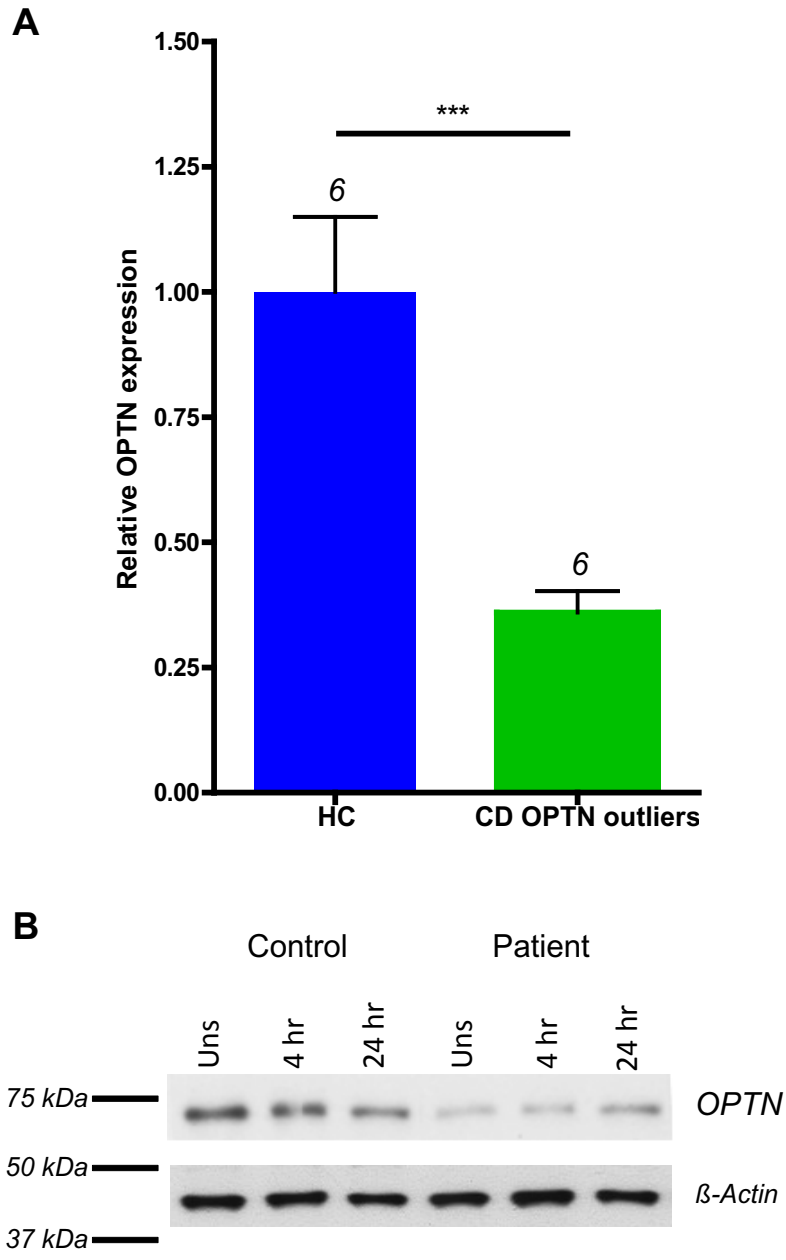


Figure 5.9 Validation of abnormal optineurin (OPTN) expression in a subset of CD patients, identified as gene expression outliers from the microarray study. **(A)** TaqMan quantitative PCR of OPTN expression in unstimulated macrophages from the patients identified as outliers, relative to randomly selected HC individuals. Numbers in each group are shown in italics. Data are presented as mean + SEM. *** represents $p<0.001$. **(B)** Western blot of macrophage OPTN expression in an outlier patient compared to a control individual. Results are shown for unstimulated samples (Uns), and 4 and 24 hours after HkEc stimulation. Position of molecular weight markers are shown.

5.2.8 Phenotype and genotypes of optineurin outlier patients

The clinical phenotypes of the CD patients with abnormal optineurin expression were surprisingly diverse (Figure 5.10A). Of the seven patients from the Illumina study, two individuals were related (individuals 1 and 6). The majority of the patients were male, although one patient with low optineurin expression was female. The average age of the seven patients was 48.3 $\pm$ 16.8 (mean $\pm$ standard deviation). All of the patients were Caucasian. Most had either ileal or ileocolonic involvement, but a wide range in age of diagnosis. Four of the patients had inflammatory disease, one had stricturing and two had fistulating disease. The seven patients were receiving a variety of medical regimens at the time of venesection, although notably, one patient was receiving no treatment and three were taking 5-ASA compounds only. The phenotypes of the UC patient, and one individual identified in the Affymetrix array cohort are also presented.

Considering the genotypes of the optineurin outlier individuals, one patient was homozygous for the G908R *NOD2* polymorphism, and the related individual was heterozygous for this variant. However, none of the other individuals in the Illumina study had any of the three CD-associated polymorphisms in *NOD2*. Interestingly, five of the individuals had an overall CD GRS (calculated from a 34-SNP genotype) that was below average for the healthy population, suggesting that the 34 polymorphisms genotyped do not play a major role in the development of CD in these patients. The two related individuals both had a GRS of 1.65 or above. The outlier patient identified in the Affymetrix study was compound heterozygous for CD-associated variants in *NOD2* and had a GRS of 9.03.

Macrophage TNF secretion after bacterial stimulation was also determined from four of the optineurin outlier patients and compared to the HC response. Median macrophage TNF release from the optineurin outlier patients after HkEc stimulation

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Patient	Microarray Cohort	Diagnosis	Gender	Age	Current smoking	Treatment	Montreal classification	*NOD2* genotype	GRS
1#	Illumina	CD	M	23	No	MTX	A1L1+4B3p	mt/wt	1.65
2	Illumina	CD	M	60	No	Steroid	A2L1B3	wt/wt	0.62
3	Illumina	CD	M	36	No	None	A2L3B2	wt/wt	0.45
4	Illumina	CD	M	34	No	5-ASA	A2L3B1	wt/wt	0.34
5	Illumina	CD	F	62	No	5-ASA	A3L2B1	wt/wt	0.7
6#	Illumina	CD	M	58	No	MTX	A2L3B1	mt/mt	2.03
7	Illumina	CD	M	65	Yes	5-ASA	A3L1B1	wt/wt	0.44
8	Illumina	UC	M	48	No	5-ASA			
9	Affymetrix	CD	M	75	No	5-ASA	A2L2B3	mt/mt	9.03

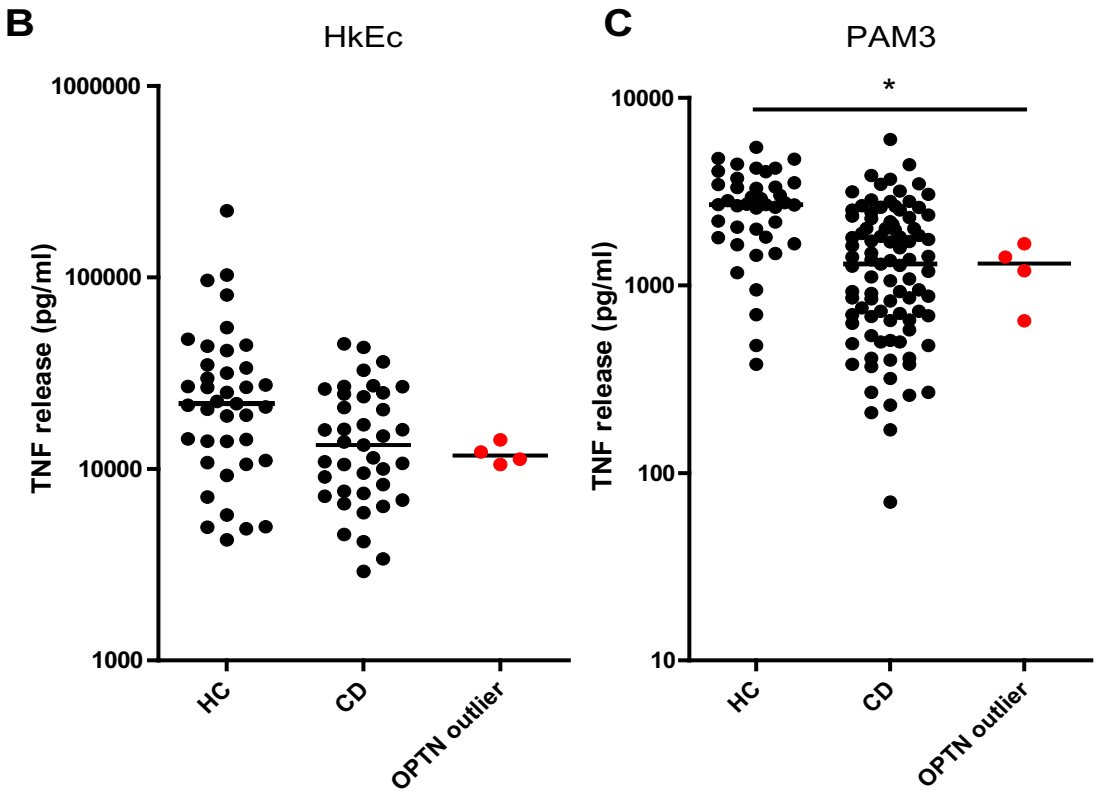


Figure 5.10 Phenotypes of optineurin outlier patients. **(A)** Details of patients identified as abnormal for optineurin (OPTN) expression by microarray gene expression outlier analysis. The microarray study in which the patients were identified is shown with demographics. Treatment regimes at the time of venesection are detailed for each patient (5-ASA = 5-aminosalicylates, MTX = methotrexate). The *NOD2* genotypes (where wt represents wild type allele, mt represents CD-associated variant allele) and calculated genetic risk score (GRS) based on a 34 SNP genotype are also shown. # indicates related individuals. **(B)** TNF release from optineurin outlier patient macrophages in response to HkEc stimulation, compared to HC and CD patients. Data are presented on a logarithmic scale **(C)** Deficient TNF release from optineurin outlier patient macrophages in response to stimulation with Pam_3CSK_4 (PAM3). Data are represented on a logarithmic scale. * represents $p<0.05$.

was 11,762 pg/ml (interquartile range 10,897-13,232 pg/ml), compared to 21,991 pg/ml (interquartile range 11,080-35,059 pg/ml) from HC individuals, however this reduction did not reach statistical significance (Figure 5.10B). In response to TLR2 stimulation, macrophage TNF release was significantly ($p<0.05$) attenuated from the optineurin outlier patients compared to HC individuals (Figure 5.10C). Therefore, the macrophage abnormality of TNF secretion in these patients is consistent with that identified in the overall CD cohort, but more striking after TLR2 than HkEc stimulation.

5.2.9 Functional relevance of abnormal optineurin expression in TNF release

In order to test whether low expression of optineurin had any functional relevance to TNF secretion after bacterial stimulation, optineurin expression was depleted in THP-1 cells using siRNA. 24 hours after transfection with specific a siRNA targeted against optineurin, cells were stimulated with HkEc and Pam_3CSK_4, and TNF release determined after 6 hours. Results were compared to mock transfected cells (without siRNA) and cells transfected with a non-targeting siRNA to control for potential off-target effects. Western blotting revealed a reduction in optineurin expression of ~60% in cells transfected with the OPTN siRNA compared to mock transfected cells. No significant difference in optineurin expression was observed in cells transfected with the negative control siRNA (Figure 5.11A, B). The proportion of viable cells after siRNA transfection was equivalent between mock, negative control and optineurin knockdown cells, as determined by propidium iodide staining and flow cytometry (Figure 5.18C).

After TLR2 stimulation with Pam_3CSK_4, TNF release was significantly attenuated from cells transfected with siRNA directed against optineurin ($p<0.01$) in comparison to mock transfected cells (Figure 5.12A). There was no significant difference between TNF release after TLR2 stimulation from mock transfected cells and

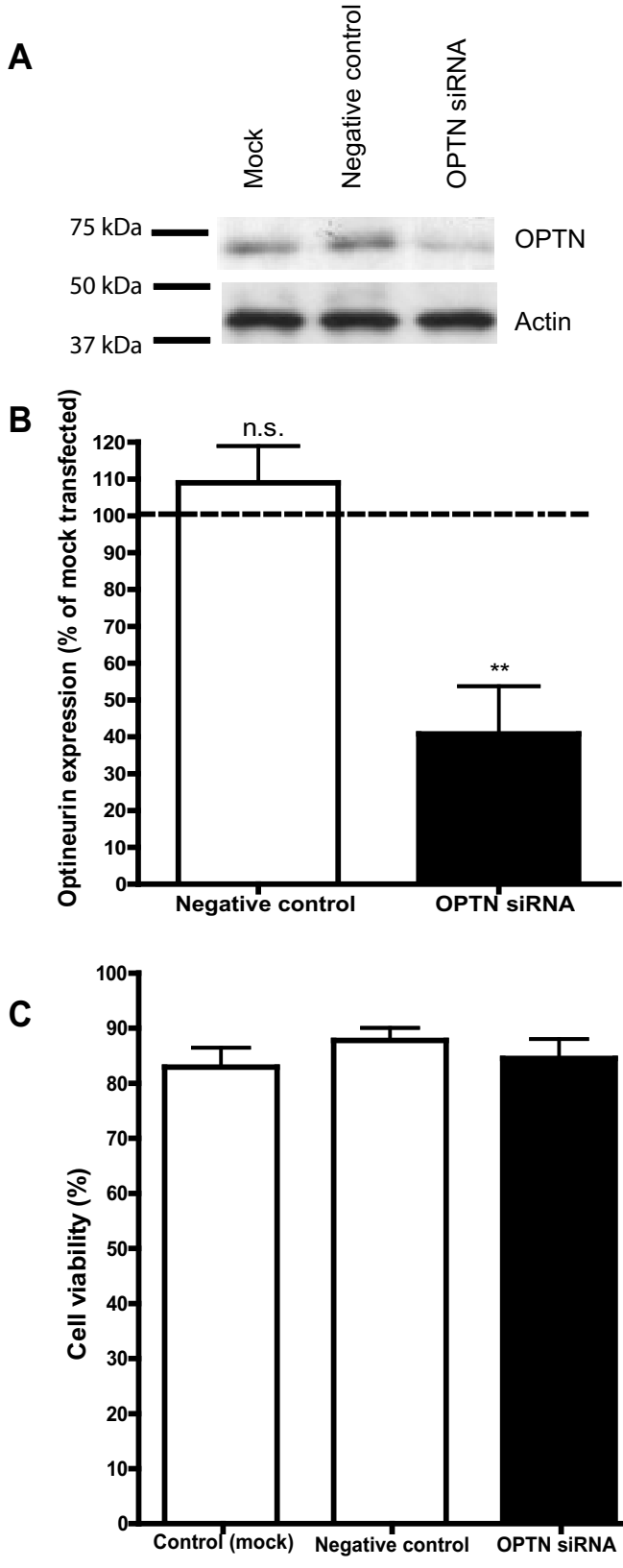


Figure 5.11 Knockdown of optineurin in THP-1 cells using siRNA. **(A)** Western blot showing depletion of optineurin (OPTN) in siRNA transfected cells in comparison to mock and control siRNA tranfected cells. OPTN is detected as a 74 kDa band. Actin loading controls are also shown. **(B)** Quantification of OPTN depletion in knockdown cells by densitometry. Results are expressed as % of mock transfected cells. Data are mean of 6 experiments with SEM. ** represents $p<0.01$. **(C)** Viability (as a % of total cell population) 24 hours after transfection was determined by propidium ioidide staining and flow cytometry. There were no significant differences in viability between mock, negative control and OPTN siRNA transfected cells.

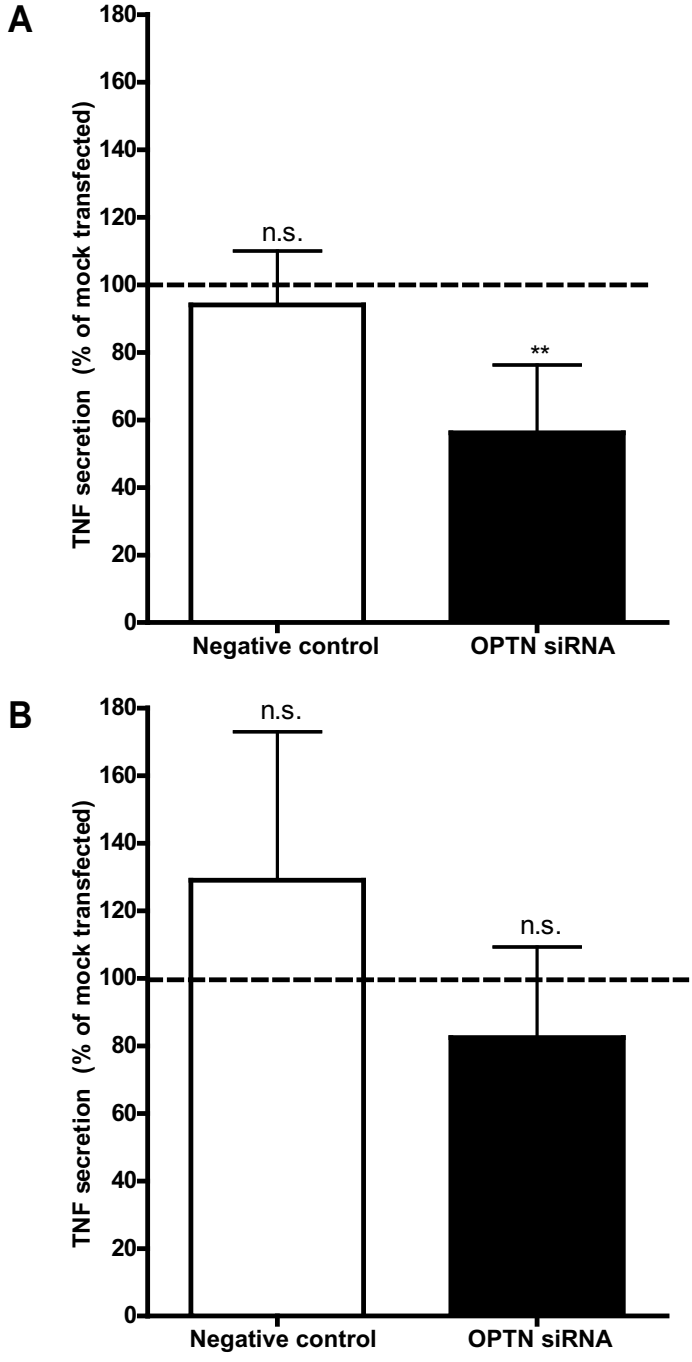


Figure 5.12 Impaired TNF release from optineurin depleted THP-1 cells after TLR2, but not HkEc stimulation. THP-1 cells were transfected with siRNA directed against optineurin (OPTN), non targeting siRNA (negative control) or mock transfected (without siRNA). After 24 hours, cells were stimulated with **(A)** Pam_3CSK_4 or **(B)** HkEc for 6 hours and amount of TNF released determined by bioassay. Results are mean + SEM of 6 experiments, presented as % of mock transfected cells. ** represents $p<0.01$ compared to mock transfected cells.

cells transfected with the control siRNA. After HkEc stimulation, no significant differences in TNF release were observed between mock, negative control and optineurin siRNA transfected cells (Figure 5.12B).

5.2.10 DNA sequence variants associated with abnormal optineurin expression

Given the deficient expression of optineurin observed in the subset of outlier patients, the *OPTN* gene was sequenced in seven of these individuals to test for the presence of mutations and polymorphisms. The *OPTN* gene is located on chromosome 10 and consists of 16 exons, 13 of which code for the optineurin protein (394). All exons and exon-flanking sequences were sequenced from the genomic DNA of optineurin outlier individuals. In addition, sequencing was conducted of ~2 kilobase (kb) regions up- and downstream of the *OPTN* gene. No novel mutations were detected in these regions in any of the patients tested; all sequence variants identified had been previously documented in dbSNP and/or the 1000 Genomes Project.

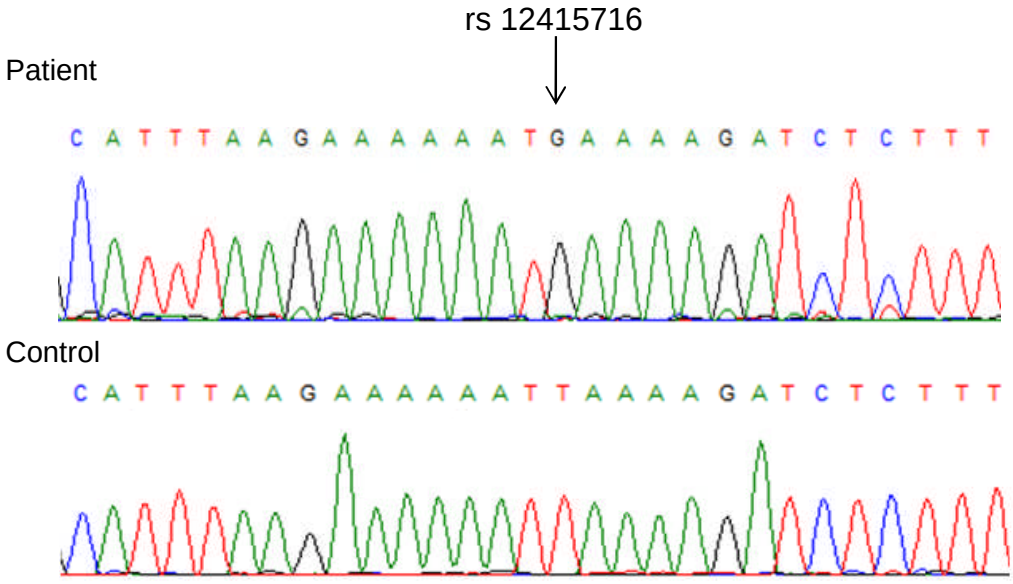
Heterozygous missense mutations in *OPTN* have been previously linked to primary open angle glaucoma, including the E50K, M98K and R545Q mutations (394). More recently, a homozygous deletion of exon 5, a nonsense mutation (Q298X) and a missense mutation (E478G) have been identified in patients with amyotrophic lateral sclerosis (ALS) (395). None of these mutations could be identified in any of the patients with abnormal optineurin expression.

Although no novel mutations were found, a number of recognised SNPs were identified in the outlier patients (Figure 5.13A, B). Notably, five patients were homozygous for the rs765884, rs12415716 and rs7098640 minor alleles, and the remaining two individuals were heterozygous for the variant SNPs. Rs765884 is located in an intronic region upstream of exon 9, whereas rs12415716 and rs7098640 are located downstream of the last exon (Figure 5.13C). Six of the seven patients also had

A

SNP	Wild type allele	Minor allele	Minor allele Frequency	OPTN outlier patient						
				1	2	3	4	5	6	7
rs3829924	G	A	0.038	G/G	G/G	A/G	G/G	G/G	G/G	G/G
rs11548142	G	A	0.2	G/G	G/G	A/A	G/G	A/A	G/G	A/A
rs10906303	A	G	0.18	G/G	G/G	A/A	G/G		G/G	A/A
rs2234968	G	A	0.17	G/G	G/G	A/A	G/G		G/G	A/A
rs4748020	G	C	0.22	C/C	G/G	G/G	C/C	G/G	C/C	G/G
rs765884	T	C	0.202	C/C	C/T	C/C	C/C	C/C	C/T	C/C
rs12415716	T	G	0.196	G/G	G/T	G/G	G/G	G/G	G/T	G/G
rs7098640	T	C	0.195	C/C	C/T	C/C	C/C	C/C	C/T	C/C

B



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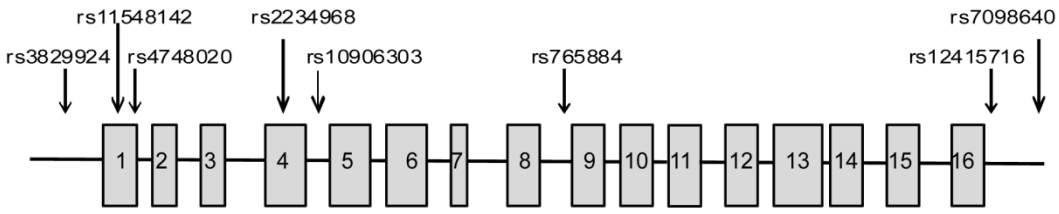

Figure 5.13 Single nucleotide polymorphisms (SNPs) identified in the *OPTN* region in outlier patients. **(A)** Recognised SNPs detected in outlier individuals . The minor allele frequency shown is derived from HapMap and/or the 1000 Genome project. Patients carrying a minor allelic variant are highlighted in red for each SNP. **(B)** Representative DNA sequence chromatogram from an outlier patient and control individual. The outlier patient is homozygous for the minor allele (G) of rs12415716; the control patient is homozygous wild type (T). **(C)** Schematic diagram of the *OPTN* gene, showing the position of the identified SNPs. Exons (grey boxes) are numbered.

sequence variants in exon 1. These included three individuals who were homozygous for the rs11548142 minor allele, and three other patients who were homozygous for a distinct polymorphism (rs4748020). One individual was heterozygous for an uncommon sequence variant in the promoter region of *OPTN* (rs3829924).

SNP annotation and proxy search (SNAP) was subsequently used to determine whether any of the identified SNPs were in linkage disequilibrium. This software package utilises genotype data from the HapMap and 1000 genome projects to calculate linkage disequilibrium data between polymorphisms, and identify proxy SNPs (271). The rs765884, rs12415716 and rs4748020 SNPs are in strong disequilibrium, indicated by the linkage disequilibrium $D^{'}$ values of 1, and r^2 correlation co-efficient values of greater than 0.8. In addition, a number of other SNPs are in linkage disequilibrium with the SNPs identified in the outlier patients. Results for rs12415716 are presented (Figure 5.14A, B). This observation suggests that the optineurin outlier patients may have a common haplotype (a combination of alleles at adjacent loci that are inherited together). Of the other SNPs identified, two were in linkage disequilibrium (rs10906303 and rs2234968, r^2=0.98). Linkage disequilibrium data were not available for rs4748020.

In order to assess whether the common haplotype identified in the optineurin outlier patients was associated with reduced optineurin expression, data from expression quantitative trait loci (eQTL) studies were interrogated using Genevar (GENe Expression VARiation) software (272). eQTL studies investigate associations between thousands of genotyped SNPs and transcriptomic data in particular cell or tissue types, derived from microarray analysis. No eQTL data were available for the SNPs directly identified in the outlier patients. However, rs17512962, a proxy SNP identified to be in linkage disequilibrium with rs12415716, was significantly associated with expression in fibroblasts (Figure 5.15A) (399).

A

SNP	Genomic position	D'	r^2
rs67121368	13214765	1	1
rs12415683	13212878	1	1
rs17512962	13209072	1	1
rs67978856	13202389	1	1
rs57199113	13200218	1	0.917
rs57057378	13200041	1	0.917
rs765884	13204338	1	0.913
rs7098640	13222655	1	0.879

B

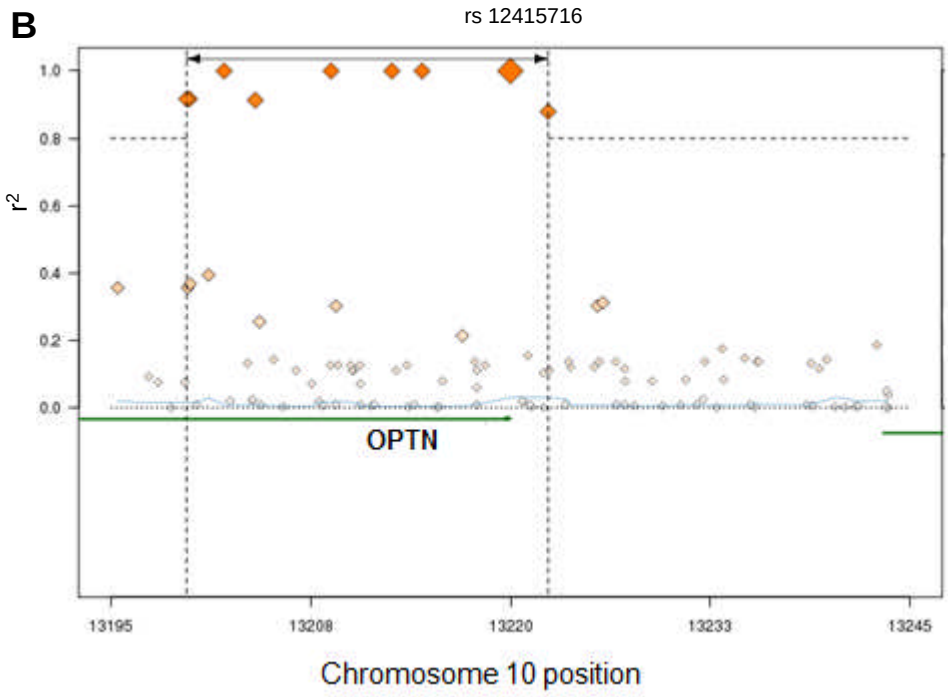

Figure 5.14 Linkage disequilibrium between single nucleotide polymorphisms identified in optineurin outlier patients. SNP annotation and proxy search software was used to determine whether SNPs detected were in linkage disequilibrium**. (A)** Polymorphisms in linkage disequilibrium with rs12415716 using a threshold r^2 of 0.8. Polymorphisms identified in the outlier individuals are highlighted in red. **(B)** Diagram of linkage disequilibrium between rs12415716 and other SNPs (highlighted in orange). The double-headed arrow shows the region where r^2 >0.8 between rs12415716 and the other SNPs. The positions of SNPs and the OPTN gene (green) on chromosome 10 are shown. Data are shown are according to human genome reference assembly GRCh37.

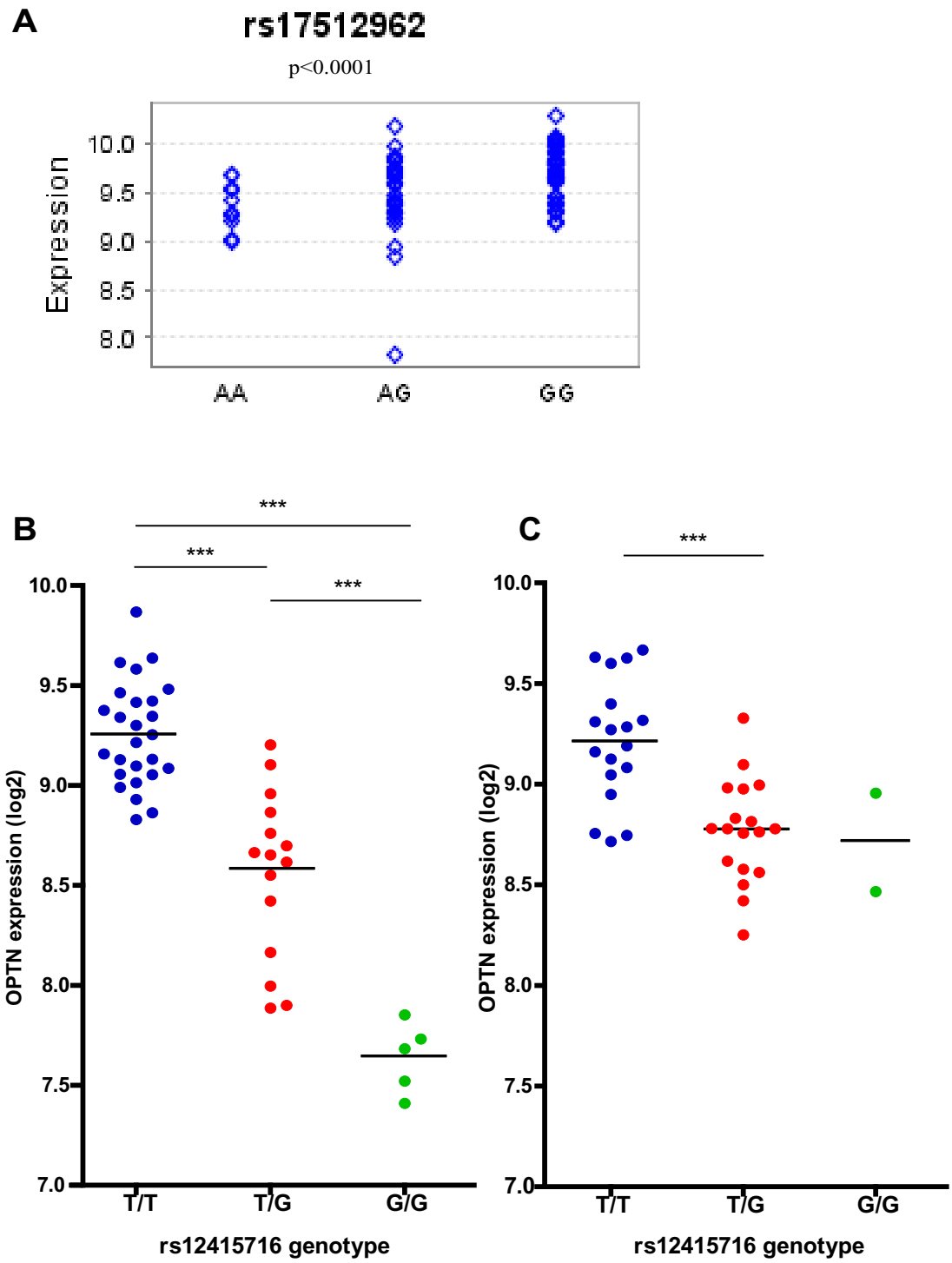

Figure 5.15 Association of single nucleotide polymorphisms in the *OPTN* region with optineurin (OPTN) expression. **(A)** Rs17512962, which is in linkage disequilibrium with rs12415716 (r^2=1), is associated with OPTN expression in fibroblasts. Data are derived from an expression quantitative trait locus study (399). **(B)** Association of rs12415716 genotype with optineurin expression (from microarray data) in the CD cohort. **(C)** Corresponding results for the HC cohort. *** represents p<0.001. Data are presented on a logarithmic (to base 2) scale.

To determine whether the haplotype was likewise associated with macrophage optineurin expression in the microarray study cohorts, CD patients (n=43) and HC individuals (n=38) were genotyped for rs12415716. In the CD cohort, 26 individuals were homozygous for the wild type allele (T), 15 individuals were heterozygous and 5 individuals were homozygous for the minor allele (G), giving an overall minor allele frequency of 0.27. The allele frequencies were similar in HC; the minor allele occurring at an overall frequency of 0.29. As expected from the eQTL studies, there was a significant difference in optineurin expression between wild type individuals, individuals heterozygous for the minor allele and individuals homozygous for the minor allele (all $p<0.001$) (Figure 5.15B).

HC individuals homozygous for the wild type allele had significantly greater expression of optineurin than individuals heterozygous for the wild type allele. Two HC individuals in the study were homozygous for the minor allele. These individuals did not display the gross abnormalities in expression observed in CD outliers, suggesting that the presence of the haplotype may be necessary but not sufficient to cause abnormal optineurin expression (Figure 5.15C).

5.3 Discussion

In this chapter, the global transcription profiles of HC, CD and UC macrophages are interrogated in an attempt to identify molecular lesions responsible for attenuated pro-inflammatory cytokine release observed in CD. Although a number of differentially expressed genes were identified between HC and CD groups which could be of functional relevance, none of these remained significant after correction for multiple testing. Furthermore, PCA analysis was unable to separate samples on the basis of disease grouping. Therefore, whilst subtle differences may exist between HC and CD, the transcription profiles of CD macrophages do not display gross abnormalities or a

unique overall expression signature. Furthermore, very few differentially expressed genes could be identified when comparing CD with UC, suggesting similarity between these two groups.

The absence of striking differences in global gene expression was surprising, given the previous reports of differentially expressed genes in PBMCs from CD patients (385), and furthermore in HkEc stimulated macrophages in a previous study conducted by our laboratory (136). In their study, Burczynski *et al*. investigated PBMC expression profiles from patients with a Crohn's Disease Activity Index (CDAI) score of 220-400; it is therefore possible that disease activity could have been a confounding factor. This is however unlikely to explain the difference between the study previously published by our laboratory and the present findings, given that both investigations excluded patients with active disease. Whilst discrepancies in gene expression can also arise from differences in sample processing (400) and cell culture conditions, in both cases macrophages were cultured in the same manner from PBMCs.

An important difference between the two studies conducted was the microarray platform utilised for expression analysis. The previous investigation interrogated genome-wide expression using Affymetrix GeneChip arrays, whereas the current study utilised Illumina bead-based arrays. There are several notable differences between the two array platforms, including the nature of the probes used to detect and quantify transcripts. Affymetrix GeneChip array probes are oligonucleotides typically 25 nucleotides in length, synthesised at predefined locations on the array (401;402). In contrast, Illumina arrays utilise long oligonucleotide probes, which are attached to microbeads and assembled randomly onto the array. Decoding algorithms are subsequently required to determine the location of each probe (402;403). Whilst studies such as the MicroArray Quality Control project have demonstrated concordance

between the results of Affymetrix and Illumina arrays (404), others have indicated subtle differences. For example, expression levels for low expressed transcripts appear to be less reproducible across platforms than abundant transcripts (402). Different methods of data processing between array platforms, such as normalisation, background subtraction and statistical thresholds employed, may also affect the observed concordance (405). It is therefore possible that the different array platforms used could contribute to the lack of concordance in the differentially expressed genes identified in the two experiments.

Patient heterogeneity is another likely reason for the different results obtained between the Affymetrix and the Illumina analysis, and may also explain the lack of differentially regulated genes observed in the present study after correction for multiple testing. CD is a complex and heterogeneous syndrome, demonstrated by the diversity in clinical phenotypes and genetic architecture between patients. It is therefore likely that molecular mechanisms giving rise to functional abnormalities in CD will be highly diverse. Analysis of the Affymetrix dataset identified 18 differentially regulated genes of known function in HkEc stimulated macrophages, nine of which participate in vesicle trafficking and cytoskeletal re-organisation (136). Interestingly, some of the genes identified as potentially abnormal in this study (without FDR correction) related to cytoskeletal control, such as *LRRC50*, and pathway analysis software also identified 'cytoskeletal remodelling' as an enriched process within this differential gene list. It is therefore plausible that whilst the identities of differentially expressed genes differ between patient cohorts, some have overlapping biological functions.

In view of the heterogeneity of CD, an 'outlier analysis' strategy was employed to identify abnormally expressed genes in individual CD patients. Using this analysis, a subset of patients was identified with abnormal expression of optineurin. The previously

described functional roles of optineurin in vesicle trafficking and protein secretion (392) implicated it as a likely candidate to influence pro-inflammatory cytokine secretion, and indeed depletion of optineurin expression in a monocytic cell line caused reduced TNF release in response to TLR2 stimulation. Together, these findings demonstrate how a 'individualised approach' to analysis of microarray data, using strategies such as the customised outlier analysis, has the potential to be highly informative in identifying rare or uncommon defects in gene expression in individual CD patients, which have pivotal functional consequences.

Optineurin is a ubiquitously expressed protein that localises to the perinuclear region and intracellular vesicles, especially those in close proximity to the plasma membrane (392). It interacts with a number of other proteins critical in intracellular trafficking and cytoskeletal control, including myosin VI, Rab8 and huntingtin (406). Given the observation of impaired post-translational handling of pro-inflammatory cytokines in CD macrophages, it is very plausible that a reduction in optineurin expression attenuates TNF release by impeding efficient intracellular trafficking processes. In further support of this, recent work has implicated key roles for optineurin in both the maintenance of Golgi structure (392) and in secretory vesicle fusion (407). Depletion of optineurin expression in cell lines attenuates secretion of proteins such as vesicular stomatitis virus glycoprotein (392), reduces the number of vesicle fusion events at the plasma membrane (407) and inhibits directed migration towards growth factors (398). Furthermore, optineurin is localised to recycling endosomes. In these vesicles, optineurin has been shown to interact with the transferrin receptor and regulate its trafficking to the juxtanuclear region (408). Interestingly, TNF and IL-6 secretion occurs via recycling endosomal compartments in cell lines (198). It is tempting to speculate that abnormal optineurin expression precipitates a dysregulation of recycling-

endosome dependent trafficking in CD macrophages, which impairs efficient TNF release on encountering bacterial stimuli.

Surprisingly, depletion of optineurin in the cell line did not strongly affect TNF secretion after HkEc stimulation. This is mirrored in cultured primary macrophages from the outlier patients. Pam_3CSK_4 is a synthetic ligand that binds TLR1/2 specifically, whereas HkEc contains ligands such as LPS capable of TLR4 activation. It is likely that the factors governing pro-inflammatory cytokine release, including the post-translational trafficking machinery, are likely to be subtly distinct between microbial stimuli. Indeed, investigations described in chapter 3 support this argument, as a relationship was detected between the GRS (calculated from a 34 SNP genotype of GWAS susceptibility loci) and macrophage TNF release in response to HkEc, but not TLR2.

As optineurin is a multifunctional protein that is also known to influence NF-κB activation by antagonising the effects of NEMO (409), further work is also required to determine the precise mechanism by which deficient optineurin expression impairs TNF release in response to TLR2. Such investigations could include incubation of control and optineurin depleted cells with inhibitors of protein trafficking and lysosomal function, and determining the effect on intracellular levels of TNF, as conducted previously in primary macrophages from CD patients. Microscopy studies of control, knockdown and primary patient macrophages could also prove extremely valuable in delineating potential mechanisms. In particular, immunocytochemistry and confocal microscopy should be performed to determine whether optineurin colocalises with TNF in vesicular compartments such as recycling endosomes. In addition, total internal reflection fluorescence microscopy (TIRF) could be employed to track vesicular transport, to determine whether abnormalities identified in optineurin deficient cell lines

are mirrored in cultured primary macrophages from the optineurin outlier patients. Furthermore, it would be interesting to investigate whether macrophages from this subset of patients have any defects in directed migration or lamellipodia formation (membrane projections that form at the leading edge of motile cells), given that *in vitro* depletion of optineurin is associated with abnormalities in these processes (398).

Recently, a key role for optineurin in autophagy of intracellular bacteria was identified (393), representing a major advance in current understanding of optineurin and its functions in innate immunity. Optineurin was shown to act as an autophagy receptor. At a molecular level, optineurin was shown to be recruited to ubiquitinated forms of *Salmonella* within the cell cytosol, where it interacts with TANK-binding kinase (TBK1). TBK1 phosphorylates optineurin, which enhances its affinity for LC3. Consequentially, clearance of intracellular *Salmonella* is promoted. Thus, cells deficient in optineurin have impaired autophagy against *Salmonella* increased intracellular bacterial proliferation (393). The autophagic handling of intracellular bacteria in the optineurin outlier patients should be further explored, in comparison to both control individuals and other CD patients. UC patients, including the one optineurin outlier patient identified in this study, should also be investigated. It is possible that the impaired release of TNF observed downstream of TLR2 stimulation mechanistically relates to impaired induction of autophagy, given that this process may have a role in TNF release downstream of TLR stimulation (237).

Heterogeneity between individual CD patients and control individuals, the potential for confounding factors, and constraints on *in vivo* testing in human subjects all present problems when attempting to prove the contribution of a specific molecule to the development of disease. Studies using knockout mice, in which the environment and mouse genetic background can be readily controlled, may be highly informative in

validating the *in vivo* significance of candidate molecules such as optineurin to disease pathogenesis. Recently, our laboratory has obtained mice with a targeted insertion in the *OPTN* gene that disrupts expression. Interestingly, these mice are viable and do not display gross ocular or neurological phenotypes. Macrophages from these mice should be cultured to validate the attenuated pro-inflammatory cytokine release observed in patient and siRNA-knockdown cells, and any other abnormalities identified in future work. Furthermore, the *in vivo* significance of optineurin deficiency in the development of bowel inflammation could be tested using classical inducers of colitis in rodents, such as DSS (410) and haptens such as trinitrobenzene sulfonic acid (411). In addition to comparing changes in weight, bleeding and histological scores in the knockout mice with littermate controls, infiltration of acute inflammatory cells such as neutrophils to the lamina propria and submucosa could be determined during the acute phase of colitis induction. This has previously been performed in studies of TLR4 knockout mice, which demonstrated more pronounced bleeding and impaired recruitment of neutrophils (148).

Optineurin has previously been linked to diverse diseases, including glaucoma (394), ALS (395) and Paget's disease (396), which may be a testament to its multiplicity of functions in a variety of different cell types. In this study, no mutations previously associated with human disease were identified, although a distinct haplotype was present in all optineurin outlier patients. The previous association of optineurin with neurological and ocular disorders does not preclude its potential relevance to other diseases such as CD. Indeed, sequencing of the *NOD2* gene has demonstrated how heterogeneous lesions affecting the same molecule can give rise to distinct syndromes. Sequence variants in the leucine-rich repeat domain of *NOD2* are associated with CD (211), whereas mutations in the nucleotide binding domain of *NOD2* cause Blau

syndrome (412). Whilst CD and Blau syndrome share some phenotypic overlap, the latter is not associated with bowel inflammation (413).

The optineurin outlier patients identified were homozygous or heterozygous for the minor allele of various SNPs in the optineurin gene region. As these SNPs are in strong linkage disequilibrium, the presence of a common haplotype is suggested. Furthermore, the results indicate that the abnormal optineurin expression is at least partially genetically determined. As multiple variants in linkage disequilibrium are present in the genomic region, it is difficult to pinpoint which of these, if any, are crucial in determining optineurin expression. It is also possible that all are in linkage disequilibrium with another variant or mutation that is as yet unidentified. Further sequencing of the *OPTN* region, including intronic sequences, together with functional data, may help clarify this issue.

If a reduction in optineurin expression gives rise to a macrophage defect of relevance to CD, and certain SNPs are associated with optineurin expression, it is surprising that polymorphisms in the *OPTN* gene have not to date been linked to CD by GWAS. Such studies compare allele frequency of polymorphisms between thousands of disease cases and controls to identify significant associations (115;116), and notably rs17512962 has been included in some of these analyses. However, although the optineurin outlier patients were either homozygous or heterozygous for several minor allelic variants, these polymorphisms were also detected in other CD patients and HC individuals within the cohort. In these individuals, macrophage optineurin expression was within normal limits. Therefore, the presence of a specific haplotype may be necessary, but not in itself sufficient, to cause deficient levels of optineurin expression. In this case, additional defects in the transcriptional regulation of the *OPTN* gene could act to compound the effect of the haplotype on optineurin expression. It is possible that

in a subset of individuals, the presence of an additional rare mutation gives rise to a profound defect in optineurin expression. Although no novel mutations could be detected in the *OPTN* exonic region, intronic genetic variation is well established to influence gene expression (414;415). Furthermore, rare alterations in trans-acting elements such as transcription factors could act in concert with a variant or mutation in cis to give rise to grossly deficient expression. Given the potentially limited power of current GWAS to identify rare abnormalities (256-258), in these scenarios it is plausible that an association with *OPTN* and CD would not be identified.

Combinations of relatively uncommon SNPs could also be the critical determinant of abnormal optineurin expression. In addition to the haplotype, SNPs were also identified in exon 1 in six of the patients; these could also exert effects on optineurin expression. One of these, rs4748020, has not been studied in GWAS and no proxy markers of this SNP exist. A combination of minor alleles (that are not usually in linkage disequilibrium) may be critical in determining abnormal optineurin expression and CD susceptibility; if the co-inheritance of these is an uncommon event, GWAS may be unable to identify the association.

In addition to optineurin, a host of other gene expression outliers in CD were identified in this study, some of which could represent important targets for future investigation. The majority of gene expression outliers were private to individual patients, which may be in keeping with the recent prediction that each person possesses on average at least 60 new mutations in their genome (416). Ten outlier genes mapped to regions of CD association by GWAS. These included *UTS2*, encoding a potent vasoconstrictor (417), and *CARD9*, encoding a caspase recruitment domain-containing protein that may have a role in NF-κB activation and apoptosis (418), and in the innate immune response to fungal stimuli (419). Given that CD is associated with diminished

blood flow at sites of subcutaneous bacterial injection, and impaired macrophage pro-inflammatory cytokine in response to various microbial stimuli (including *C. albicans*, as demonstrated in chapter 3), UTS2 and CARD9 could be relevant candidates for future study. It would be interesting to conduct sequencing of the relevant genomic locus in these individuals, to genotype for the relevant susceptibility polymorphism and identify any concurrent mutations or structural abnormalities. Preliminary studies conducted by our laboratory have validated the gross overexpression of urotensin 2 in a subset of individuals, and revealed that all urotensin 2 outlier individuals are homozygous for the risk-associated allele in this region. Further sequencing of the *UTS2* genomic locus in these patients is therefore now a priority, using techniques such as next generation sequencing.

In a wider context, a personalised approach to understanding transcriptomic abnormalities in individual patients could be a powerful means of investigating heterogeneous disorders, especially when integrated with other techniques such as DNA sequencing and functional assays. Previously, analogous strategies have been attempted in oncology (383) and psychiatric research (420). Whilst the basic premise was the same, each of the studies employed distinct methods for detecting gene expression outliers in individuals or subsets of patients. One utilised an 'extreme values' analysis based on fold change from mean expression in a control cohort, using data from multiple cortical regions of the brain as an internal validation to identify relevant gene expression outliers (420). In oncology, the COPA algorithm has been employed with considerable success. This identified a subset of prostate cancer samples that displayed overexpression of the ETS transcription factor genes *ERG* and *ETV1*, leading to the important discovery of *TMPRSS2-ETS* chromosomal translocations in a subset of the samples (383). COPA identifies pairs of genes that have the largest number of mutually exclusive outliers in the disease group (ie samples that are outliers for gene X and

samples that are outliers for gene Y, but never both). The threshold set for outlier detection is based upon median absolute deviation of expression within the disease grouping (382). Whilst this is a powerful tool for detecting chromosomal translocations, the strategy may be less applicable to complex disorders. The 'outlier analysis' technique developed here compares the transcriptomic profile of an individual patient with a panel of control individuals independently of other samples in the disease grouping, facilitating the identification of rare defects in expression in individual patients.

Whilst providing interesting results, the microarray outlier analysis should be regarded primarily as a 'hypothesis-generating' tool, whereby abnormal gene expression in individual patients is interrogated to identify candidates for future functional investigations, as demonstrated for optineurin. The strategy may well be applicable to other heterogeneous disorders. Important prerequisites for such studies include availability of mRNA for quantitative PCR analysis, to validate that the observed changes in gene expression are genuine and not artefacts of analysis or poor probe hybridisation. Furthermore, knowledge of functional abnormalities in the cell or tissue in question is crucial, to enable prioritisation of candidates and testing of their functional relevance. Nevertheless, when combined with other strategies, the outlier analysis strategy could be of considerable use in identifying rare defects of biological significance in individual patients, and potentially understanding the nature of some of the 'missing heritability' of complex disorders.

Chapter 6: General Discussion

6.1 Summary of investigations conducted and novel findings

CD is a complex, heterogeneous condition predominately affecting the gastrointestinal tract. The CD phenotype is characterised by a systemic defect in neutrophil recruitment to acute inflammatory sites, and delayed bacterial clearance. Macrophages from CD patients were previously found to release attenuated levels of certain pro-inflammatory cytokines in response to HkEc. Sub-optimal release of these critical inflammatory factors was related to their abnormal post-translational trafficking and degradation in lysosomal compartments (136).

In chapter 3, the selective impairment in macrophage pro-inflammatory cytokine release in response to HkEc was validated in a new cohort of CD patients. TNF and IFN-γ release were attenuated, whilst secretion of other pro-inflammatory cytokines such as IL-2 and IL-1β, the chemokine IL-8, and anti-inflammatory cytokine IL-10 were normal, in concordance with previous findings. TNF release was depressed in response to multiple microbial stimuli, including TLR2 and TLR4 ligands and the yeast *C. albicans*, demonstrating that the phenomenon is not restricted to the response to HkEc. Defective TNF release was unrelated to the age of patient, gender, smoking status and use of 5-ASA or immunosuppressant medication. The impairment in TNF release downstream of TLR2 was more severe in patients with stricturing disease than those with inflammatory disease, whereas for TLR4, the defect was more pronounced in patients with colonic disease.

Genetic studies such as GWAS have identified SNPs that are associated with CD. No significant associations could be identified between the genotype for any individual polymorphism and the secreted levels of TNF in response to TLR2 or HkEc

stimulation. Calculation of an overall GRS, based on the genotypes of the 34 SNPs, revealed several differences between patients and HC individuals with a greater overall burden of GWAS risk alleles compared to those with a lower burden. Macrophages from patients with a GRS of greater than 1 released significantly less TNF in response to HkEc than those with a GRS of less than 1; furthermore, a weak negative correlation was observed between TNF release and GRS. Surprisingly, this relationship was not evident for TLR2 stimulation and somewhat paradoxically, macrophages from HC individuals with a GRS above 1 released greater amounts of TNF downstream of TLR2 than those with a GRS less than 1.

In chapter 4, it was hypothesised that abnormalities in the cellular sphingolipid and phospholipid composition could account for the defective macrophage function observed in CD. However, no differences in endogenous ceramide, dihydroceramide, sphingomyelin, PC, PS and PI species were detected in CD macrophages. Furthermore, studies using deuterated choline, serine and inositol revealed equivalent rates of overall metabolic flux through the PC, PS and PI synthesis pathways between HC and CD cells, therefore excluding gross defects in the global rates of synthesis of these lipids as the underlying mechanism of impaired cytokine secretion from CD macrophages. However, a reduced molar percentage of one specific PI species (PI 16:0/18:1) was synthesised over a defined time period in CD macrophages; reduced levels of this same species were detected in ileal biopsies from CD patients.

A ‘hypothesis-generating’ approach was subsequently employed to identify candidate molecular lesions in CD macrophages, by analysis of transcriptomic data from microarray experiments. Although no striking differences were apparent in the expression profiles of CD macrophages using classical group comparisons of gene expression, a customised outlier analysis strategy identified distinct abnormalities in

individual CD patients. Several interesting outliers were identified in different patients. The most commonly under-expressed outlier gene in the CD cohort was optineurin, a molecule implicated in vesicle trafficking, autophagy and intracellular bacterial clearance. The reduction in optineurin expression was validated in this subset of patients, and was associated with the presence of a specific haplotype in the optineurin genomic region. Depletion of optineurin in THP-1 cells resulted in reduced secretion of TNF in response to Pam_3CSK_4. This investigation therefore identified a subgroup of CD individuals with defective macrophage optineurin expression, of relevance to the impaired pro-inflammatory cytokine release observed after TLR stimulation. This 'individualised approach' to analysis of transcriptomic data may represent a powerful strategy to identifying molecular lesions of functional significance in other heterogeneous disorders.

6.2 Discussion of novel findings, implications and study limitations

6.2.1 Impaired macrophage function in Crohn's disease

Numerous theories have been proposed as to the underlying cause of CD, including abnormal mucosal barrier function, infection with microbial agents such as MAP and adherent, invasive *E. coli*, autoimmunity and immunodeficiency (421). This study provides further evidence for disordered innate immune function in CD in response to multiple microbial stimuli, and indicates the likely complexity and heterogeneity of molecular lesions underlying this abnormality.

In vivo studies have demonstrated a depressed acute inflammatory response to bacterial stimuli in CD. Whilst neutrophil accumulation to sites of acute inflammation is impaired (134-136), the function of these cells for the most part appears normal (179) (with the exception of respiratory burst activity, which is marginally reduced after stimulation with PMA (185)). Macrophages are phagocytic cells that are also pivotal in

acute inflammation, secreting cytokines and chemokines that enable recruitment of leukocytes from the bloodstream to acute inflammatory sites. A critical function of TNF is upregulation of adhesion molecules on endothelial cells, facilitating interaction with leukocytes and their extravastation through the capillary wall (285). Defective release of pro-inflammatory cytokines such as TNF by macrophages in response to a range of microbial stimuli therefore provides an attractive explanation for the sub-optimal recruitment of neutrophils observed in CD. Consequentially, delayed neutrophil recruitment would result in impaired clearance of material at sites of ingress of the bowel wall. The persistence of this material would act as a trigger for adaptive immunological responses, which develop to compensate for the initial failure of microbial clearance. This model is consistent with a number of animal studies, where absence of TNF or innate immune receptors such as TLR4 is detrimental in the early phases of DSS-induced colitis (148;286).

It should also be noted that defective release of pro-inflammatory cytokines by macrophages could have pleiotropic consequences beyond impaired neutrophil recruitment, potentially contributing to CD pathogenesis. In particular, recognition of bacterial components via TLRs may have an important role in the maintenance of intestinal barrier homeostasis, as demonstrated by animal studies (422). The severe intestinal injury observed in MyD88 knockout mice after DSS administration (422) was proposed to relate to defective induction of pro-inflammatory cytokines such as IL-6, which may have a role in protection of the epithelium by regulation of trefoil factors (423). Furthermore, in the present study, patients with stricturing forms of CD had a more pronounced abnormality in TNF secretion in response to TLR2 stimulation. It is therefore possible that an impaired macrophage response downstream of TLR2 could be a contributing factor in stricture pathogenesis. This could be an indirect association, for example by predisposing to a more severe disease course, or alternatively, the

macrophage response to TLR2 could influence the process of stricture formation directly. The pathogenesis of stricture formation is incompletely understood, but could relate to an increased proliferation and/or reduced migratory potential of intestinal myofibroblasts (293). TNF has been shown to influence migration of fibroblasts (424;425), although the effects are complex and dependent on the tissue, stimulus dose and timing. Polymorphisms in the PRR *NOD2* have been associated with a stricturing disease course (294), suggesting that an innate immune response to microbes could be important in the development of strictures, although further studies are required to fully establish the link.

An inherent failure of macrophage TNF secretion in response to bacterial stimuli would initially seem inconsistent with the current management of CD, given the efficacy of immunosuppressants and TNF antagonists such as infliximab. There are several important points when considering this apparent paradox. Firstly, CD is proposed to arise from an initial failure of neutrophil recruitment and bacterial clearance (136). Pathological lesions are hypothesised to develop in a subsequent, temporally distinct phase, characterised by chronic inflammation, giving rise to the symptoms experienced by the patient (151). Given the leukocytic infiltration and dramatic elevation in pro-inflammatory cytokine levels observed in the bowel wall at this later stage, it is unsurprising that immunosuppressive therapies, which suppress NF-κB activation and inhibit lymphocyte proliferation are beneficial, whilst unlikely to be influencing the putative upstream defect. Furthermore, certain immunosuppressants such as steroids, whilst efficacious for induction of remission, are less effective for prevention of recurrence (11).

In addition, the mechanism by which TNF antagonists exert benefit in CD may be more complex than originally thought (80). Infliximab and adalimumab are

humanised monoclonal antibodies directed against TNF. However, these therapies are also known to stimulate apoptosis of lamina propria T lymphocytes (possibly by binding to transmembrane forms of TNF) (208), a process that may also be defective in CD (205;207). Interestingly, etanercept, a recombinant fusion protein directed against TNF, does not show the same capacity to induce apoptosis (426), and failed to demonstrate significant clinical benefit in CD in a randomised controlled trial (427). Furthermore, the onset of CD has been documented in patients with rheumatological conditions following etanercept administration (428). Registry data collated over a 10 year period suggests an increased incidence of IBD in juvenile idiopathic arthritis patients treated with etanercept compared to the general paediatric population; the majority of these patients had histopathological features consistent with CD (429).

A significant difficulty in the investigation of complex syndromes such as CD is the discrimination of primary or initiating factors from those that are related to the disease process itself, or secondary to factors such as treatment regimens. In this study, defective macrophage cytokine secretion was observed in patients with quiescent disease and was unrelated to use of medication at the time of sample collection, arguing in favour of a primary abnormality. Furthermore, macrophages cultured from patients with UC do not display the same abnormalities in cytokine secretion, as shown in chapter 3 and elsewhere (163), indicating that the defect is not simply consequential to a chronic inflammatory condition. The relationship between an increased burden of GWAS risk alleles and diminished cytokine release in response to HkEc indicates that this response is at least partially genetically determined, again consistent with a primary functional abnormality in CD. Further work to clarify whether cytokine secretion represents a primary defect could include investigation of macrophage cytokine release from healthy relatives of affected patients with CD, in a similar manner to studies of intestinal permeability (44) and ASCA (96). Large scale population studies

investigating macrophage cytokine release with the development of CD would be valuable, but are likely to be infeasible without considerable adaptations to the current methodology. Examination of concordance of macrophage cytokine release between monozygotic and dizygotic twins would help delineate the contribution of genetic and environmental factors to this process.

Macrophages display plasticity and functional heterogeneity, which is partly dependent on their surrounding environment. The present study investigated pro-inflammatory cytokine release from peripheral blood monocyte-derived macrophages in response to microbial stimuli, and candidate molecular lesions responsible for the observed abnormalities. In support of investigating this cell type, the impaired acute inflammatory response observed in CD is a systemic abnormality, as demonstrated by various *in vivo* studies, including skin windows (134), epithelial trauma models in the bowel (135), and subcutaneous injection of killed *E. coli* (135;136). Furthermore, resident intestinal macrophages in the bowel are derived and continually replenished from peripheral blood derived monocytes (430).

In spite of this, intestinal macrophages within the mucosa may have a distinctive phenotype from that of peripheral blood mononuclear cells under normal physiological conditions. Intestinal macrophages isolated from intestinal tissue sections have been reported to express low levels of CD14 and other innate immune receptors. Cultures of these cells secrete reduced levels of pro-inflammatory cytokines in comparison to blood monocytes upon LPS stimulation, whilst retaining phagocytic capacity (431). Other studies have indicated that the mucosal situation may in fact be more complex than this. Recently, a subset of pro-inflammatory, CD14 positive macrophages was identified in healthy intestinal mucosal tissue of control individuals and patients with IBD. These cells secrete higher levels of pro-inflammatory cytokines such as TNF, IL-23 and IL-6

after bacterial stimulation than the low CD14 expressing macrophages previously described (432). Further work to clarify the extent to which intestinal macrophage subsets recapitulate the abnormalities identified in peripheral blood monocyte-derived macrophages would be helpful in validating the relevance for CD pathogenesis. Availability of tissue samples from patients with quiescent disease, and the number of cells that can be obtained, are however significant limiting factors in such studies.

Finally, it should be remembered that whilst the defect in cytokine release in cultured CD macrophages *ex vivo* was unrelated to certain environmental factors such as age of patient and smoking, the *in vivo* relevance of such factors cannot be disregarded. Smoking is associated with increased risk of CD (2), and it is possible that cigarette components could worsen an underlying abnormality in macrophage function *in vivo*. Nicotine, a constituent of cigarette smoke, has been reported to suppress macrophage pro-inflammatory cytokine production via nicotinic acetylcholine receptors (433;434). Emerging evidence also suggests intestinal bacterial components may themselves modulate macrophage function. Bifidobacteria, which are reduced in CD (118), are understood to enhance innate immune cell function, including pro-inflammatory cytokine release (119). In contrast, other microbial species could exert detrimental effects. For example, microbial mannans (present in yeast and certain mycobacteria) may have a suppressive effect on the ability of macrophages to kill mucosal strains of *E. coli* (98). The contribution of such factors to the impairment in cytokine release observed in cultured macrophages has not been directly addressed in this study. Whilst macrophages in this study were cultured *ex vivo* from peripheral blood monocytes for 5 days, making a strong influence of bacterial components and serological factors seem unlikely, potential effects cannot be ruled out.

6.2.2 Molecular mechanisms underlying impaired cytokine release and macrophage function

Identification of the molecular defects responsible for impaired cytokine release in CD macrophages is a considerable challenge, for several reasons. Firstly, CD is a complex and heterogeneous syndrome. A number of findings presented in this thesis illustrate this heterogeneity, including the wide variation in macrophage TNF release and GRS between patients, the stimulus specific differences in TNF release between phenotypes, and the lack of unifying transcriptomic abnormalities in the CD group. It is therefore likely that the underlying molecular lesions will be highly variable between patients.

Secondly, given that no abnormality has been observed in the control of cytokine gene transcription in CD macrophages, the proposed defect relates to perturbations in post-translational trafficking and mistargeting of cytokines to lysosomal compartments (136). The pathways of cytokine secretion remain relatively undefined, in stark contrast to intracellular signalling pathways. Whilst work in cell lines and murine macrophages have elucidated a number of molecules and vesicle subsets involved (189), the direct applicability to primary human macrophages has not yet been confirmed. In this study, a correlation was observed between release of TNF and IL-6 and IFN-γ indicating that the attenuated secretion is related. Interestingly, TNF and IL-6 release from cell lines is understood to involve recycling endosomes (198), suggesting this vesicle could be abnormal in CD macrophages.

A correlation was also observed between defective release of TNF downstream of TLR2, TLR4 and HkEc stimulation, indicating some likely degree of overlap in the mechanisms of impaired cytokine release between these stimuli. However, despite this potential overlap, other evidence presented in this thesis in fact suggests subtly distinct

molecular defects may be important in individual patients downstream of each microbial stimulus. Firstly, there are a number of stimulus-specific differences in macrophage TNF release between patient phenotypes. Secondly, a relationship between overall GRS and TNF release downstream of HkEc, but not TLR2 stimulation, is observed. Finally, abnormal optineurin expression in a subset of patients appears to be more strongly associated with abnormal release of TNF downstream of TLR2 rather than HkEc stimulation, a pattern that is mirrored in cell lines depleted of optineurin expression.

In spite of various strands of evidence suggesting lipid abnormalities in CD, and the established roles of sphingolipids and phospholipids in membrane structure, cytokine secretion and autophagy (197;317), no gross defects in the macrophage lipid composition could be identified in this study. Furthermore, the overall rate of metabolic flux through the PC, PS and PI synthesis pathway was normal in CD macrophages. This indicates that a gross abnormality in these lipid species is not likely to be the underlying mechanism of impaired cytokine release, although the possibility of more subtle defects (such as in particular subcellular macrophage compartments) and/or defects in individual patients cannot be excluded. Although no differences in endogenous composition of PI were observed in CD macrophages, unstimulated CD macrophages synthesised a reduced relative percentage of PI 16:0/18:1 over a 3 hour period. Interestingly, this species was also reduced in ileal biopsies from CD patients. The differences may indicate abnormal turnover of this PI species in CD, due to an abnormality in fatty acid synthesis or desaturation. Further work is required to clarify the mechanism and relevance for macrophage or intestinal barrier function.

Classical group comparisons of differential gene expression between CD macrophages and HC and UC patients did not identify striking abnormalities. This is

not entirely surprising, given the heterogeneity in clinical presentation, genetic architecture and functional defects observed in CD. In view of this heterogeneity, an outlier analysis of transcriptomic data was subsequently undertaken to identify abnormalities in gene expression in individual patients. This identified a subset of patients with significantly reduced expression of optineurin, which may account for the attenuated TLR2 response observed in macrophages from these patients. Given the previously described roles of optineurin in trafficking of vesicles such as recycling endosomes (407;408), it is very plausible that optineurin has a role in post-translational trafficking of cytokines from the Golgi apparatus to plasma membrane; when optineurin expression is reduced, delivery of these molecules to the plasma membrane is impaired. Further studies are needed to test this hypothesis, using inhibitors of protein trafficking and macrophages isolated from optineurin knockout mice. In addition to optineurin, various other genes were identified as common outliers between patients, including *ADAMDEC1*, a disintegrin and metalloprotease. Both optineurin and *ADAMDEC1* are currently being investigated as potential candidates using knockout mice, in two ongoing clinical fellowships in this laboratory.

CD is a multifactorial condition, which may involve both temporally distinct stages and dysregulation of a variety of cells and tissues (151). The molecular mechanisms underlying the functional abnormalities that characterise CD are likely to overlap. This study has not directly addressed whether the identified defects represent 'macrophage specific' phenomena, or more generalised abnormalities in multiple cell types (which could also be important in the disease pathogenesis). An interesting avenue of investigation would be determination of whether monocytic cells display some of the functional and molecular abnormalities identified in cultured macrophages. Identification of defects common to both monocytes and macrophages would facilitate

larger scale screening projects of CD patient cohorts, eliminating the requirement for tissue culture in such studies.

In addition, gastrointestinal epithelial cells also have a role in the host response to microbial components (435). They express TLRs and NLRs such as NOD2 (436), enabling induction of protective responses such as autophagy (241) and defensin secretion (436) on encountering microbes. Epithelial cells also secrete low levels of certain cytokines and chemokines in response to bacterial stimuli (437;438), and indeed stimulation of epithelial TNF release has been proposed to account for the protective effects of probiotic administration in the SAMP mouse model of ileitis (121). Furthermore, epithelial cells, and especially specialised M cells, are important in controlling the ingress of luminal contents to the underlying tissues (51). Interestingly, optineurin and its binding partner myosin VI are expressed in polarised epithelial cell lines, where they play a role in sorting of basolateral membrane proteins (439). Future studies could address whether the optineurin outlier patients have similarly reduced expression of this molecule in the epithelium, and any functional consequences of this. Determination of intestinal barrier function and permeability in the optineurin knockout mice would also be helpful in answering this question.

Over 70 distinct genetic loci have been associated with CD by GWAS (115;116). The precise mechanisms by which the associated variants influence disease pathogenesis remain incompletely understood, although many of the genes located in the susceptibility loci are known or suspected to have critical roles in innate immunity. Whilst no single variant was significantly associated with macrophage TNF release in this study, patients with a greater burden of GWAS risk alleles released reduced TNF in response to HkEc than those with a lower overall burden. This suggests that CD-associated risk variants could have a collective effect to impair TNF release in response

to HkEc. Furthermore, the results of GWAS studies appear to be consistent with functional demonstrations of defective innate immunity in CD, and specifically macrophage pro-inflammatory cytokine release in response to HkEc. Future studies should clarify which of the variants are responsible for the relationship, whether the association is direct or indirect, whether they act together in pathways, and functional mechanisms.

In contrast, this relationship was not observed for TLR2 stimulation, suggesting that the identified susceptibility loci do not play a significant role in the attenuated TNF release in response to TLR2. Other molecular abnormalities, including deficient optineurin expression, are likely to play a role in this defective response in individual patients. On one hand, attenuated TNF release downstream of TLR2 could in itself be critical in the development of CD, particularly if stimuli that are primarily detected by this receptor were to penetrate into the bowel wall. At the other end of the spectrum, a strong TLR2 response may act to compensate for other abnormalities, such as an increased burden of GWAS risk alleles, enabling a greater amount of TNF to be released and thus facilitating adequate neutrophil recruitment and clearance of microbial material. This could be important if a high load of bacteria such as *E. coli* were to reach the underlying tissues. Whilst deficient optineurin expression may act to impair TNF release downstream of TLR2 activation in a subset of patients, the molecular mechanisms underlying a vigorous, compensatory response in a subset of HC individuals, and the attenuated TNF release observed in other CD patients with normal optineurin expression, are not yet clear.

Taken together, the 71 GWAS variants identified to date are estimated to account for approximately 20% of the heritability of the disease (115). The nature and strategies for discovering the genetic variation that makes up the ‘missing heritability’

remain hotly debated (256;257). It is evident that simply conducting larger scale GWAS in their current form is unlikely to solve the problem. It is likely that much of the undiscovered genetic variation could exert powerful biological effects, including on macrophage function and bacterial clearance. Large scale sequencing projects are already under way in CD; the interpretation of these are helped by the 1000 Genomes project (440). It is likely that such projects will identify mutations and rare variants in CD patients; it will be important to test the functional effects of these on macrophage pro-inflammatory cytokine release downstream of different stimuli.

However, the relatively large number of private mutations in each individual (416), the capacity of multiple variants to exist in linkage disequilibrium, and the fact that large scale sequencing can generate false positive results due to sequencing errors (441), all make identification of the true causal mutations difficult. It is likely that integrated approaches, utilising both data from GWAS studies, whole genome sequencing projects and transcriptomic information could facilitate the determination of which variants are likely to be biologically relevant. Furthermore, in an era of 'personalised medicine', integrating genomic sequence information with knowledge of the abnormally expressed genes in an individual patient, and understanding their functional consequences, could be a worthwhile approach to deciphering the molecular pathogenesis of heterogeneous disorders and identifying relevant biomarkers in individual patients.

6.2.3 Implications for CD pathogenesis and therapeutic strategies

The findings presented in this thesis have a number of implications for the pathogenesis of CD and future therapeutic directions. Whilst the development of CD is likely to occur in temporally distinct phases in conjunction with abnormalities in a multiplicity of

cellular components, this study further establishes the concept that sub-optimal innate immune interactions with microbial stimuli may be central to the disease pathogenesis.

Penetration of luminal contents into the bowel wall is likely to be the initial triggering event in CD, which could be due to abnormal transcellular and paracellular permeability and/or interactions of the intestinal microflora with the mucosa. Permeability is likely to be influenced by exogenous factors, including emulsifying agents. Furthermore, the balance of mucosal lipid composition may also play a role in this process, although further work is required to establish this.

Subsequently, in healthy individuals, strong macrophage pro-inflammatory cytokine secretion ensures adequate neutrophil recruitment to sites of bacterial ingress in the bowel, enabling clearance of microbial material in a timely fashion. In patients with CD, pro-inflammatory cytokine secretion from macrophages is impaired in response to this material. This at least in partly due to mistargeting of pro-inflammatory cytokines to lysosomal compartments.

Heterogeneous molecular lesions underlie the impaired pro-inflammatory cytokine release. These may influence bacterial recognition (such as NOD2 variation), cytokine induction and secretion (products of GWAS loci, defective optineurin expression), and autophagy. These abnormalities are highly variable between patients. Furthermore, the most pertinent defects are likely to be different depending on the load and nature of the penetrating stimulus. Additional mechanisms (including a strong TLR2 response and the IL-23 axis), with modifying influences from environmental factors and microbial stimuli, may also be vital in compensating or compounding the defect in macrophage function *in vivo*. The attenuated macrophage pro-inflammatory cytokine release in CD results in delayed neutrophil recruitment and bacterial clearance

at sites of ingress. The persistence of this material would provoke a chronic inflammatory response (Figure 6.1).

If CD arises from impaired phagocyte function, therapies directed at stimulating innate immune function may be useful in CD. A randomised, placebo controlled trial of sargramostim (GM-CSF) published in 2005 indicated some potential benefit in active CD. Whilst the primary endpoint (decrease in CDAI score of at least 70 points at the end of treatment) was not significantly different between the sargramostim and placebo arms, significant improvement was observed as defined by secondary endpoints, including remission rate and fall in CDAI score by 100 points (18). Other immunostimulatory cytokines, such as IFN-γ, could also be useful but have yet to be fully evaluated. Such studies should take into account the clinical, genetic and functional heterogeneity between individual patients. Interestingly, the results of a phase 1 trial of sargramostim in paediatric CD were encouraging (17), but larger scale studies are required before translation into routine clinical practice. In the longer term, further study and characterisation of the gene expression outliers identified in this study could lead to novel therapeutic targets in subsets of patients, and new biomarkers for the diagnosis of CD and response to treatment.

6.3 Future directions

The studies conducted in this thesis have identified a number of interesting areas for future investigation, including:

6.3.1 Macrophage cytokine secretion and genotype correlations

- Correlation of defective TNF release in response to HkCa with the presence and titre of ASCA in patients with CD and their healthy relatives. A dysregulated innate immune response to *C. albicans* could provide a potential mechanistic

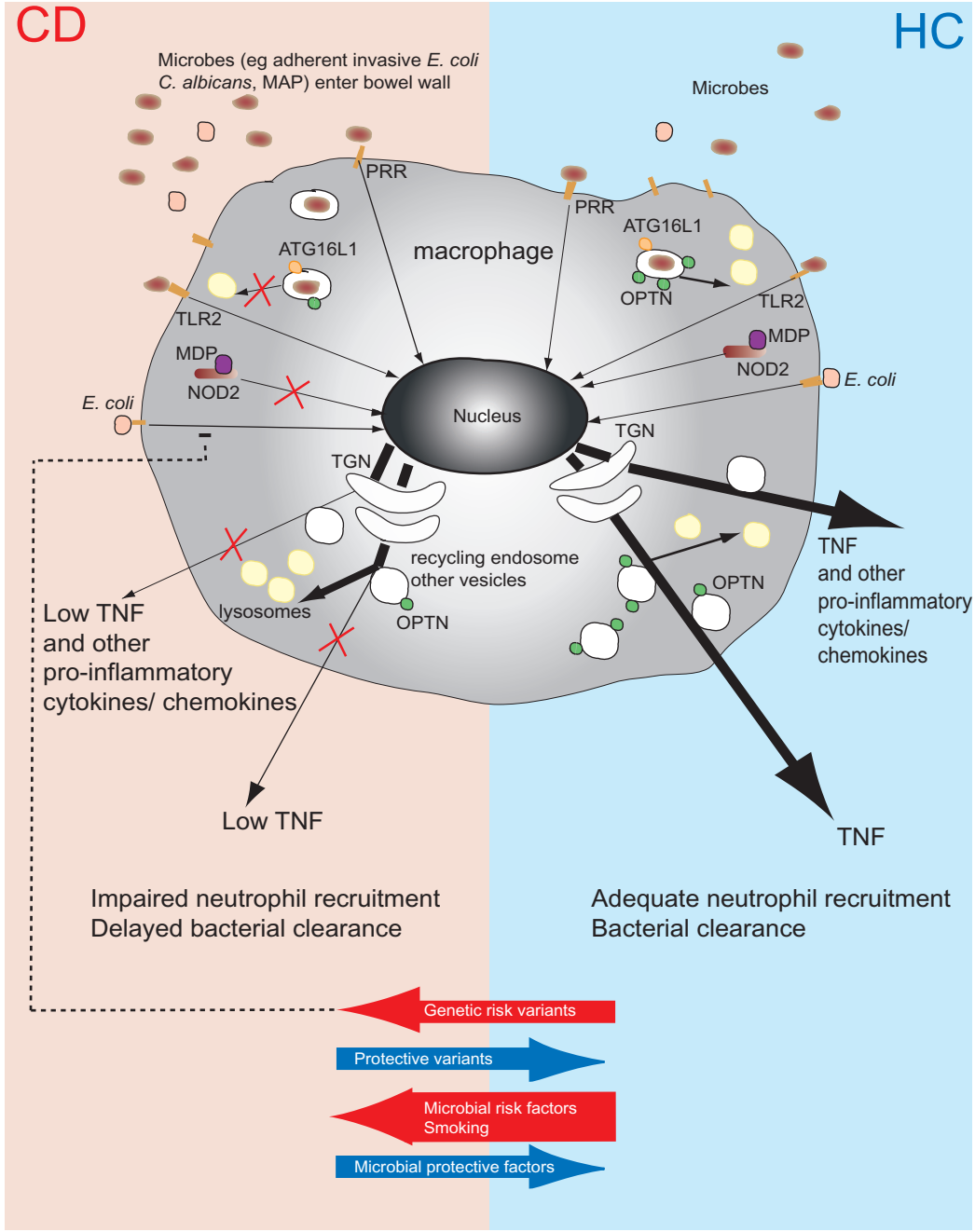

Figure 6.1 Defective macrophage function in Crohn's disease: a proposed pathogenic schema. Attenuated macrophage release of TNF occurs downstream of microbial stimuli in CD, including *E. coli,* TLR2 and TLR4 ligands and *C. albicans*, resulting in impaired neutrophil recruitment and delayed bacterial clearance when these microbial ligands penetrate into the bowel wall. The impaired pro-inflammatory cytokine release largely relates to aberrent post-translational trafficking of these molecules in CD macrophages, possibly involving recycling endosomes. Heterogeneous and complex molecular defects are likely to underly the defective cytokine release. In a subset of patients, reduced optineurin (OPTN) expression results in attenuated TNF release downstream of TLR2 activation. Deficient optineurin expression may also impair autophagy and intracellular bacterial clearance. Genetic factors linked to CD by GWAS also influence the macrophage response, including *NOD2* variation in the case of MDP stimulation. Furthermore, an increased burden of GWAS risk alleles has a cumulative effect to depress TNF release in response to *E. coli*. In HC individuals, a strong TLR2 response may be able to compensate for a high load of GWAS risk alleles. Environmental factors such as smoking and microbial components are also likely to influence the macrophage response *in vivo.*

connection between the colonisation of *C. albicans* and ASCA in CD patients and their healthy relatives, which warrants further study. Subject to ethical permission, the response to subcutaneous injection of HkCa could also be examined in CD patients. Recently, polymorphisms in the *CARD9* locus have been associated with CD (115), and several patients were identified as gene expression outliers for CARD9 in the transcriptomic study. As CARD9 may be involved in the innate immune response to fungi (419), future work could interrogate a possible relationship between the polymorphism, defective expression in the outlier patients and abnormal pro-inflammatory cytokine release to *C. albicans*.

Given that the mannan epitope for ASCA is also present in mycobacterial species such as MAP (98), and defective macrophage TNF release may also occur in response to these species (187), it would be interesting to perform parallel comparison studies of the response to these two organisms.

- Determination of intestinal macrophage cytokine secretion in response to microbial stimulation. Whilst a well designed experiment should ideally include investigation of intestinal macrophages isolated from patients with quiescent CD on minimal treatment, such studies are unlikely to be feasible. The serial biopsy technique utilised previously (135) could be adapted specifically to investigate TNF production at sites of repeated trauma in the bowel.
- Further investigation of the role of CD-susceptibility variants in macrophage pro-inflammatory cytokine secretion after bacterial stimulation. The present study indicated a relationship between attenuated TNF release after HkEc stimulation and overall GRS. Larger scale studies may be required to identify specific variants responsible for this association; which should be interpreted in the light of functional investigations in primary macrophages and macrophage

cell lines. A trend towards reduced TNF release after HkEc stimulation was observed in CD and HC individuals homozygous for CD-associated variants in *NOD2*; extension of the study to test larger numbers of individuals with these variants would increase the power to detect a small difference. Furthermore, 39 new susceptibility loci were identified in a more recent GWAS meta-analysis of CD. Several of these contain genes with putative roles in vesicle trafficking, including *VAMP3* and *SCAMP3* (115). It would be interesting to genotype patients and controls for these additional variants and investigate associations with macrophage TNF release. However, given the prevalence of these alleles in the HC population and their small contribution to the overall heritability of CD, it is likely that any effect will be subtle.

- Investigation of macrophage cytokine secretion in unaffected relatives of CD patients, and mono- and dizygotic twins. This would help prove whether diminished macrophage cytokine secretion is a primary abnormality, and the relative contribution of genetic and environmental factors to the phenomenon. Genotyping and calculation of the genetic risk score should be performed in these individuals, and any correlation with macrophage cytokine release, and other parameters such as intestinal permeability determined.
- Parallel investigation of vesicle trafficking in CD macrophages. Such studies are currently in progress in our laboratory. Findings presented here, coupled with recent work in macrophage cell lines point to the recycling endosomal compartment as potentially defective in these cells. This could be further investigated, for example, by tracking transferrin receptor recycling. Furthermore, the observation of abnormal optineurin expression in a subset of patients could be indicative of defects in vesicle subsets (discussed in 6.3.3) which warrant further investigation.

6.3.2 Lipid investigations in CD

- Determination of fatty acid synthesis and metabolism in cultured macrophages from CD patients. A reduced molar percentage of PI 16:0/18:1 was synthesised in CD macrophages, which may relate to an underlying defect in fatty acid synthesis, desaturation or metabolism. Such studies could employ stable isotope labelled acetate to determine synthesis over defined time points. Patients could also be genotyped for CD-associated variants in *FADS1*, *SPTLC2* and *ACSL6*, and relationships between the genotype and fatty acid synthesis could be determined.
- Further investigation of the PI changes apparent in the intestinal mucosa in CD. This could include characterisation of PI species present within colonic and ileal mucus, and investigation of PI composition and synthesis in cellular components isolated from mucosa, such as intestinal macrophages.

6.3.3 Macrophage transcriptomic abnormalities in CD

- Further study of the role of optineurin in macrophage TNF release, and the functional consequences of abnormal expression in CD. To clarify the mechanism by which optineurin influences TNF release, optineurin depleted cells could be incubated with inhibitors of intracellular trafficking such as brefeldin-A, monensin, ammonium chloride and chloroquine (as previously performed in primary CD macrophages (136)) and intracellular levels of pro-inflammatory cytokines quantified by western blotting. The possibility of colocalisation of TNF with optineurin in vesicles could be examined by confocal microscopy. In primary macrophage cultures from the outlier patients, TIRF could be used to follow vesicle transport and quantify fusion events at the plasma membrane. Furthermore, investigation of autophagy, directed migration

and lamellipodia projection is warranted, given the ascribed roles of optineurin in these processes.

- Investigation of optineurin expression in other cell types from the outlier patients, such as monocytes, to clarify whether the abnormality is macrophage specific or global. More generally, optineurin expression could also be determined in other cellular and tissue samples from CD patients, such as intestinal biopsy samples. The functional consequences of abnormal optineurin expression on handling of Gram positive and Gram negative bacteria could be determined in a variety of cell types.
- Investigation of the *in vivo* functional significance of abnormal optineurin expression. Our laboratory has obtained mice with a targeted insertion within the *OPTN* locus, that acts to disrupt expression of this gene. These mice are viable, and interestingly do not appear to develop spontaneous neurological, ocular or gastrointestinal phenotypes (unpublished finding). In order to determine whether optineurin deficiency impacts on the development and course of intestinal inflammation, these mice could be treated with DSS and TNBS, both of which are classical inducers of colitis. Given that CD involves an abnormal immune response to luminal microbes, investigation of infection-induced colitis models, such as *Citrobacter rodentium* may also be relevant. Finally, macrophages from these mice should be cultured and pro-inflammatory cytokine release examined in response to TLR stimulation, to validate findings in THP-1 cells. Similar studies are also in progress for *ADAMDEC1*, which was under-expressed in six patients with CD.
- Further sequencing of the *OPTN* genomic region in the optineurin outlier patients, including intronic regions to identify possible mutations in linkage

disequilibrium with the variants identified in this study, and their assessment as potential candidates.

- Sequencing of *UTS2, CARD9* and other outlier genes located in regions of CD susceptibility. A number of individuals were identified as gene expression outliers for genes located in CD susceptibility loci. Given the limited capacity of GWAS to identify rare variants, it is possible that these patients have a rarer variant or structural abnormality in this region in linkage disequilibrium with a common variant that leads to a gross abnormality in expression.
- Extension of the gene expression outlier strategy to identify abnormally expressed genes in individuals with other heterogeneous disorders, and validation of their functional relevance. Specifically, transcriptomic data has already been obtained from 45 individuals with UC. Macrophages from UC patients release abnormal levels of CXCL10 on LPS stimulation (163); this dataset should be fully interrogated for abnormalities in gene expression that could provide a molecular basis for this exaggerated immune response.
- Correlation of abnormally expressed genes in CD macrophages with GWAS susceptibility polymorphisms. A number of differentially expressed genes (before correction for multiple testing) identified in CD macrophages have been previously associated with GWAS susceptibility. Correlation of this abnormal expression with genotype information from these patients may shed light on the mechanisms by which these variants influence CD pathogenesis.

6.4 Conclusion

CD is a heterogeneous, multifactorial syndrome that is associated with considerable lifelong morbidity. Whilst its cause remains controversial, a considerable body of evidence now suggests that impaired acute inflammation and defective bacterial clearance may be critical factors. This thesis has provided a number of novel insights into the molecular pathogenesis of this chronic condition. Macrophages from CD patients release deficient levels of TNF in response to a range of microbial stimuli, including HkEc, TLR agonists and HkCa. The macrophage defect does not result from gross abnormalities in macrophage sphingolipid or phospholipid composition. In the case of HkEc, this attenuation of TNF release is partially related to an increased burden of GWAS risk alleles. A subset of CD patients also display abnormal macrophage expression of optineurin, which results in defective TNF release downstream of TLR2 stimulation. These findings increase our understanding of CD as a condition characterised by sub-optimal macrophage function, and indicate some of the likely molecular defects responsible in individual patients. In the future, this may facilitate the development of novel therapeutic strategies for CD.

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Appendix 1

Demographics of patients and volunteers

A

	HC	CD	UC
Number	39	43	25
Gender (M:F)	23:16	20:23	13:12
Mean age	36.7	38.3	41.5
Age standard deviation	11.3	13.6	15.8
Age range	21-63	20-65	22-74
Smokers	3	4	3
Treatment			
No medication		14	12
5-ASA only		21	11
Immunosuppressants		8	2
Disease location			
L1		13*	
L2		16	
L3		12*	
L4		2	
Age of onset			
A1		11	
A2		27	
A3		4	
Unknown		1	
Disease behaviour			
B1		22	
B2		12	
B3		9	
Perianal involvement		2	
Genotype information	37	41	

B

	HC	CD
Number	41	101
Gender (M:F)	21:20	37:64
Mean age	32.8	41.6
Age standard deviation	9.93	15.3
Age range	20-59	18-75
Smokers	5	20
Treatment		
No medication		25
5-ASA only		76
Immunosuppressants		0
Disease location		
L1		32
L2		44
L3		24
L4		1
Age of onset		
A1		15
A2		65
A3		21
Disease behaviour		
B1		63
B2		23
B3		15
Perianal involvement		9
Genotype information	36	97

C

	HC	CD
Number	16	18
Gender (M:F)	6:10	7:11
Mean age	38.4	39.4
Age standard deviation	11.3	14.5
Age range	23-57	20-65
Smokers	1	2
Treatment		
No medication		6
5-ASA only		10
Immunosuppressants		2

Appendix Table I. Demographics and Montreal classification of patients included in macrophage cytokine secretion assay studies (chapter 3). **(A)** Study investigating response to HkEc. **(B)** Study investigating macrophage response to TLR agonists. * One patient had ileal (L1) disease with upper GI (L4) involvement, another patient had ileocolonic (L3) disease with upper GI (L4) involvement. **(C)** Study investigating macrophage response to HkCa.. Immunosuppressants include methotrexate, azathioprine or 6-mercaptopurine.

A

	Unstimulated		HkEc stimulated	
	HC	**CD**	**HC**	**CD**
Number	7	8	7	12
M:F	5:2	4:4	5:2	6:6
Mean age	44.1	36.2	44.1	39.7
Age standard deviation	15.6	12.9	15.6	14.8
Age range	23-63	19-63	23-63	19-65
Smokers	0	0	0	1
Treatment				
No medication		2		4
5-ASA		6		8

B

	Unstimulated		HkEc stimulated	
	HC	**CD**	**HC**	**CD**
Number	10	13	7	8
M:F	5:5	6:7	5:2	4:4
Mean age	33.5	36.5	34.8	35.1
Age standard deviation	9.2	13.7	10.4	11.6
Age range	22-55	23-70	22-55	23-61
Smokers	1	1	0	1
Treatment				
No medication		6		3
5-ASA		7		5
Mean BMI	23.7	23.3	23.3	23.3
BMI standard deviation	3.5	3.0	3.8	3.7
BMI range	17.8-30.5	19.4-29.4	17.8-30.5	19.4-29.4

C

	HC	**CD**
Number	5	5
M:F	1:4	3:2
Mean age	40.0	33.1
Age standard deviation	17.8	10.1
Age range	21-54	20-46
Smokers	0	0
Treatment		
No medication		3
5-ASA		1
Methotrexate		1
Previous resection		2
Active disease		2

Appendix Table II Demographics of patients included in lipid studies (chapter 4). **(A)** Demographics of patients included in sphingolipid study. **(B)** Demographics of patients included in phospholipid study with body mass index (BMI). **(C)** Demographics of patients included in shotgun lipidomics study.

	HC	CD	UC
Number	43	61	45
Gender (M:F)	24:19	31:30	20:25
Mean age	36.8	39.0	40.4
Age standard deviation	10.9	14.2	13.2
Age range	21-61	18-69	21-74
Current smokers	4	7	4
Treatment			
No medication		16	31
5-Aminosalicylates		32	13
Immunosuppressants		8	1
Steroid		1	0
Biologic		4	0
Site of disease			
L1		19*	
L2		15	
L3		25*	
L4		2	
Age of onset			
A1		14	
A2		39	
A3		8	
Disease behaviour			
B1		32	
B2		12	
B3		17	
Perianal involvement		9	

Appendix Table III. Demographics and Montreal classification of patients included in microarray studies. * 1 patient with ileal disease had upper GI (L4) involvement, and 2 patients with ileocolonic disease had upper GI involvement. Immunosuppressants include azathioprine, 6 mercaptopurine or methotrexate. Biologic therapies include infliximab and adalimumab.

Appendix 2

Publications and presentations

Publications

Hayee B., Antonopoulos A., Murphy E.J., Rahman F.Z., **Sewell G**., Smith B.N., McCartney S., Furman M., Hall G., Bloom S.L. et al. 2011. G6PC3 mutations are associated with a major defect of glycosylation: a novel mechanism for neutrophil dysfunction. *Glycobiology.* 2011.

Hayee B., Rahman F.Z., **Sewell G**., Smith A.M., Segal A.W. 2010. Crohn's disease as an immunodeficiency. *Expert Rev Clin Immunol*.;6(4):585-96.

Palmer C.D., Rahman F.Z., **Sewell G.W**., Ahmed A., Ashcroft M., Bloom S.L., Segal A.W., Smith A.M. 2009. Diminished macrophage apoptosis and reactive oxygen species generation after phorbol ester stimulation in Crohn's disease. *PLoS ONE*.;4(11):e7787.

Sewell G.W., Marks D.J., Segal A.W. 2009. The immunopathogenesis of Crohn's disease: a three stage model. *Curr Opin Immunol.*;21(5):506-13.

Smith A.M., Rahman F.Z., Hayee B., Graham S.J., Marks D.J., **Sewell G.W.**, Palmer C.D., Wilde J., Foxwell B.M., Gloger I.S., Sweeting T. et al. 2009. Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J Exp Med*.;206(9):1883-97

Marks D.J, Rahman F.Z., **Sewell G.W**., Segal A.W. 2009. Crohn's disease: an Immune Deficiency State. *Clin Rev Allergy Immunol.*

Poster presentations

Sewell G.W., Smith A.M, Bielawski J., Hannun Y.A., Rahman F.Z., Roden D., Marks D.J., Hayee B.H., Bloom S.L., Walker A.P., Segal A.W. Evaluation of sphingolipids as candidate molecules for impaired macrophage-mediated acute inflammation in Crohn's disease. *The Macrophage: Intersection of pathogenic and protective inflammation*. Keystone Symposia Conference 2010.

Hayee, B., **Sewell, G**., Rahman, F. Z., Bloom, S. L., Smith, A. M., Segal, A. W. (2010). Differential bacterial clearance and cytokine secretion by macrophages explains localisation of Crohn's disease to the gut. Gut. (Vol.59 pp.A61-A61).

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Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease

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The cause of Crohn's disease (CD) remains poorly understood. Counterintuitively, these patients possess an impaired acute inflammatory response, which could result in delayed clearance of bacteria penetrating the lining of the bowel and predispose to granuloma formation and chronicity. We tested this hypothesis in human subjects by monitoring responses to killed *Escherichia coli* injected subcutaneously into the forearm. Accumulation of ^{111}In-labeled neutrophils at these sites and clearance of ^{32}P-labeled bacteria from them were markedly impaired in CD. Locally increased blood flow and bacterial clearance were dependent on the numbers of bacteria injected. Secretion of proinflammatory cytokines by CD macrophages was grossly impaired in response to *E. coli* or specific Toll-like receptor agonists. Despite normal levels and stability of cytokine messenger RNA, intracellular levels of tumor necrosis factor (TNF) were abnormally low in CD macrophages. Coupled with reduced secretion, these findings indicate accelerated intracellular breakdown. Differential transcription profiles identified disease-specific genes, notably including those encoding proteins involved in vesicle trafficking. Intracellular destruction of TNF was decreased by inhibitors of lysosomal function. Together, our findings suggest that in CD macrophages, an abnormal proportion of cytokines are routed to lysosomes and degraded rather than being released through the normal secretory pathway.

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Abbreviations used: Bref-A, brefeldin A; CD, Crohn's disease; cDNA, complementary DNA; DSS, dextran sodium sulfate; FDR, false discovery rate; GWAS, genome-wide association studies; HC, healthy control; HkEc, heat-killed *E. coli*; mRNA, messenger RNA; UC, ulcerative colitis.

Crohn's disease (CD) is a chronic inflammatory disorder, primarily affecting the gastrointestinal tract. It arises through an aberrant interaction between the bowel contents and the immune system, although the mechanistic basis of disease pathogenesis remains poorly understood. To date, numerous hypotheses have been proposed including atypical infection, the presence of abnormal particulate material within the bowel, and autoimmunity (Marks and Segal, 2008). Most current theories center on disordered T lymphocyte activation (Xavier and Podolsky, 2007). Although T lymphocytes are important in the induction and maintenance of the chronic inflammatory phase, their involvement in the induction of CD lesions remains unproven.

Recent genome-wide association studies (GWAS) have generated considerable interest after the identification of >30 polymorphisms that increase susceptibility to CD (Wellcome Trust Case Control Consortium, 2007; Barrett et al., 2008). Several of these refocus attention on the innate immune system.

Dr. Foxwell died on 17 December 2008

A.M. Smith and F.Z. Rahman contributed equally to this paper.

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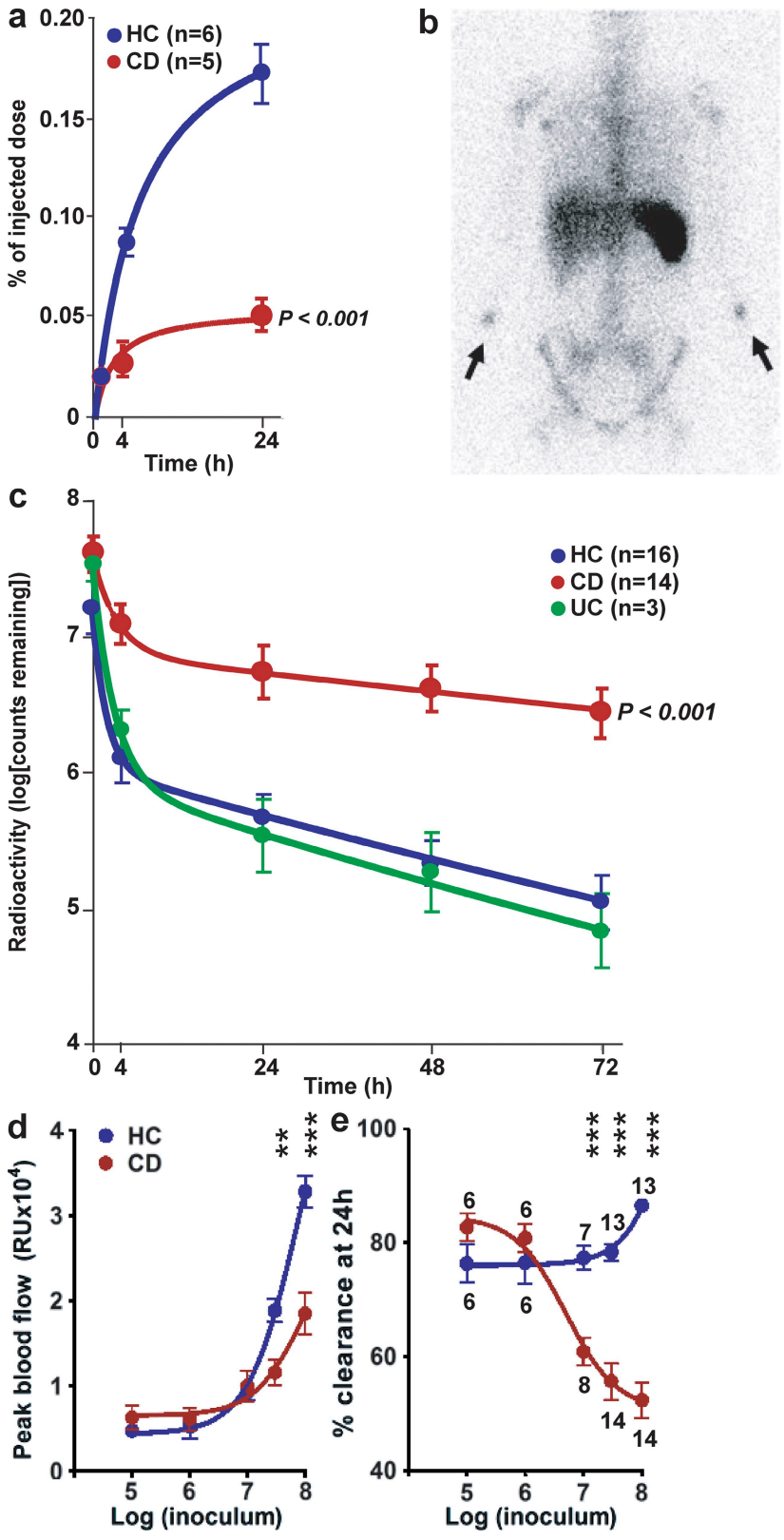

Figure 1. Neutrophil accumulation and subsequent clearance of *E. coli* from the tissues is markedly delayed in a dose-dependent manner in CD. ^{111}Indium-labeled autologous neutrophils were injected intravenously at the same time as killed *E. coli* were injected subcutaneously into each forearm. (a) Radioactivity measured over the injection sites showed a much smaller proportion of labeled cells accumulating in CD subjects. (b) γ-Camera image of a CD patient at 24 h after injection, demonstrating focal accumulations of radioactivity at bacterial injection sites (arrows) and confirming lack of bowel

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The *CARD15* (*caspase-recruitment domain 15*) gene remains the best studied and encodes a pathogen-recognition protein primarily expressed within phagocytes (Hugot et al., 2001; Ogura et al., 2001). Its product, NOD2, responds to bacteria-derived peptidoglycan by stimulating proinflammatory cytokine release, a function attenuated by the described polymorphisms. Other significantly replicated associations include the *ATG16L1* and *IRGM* genes coding for proteins involved in autophagy.

Although these associations are highly significant when comparing very large population groups and are valuable in providing an indication of cellular processes and pathways involved in CD pathogenesis, the effect sizes at any individual locus are weak (the majority incurring odds ratios <1.6), and polymorphisms are common in healthy control (HC) subjects. For example, the incidence of one or two CD-related mutations in the gene for NOD2 in the general population is ∼15 and 0.5%, respectively, whereas in CD the figures are 28 and 8% (Cuthbert et al., 2002; Hugot et al., 2007). However, only 1 in 1,000 of the population develop CD, which means that in a general population of 100,000 individuals, roughly 15,000 will have one relevant mutation and 500 will have two such mutations. This population will have ∼100 cases of CD, in whom there will only be 28 single and 8 double mutations on the NOD2 gene. The numbers are even more dramatic when examining mutations at the ATG16L1 (autophagy) locus with 27,000 of the general population having the GG CD-related genotype as compared with 37 patients (Hampe et al., 2007). GWASs have been informative but have failed to explain the cause of the diseases under investigation (Donnelly, 2008). All 32 CD-associated genes combined have been calculated to contribute <20% of the heritable risk (Barrett et al., 2008), leading to the concept of the "missing heritability" (Maher, 2008).

It was realized >30 yr ago that the acute inflammatory response in respect to neutrophil recruitment was defective in CD (Segal and Loewi, 1976). More recently, we have documented defective neutrophil accumulation and cytokine levels at sites of intestinal and skin trauma (Marks et al., 2006). These defects in acute inflammation were independent of the *CARD15* genotype. Further investigation demonstrated reduced blood flow in the skin upon bacterial challenge that could be partially corrected by the administration of sildenafil. We concluded that a general phenotypic abnormality can be revealed in CD when the innate immune system is stressed in vivo, namely that acute inflammation is generally and severely impaired. We propose that the consequence of a weak initial reaction to the ingress of gut bacteria into the tissues would be their defective removal and that this persistence could drive secondary chronic inflammation. A unique set of experiments were performed to test this hypothesis directly by measuring the accumulation of neutrophils and clearance of bacteria from the tissues of CD patients. In addition, in vitro studies were performed using monocyte-derived macrophages to identify a defective mechanism, which could explain the diminished acute inflammatory response in CD patients. In the absence of a single causative gene and, hence, an accurate animal model of CD, these experiments had to be conducted on human subjects.

RESULTS

Neutrophil recruitment to sites of bacterial injection

We first determined whether the previously observed impaired recruitment of neutrophils to sites of local trauma in CD extended to the inflammation induced by bacteria in the tissues (Segal and Loewi, 1976; Marks et al., 2006). Autologous neutrophils were labeled with ^{111}indium (Segal et al., 1981) and injected intravenously at the same time as *Escherichia coli* were inoculated into the volar aspect of each forearm in HC ($n = 6$) and CD ($n = 5$) subjects. The labeled neutrophils accumulated at the sites of bacterial injection (Fig. 1 a) in addition to the previously described distribution to the spleen, liver, and bone marrow (Fig. 1 b).

In HC subjects, significant cellular recruitment could be measured at 4 h (889 ± 53 cpm; $n = 6$), and this increased over a 24-h period (1,738 ± 98 cpm; $n = 6$), at which time ∼0.17 ± 0.014% of the injected dose of radioactivity was present at each injection site (Fig. 1 a). Assuming a whole body pool of roughly 10^{11} neutrophils (Cartwright et al., 1964), this represents $\sim 10^8$ cells to deal with the 3×10^7 bacteria injected. The results were very different in CD subjects ($P < 0.0001$; Fig. 1 a), with less than one-third of the normal numbers of neutrophils accumulating at 4 h (290 ± 52 cpm; $n = 5$) and 24 h (555 ± 78 cpm; $n = 5$).

Clearance of ^{32}P-labeled *E. coli*

A functional consequence of reduced neutrophil influx was assessed by measuring the clearance of ^{32}P-labeled *E. coli* from the forearm in HC ($n = 16$), CD ($n = 14$), and ulcerative colitis (UC; $n = 3$) subjects. Immediately after inoculation, surface counts at each injection site were 2,688 ± 160 cpm (mean ± SEM). In all groups, clearance followed biphasic nonexponential kinetics, with a rapid initial phase followed by a slower secondary phase (Fig. 1 c). An overall test for equality demonstrated that clearance curves for HC and CD subjects were significantly different ($P = 9 \times 10^{-6}$), with a marked delay in CD compared with HC. UC subjects demonstrated clearance profiles similar to those of HC. Extrapolating clearance curves

inflammation. (c) ^{32}P-labeled killed *E. coli* were injected into the subcutaneous tissues of the forearm and radioactivity was measured at the skin surface. Clearance of radioactivity was much slower in CD than in HC or UC. Extrapolating these curves indicated that almost complete removal (99%) would take 10.2 and 7.1 d in HC and UC subjects, respectively, compared with 44.3 d in CD. (d and e) Effect of increasing bacterial dose from 10^5 to 10^8 on blood flow at injection site (d) and bacterial clearance (e). The numbers of subjects studied in the dose response experiment are depicted in e. All results are expressed as mean ± SEM (**, $P < 0.01$; ***, $P < 0.001$).

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to a point where 99% of inoculated material would have been cleared gave total clearance times of 10.2 d (95% confidence interval: 8.3–13 d) in HC, 7.1 d (5.4–10.4 d) in UC, and 44.3 d (21.8–∞ d) in CD. Patients were subdivided into those with ileal or colonic involvement, as CD is a syndrome with both clinical and genetic evidence to suggest that precise pathogenic mechanisms operating in each region of the bowel may differ. Clearance appeared to be faster in the ileal ($n = 5$) than in the colonic ($n = 9$) CD subjects, but the differences were not statistically significant (Fig. S1 a). In addition, the presence of CD-associated mutations in *CARD15* had no effect on bacterial clearance (Fig. S1 b).

The numbers of bacteria injected had an important effect on local blood flow and bacterial clearance. In HC subjects, blood flow increased about sevenfold between doses of 10^5 and 10^8 organisms (Fig. 1 d). Blood flow levels in CD patients were similar to those in HC between dosages of 10^5 and 10^7 bacteria. Doses $>3 \times 10^7$ resulted in significantly reduced blood flow in CD subjects, with levels approximately half those recorded for HC. Bacterial clearance was dramatically different between the two groups (Fig. 1 e). In HC subjects, clearance rates were relatively stable between 10^5 and 3×10^7 organisms and actually increased at the highest dose, whereas in CD, although it was normal at 10^5 and 10^6, there was a dramatic and highly significant ($P < 0.0001$) drop in clearance at and above 10^7 bacteria.

Macrophage cytokine secretion

Dramatic differences were observed in the secretion of a panel of inflammation-related cytokines and chemokines by peripheral blood monocyte-derived macrophages after 24 h of stimulation with heat-killed *E. coli* (HkEc; Fig. 2). We observed distinct profiles in patients with ileal and colonic CD. The proinflammatory cytokines TNF, IL-4, IL-5, IL-13, IL-15, and IFN-γ were reduced in all CD patients, whereas IL-6, IL-12(p70), G-CSF, and GM-CSF were only low in colonic CD and IL-17 was only low in ileal CD. Direct comparisons between macrophages from ileal and colonic CD subjects identified IL-6, IFN-γ, and G-CSF as significantly different between the two phenotypes. All three were released at lower levels by colonic compared with ileal CD macrophages. In contrast, macrophages from patients with UC secreted proinflammatory cytokines at levels similar to, or greater than, HC subjects. All patients released normal levels of the antiinflammatory cytokines IL-1Ra and IL-10 and the chemokines RANTES, IP-10, MCP-1, and IL-8.

In addition, we examined the secretion of IL-5, IL-6, IL-10, IL-17, GM-CSF, MCP-1, and IP-10 at 24 h by macrophages after stimulation with the TLR2 ligand Pam_3CSK_4. Secretion of IL-5 and GM-CSF were both reduced irrespective of CD phenotype, whereas secretion of IL-6 and IL-17 were low from patient macrophages with colonic or ileal CD, respectively (Fig. S2 a). Previously, we have extensively studied CD patients with NOD2 mutations using muramyl dipeptide and reported transcription profile and cytokine secretion abnormalities (Marks et al., 2006). We have now examined the influence of NOD2 status on TNF secretion by macrophages in response to TLR2 or TLR4 agonists. The presence of one or two CD-associated mutations in the NOD2 gene had no effect on TNF secretion (Fig. S2 b).

Differences in monocyte/macrophage populations and maturation were investigated in the three groups (Fig. S3). The initial M1 ($CD14^+/CD16^-$) and M2 ($CD14^+/CD16^+$) monocyte population were similar in the all three groups, as were messenger RNA (mRNA) levels of the macrophage maturation markers F4/80, L-selectin, and ICAM-1 after in vitro differentiation.

Macrophage mRNA transcription profiles

To account for impaired cytokine secretion, we examined global transcription profiles of macrophages. RNA was examined from macrophages from HC ($n = 26$), ileal ($n = 10$), colonic CD ($n = 16$), and UC ($n = 20$) subjects, before and after exposure to HkEc. The proinflammatory cytokines GM-CSF, IL-6, IFN-γ, and TNF, which had demonstrated diminished secretion, were tested by quantitative PCR and found to have mRNA levels equivalent to those of HC after HkEc stimulation (Table S1). However, disease-specific changes were observed and are presented in Fig. 3. A four-way Venn diagram shows the numbers of probe sets differing in expression level ($P < 0.01$ and fold change >1.2) before and after stimulation between either group of CD patients and HC subjects (Fig. 3 a). Significant changes from HC levels were observed in the expression of hundreds of genes in the different groups, which were internally consistent. Surprisingly little overlap was observed between profiles of HkEc-stimulated macrophages in ileal and colonic CD.

The identities of the differentially regulated genes common to ileal and colonic CD, and the cellular processes in which they are involved, are shown in Fig. 3 (d and i). The 27 probe sets represent 18 genes of known function. Of these, nine have previously been reported as participating in vesicle trafficking and cytoskeletal organization, suggesting that the problem may lie in the posttranscriptional processing, storage, or secretion of cytokines. The data were also analyzed by determining the most significantly induced or repressed genes in individual groups, with false discovery rate (FDR) correction applied. These gave similar results to those shown in Fig. 3 (Fig. S4; Tables S2 and S3), demonstrating an overrepresentation of genes involved in vesicle trafficking and cytoskeletal organization.

To control for nonspecific effects resulting from chronic bowel inflammation, we conducted similar analyses on macrophages from UC patients. In UC, a total of 354 and 554 probe sets differed from HC, before and after HkEc stimulation, respectively. There was very little overlap between differentially expressed genes in CD and UC (Fig. 3, b and c). Of those genes common to both, a relatively large proportion was also involved in vesicle trafficking and cytoskeletal organization, as well as RNA metabolism and cellular signaling (Fig. 3 d, ii-iv). However, although most of the differentially expressed genes common to both CD

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groups were similarly up- or down-regulated in comparison with HC (Fig. 3 d, i), the majority of those common to UC and CD changed reciprocally (Fig. 3 d, ii-iv), highlighting the fact that UC and CD are distinct diseases, possibly at opposite ends of the inflammatory response (Marks and Segal, 2008).

a

pg/ml	SEM	n=				
22177	3206	17	9	12	12	TNF-α
34	2	5	4	8	11	IL-4
11	2	5	4	8	11	IL-5
64	16	20	13	18	11	IL-13
11	2	5	4	8	11	IL-15
2964	131	5	4	8	11	IL-17
545966	250666	15	9	10	11	IL-6
590	62	20	13	18	11	IL-12(p70)
11192	2221	20	13	18	11	IFN-γ
717	76	20	13	18	11	GM-CSF
10621	3506	15	9	10	11	G-CSF
122	38	5	4	8	11	IL-1β
22407	8055	15	9	10	11	RANTES
89345	16444	15	9	15	11	IP-10
7048	1348	15	7	10	11	MCP-1
274635	35575	19	7	14	17	IL-8
7623	1242	20	13	17	11	IL-10
21909	4182	20	13	17	11	IL-1Ra
		HC	Ileal CD	Colonic CD	UC	

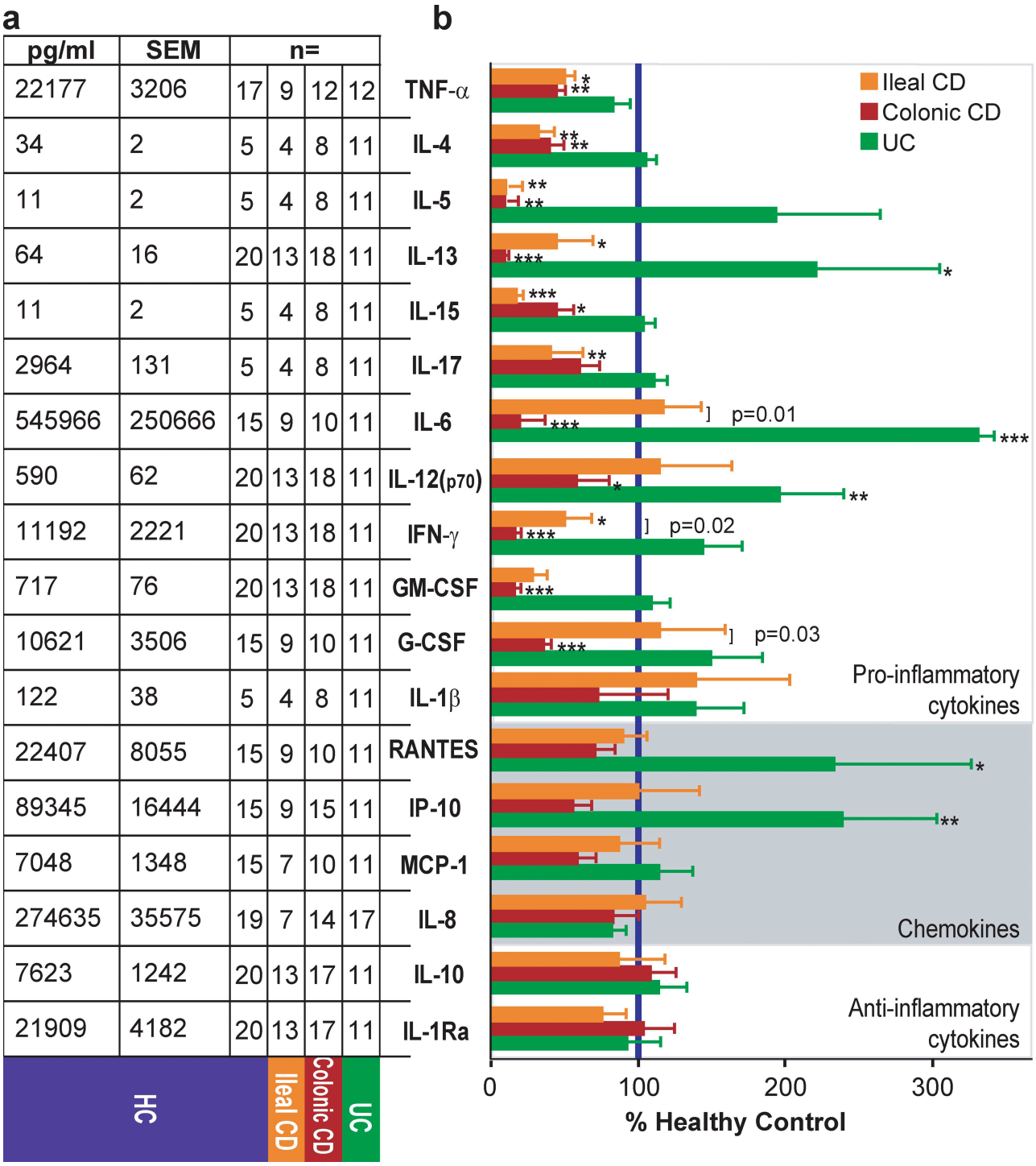

Figure 2. Proinflammatory cytokine secretion by macrophages from CD patients is deficient in response to *E. coli*. Supernatants from macrophages stimulated for 24 h with HkEc were tested for the levels of cytokines and chemokines. (a) Macrophages from HC subjects released varying amounts of cytokines and chemokines after HkEc stimulation. (b) Cytokine and chemokine release expressed as a percentage of that secreted by HC cells (blue bar) from ileal and colonic CD patients. The numbers of subjects in each group are shown on left; each patient was used once. All results are expressed as mean ± SEM (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

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Transcription, translation, and secretion of cytokines by macrophages

Our observations of diminished proinflammatory cytokine secretion, equivalent cytokine steady-state mRNA levels, and a high proportion of abnormally expressed genes associated with vesicle trafficking and cytoskeletal organization in CD led us to investigate further aspects of RNA metabolism and intracellular protein handling. Cytokine transcriptional activation was investigated using macrophages transfected with a TNF promoter–luciferase reporter. TNF gene transcription occurred in CD (n = 7) and HC (n = 8) macrophages after HkEc stimulation at equivalent levels (Fig. 4 a). TNF mRNA stability was determined using actinomycin-D (an inhibitor of RNA polymerase) and was identical between HC and CD macrophages after HkEc stimulation (Fig. 4 b).

Cell lysates were prepared from macrophages from HC (n = 6) and colonic CD (n = 6) subjects, and intracellular TNF was quantitated by Western blotting (Fig. 4, c and d). The level of intracellular TNF in HkEc-stimulated macrophages was lower in CD ($P < 0.05$) than HC (Fig. 4, c and d). The

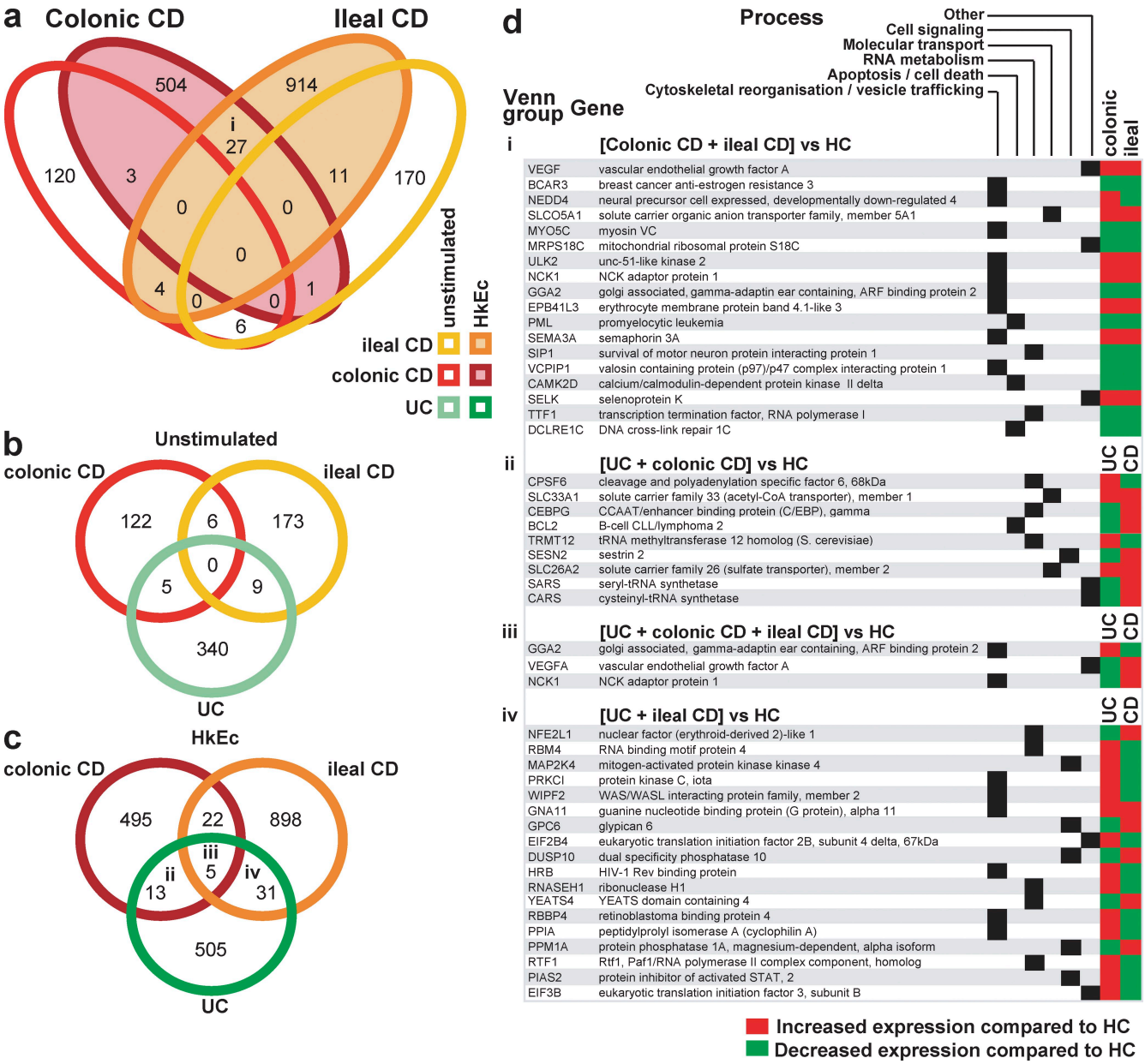

Figure 3. Abnormal transcriptional profiles of CD macrophages on Affymetrix gene arrays. (a) Venn diagram shows the number of probe sets differing in expression ($P < 0.01$) between ileal and colonic CD versus HC macrophages in the unstimulated state and after exposure to HkEc. (b and c) As in a, but comparing differentially expressed probe sets between ileal and colonic CD and UC versus HC in unstimulated (b) and HkEc-stimulated (c) cells. (d) i-iv designate overlaps of differentially expressed genes of known function after HkEc stimulation between disease groups, as labeled in Venn diagrams.

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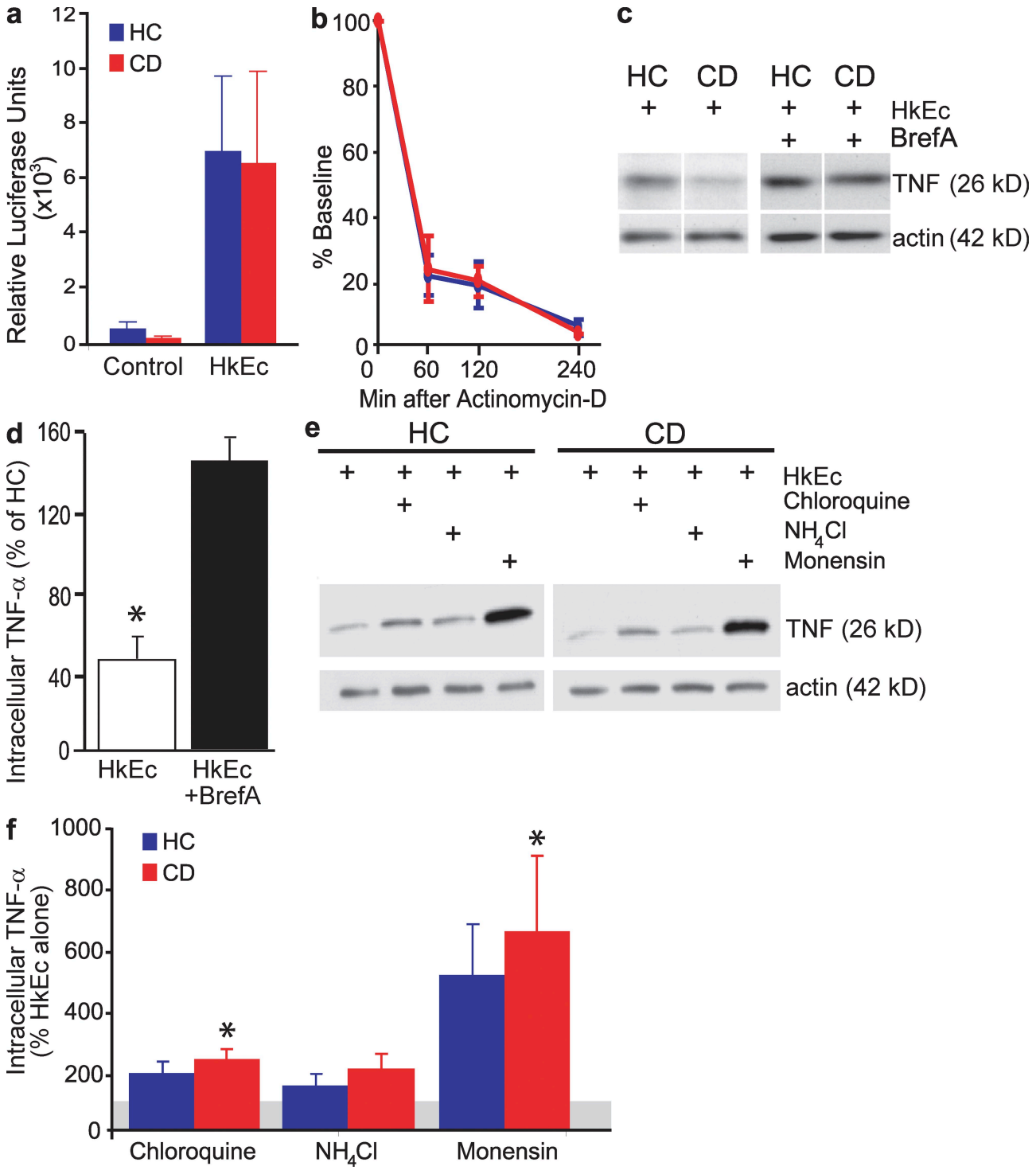

Figure 4. Intracellular levels of TNF are lower in CD, despite normal transcription and mRNA stability, but restored to normal in the presence of Bref-A and lysosome inhibitors. (a) Macrophages transfected with an adenoviral vector containing a TNF promoter and luciferase reporter demonstrated equivalent TNF transcription levels in HC ($n = 8$) and CD ($n = 7$) subjects. (b) TNF mRNA stability is comparable in HC and CD macrophages after stimulation with HkEc. (c) Intracellular levels of TNF after HkEc stimulation with or without Bref-A were determined by Western blotting. (d) CD contained significantly lower levels of intracellular TNF than HC macrophages after HkEc stimulation but returned to normal levels with the inclusion of Bref-A ($n = 6$ in all groups). (e) Intracellular levels of TNF after HkEc stimulation with or without lysosomal inhibitors were determined by Western blotting. (f) Inhibitors of lysosomal proteolysis increase intracellular TNF levels in HC and CD macrophages ($n = 4$ in all groups; gray line denotes level of HkEc alone). Significance levels are compared with HkEc alone. All patients in these studies had colonic CD and subjects were used once per assay. Results are shown as mean ± SEM (*, $P < 0.05$).

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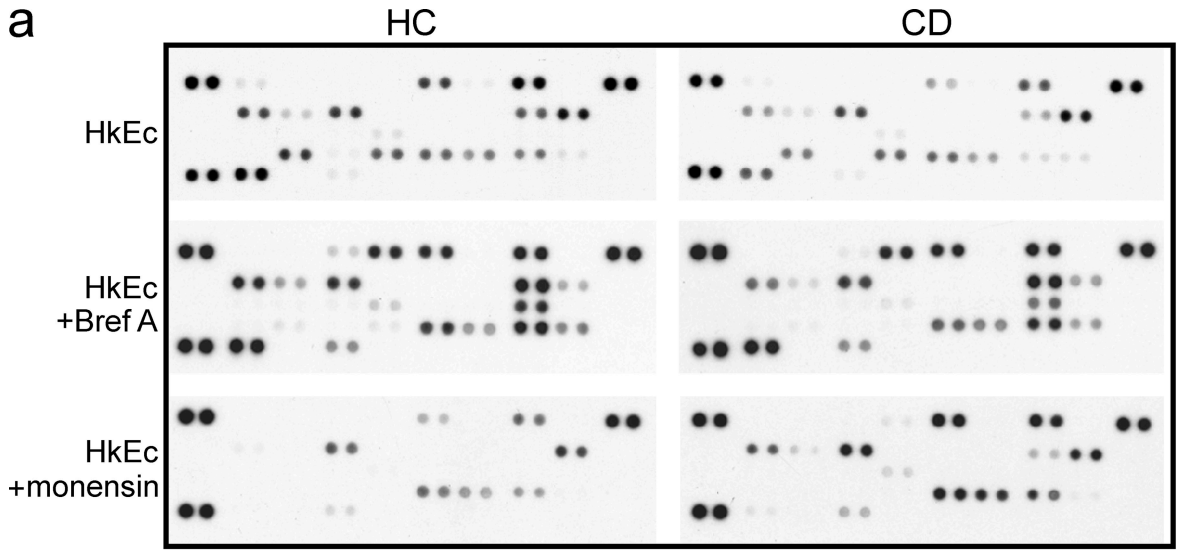

b

+	C5a	CD40L	G-CSF	GM-CSF	GROa	I-309	sICAM1	IFN-g	+
	IL-1a	IL-1b	IL-1ra	IL-2	IL-4	IL-5	IL-6	IL-8	
	IL-10	IL-12p70	IL-13	IL-16	IL-17	IL-17E	IL-23	IL-27	
	IL-32a	IP-10	I-TAC	MCP-1	MIF	MIP-1a	MIP-1b	serpin E1	
+	RANTES	SDF-1	TNF-a	sTREM-1					-

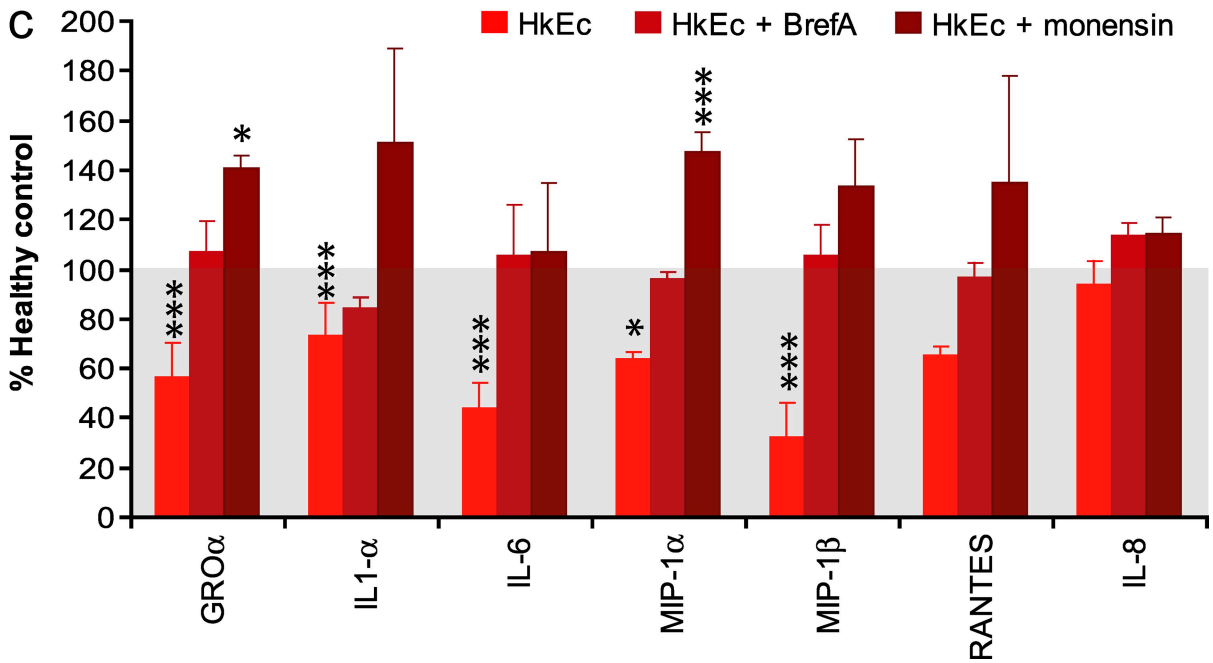

Figure 5. Intracellular levels of chemokines and cytokines after HkEc stimulation in the presence of vesicle trafficking and lysosomal inhibitors. (a) Intracellular cytokine array profiles obtained from macrophages stimulated with HkEc in the presence of absence of either Bref-A or monensin.

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inclusion of brefeldin A (Bref-A), a potent inhibitor of ER-to-Golgi protein transport during macrophage HkEc stimulation resulted in approximately equivalent levels of intracellular TNF in both CD and HC (Fig. 4, c and d). The results obtained with Bref-A demonstrated that TNF translation occurs normally in CD macrophages.

We investigated the role of the lysosome in intracellular cytokine trafficking with the inclusion of monensin, NH_4Cl, or chloroquine during macrophage HkEc stimulation. NH_4Cl and chloroquine partition into acidic compartments and elevate the pH, whereas monensin is a proton ionophore with similar but more potent effects. In HkEc-stimulated macrophages from colonic CD patients ($n = 4$), all three inhibitors of lysosomal function increased intracellular levels of TNF by between twofold and sevenfold (Fig. 4, e and f). Incubating macrophages with the proteosome inhibitor MG132 was without effect (unpublished data).

In addition to TNF, the intracellular levels of GRO-α, IL-1-α, IL-6, MIP-1α, and MIP-1β were also depressed in CD macrophages after HkEc stimulation (Fig. 5). The inclusion of Bref-A resulted in the normalization of intracellular levels of all the cytokines and chemokines tested (Fig. 5, a and c). In comparison, monensin either normalized the intracellular levels (IL-1α, IL-6, and MIP-1β) or resulted in a significant elevation compared with HC ($n = 4$; GRO-α and MIP-1α; Fig. 5, a and c).

DISCUSSION

In contrast to the relatively weak genetic effects identified in the GWAS (Wellcome Trust Case Control Consortium, 2007), a common major phenotypic abnormality becomes apparent in CD when the innate immune system is stressed in vivo. This is the result of a severely impaired acute inflammatory response in these individuals (Marks et al., 2006). We have suggested that the intensity of the inflammatory response in the whole population follows a normal Gaussian distribution. Individuals at the extreme lower tail of this spectrum, as a consequence of the additive effect of many gene variants, are at risk of CD. NOD2, and potentially other systems, provide a compensatory boost to acute inflammation, which is protective in this setting (Marks and Segal, 2008).

These studies provide the first demonstration in CD that there is a profound defect in the recruitment of neutrophils after the introduction of *E. coli* into the tissues and that the subsequent clearance of these organisms is grossly delayed in this condition. This bacterium was used in these studies because it is the major aerobic component of the bowel flora and present in those regions of the gut affected by CD (Finegold, 1969). *E. coli* have been implicated in the pathogenesis of CD (Rhodes, 2007), have been found within macrophages in Crohn's tissue (Liu et al., 1995), and have also been cultured from draining mesenteric lymph nodes (Ambrose et al., 1984b). Granulomas removed from CD tissue have been shown by PCR to contain *E. coli* DNA (Ryan et al., 2004), and an ileal CD *E. coli* isolate (LF82) was capable of inducing granuloma formation in vitro (Meconi et al., 2007). In addition, we had previously shown that there was an important abnormality of acute inflammation in response to *E. coli* in CD (Marks et al., 2006).

The observed abnormalities in CD did not occur as a consequence of the inflammatory process, drug therapy, or bowel ulceration. All the patients were in remission and most were not on therapy, with a minority receiving 5-aminosalicylic acid–containing drugs. Bacterial clearance was normal in the control group of subjects with UC, who were also in remission and on 5-aminosalicylic acid or no treatment.

The rate at which neutrophils accumulate at sites of bacterial ingress into the tissues is crucial to the final outcome. It has previously been demonstrated that in a three-dimensional tissue matrix, bacterial phagocytosis is directly related to neutrophil numbers (Li et al., 2004). In the absence of adequate neutrophil recruitment, monocytes and macrophages might function in a containing role, such that material remaining after inoculation is surrounded and only slowly degraded (Issekutz et al., 1981). This is supported by the recovery of bacterial products from Crohn's granulomata (Ryan et al., 2004).

The consequence of the delayed neutrophil accumulation is that foreign fecal material is not cleared adequately from the tissues, leading to granulomatous inflammation. Congenital monogenic innate immunodeficiencies, in which neutrophil dysfunction predominates, frequently manifest bowel inflammation that is indistinguishable from CD (Rahman et al., 2008). These disorders are also associated with major defects in the clearance of foreign material caused by failure of neutrophils to accumulate in sufficient numbers (congenital neutropenias and leukocyte adhesion deficiency), poor digestion within excessively acidic phagolysosomes (chronic granulomatous disease and glycogen storage disease-1b), and impaired vesicle trafficking and fusion of the granules and phagosomes (Chediak-Higashi and Hermansky-Pudlak syndromes). The genes underlying these disorders have not been detected by GWAS of CD because of their extreme rarity.

In the absence of a monogenic lesion in the immune system, CD patients might also be unduly susceptible to bacterial infection, although the necessary large-scale studies have not been performed (Kyle, 1980; Ambrose et al., 1984a; Kaplan et al., 2007). It becomes more difficult to assess the incidence of disease-specific infection once a diagnosis of CD has been made because of confounding factors including surgery, malnutrition, immunosuppressant therapy, and bowel-related complications such as abscesses and fistulae. Another consideration is bacterial load. Most acute infections originate from

(b) Cytokine array map. (c) Various chemokines and cytokines demonstrate reduced intracellular levels in CD macrophages after HkEc stimulation and are either normalized or elevated after Bref-A or monensin treatment ($n = 4$ in all groups). All patients studied had colonic CD and subjects were used once per assay. Results shown are mean ± SEM (*, $P < 0.05$; ***, $P < 0.001$).

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a small number of inoculating organisms, which even the dampened immunity in CD appears to be able to control. Our findings of the relationship between bacterial dose and clearance are important in this respect because they demonstrate that CD patients can deal efficiently with small numbers of organisms in the tissues but that the clearance mechanisms are overwhelmed by a large bolus of bacteria. CD occurs almost exclusively in the terminal ileum and colon, which respectively contain $\sim 10^8$ and 10^{11} bacteria/ml, higher than the 10^5 bacteria/ml or less in other regions of the small bowel (Farthing, 2004). Reflux through the ileocaecal valve carries caecal contents into the terminal ileum, elevating the microbial levels in this region (Malbert, 2005). After surgery, recurrence is seen in ~80% of cases, and where small bowel has been resected, recurrence generally occurs in the small bowel just proximal to the anastomosis. This indicates that recurrence is highly dependent on the proximity to the large bowel contents rather than a specific abnormality associated with the terminal ileum (Cameron et al., 1992). Therefore, the large bowel and adjacent ileum provide a unique environment in the body that contains a massive bacterial load, which can gain instant access to the tissues if the mucosal barrier is breeched.

The detrimental effect of fecal content in the tissue might not primarily result from the infectious nature of the organisms, as many are not particularly virulent, but by the quantity of organic material that must be removed before resolution can occur. Difficulty in clearing exogenous organic material could also account for the false-positive results in CD to Kveim tests, which were conducted to diagnose sarcoidosis by injecting a preparation of the spleen of a patient into the skin, which was subsequently examined for a granulomatous response (Mitchell, 1971).

Clearly, the retention of fecal material in the tissues of the wall of the bowel will have major local and systemic inflammatory, immunological, and constitutional consequences. Many of the immunological studies conducted on Crohn's patients, such as those implicating adaptive immunity in local tissue damage (Xavier and Podolsky, 2007), might be measuring secondary immunological responses to this foreign material.

We then turned our attention to identifying the mechanism underlying impaired neutrophil accumulation. CD is caused by the complex interplay of many genes coupled with bacterial exposure, which makes it difficult, if not impossible, to identify specific molecular lesions common to these patients, and GWASs clearly indicate that there are no dominant causative genes. However, the characteristic pathological features of the condition are suggestive of a common cellular pathology produced by the various additive molecular aetiologies. The neutrophils themselves appear functionally normal (Moráin et al., 1981), and their diminished migration to acute inflammatory sites in the skin or bowel has been attributed to defective local cytokine production (Marks et al., 2006). These mediators are primarily secreted by resident tissue macrophages (Medzhitov, 2008), which, in the bowel, are derived and continually replenished from peripheral blood monocytes (Smythies et al., 2006). We therefore measured the secretion of a panel of inflammatory cytokines and chemokines by macrophages in response to stimulation with *E. coli* or TLR ligands and observed the production of dramatically low levels of proinflammatory cytokines in cells from CD patients. In our previous study, we found local levels of IL-8 and IL-1β to be low after trauma to the skin and bowel (Marks et al., 2006). In the current study, we have found that macrophages secrete normal amounts of IL-8 after stimulation with bacteria. This discrepancy between tissue levels and isolated macrophages in response to *E. coli* could lie in the type of cells producing IL-8 in skin windows and bowel biopsies, and/or in a different agonist. Neutrophils themselves produce copious amounts of IL-8 and IL-1β, so the lower levels in skin windows and bowel biopsies in CD are consistent with the reduced recruitment at these sites (Fujishima et al., 1993; Greten et al., 2007). In the study by Marks et al. (2006), the release of IL-8 by macrophages from CD patients was diminished after stimulation with wound fluid, TNF, and C5a but unaltered after stimulation with the TLR4 ligand lipopolysaccaride. IL-8 release is normal after bacterial stimulation (whole *E. coli* as well as TLR2 stimulation), so the lower levels in the in vivo study could be the result of activation of macrophages by nonmicrobial agonists.

To explain the cause of impaired cytokine secretion by macrophages from CD patients, we examined the profiles of RNA expression against a chip containing fragments of genes representing the whole human transcriptome. The first important observation was that macrophages from patients with colonic or ileal CD and UC exhibited distinct expression profiles when compared with HC, identifying these as three separate disease clusters. Second, levels of mRNA encoding proinflammatory cytokines were normal in unstimulated and HkEc stimulated macrophages, providing evidence that the defective secretion was not at the level of gene transcription.

A possible mechanism responsible for diminished proinflammatory cytokine release was provided by examining the abnormally expressed genes common to both types of CD after HkEc stimulation. Approximately half of the genes had some association with cellular secretory systems, suggesting that impaired macrophage secretion is responsible for the diminished proinflammatory cytokine release. The failure of a secretory system would provide a generic mechanism for the impaired release of several functionally related cytokines. Hermansky-Pudlak syndrome and Chediak-Higashi disease, both of which are associated with noninfectious granulomatous enterocolitis, are the result of primary abnormalities in the cytoskeletal transport of vesicles (Rahman et al., 2008). These findings are particularly compelling given the recent descriptions of variants in autophagy genes which are associated with CD susceptibility (Hampe et al., 2007; Parkes et al., 2007; Rioux et al., 2007). Autophagy involves vesicle trafficking in the formation of double-membrane vesicles that deliver cytoplasmic material to the lysosomes (Levine and Deretic, 2007). Additionally, autophagy is required to kill and remove intracellular organisms in macrophages, so its dysfunction is consistent with the theme we have developed:

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that CD results from a failure to digest and remove microbial and other foreign material.

Interestingly, there was no other direct relationship between the collections of genes identified in this study in macrophages and the polymorphisms highlighted by the previous GWAS (Wellcome Trust Case Control Consortium, 2007; Barrett et al., 2008), with the exception of HLA-G and SPTLC2, which were up-regulated and down-regulated, respectively, in colonic CD (Table S3). This is not entirely unexpected because association studies interrogate the whole genome in a nonselective manner, whereas we have focused on RNA expression in a single cell type, which plays a pivotal role in inflammation, in resting cells and those after activation with a naturally occurring agonist.

Several microarray expression studies have previously been performed in CD and UC. However, their experimental approach differed greatly from ours, as did the results they obtained. This is not surprising in view of the very diverse cell populations and variable states of stimulation in their samples. Those on blood cells were performed on freshly isolated unstimulated mixed cell populations consisting of lymphocytes and monocytes with granulocyte contamination (Mannick et al., 2004; Burczynski et al., 2006). The majority used bowel biopsies, in which a wide spectrum of cells is present in noninflamed tissue. These are further supplemented by innate and adaptive immune cells when inflammation is present (Lawrance et al., 2001; Costello et al., 2005; Hughes, 2005).

To understand the failure of macrophages to secrete normal quantities of proinflammatory cytokines in CD, we used TNF as a model, because this molecule has been extensively studied and experimental tools are readily available. We found that gene transcription and mRNA stability are normal in CD macrophages. Bref-A prevents vesicular movement between the ER and the Golgi, resulting in accumulation of proteins within the former. Stimulation in the presence of Bref-A revealed normal cytokine synthesis by CD macrophages. Normal synthesis coupled with deficient secretion and low intracellular levels of the cytokines indicate accelerated degradation. The accumulation of normal amounts of TNF and other proinflammatory cytokines after the addition of lysosomal inhibitors to CD cells indicates that this aberrant breakdown occurs in the lysosomal compartment.

The observation that macrophage secretion of TNF in CD is abnormally low might at first seem paradoxical, given the therapeutic efficacy of TNF blockade in many of these patients (van Deventer, 1999). However, we are comparing two temporally distinct events, acute inflammation over a time course of hours with chronic inflammation over weeks to months. The acute inflammatory response, dependent on the acute release of proinflammatory cytokines, is important for the clearance of bacteria and fecal material from the tissues, which takes place over a few hours. The lack of TNF after the administration of therapeutic antagonists for the treatment of other chronic inflammatory diseases has been reported to precipitate the development of CD (Charach et al., 2008; Yazisiz et al., 2008).

Failure to clear foreign material from the tissues induces a chronic granulomatous reaction around the retained organic material over a much longer time frame. The accumulation of macrophages and T lymphocytes in these granulomata leads to local tissue damage and constitutional symptoms through the sustained secretion of cytokines (Bazzoni and Beutler, 1996). Even if the net production of cytokines by each cell were lower than normal, the overall number of cells is so great that damaging concentrations are produced. In this setting, drugs directed against TNF can prove beneficial by direct cytokine blockade, by inducing leukocyte apoptosis, and by stimulating the effect of T_{reg} cells, which suppress inflammation (Wong et al., 2008). A clear example of the dichotomous effect of TNF is provided by the dextran sodium sulfate (DSS) bowel inflammation model in TNF knock-out mice. Contrary to initial expectations, mice deficient in this cytokine are more susceptible to the induction of bowel inflammation by DSS, whereas TNF inhibition is effective in ameliorating established DSS colitis in wild-type animals (Naito et al., 2003).

The relationship of impaired cytokine secretion to disordered packaging and vesicle transport, rather than defects in their production or stability, provides a novel insight into the mechanisms underlying the pathogenesis of CD. Similar mechanisms could lead to abnormalities in other relevant cell types, such as the Paneth cell (Cadwell et al., 2008). A basic abnormality in macrophage biology could also explain extraintestinal manifestations of CD, such as arthritis, and lesions in the eyes, skin, lungs, and other tissues (Ephgrave, 2007). There may be many other diseases that present with exuberant granulomatous inflammation as a result of an underlying failure of acute inflammation and innate immunity, leading to defective clearance of the initiating agent.

MATERIALS AND METHODS

Patients. These studies were approved by the Joint University College London (UCL)/UCL Hospitals (UCLH) Committee for the Ethics of Human Research (project numbers 02/0324 and 04/Q0502/29) and the Administration of Radioactive Substances Advisory Committee (RPC597-790). Patients from the Gastroenterology outpatient clinic at UCLH who met inclusion criteria were recruited to participate in the study. All patients had definitive diagnoses of CD or UC, which were confirmed using standard diagnostic criteria, with quiescent disease (Harvey-Bradshaw or Mayo score <3; Harvey and Bradshaw, 1980; Schroeder et al., 1987). Patients on either no medication or a stable maintenance dose of 5-aminosalicylates (2.5 g/d) for the previous 3 mo were included. None of the patients had received corticosteroid, immunosuppressant, anti-TNF, or metronidazole therapy within 3 mo of enrollment. HC subjects approximately matched for age, sex, and smoking history were recruited from the Department of Medicine, UCL. Written informed consent was obtained from all volunteers. No subject was studied more than once in each of the different experiments. Details of patients and HC subjects included in in vivo experiments are provided (Table S4). CD patients were genotyped for the three common polymorphisms in *CARD15* as previously described (Table S4; Marks et al., 2006). In some studies, we compared the results obtained from CD patients with ileal or colonic involvement, as there is evidence that different pathogenic mechanisms might operate in these regions of the bowel (Cuthbert et al., 2002). We excluded the ~30% of CD patients with mixed ileocolonic disease to simplify phenotyping and analysis.

Neutrophil accumulation studies. Details of controls and patients are provided in Table S4. No patient had received corticosteroid, immunosuppressant,

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anti-TNF, or nonsteroidal drug therapy within 3 mo of enrolling in the study, although patients on maintenance 5-aminosalicylates with no change in their dose in the previous 3 mo were included. Patients with a history of skin disease were not enrolled for any injection studies.

A fully antibiotic-sensitive clinical isolate of *E. coli* (NCTC 10418) was grown overnight at 37°C in DME (Sigma-ALdrich) and 1.25 g/liter sterile yeast extract (Oxoid). Bacteria were then killed by exposure to a UV source (ChemiDoc, trans-UV mode; Bio-Rad Laboratories) for 60 min, and washed multiple times in sterile saline. Bacterial concentrations were determined by optical density (OD_{600} = 0.365 equates to 10^8 *E. coli*/ml [Yourassowsky et al., 1989]). Sterility of the final sample was confirmed by multiple cultures.

Peripheral venous blood was collected from subjects into syringes containing 5 U/ml heparin. Separate blood samples were taken for full blood count and routine serum biochemistry. Neutrophils were isolated by centrifugation through Lymphoprep (Axis Shield), and then erythrocytes were removed by sedimentation with 10% dextran followed by hypotonic lysis. The neutrophils were then washed once in sterile injection-grade normal saline and incubated with 6 MBq ^{111}In-oxine (GE Healthcare) for 20 min at room temperature (Segal et al., 1981). A mean ± SEM of 3.40 ± 0.35 × 10^7 cells labeled with 5.15 ± 0.17 MBq (n = 11) were resuspended in 5 ml of normal saline. These were injected intravenously through a cannula in a vein in the antecubital fossa, which was subsequently flushed with a further 10 ml of normal saline.

Immediately after neutrophil injection, 3 × 10^7 UV-killed *E. coli* suspended in 100 µl of normal saline were injected subcutaneously into the volar aspect of each forearm. Radioactivity was counted using a β counter in scaler mode (RadEye B20; Thermo Fisher Scientific) over these injection sites at baseline and at 1, 4, and 24 h after injection. These sites were shielded from the remainder of the body during counting to avoid detection of label accumulating in the liver or spleen. To control for background radioactivity, this was counted over a control site in the left calf and these counts were subtracted from counts over the injection sites to obtain specific accumulations of radioactivity produced by the bacterial injections. The absolute radioactivity at the injection sites was determined by calibration of the meter against serial dilutions of a sample of free ^{111}In-oxine with known activity. The 24-h counts over the injection sites in the six HC subjects were 1,738 ± 98 cpm. We did not study control subjects with UC because we felt that the additional information we would obtain did not justify the theoretical risk of administering radioactivity systemically to these subjects.

Bacterial clearance studies. Details of controls and patients included in each set of experiments are provided in Table S4. *E. coli* were grown, as before, overnight at 37°C in 9 ml of phosphate-free DME (Invitrogen) supplemented with 1 ml of phosphate-containing DME (Sigma-Aldrich), 1.25 g/liter of sterile yeast extract, and 18.5 MBq ^{32}P-orthophosphate PBS (GE Healthcare). This isotope was chosen because it is a β emitter with a short half-life (~14.7 d) but of sufficient energy to emit through skin and readily and stably incorporate into bacteria (Jordan, 1970). Labeled bacteria were killed by UV exposure for 60 min and washed multiple times in sterile saline to remove unbound radiolabel. A specific activity ± SEM of 6.39 ± 1.52 MBq per 10^8 bacteria was achieved. Unlabeled bacteria from the same reference sample were also grown and killed by UV exposure for 60 min, in the absence of radiolabel and in phosphate-containing DME supplemented with sterile yeast extract.

A preparation of *E. coli* containing 1 kBq of radioactivity was mixed with unlabeled bacteria to produce a dose range of 10^5, 10^6, 10^7, 3 × 10^7, and 10^8 bacteria in 100 µl of sterile saline for inoculation (different doses were used depending on the experiment) and injected into the volar aspect of both forearms. Surface radioactivity was counted over a 3-min period using a β counter in scaler mode (RadEye B203). Initial surface counts per minute at each site were 2,688 ± 160 (mean ± SEM). Counts were subsequently repeated at 4, 24, 48, and 72 h. Correlations between the readings in each arm were r^2 = 0.67 ($P < 0.005$).

Radiation dose in bacterial clearance studies. Using values published for ^{32}P in the MIRDOSE 3.1 spherical tumor model (Stabin, 1996), with a presumed tissue dispersion volume of 0.1–3.9 ml, a dose of 1 kBq was calculated to deliver an absorbed dose of 0.06–1.00 Gy if no clearance occurred. If a biological half-life of 3 h was presumed, the absorbed dose was estimated at 0.5–10.0 mGy.

The threshold dose of ^{32}P resulting in erythema after extravasation has been determined as 10 Gy (Castronovo et al., 1988). A dose of 1 kBq was therefore selected as producing sufficient emitted radioactivity to be recordable at the skin surface but falling well within acceptable established safety limits and ensuring that inflammation generated was solely the result of the presence of bacteria rather than isotope.

Macrophage isolation and culture and stimulation. Peripheral venous blood was collected from subjects into syringes containing 5 U/ml heparin. Mononuclear cells were isolated by differential centrifugation (900 g for 30 min at 20°C) over Lymphoprep and washed twice with sterile PBS (Invitrogen) at 300 g (5 min at 20°C). Cells were resuspended in 10 ml RPMI-1640 medium (Invitrogen) supplemented with 100 U/ml of penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), and 20 mM Hepes, pH 7.4 (Sigma-Aldrich) and plated at a density of ~5 × 10^6 cells/ml in 8 cm^2 Nunclon Surface tissue culture dishes (Nunc) at 37°C in 5% CO_2. After 2 h, nonadherent cells were discarded and 10 ml of fresh RPMI supplemented with 10% FBS (Sigma-Aldrich) was added to each tissue culture dish. Cells were then cultured for 5 d at 37°C in 5% CO_2, with the addition of a further 10 ml of fresh 10% FBS/RPMI after 24 h.

Adherent cells were scraped on day 5 and replated in 96-well culture plates (Nunc) at equal densities (10^5/well) in X-Vivo-15 medium (Cambrex). These primary monocyte-derived macrophages were incubated overnight at 37°C in 5% CO_2 to adhere and were then stimulated for up to 24 h, depending on the experiment, with 2.5 × 10^5 HkEc, prepared as previously described (Marks et al., 2006), 2 µg/ml Pam_3CSK_4 (Enzo Biochem, Inc.), or 200 ng/ml LPS (Enzo Biochem, Inc.).

Cytokine secretion assays. Macrophage supernatants were collected after 24-h stimulation of primary monocyte-derived macrophages with HkEc as described in the preceding section. The expression profile of a panel of cytokines in macrophage supernatants was measured using the Beadlyte Bio-Plex human cytokine assay (Bio-Rad Laboratories) according to the manufacturer's instructions. Our assay was customized to detect and quantify IL-1Ra, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-15, IL-17, G-CSF, GM-CSF, IFN-γ, IP-10, and MCP-1. IL-8 secretion was measured using an ELISA kit (R&D Systems). The assay was conducted according to the manufacturers' instructions with recombinant human standards.

TNF release was measured using a cytotoxicity bioassay (obtained from B. Beutler, The Scripps Institute, La Jolla, CA) as previously described (Aggarwal et al., 1985). Mouse L929 fibroblast cells were grown in DME (Invitrogen), supplemented with 10% FBS (Sigma-ALdrich), 100 U/ml of penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen) at 37°C in 5% CO_2. A confluent monolayer of mouse L929 fibroblasts was trypsinized and resuspended to 4 × 10^5 cells/ml in DME. L929 cells were seeded into 96-well flat bottomed tissue culture plates (4 × 10^4 cells/well) and incubated overnight at 37°C in 5% CO_2. After overnight culture, the medium was discarded, replaced by 50 µl DME containing 0.04 mg/ml cyclohexamide, and incubated for 20 min at 37°C in 5% CO_2. 50 µl of cell-free supernatant (diluted 1:50 in DME), collected from primary macrophages as already described, were added to individual wells. Serially diluted recombinant human TNF (100–0 pg/ml; R&D Systems) was used to determine the standard curve for the assay.

Cytokine release in culture supernatants was normalized for the numbers of viable cells in each well, ascertained with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, tetrazolium salt) assay (Boehringer Ingelheim). 20 µl of 2.5 ng/ml MTT was added to each well and incubated for 4 h at 37°C in 5% CO_2. Supernatants were carefully discarded and 100 µl/well of lysis solution (90% isopropanol, 0.5% SDS, 0.04 N HCl, and 10% H_2O) was added to each well for 1 h at room temperature. The absorbance was read at 570 nm using a microplate reader (Anthos Labtec Instruments).

RNA purification. Total RNA was prepared from monocyte-derived macrophages, either unstimulated or stimulated for 4 h with HkEc, using the RNeasy Mini kit with RNase-free DNase treatment (QIAGEN). Optical

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density readings were determined for OD_{260}/OD_{280} and OD_{260}/OD_{230} using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) to assess protein and solvent contamination, respectively. Before GeneChip studies, RNA quality was further analyzed by assessing ribosomal RNA bands 28S/18S ratios using a Bioanalyzer (Agilent Technologies) high resolution electrophoresis system.

Whole genome GeneChip expression analysis. RNA samples were anonymized and randomized by disease group, treatment, and gender into three balanced batches. Linear amplification of RNA samples was performed using the standard Ovation RNA Amplification System 2 (NuGEN) as one batch on two 96-well plates. Samples were then fragmented, biotin labeled, and hybridized in three separate batches to the human U133 plus 2 GeneChip (Affymetrix) for 16 h to generate the transcriptomic data. The microarrays were washed and scanned according to Affymetrix protocols. Quality was assessed using report files generated in GCOS (GeneChip Operating System; Affymetrix) and checked against in-house criteria (GlaxoSmithKline) for probe and hybridization quality by analysis of the data for background, percentage of present calls, background standard deviation, raw Q (noise), scaling factor, GAPDH 3′ to 5′ ratio, and β-actin 3′ to 5′ ratio (Heber and Sick, 2006). The data were assessed for homogeneity of quality control metrics by principal component analysis (PCA) of gene expression using Array Studio (OmicSoft Corporation). To ensure that all samples corresponded to the annotated gender, expression was analyzed for gender-specific genes (XIST and USP9Y). Data from a total of 66 microarrays were therefore analyzed: HC, 16 unstimulated and 12 HkEc; ileal CD, 9 unstimulated and 6 HkEC; and colonic CD, 13 unstimulated and 10 HkEc. Global gene expression of the normalized probe intensity data were initially analyzed using Array Studio for the visual assessment of key trends by PCA. Further analysis of the intensities used a General Linear Model (GLM) performed in Array Studio. The GLM was used to build comparisons of HCs versus each disease group by treatment. A second experiment was then performed in which comparisons were made between macrophages from HC (7 unstimulated and 5 HkEc) and UC (19 unstimulated and 20 HkEc). Genes were considered to be differentially expressed between groups where $P < 0.01$, fold change was ≥1.2, and expression level was greater than the median cut of 5.0 fluorescence units. Where appropriate, the p-value was corrected for multiple comparisons (FDR correction, Benjamini and Hochberg [1995] method). For the overlaps of the Venn diagrams where a significant result was obtained independently in two separate experiments, the FDR correction was not applied; for assessment of the most significantly regulated genes, both raw p-value and FDR-corrected p-value are presented. Microarray data in MIAME format can be found at http://www.ebi.ac.uk/microarray-as/ae/ under accession nos. E-TABM-733 and E-TABM-734.

TaqMan protocol. Expression levels of selected genes found to be differentially regulated by the transcriptomic array data were validated by real-time RT-PCR TaqMan analysis using the ABI Prism 7900 Sequence Detector System (Applied Biosystems). 200 ng of total RNA were reverse transcribed to complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the standard protocol. The equivalent of 10 ng RNA per well was arrayed into 384-well plates using a Biomek FX robot (Beckman Coulter), and quantitative RT-PCR was performed using a 7900HT Sequence Detector System (Applied Biosystems) in a 10-µl reaction volume.

The following TaqMan primers and probes used: GMCSF (forward, 5′-AGCCTCACCAAGCTCAAGGG-3′; reverse, 5′-GGGTTGGAGGGCAGTGCT-3′; probe, 5′-CCCTTGACCATGATGGCCAGCC-3′), IL6 (forward, 5′-TGACCCAACCACAAATGCCA-3′; reverse, 5′-CATGTCCTGCAGCCACTGG-3′; probe, 5′-CTGTGCCTGCAGCTTCGTCAGCA-3′), IFN-γ (forward, 5′-CCAACGCAAAGCAATACATGAAC-3′; reverse, 5′-ACCTCGAAACAGCATCTGACTCC-3′; probe, 5′-TCATCCAAGTGATGGCTGAACTGTCGC-3′), and TNF (forward, 5′-TCCTCTCTGCCATCAAGAGCC-3′; reverse, 5′-GTCGGTCACCCTTCTCCAGC-3′; probe, 5′-TGGAAGACCCCTCCCAGATAGATGGGGC-3′). The final optimized concentrations for the forward and reverse primers plus the probe were 900 nmol/l, 900 nmol/liter and 100 nmol/liter, respectively. A standard curve was generated using human genomic DNA (Promega). PCR parameters were the following: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Data were acquired and processed with Sequence Detector 2.3 software (Applied Biosystems). A linear regression line calculated from the standard curves of serially diluted genomic DNA allowed relative transcript levels in RNA-derived cDNA samples to be calculated from the fluorescent signal in each run.

The data generated by the real-time PCR TaqMan were analyzed in Array Studio. The genes selected for RT-PCR were normalized using a covariate to account for any change in expression caused by RNA loading of the samples. This covariate was represented by the scores from the first principal component obtained from a PCA of the two housekeepers, β-actin and cyclophilin-α, and β_2-microglobulin (identified from the microarray data analyses as having invariant expression across all samples). A general linear model was built to look at the comparisons of HCs versus each disease group by treatment, and unstimulated versus stimulated samples by disease group. An FDR correction was applied using the Benjamini and Hochberg (1995) methods.

TNF reporter studies. Recombinant replication-deficient adenoviral constructs encoding pAdTrack-TNF 5′ promoter-luciferase-3′ UTR (AdTNF 5′/3′) and pAdTrack-TRF 5′ promoter-luciferase (AdTNF 5′ only) were generated as previously described (Horwood et al., 2003). Macrophages were plated in 96-well plates (Nunc) at 10^5 cells/well and allowed to express adenoviral transgenes for 24 h before stimulation with HkEc for 4 h. Luciferase reporter gene assays were performed as previously described (Palmer et al., 2008). Before lysis, viral infection rates were measured by GFP fluorescence (excitation, 485 nm; emission, 520 nm) using a FLUOstar Omega machine (BMG LABTECH) and software according the manufacturer's instructions, and luciferase activity was normalized to these levels.

mRNA stability. Macrophages were stimulated with HkEc for 4 h followed by the addition of 2 µg/ml actinomycin D. Total RNA was purified as described in RNA purification at 0, 1, 2, and 4 h. TNF mRNA levels were determined by RT quantitative PCR and normalized to GAPDH and ribosomal 18S.

Cytokine protein arrays. 5×10^5 macrophages were incubated with HkEc alone or in combination with 2.5 µM Bref-A (Merck) or 2.5 µM monensin (Sigma-Aldrich) for 4 h. Cells were lysed in TBS containing 0.1% NP-40 and protease inhibitors, sonicated (3× 3-s bursts), and then interrogated with commercially available antibody arrays (Human Cytokine Array Panel A; R&D Systems), according to the manufacturer's instructions. Processed arrays were exposed to x-ray film, and the signal was determined and normalized to the internal positive control spots by densitometry using ImageJ software (National Institutes of Health).

Lysosomal inhibition and cytokine production. 2×10^5 macrophages from patients with CD and HC subjects were prepared as previously described and stimulated for 4 h with 5×10^5 HkEc plus either cell media, or media with 2.5 µM monensin, 10 mM NH_4Cl, or 100 µM chloroquine. Macrophages were lysed and run on 10% tricine PAGE gel followed by Western blotting. Membranes were incubated with goat anti-TNF (R&D Systems) 1:1,500 overnight at 4°C. After washing, the membranes were incubated with anti–goat-HRP (Vector Laboratories) at 1:2,000 for 1 h at room temperature, washed again, and then visualized with ECL plus detection reagent (GE Healthcare). All gels were subjected to ImageJ software analysis and normalized to actin.

Online supplemental material. Fig. S1 shows bacterial clearance in CD and HC subjects subdivided according to disease phenotype and *CARD15* genotype. Fig. S2 shows macrophage cytokine release after 24-h stimulation with TLR2 and TLR4 ligands. Fig. S3 depicts macrophage phenotype analysis. Fig. S4 shows functional groupings of the most significantly up-regulated

and down-regulated genes in macrophages stimulated with HkEc. Table S1 shows cytokine mRNA levels after HkEc stimulation. Table S2 depicts differentially expressed genes in ileal CD macrophages stimulated with HkEc. Table S3 depicts differentially expressed genes in colonic CD macrophages stimulated with HkEc. Table S4 provides demographics of subjects used in the injection studies. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091233/DC1.

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Diminished Macrophage Apoptosis and Reactive Oxygen Species Generation after Phorbol Ester Stimulation in Crohn's Disease

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Abstract

Background: Crohn's Disease (CD) is a chronic relapsing disorder characterized by granulomatous inflammation of the gastrointestinal tract. Although its pathogenesis is complex, we have recently shown that CD patients have a systemic defect in macrophage function, which results in the defective clearance of bacteria from inflammatory sites.

Methodology/Principal Findings: Here we have identified a number of additional macrophage defects in CD following diacylglycerol (DAG) homolog phorbol-12-myristate-13-acetate (PMA) activation. We provide evidence for decreased DNA fragmentation, reduced mitochondrial membrane depolarization, impaired reactive oxygen species production, diminished cytochrome c release and increased IL-6 production compared to healthy subjects after PMA exposure. The observed macrophage defects in CD were stimulus-specific, as normal responses were observed following p53 activation and endoplasmic reticulum stress.

Conclusion: These findings add to a growing body of evidence highlighting disordered macrophage function in CD and, given their pivotal role in orchestrating inflammatory responses, defective apoptosis could potentially contribute to the pathogenesis of CD.

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Introduction

Crohn's disease (CD) is a chronic relapsing inflammatory disease of the gastrointestinal tract associated with considerable lifelong morbidity[1]. It is characterized by granulomatous inflammation that most frequently affects the terminal ileum and colon. The incidence of CD has risen dramatically since the latter part of the 20th century for reasons that remain poorly understood[2].

Despite tremendous advances in our understanding of the immunology of the gastrointestinal tract, the pathogenesis of CD remains elusive and highly contentious. Patient heterogeneity supports the complex nature of this disease and is a major difficulty in defining its cause. Various hypotheses concerning the pathogenetic mechanisms have been proposed over the years[3]. Most implicate a dysregulated mucosal immune response to intestinal luminal contents in those with a susceptible immunological background. The etiology of CD is almost certainly multifactorial, with numerous genetic and environmental factors that differ between individuals giving rise to a common syndrome.

We have previously shown a failure of acute inflammation in CD that is systemic and operates at the level of the macrophage[4,5]. This defect results in diminished pro-inflammatory cytokine release, reduced neutrophil recruitment and the persistence of bacterial products within the tissue, which can potentially drive chronic inflammation. Other groups have previously shown abnormal apoptosis in both neutrophils and T-lymphocytes from CD patients under a variety of conditions[6,7], and both anti-TNF and 5-aminosalicylic acid (5-ASA) therapy have been shown to induce apoptosis in leukocytes from CD patients[8–10]. These observations lead us to investigate whether CD macrophages also exhibit an apoptotic defect which may contribute to the immunopathology of CD.

Apoptosis is a tightly-regulated mechanism in controlling tissue homeostasis that can be initiated by a variety of signals and stress factors; Its physiological and pathological importance is highlighted by the fact that dysregulated apoptosis underlies many cancers and malignancies[11]. Concurrently, it has been shown that CD can predispose to an increased risk of developing colorectal cancers[12]. Studies in mice showed that neutrophil and macrophage apoptosis were characteristics of the resolving phase of inflammation[13], suggesting an important role for apoptosis in the resolution of inflammation, which is defective in many chronic

inflammatory diseases[14]. Induction of apoptosis can occur via extrinsic factors (through death domain-containing receptors) or via intrinsic factors such as activation of tumor suppressor protein p53, which is activated in response to DNA damage, UV radiation and a range of chemotherapeutic drugs, and induces apoptosis-regulating pathways involving the mitochondria[15,16]. Such intrinsic factors also include reactive oxygen species (ROS), which was shown to induce apoptosis in RAW264.7 macrophages and are posited to function via the mitochondria[17,18]. Furthermore, studies in murine hepatocytes have shown that ROS-induced apoptosis required mitochondrial involvement in a protein kinase C (PKC)-dependent manner[19]. PKCs are a group of kinases that have been widely associated with apoptotic signaling[20]. Studies have shown that the regulation of PKC activity is highly complex, involving both a variety of phosphorylation events at different amino acid residues and conformational changes/cleavages conveying different states of (de)activation, depending on isoform, cell type and stimulus[21–23]. In particular, novel isoforms PKCδ and PKCε have been implicated in regulating cell survival and apoptosis[22], by interacting with a variety of proteins from the apoptotic machinery, including mitochondria-associated genes and caspases during apoptotic signaling processes[24,25].

In this study, we demonstrate that stimulation with the DAG-homologue PMA[22] induces an abnormal apoptotic response, reduced NADPH oxidase activation and elevated IL-6 secretion in macrophage from CD patients. These findings add to a growing body of evidence highlighting disordered macrophage function during the acute inflammatory response in CD, providing further insight about the pathogenesis of this chronic disorder.

Materials and Methods

Ethics Statement

These studies were approved by the Joint UCL/UCLH Committee for the Ethics of Human Research (project number 04/0324). Written informed consent was obtained from all volunteers.

Patients

Patients with endoscopically- and histologically-proven CD were identified through the gastroenterology outpatient clinics at University College London Hospitals (UCLH). All patients had quiescent disease at time of venesection (Harvey-Bradshaw Activity $\leq$3). Healthy controls were identified through the Department of Medicine, University College London (UCL). No subject was studied more than once in each of the different sets of experiments.

Macrophage Isolation, Culture and Stimulation

Peripheral venous blood was collected from subjects into syringes containing 5 U/ml heparin. Mononuclear cells were isolated by differential centrifugation (2000 rpm, 30 min) over Lymphoprep (Axis-Shield, Oslo, Norway) and macrophages differentiated as previously described[5]. Adherent cells were scraped on day 5 and re-plated at densities (10^6 cells/ml) in X-Vivo-15 medium (Cambrex, MD, USA). Plated macrophages were incubated overnight at 37°C, 5% CO_2, and then stimulated as appropriate. Stimuli used were PMA at 1 μg/ml, 1 μM RITA (2,5-bis (5-hydroxymethyl-2-thienyl) furan, obtained from the National Cancer Centre, Drug Therapeutic Program, Frederick MD (NSC-652287)), 1 μM Thapsigargin (Sigma-Aldrich, UK), and 2.5 mM *N*-Acetyl-L-cysteine (NAC) (Sigma-Aldrich, UK), heat-killed *E. coli* (HkEc), prepared as previously described (4) at a ratio of 2.5 HkEc/macrophage, 2 μg/ml Pam_3CSK_4 (P_3C) (Alexis Biochemicals, San Diego), 200 ng/ml LPS (Alexis). 1 μg/ml Infliximab (Remicade®) anti-TNF neutralizing antibody, human recombinant TNF at 25 ng/ml (Calbiochem, CA, USA), and human recombinant IL-6 at 10 ng/ml (R&D Systems, Abingdon, UK). Inhibitors used were 1 μM PKC inhibitor Bisindolylmaleimide I (BIM), (Calbiochem) and 25 μM topoisomerase II inhibitor etoposide phosphate (Sigma-Aldrich, UK).

MTT Cell Viability Assay

Cell viability was ascertained by MTT assay (Boehringer Ingelheim, Berkshire, UK). Briefly, 20 μl of 2.5 ng/ml MTT were added to each well and incubated for 18 hours (h) at 37°C, 5% CO_2. Supernatants were discarded and 100 μl/well of lysis solution (90% Isopropanol, 0.5% sodium dodecyl sulphate (SDS), 0.04 M NH_4Cl, 9.5% H_20) added to each well for 1 h at room temperature. The absorbance was read at 570 nm using a FLUOstar OMEGA microplate reader and software (BMG LABTECH Ltd., Aylesbury, UK).

DNA Fragmentation Assay

Macrophages were stimulated and cells permeabilized in 0.1% Triton X-100/PBS with 2 μM propidium iodide (PI) (Sigma-Aldrich, UK) for 1 h in the dark. DNA fragmentation was assessed by flow cytometry as previously described [26] using a FACSCalibur flow cytometer (BD Biosciences, NJ, USA), and analysis performed using the Cellquest™ software. The proportion of DNA giving fluorescence below the $G_{1\text{-}0}$ peak (gated as M1) was used as a measure of apoptosis.

Beadlyte Cytokine Secretion Assays

The expression profile of a panel of cytokines in macrophage supernatants was measured using the Beadlyte Bio-Plex™ human cytokine assay (Bio-Rad Laboratories, Hemel Hempstead, UK), according to the manufacturer's instructions. Our assay was customized to detect and quantify IL-1ra, RANTES, IL-6, GM-CSF and MCP-1.

ELISA

The concentrations of human IL-10 (PeproTech, Inc., NJ, USA), IL6 (BD Biosciences) and IL-8 (PeproTech) were determined by ELISA according to the manufacturers' instructions. Cytochrome C concentrations in cell lysates were quantified using the Quantikine® Human Cytochrome C Immunoassay (R&D Systems) according to the manufacturer's instructions. Absorbance was read and analyzed at 450 nm on a FLUOstar OMEGA microplate reader and software (BMG LABTECH Ltd., Aylesbury, UK).

TNF Bioassay

Release of bioactive TNF was measured using a cytotoxicity bioassay (obtained from Prof. B. Beutler, The Scripps Institute, CA, USA) as previously described[27]. Serially diluted rhTNF (100–0 pg/ml) (Calbiochem) was used to determine the standard curve for the assay.

Amplex® Red Reactive Oxygen Intermediate (ROI) Release Assay

Release of H_2O_2 by PMA-stimulated HC and CD macrophages was assessed by Amplex® Red fluorometric assay alongside a standard curve. Cells were plated in a 96-well flat-bottomed plate at a density of 10^5 cells/well. For inhibitor studies, cells were pre-incubated with 1 μM inhibitor for 1 h. H_2O_2 production was measured at 37°C in the presence of 4 μM Amplex® Red

(Molecular Probes), 0.1 U/ml horseradish peroxidase (HRP) (Sigma-Aldrich) and, where appropriate, 1 μg/ml PMA and 1 μM BIM in sterile PBS using the FLUOstar OMEGA microplate reader (BMG LABTECH Ltd.). Excitation was set at 544 nm and emission was set at 590 nm, with measurements taken at 30 sec intervals. Rate of H_2O_2 production per hour (nM/h) was calculated over the first seven minutes (exponential phase) using the FLUOstar OMEGA software (BMG LABTECH Ltd.).

Mitochondrial Membrane Potential Detection Assay

Loss of mitochondrial membrane potential in CD and HC macrophages was measured by flow cytometry using the APO LOGIX^{TM} JC-1 kit (Peninsula Laboratories, LLC, CA, USA) according to the manufacturer's instructions. Fluorescence was measured using FACSCalibur flow cytometer (BD Biosciences, NJ, USA), and analysis performed using the Cellquest^{TM} software.

Statistical Analysis

Data were analyzed using paired or unpaired t-test using the GraphPad Prism 5 software.

Results

Abnormal PMA-Induced Cell Death Is Associated with Macrophages from CD Patients

Abnormal apoptosis has been previously described in neutrophils and T lymphocytes isolated from CD patients[6,7,28–30]. We wanted to investigate the affects of numerous apoptotic stimuli on macrophages as these cells have been shown to be defective in patients with CD[5,4]. Cell survival was determined by measuring the amount of DNA fragmentation before and after stimulation. In contrast to a published report on T lymphocytes apoptosis levels, baseline rate of DNA fragmentation was not significantly different between CD and HC macrophages (Figure 1). Macrophages were stimulated for 24 h with an panel of apoptosis-inducing agents: RITA (p53-activating small molecule)[31,32], PMA, etoposide (topoisomerase II inhibitor), thapsigargin (sacro/endoplasmic reticulum (ER) Ca^{2+} ATPase inhibitor, induces ER stress), bacterial stimulation or TNF (Figure 1A). Increased DNA fragmentation was observed after RITA, PMA and thapsigargin stimulation in macrophages from HC and CD. Macrophages from CD patients were more resistant to PMA-induced DNA fragmentation (23.4±10.7 %) compared to those from HC (36.5±11.6 %, $p<0.0001$) (Figure 1A and B). The addition of TNF resulted in a moderate decrease in DNA fragmentation in macrophages from CD subjects (13±1.1 %) compared to unstimulated cells (15.4±2 %, $p<0.05$) and TNF treated HC macrophages (20±3 %, $p<0.05$). Etoposide and bacteria exposure had no effect on the level of DNA fragmentation compared to unstimulated macrophages. Macrophages from CD patients therefore undergo PMA-induced apoptosis, but the level of DNA fragmentation is only 64±4.4 % of that seen in HC. Decreased rate of apoptosis in PMA-stimulated CD macrophages was independent of disease phenotype (Figure S1), age ($R^2 = 0.03$) and gender ($p = 0.1399$) (Table S1). These data show that the induction of apoptosis via p53 and ER stress pathways are unaffected in macrophages from CD patients. In contrast, abnormal macrophage apoptosis levels are evident after stimulation with PMA and to a lesser extent TNF.

In order to determine if decreased PMA-induced DNA fragmentation in macrophages from CD patients was the result of alterations in cell viability, a MTT assay was performed (Figure 1C). Macrophage survival 24 h after PMA stimulation was significantly higher in CD (99.2±3.4 %) than in HC (70.9±4.9 %, $p<0.0001$). These data show that macrophages from CD patients are much more resistant to PMA-induced apoptosis resulting in increased survival compared to HC. These results contrast with our findings in patients with chronic granulomatous disease (CGD)[33], who show normal PMA-induced macrophage apoptosis despite frequently presenting with granulomatous enterocolitis indistinguishable from CD [34,35]. In addition, macrophages from patients with ulcerative colitis demonstrate a decrease in viability after PMA exposure (78.8 ±4.6 %, $n = 13$) that was equivalent to HC ($p = 0.29$) and significantly different to CD ($p = 0.018$) (Figure S2). Defective macrophage viability after PMA stimulation does not seem to be a consequence of general chronic inflammation, but specific to patients with CD.

Loss of Mitochondrial Membrane Potential Is Impaired in PMA-Stimulated CD Macrophages

Cell death via the apoptotic pathway results from a number of key steps which include permeabilization of the mitochondrial membrane and loss of membrane potential, cytochrome C release and caspase 3 activation[36]. Ultimately these lead to DNA fragmentation, membrane blebbing and apoptosis. Loss of mitochondrial membrane potential in macrophages from CD and HC following PMA stimulation for 24 h was measured (Figure 2A and B). A significant loss of mitochondrial membrane potential after PMA exposure was evident in both HC ($p<0.001$) and CD ($p<0.05$) macrophages. The mean percentage of macrophages demonstrating loss of mitochondrial membrane potential was significantly lower in CD patients (10.3±2.2 %) compared to HC (44.0±6.4, $p<0.01$) (Figure 2A and B). Macrophages were incubated with RITA in order to investigate p53 signaling which also results in loss of mitochondrial membrane potential during the induction of apoptosis (Figure 2B)[31,15,32]. Stimulation with RITA induced a loss of mitochondrial membrane potential in macrophages from both HC ($p<0.01$) and CD ($p<0.01$), with no significant difference between the two groups ($p = 0.3553$). These findings further supporting the concept that the intrinsic apoptotic pathway downsteam of p53 is able to operate normally in macrophage from CD patients. Loss of mitochondrial membrane potential results in the release of cytochrome C[16], which was measured after stimulation. Intracellular cytochrome C levels in macrophages from CD were significantly lower than those in HC macrophages after PMA stimulation ($p<0.01$, Figure 2C). Resistance to apoptosis in mucosal T lymphocytes from CD patients has previously been shown to correlate with decreased Bax expression[24]. We therefore assessed Bax mRNA levels in HC and CD macrophages following PMA stimulation by quantitative PCR. PMA exposure resulted in the upregulation of Bax in HC macrophages (Figure 2D). The upregulation of Bax was significantly lower in macrophages from CD patients after PMA stimulation compared to HC subjects (Figure 2D). These results are consistent with the diminished apoptosis observed thus far, and provide further evidence for a general dysregulation of PMA-induced responses in macrophages from CD patients.

Defective PMA-Induced Reactive Oxygen Species (ROS) Production in CD Macrophages

In addition to an apoptotic response, PMA stimulation also induces the production of ROS in macrophages through the activation of the NADPH oxidase system[37]. PMA-induced ROS production, determined by H_2O_2 generation, in HC and CD macrophages was assessed (Figure 3A). The rate of H_2O_2 production was significantly decreased in CD macrophages

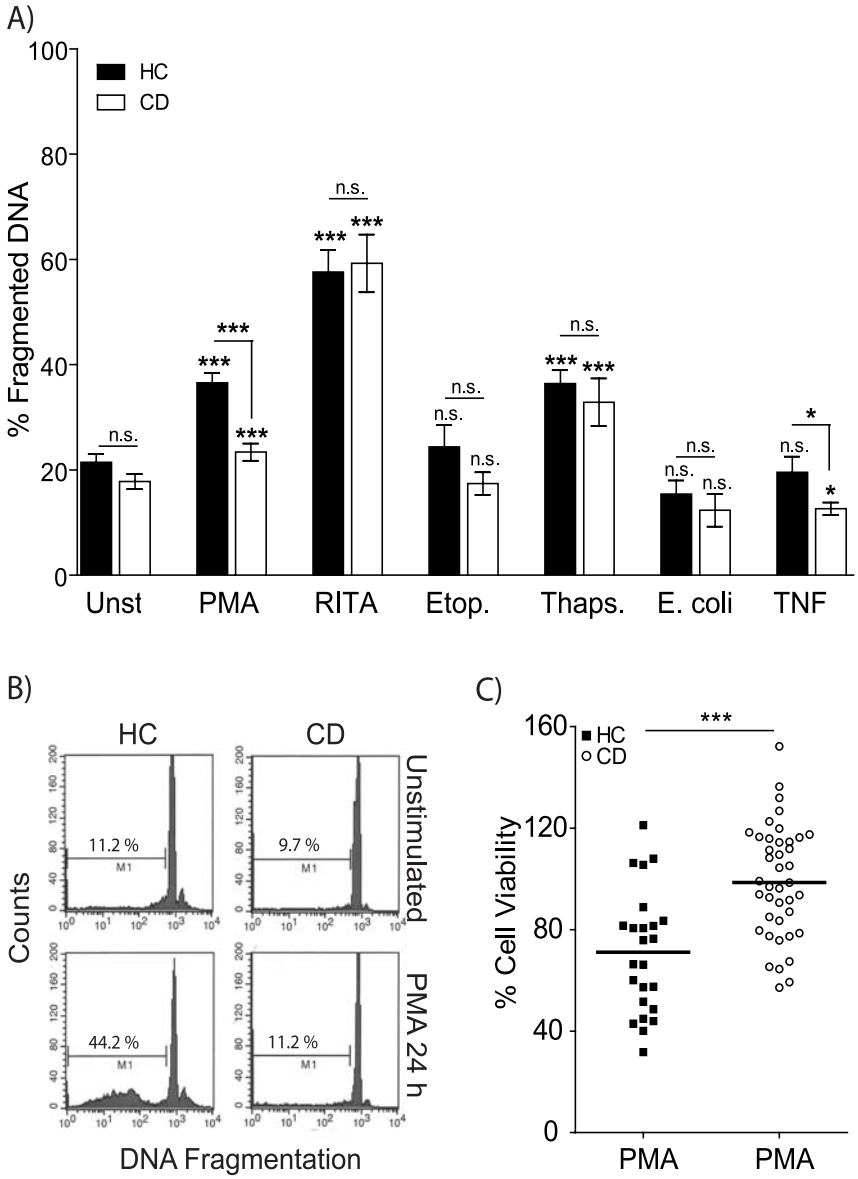

Figure 1. Defective apoptosis in macrophages from CD subjects. A Macrophages from HC and CD patients were untreated or stimulated with 1 μg/ml PMA (HC n = 39, CD n = 44), 1 μM RITA (HC n = 10, CD n = 7), 25 μM Etoposide (HC n = 7, CD n = 7), 1 μM Thapsigargin (HC n = 9, CD n = 8) for 24 h, *E. coli* (HC n = 10, CD n = 12) or 25 ng/ml recombinant TNF. DNA fragmentation (events below the $G_{1\text{-}0}$ peak) was assessed by flow cytometry. Mean percentage ± SEM of apoptosis for HC (black bars) and CD (open bars) are shown (° significance between stimulated and untreated, * between HC and CD). **B** Representative histograms for HC unstimulated (upper left panel), HC plus PMA 24 h (lower left), CD unstimulated (upper right) and CD plus PMA 24 h (lower right). **C** Viability for CD and HC macrophages following stimulation with 1 μg/ml PMA for 24 h was assessed by MTT assay. Data are presented as percent of untreated cells for HC (black squares, n = 24) and CD (open circles, n = 41) with group mean. *Statistical analysis:* Paired or unpaired t-test. *Symbols*: $p<0.05$ (* or °), $p<0.001$ (*** or °°°).
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(17.1±1.4 nM/h) compared to HC (23.7±1.9 nM/h, $p<0.01$). In order to assess whether there was a causal link between reduced H_2O_2 production and decreased apoptosis in macrophages from CD patients in response to PMA, cells were treated with the antioxidant *N*-acetyl-L-cysteine (NAC). Macrophages from HC pre-treated with NAC reduced the amount of free H_2O_2 after PMA-stimulation by 46.7 %, which was even greater than the 17% observed with macrophages from CD patients (Figure 3B). However, reduced H_2O_2 levels in the presence of NAC had no effect on PMA-induced DNA fragmentation (Figure 3C) or mitochondrial membrane potential (Figure 3D). This indicates that the decreased ROS levels observed in macrophages from CD patients are not associated with, and consequently not responsible for the resistance to PMA induced loss in cell viability.

Macrophages from CD patients demonstrate two distinct abnormalities after PMA stimulation: increased resistance to apoptosis and diminished NADPH oxidase activity. These results are in line with our previous findings showing normal levels of PMA-induced apoptosis in macrophages from CGD patients who generate no ROS due to a complete absence in NADPH oxidase activity[33].

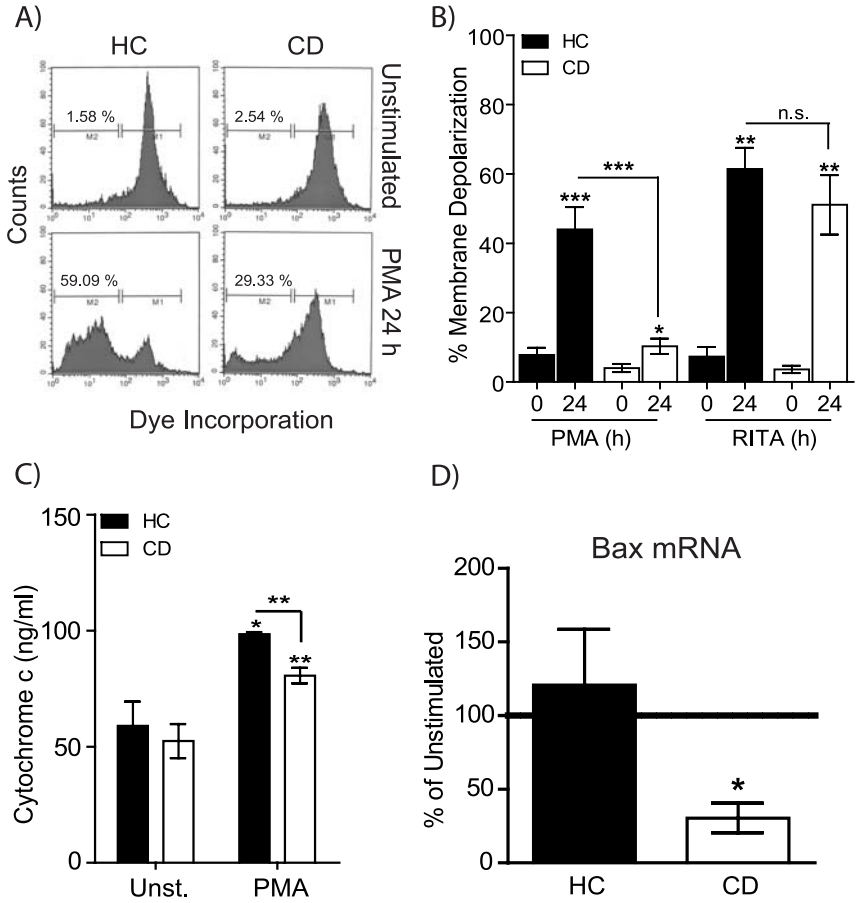

Figure 2. Mitochondrial membrane depolarization, cytochrome c and BAX expression are abnormal in CD macrophages after PMA activation. Macrophages were stimulated with 1 μg/ml PMA for 24 h and the effects on mitochondrial membrane potential, cytochrome c and BAX expression determined. **A** Representative histograms of macrophage population for HC unstimulated (upper left panel), HC PMA 24 h (lower left), CD unstimulated (upper right) and CD PMA 24 h (lower right) are shown. Gated populations show cells with an intact mitochondrial membranes (M1) and cells which have lost mitochondrial membrane integrity (M2). **B** Proportion of macrophages with mitochondrial membrane depolarization are shown as mean percentage ± SEM after either PMA (HC n = 9, CD n = 10) or RITA (HC n = 5, CD n = 5) stimulation. **C** Intracellular cytochrome C levels in macrophages stimulated with 1 μg/ml PMA for 24 h were measured by ELISA. Cytochrome C production (ng/ml) at 24 h is shown for HC (n = 4, black bars) and CD (n = 8, open bars). **D** Macrophages were stimulated with 1 μg/ml PMA for 4 h followed by total RNA isolation. Bax mRNA levels were determined by qPCR and expressed as the change in expression compared to unstimulated cells. *Statistical analysis:* Paired or unpaired t-test. *Symbols*: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).
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Increased IL-6 Production in Macrophages from CD Patients in Response to PMA

Previously, we have reported a defective acute inflammatory response to microbial challenge associated with macrophages from CD patients[5,4]. We were therefore interested in determining the effects of chronic activation and reduced apoptosis with the DAG homolog-PMA on cytokine generation by macrophages from CD subjects. Analyses of cytokines produced in response to PMA did not reveal the same dramatic difference in cytokine release seen with bacterial and toll-like receptor stimulation [5]: TNF, MCP-1, IL-8, IL-1Ra, IL-10, GM-CSF and RANTES were released at levels not significantly different from HC macrophages (Figure 4A). Macrophages from CD patients produced significantly more IL-6 than HC at 24 h after PMA stimulation (Figure 4A, $p<0.05$). Increased secretion of IL-6 by CD macrophages after PMA stimulation is consistent with the elevated serum levels previously reported for patients with active disease[38]. There is also a direct correlation between IL-6 serum levels and disease severity[39]. IL-6 has also been shown to produce anti-apoptotic effects via gp130 and the activation of the JAK/STAT3 pathway[40]. In order to ascertain whether elevated IL-6 secretion exerted any effect on the apoptotic response, HC and CD macrophages were stimulated with recombinant IL-6 in the presence or absence of PMA. Addition of IL-6 to HC and CD macrophages did not alter the basal level of apoptosis seen in unstimulated cells (Figure 4B). As shown previously, the macrophages from CD subjects were more resistant to DNA fragmentation than HC after PMA activation. The inclusion of IL-6 had no effect on the rate of DNA fragmentation in either the HC or CD subjects with or without PMA. STAT3 phosphorylation was also similar between the two groups (data not shown). It therefore seems unlikely that the elevation in IL-6 is responsible for the reduced apoptosis in macrophages from CD patients.

PMA Induced Apoptosis and ROS Generation Signals through a Bisindolylmaleimide Sensitive Pathway

PMA has been previously shown to activate a number of intracellular signaling molecules which are sensitive to DAG

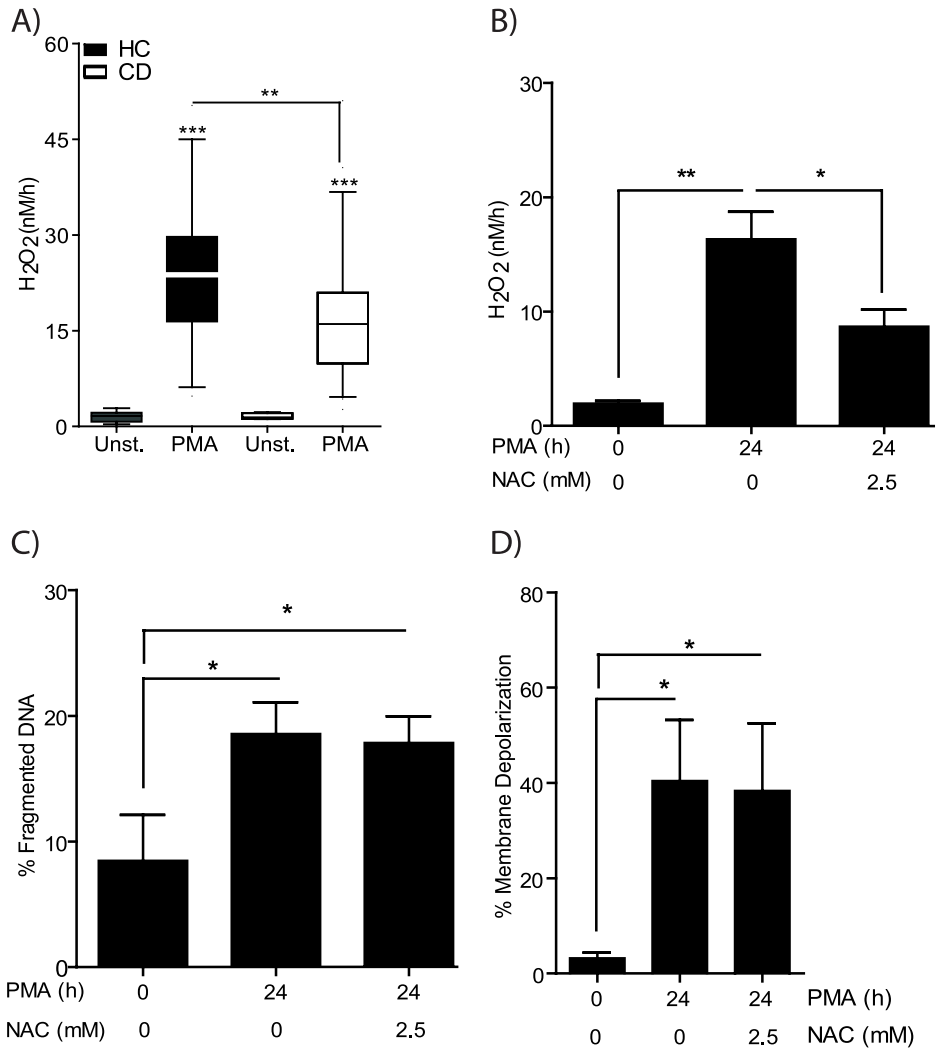

Figure 3. Decreased PMA-induced reactive oxygen species production in CD macrophages. NADPH oxidase activity was assessed by measuring the generation of H_2O_2 by macrophages from HC and CD subjects after stimulation with 1 μg/ml PMA. **A** Production of H_2O_2 (nM/h) was elevated after PMA stimulation in both HC (n = 29) and CD (n = 47) groups. Macrophages from CD patients demonstrated reduced H_2O_2 release than HC. **B** H_2O_2 levels were determined after HC macrophages (n = 4) pre-incubated with 2.5 mM *N*-Acetyl-L-cysteine (NAC) for 1 h followed by PMA stimulation. The presence of NAC resulted in reduced H_2O_2 levels. **C** DNA fragmentation and **D** mitochondrial membrane potential were unaltered by the presence of NAC. *Statistical analysis:* Paired or unpaired t-test. *Symbols*: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), HC (black bars), CD (white bars).
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generation downstream of phospholipase C. These include the classical and novel protein kinase C (PKC) family as well as a host of other DAG responsive molecules[22]. The effects on apoptosis and ROS generation in the presence of bisindolylmaleimide I (BIM), a potent but none-selective inhibitor of PKC, were assessed[41]. Pre-incubation with BIM significantly inhibits PMA-induced DNA fragmentation (Figure 5A), mitochondrial membrane depolarization (Figure 5B) and ROS generation (Figure 5C) in macrophages from HC and from CD subjects. The exact PMA sensitive molecules responsible for the abnormalities are still unknown, but our findings suggest that the abnormalities in macrophages from CD are downstream of a BIM-sensitive PMA inducible pathway.

Discussion

This study identified several defects associated with macrophages from CD patients: 1) Macrophages show increased viability and decreased apoptosis downstream of the DAG homolog PMA. 2) The impaired apoptotic response is PMA-specific, as activation of apoptosis via p53 and ER stress are normal in CD macrophages. 3) PMA-induced NADPH oxidase activity is also impaired in CD macrophages. 4) PMA exposure also results in elevated IL-6 release. In addition to these observations we have recently described a major defect in the innate immune response of CD patients in response to bacterial stimulation, which results in impaired clearance as a consequence of defective cytokine secretion from macrophages[5]. We therefore propose that defective macrophage function plays a major role in the abnormal acute inflammatory response and subsequent chronic granulomatous inflammation in CD.

Studies investigating apoptosis in CD have thus far concentrated on lymphocytes and neutrophils[7,6,30,29,28], and this is the first time that a defect in CD macrophage apoptosis has been shown. Interestingly, aberrant apoptosis in T-lymphocytes was observed in response to numerous apoptotic stimuli, including IL-

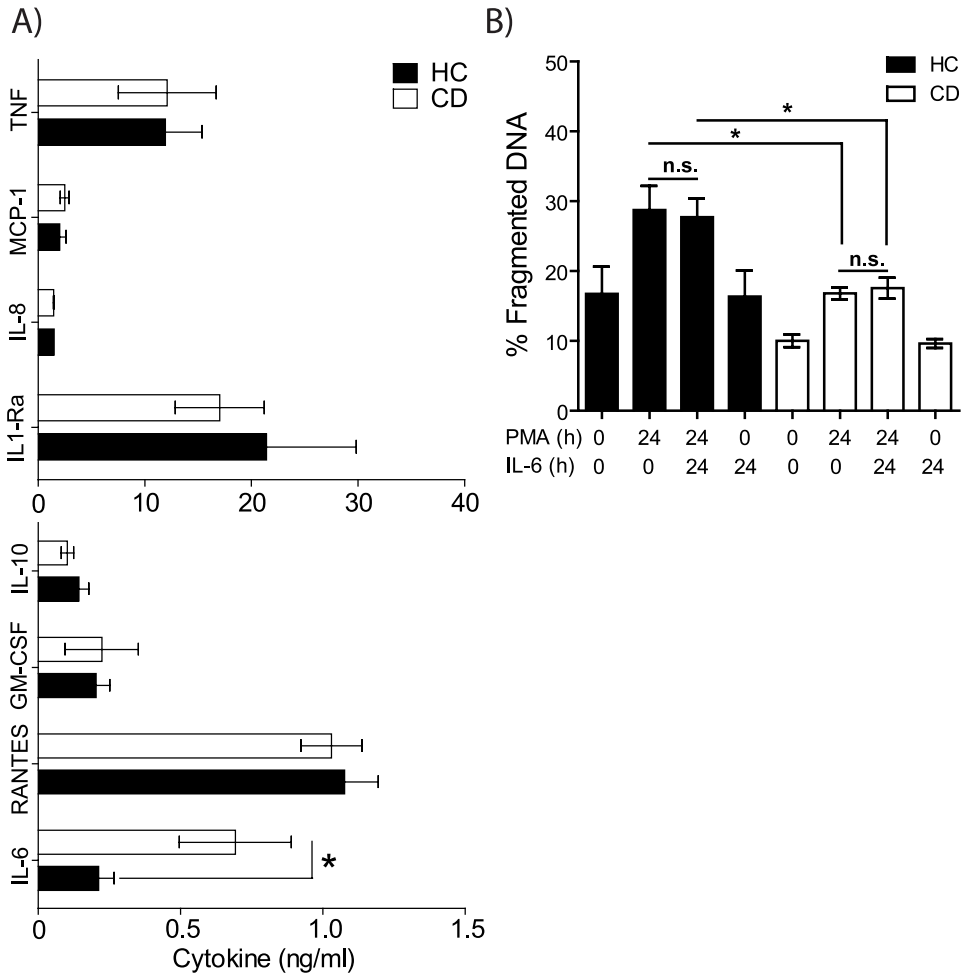

Figure 4. Dysregulated PMA-induced IL-6 production in CD macrophages. A Cytokine secretion from HC (n = 7) and CD (n = 10) macrophages following stimulation with 1 μg/ml PMA for six hours was assessed. IL-6 production was significantly elevated in macrophages from CD patients compared to HC subjects. **B** Measuring the effect of elevated IL-6 (1 ng/ml) on DNA fragmentation in the presence or absence of PMA stimulation. DNA fragmentation was unaltered by the inclusion of IL-6 in both HC (n = 7) and CD (n = 7) macrophages. *Statistical analysis:* Paired or unpaired t-test. *Symbols*: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***).
doi:10.1371/journal.pone.0007787.g004

2 deprivation, Fas ligand binding, and nitric oxide (NO) exposure, as well as lower spontaneous apoptosis in CD biopsy tissue explants[30]. These data contrast with our findings on several accounts. Firstly, baseline (spontaneous) macrophage apoptosis was comparable in HC and CD subjects. Secondly, apoptotic responses following p53 activation and ER stress induction were normal, suggesting a more specific defect in macrophages compared to T lymphocytes in CD. These cell-type-specific differences are further highlighted by the fact that CD neutrophils exhibit delayed apoptosis in suspension and accelerated apoptosis following adhesion to fibronectin[30], and support our hypothesis of CD as a multi-factorial syndrome that has multiple interacting cellular and tissue components[5,42].

PMA acts through the activation of DAG responsive proteins and targets a number of potential pathways in human macrophages. Protein kinase C (PKC) family members are especially sensitive to PMA activation and have been implicated in the induction of both apoptosis as well as NADPH oxidase activity[43,44]. The conventional or classical PKCs (α, βI, βII and γ) and the novel PKCs (δ, ε, η and θ) are all activated by the additional binding of DAG or its homologue PMA[44]. Due to this complexity we were unable to identify abnormal PKC activity using isotype specific activation antibodies, but the inclusion of BIM, a non-selective PKC inhibitor provided some evidence to support their involvement. Furthermore, abnormal PKC activity has previously been identified in CD patients during acute inflammation[45]. Defective apoptosis and NADPH oxidase activity may result from abnormal PKC activity but equally it may depend on downstream events. The fact that apoptosis occurs normally when p53 and ER stress are activated directly suggest that the machinery required for programmed cell death functions normally in CD macrophages. Further work is needed to identify the specific PMA inducible defects in macrophages from CD patients.

We have previously shown that macrophages from CGD patients that completely lack NADPH oxidase activity apoptose normally after PMA activation[33]. The inclusion of the ROS sequester NAC resulted in decreased levels of H_2O_2 but had no apparent effect on the apoptotic response in macrophages from HC. These observations suggest that reduced NADPH oxidase activity does not confer protection against apoptosis after PMA stimulation in primary human macrophages.

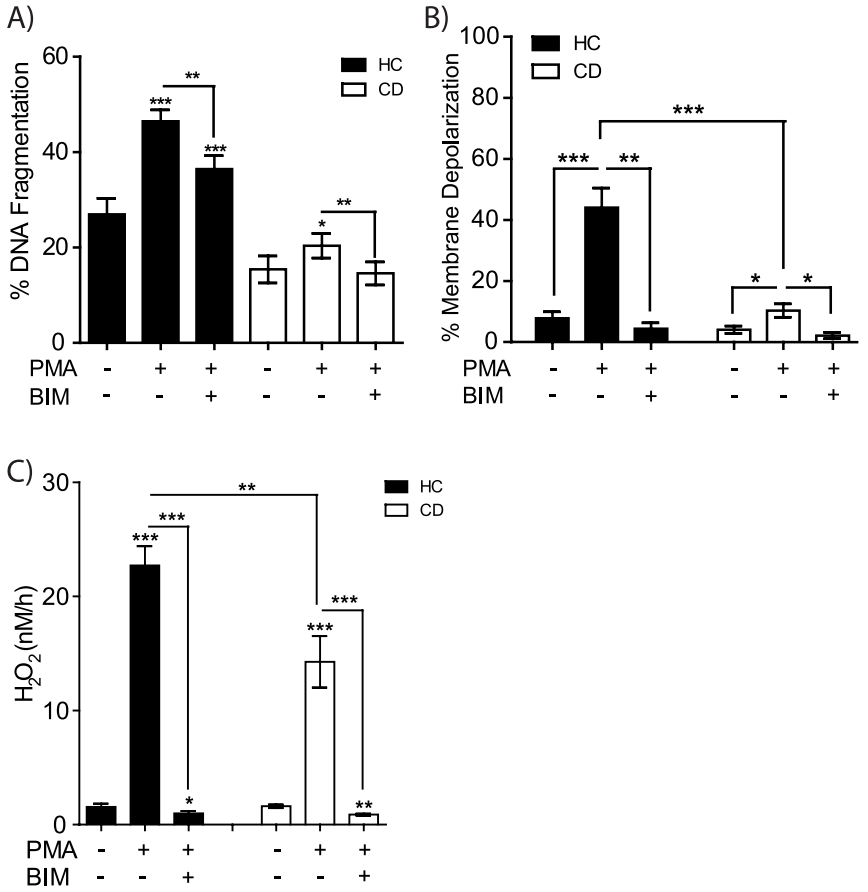

Figure 5. PMA induced apoptosis, NADPH oxidase activity and mitochondrial membrane depolarization are all inhibited by Bisindolylmaleimide I. Macrophages from HC (n = 5–10) and CD (n = 5–10) were left untreated, or pre-incubated with 1 μM Bisindolylmaleimide I (BIM) for 1 h followed by PMA stimulation. BIM significantly reduced **A** DNA fragmentation, **B** mitochondrial membrane depolarization and C H_2O_2 release in both HC and CD. Paired or unpaired t-test. *Symbols*: $p<0.05$ (*), $p<0.01$ (**), $p<0.001$ (***), HC (black bars), CD (white bars).
doi:10.1371/journal.pone.0007787.g005

We have also shown dysregulation of PMA-induced secretion of IL-6, a cytokine which has previously been associated with the chronic phase of CD[38,46]. Macrophages are thought to be one of the main cell types responsible for elevated intestinal IL-6 levels[47], and although our results show that IL-6 production does not have an autocrine effect on macrophage apoptosis, it is nonetheless possible that increased IL-6 levels in active CD contributes to the pathogenesis. Current therapies for CD are being developed that specifically target IL-6 and its receptor gp130 and the results from these studies will help to determine the precise role IL-6 plays in the pathophysiology of CD[48]. Our previous research has clearly shown that microbial stimulation resulted in significantly reduced pro-inflammatory cytokine secretion, including IL-6, in patients with CD[4,5]. This defect results in reduced neutrophil recruitment and the retention of bacteria within the tissue. It is plausible that chronic inflammation in CD is driven by the residual bacteria/bowel content in combination with defective macrophage apoptosis. This could result in the persistence of the pro-inflammatory stimuli, prolonged cytokine secretion, a failure in resolution, defective wound healing, granulomatous tissue formation, angiogenesis, fibrosis and scar formation; all of which are hallmarks of the chronic inflammatory phase in CD.

The precise mechanisms involved in acute inflammation and its subsequent resolution remain poorly understood, although it has become apparent that apoptosis plays an important role in the resolution phase. Initial work identified neutrophil apoptosis as a critical factor in switching off inflammation and inducing the resolution phase[49]. More recently, a role for macrophage apoptosis in the resolution phase of acute inflammation has been described[13]. It now seems that the induction and subsequent resolution of an acute inflammatory response require complex coordinated phases with regards to cellular recruitment and clearance of inflammatory cells. It is highly probable that defects affecting these processes contribute to the onset of human inflammatory diseases and specifically CD.

The importance of defective apoptosis in the immuno-pathology of CD is further substantiated by evidence that several efficacious CD therapies have the potential to induced apoptosis. Several different TNF-antagonists show clinical efficacy in inflammatory diseases[50]. These can broadly be divided into neutralizing antibodies (infliximab and adalimumab) and recombinant receptors (etanercept). Whilst both classes are equally clinically efficacious in rheumatoid arthritis, the recombinant TNF receptor/immunoglobulin G fusion protein etanercept is not effective in CD[51]. Studies in peripheral and lamina propria T lymphocytes have attributed this to the fact that, whilst both classes therapeutics neutralize TNF *in vitro*, only the neutralizing antibodies are capable of inducing apoptosis in these cells[8,50,52]. It has thus been proposed that the beneficial effects of anti-TNF therapies in active CD relate not to direct binding and

sequestering soluble TNF, but cross-linking of membrane-bound forms and induction of leukocyte apoptosis[50]. Thiopurines and methotrexate are immunosuppresants widely used in the treatment of moderate to severe CD and other chronic inflammatory conditions. Thiopurines have been shown to induce apoptosis via induction of a mitochondrial pathway[53]. Methotrexate has been shown to induce apoptosis as well as elevating ROS generation[54,55]. There is also evidence that 5-ASAs, another commonly used group of drugs used in mild CD, may have the ability to induced apoptosis in leukocytes[10,56,57]. Probiotics have recently attracted much interest as a potential treatment for CD. A recent study has shown that the administration of the probiotic *Lactobacillus casei* resulted in an increase in the number of intestinal lymphocytes undergoing apoptosis in active CD[58]. Collectively, these observations suggest that the clinical efficacy of commonly used CD therapies might at least in part be due to restoration of the apoptotic responses in macrophages and other leukocytes. The association between therapeutic success in CD and activation of apoptosis continue to increase and may be an important consideration in future drug development for this chronic inflammatory disease.

Supporting Information

Table S1 Patient demographics. All the CD patients used in this study have been listed with gender, age, ethnicity, phenotype, current treatment and smoking status if known. m = male, f = female, TI = terminal ileal, MTX = methotrexate, * = data not available.
Found at: doi:10.1371/journal.pone.0007787.s001 (0.01 MB PDF)

Figure S1 Altered apoptotic response in CD macrophages is independent of disease location. Data from CD macrophages presented in Figure 1B are presented as percent of apoptotic cells for each donor (HC n = 39; CD n = 44) sub-divided into disease location: colonic disease (col) (n = 15), ileocolonic disease (I/C) (n = 18) and terminal ileal disease (TI) (n = 11). Statistical analysis: Unpaired t-test. Symbols: $p<0.001$ (***), HC (black squares), col CD (open triangles), I/C CD (grey triangles) and TI CD (black triangles).
Found at: doi:10.1371/journal.pone.0007787.s002 (1.29 MB EPS)

Figure S2 Abnormal macrophage response to PMA stimulation is specific for CD patients. Viability assay for macrophages from CD, HC and ulcerative colitis subjects following stimulation with PMA for 24 h. Data are presented as percent of untreated cells for CD (blue, n = 41), HC (red, n = 24) and UC (black, n = 13). Statistical analysis: Unpaired t-test.
Found at: doi:10.1371/journal.pone.0007787.s003 (0.03 MB JPG)

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Author Contributions

Conceived and designed the experiments: CDP AA MA AMS. Performed the experiments: CDP FZR GWS AMS. Analyzed the data: CDP FZR AWS AMS. Contributed reagents/materials/analysis tools: AA MA SLB AWS. Wrote the paper: CDP FZR AMS.

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