Acetylation: a critical factor in maintaining intestinal inflammation?

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Acetylation: A Critical Factor in Maintaining Intestinal Inflammation?

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Acknowledgements

Abstract

In inflammatory bowel disease (IBD), both chronic pro-inflammatory pathways and failure of anti-inflammatory (healing) mechanisms sustain disease. The two major anti-inflammatory gut cytokines are transforming growth factor (TGF)-β and interleukin (IL)-10. Acetylation of regulatory proteins may play a role in the activation of both pathways. In IBD there is excess production of pro-inflammatory cytokines such as IL-1β and under-expression of IL-10. Fibroblasts also over-produce matrix metalloproteinases (MMP), mediating tissue destruction. Curcumin, a component of the spice turmeric and a known inhibitor of acetylation, shows clinical benefit in IBD in early trials. **Objectives:** To assess the anti-inflammatory effects of curcumin in the gut of children and adults with IBD. **Methods:** Intestinal mucosal tissue biopsies, mononuclear cells and colonic myofibroblasts from children and adults with active IBD were cultured *ex vivo* with curcumin. p38 MAPK, NF-κB and MMP-3 were measured by immunoblotting. IL-1β, interferon (IFN)-γ and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA). **Results:** We have shown favourable modulation of the cytokine profile by curcumin, with enhanced IL-10 expression and decreased IL-1β, and we have demonstrated reduced p38 MAPK activation in intestinal mucosal tissue. We have also shown dose-dependent suppression of MMP-3 expression in colonic myofibroblasts (CMF) with curcumin, by a mechanism which appears to be acetylation-dependent. **Conclusion:** Curcumin, a naturally occurring

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food substance with no known human toxicity, holds promise as a novel therapy in

IBD.

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Abbreviations

5-ASA	5-Aminosalicylic acid
AA	Anacardic acid
AP-1	Activator protein 1
APC	Antigen presenting cell
ATG16L1	Autophagy related 16-like 1
BAFF	B cell activation factor
BCA	Bicinchonic acid
BLP	Bacterial lipoprotein
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CARD15	c-terminal caspase recruitment domain 15
CBP	CREB binding protein
CCL25	Chemokine (C-C motif) ligand 25
CCR	Chemokine (C-C motif) receptor
CD	Crohn's disease
CD40L	CD40 ligand
CFTR	Cystic fibrosis transmembrane regulator
CMF	Colonic myofibroblasts
COX2	Cyclo-oxygenase 2
CREB	cAMP response element binding
CRP	C reactive protein

DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DLG5	*Drosophila* long disc homologue gene 5
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
egr-1	Early growth response factor 1
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electromobility shift assay
ERK	extracellular signal-regulated kinase
ESR	Erythrocyte sedimentation rate
GNAT	Gcn5-related N-acetyl transferase
HBSS	Hanks balanced salt solution
HCC	Hepatocellular carcinoma
β-HCG	β-human chorionic gonadotrophin
HDAC	Histone deacetylase
HEK	Human embryonic kidney cells
HIMEC	Human intestinal microvascular endothelial cells
HIF-1α	Hypoxia-inducible factor 1α
HIV	Human immunodeficiency virus
HO-1	Heme oxygenase 1
HRP	Horseradish peroxidise

HUEVC	Human umbilical endothelial vein cells
IBD	Inflammatory bowel disease
ICAM-1	Intracellular adhesion molecule 1
ICE	Interleukin converting enzyme
IFN-γ	Interferon γ
IGFBP-3	Insulin-like growth factor binding protein 3
IKK	IκB kinase
IL	Interleukin
IL-1RI	IL-1 receptor type I
IRAK	IL-1 receptor associated kinase
IRGM	Immunity related GTPase family M
iv	Intravenous
JAB1	Jun activation domain-binding protein 1
Jak1	Janus family kinase 1
JNK	c-Jun N-terminal kinase
LPMC	Lamina propria mononuclear cell
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTβR	Lymphotoxin β receptor
MadCAM-1	Mucosal addressin cellular adhesion molecule 1
MAPK	Mitogen-activated protein kinase
MCP-1	Mast cell protein 1
MDP	Muramyl dipeptide

MDR	Multidrug resistant gene
MHC II	Major histocompatibility complex type II
MKK	Mitogen-activated protein kinase kinase
MMP	Matrix metalloproteinase
MRSA	Methicillin resistant *Staphylococcus aureus*
MST1	Macrophage stimulating 1
Myd88	myeloid differentiation marker 88
MYST	MOZ, YBF2/SAS3 and TIP60 groups
NEMO	Nuclear factor-κB essential modulator
NF-κB	Nuclear factor-κB
NHL	Non-Hodgkin's lymphoma
NK cell	Natural killer cell
NOD2	Nucleotide-binding oligomerisation domain containing 2
(i)NOS	(inducible) nitric oxide synthase
NS	Non-significant
OCT	Organic cation transporter
ODC	Ornithine decarboxylase
OFG	Orofacial granulomatosis
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCAF	p300 CREB associated factor
PKC	Protein kinase C

PML	Promyelocytic leukemia
PPAR-γ	Peroxisome proliferator-activated receptor-γ
RIPA	Radio-immuno precipitation assay
(m)RNA	(messenger) ribonucleic acid
(si)RNA	(short interfering) ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCFA	Short chain fatty acid
SDS/PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERCA pump	Sarcoplasmic endoplasmic reticulum calcium pump
SLC22A5	Solute carrier family 22 member 5
Smurf	Smad-ubiquitin regulatory factor
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SOD	Superoxide dismutase
STAT	Signal transducer and activator of transcription
TGF-β(R)	Transforming growth factor β (receptor)
Th1	T helper cell type 1
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
TNF-α	Tumour necrosis factor alpha
Tollip	Toll-interacting protein

UC	Ulcerative colitis
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

1.0   Introduction

1.1   Inflammatory Bowel Disease

1.1.0   Epidemiology of Inflammatory Bowel Disease in Adults and Children

Inflammatory bowel disease (IBD) is a source of considerable morbidity in children, adolescents and adults. It is characterised by chronic inflammation of the gastrointestinal tract, which follows a lifelong relapsing and remitting course. Its incidence in children under sixteen in the United Kingdom is 5.2 per 100,000 per year [1]. This comprises 3.0 cases of Crohn's disease (CD), 1.5 cases of ulcerative colitis (UC), 0.6 cases of indeterminate colitis and 0.1 cases of orofacial granulomatosis (OFG). In adults the incidence in northern Europe is estimated at between 10-20 cases per 100,000 per year, with an approximately equal split between CD and UC [2]. The commonest age of onset is in the third decade of life, with a peak around the age of 30 years when assessed in 2004 [3]. However the incidence of IBD, particularly CD, has been increasing over the past decade, and new cases presenting in teenage years seem to account for a significant portion of this increase [2]. Although the overall population incidence is rising as well, this changing pattern may lower the average age of onset of IBD, particularly CD, possibly to be reflected in future epidemiological studies.

As well as the changing incidence, and the preponderance of CD over UC in childhood, other unexplained differences exist between childhood and adult onset IBD. UC more frequently occurs in a severe phenotype when presenting for the first time in childhood compared to adulthood. A much higher proportion of children than adults have disease affecting the entire colon (pancolitis) at the time of initial diagnosis [1], and of those presenting with disease only affecting the rectum (proctitis), a greater proportion of children than adults go on to develop extensive UC [4]. In CD there is an overall preponderance of females; yet of those diagnosed in childhood it is commoner amongst males [1, 5]. Finally genetic susceptibility appears to be more important in childhood onset IBD [6, 7]. This may be logical in a multi-factorial illness, since in childhood there is comparatively less time for exposure to environmental factors, therefore genetic influences account for a greater part of the aetiology.

The largest disease burden of IBD is in the industrialised world and the prevalence is generally higher in more northerly countries. Until recently IBD was hardly recognised in the developing world, although rising numbers of cases in India and Asia are now being seen [8] [9] [10]. When families migrate from East to West, their children take on a risk at least equal to the local IBD incidence in the first generation [11]. These observations suggest that environmental factors related to industrialised living are important in the aetiology of IBD. The study of a dietary agent, especially one such as curcumin whose natural levels of consumption are much higher in Asia than the UK, therefore holds particularly intriguing relevance.

1.1.1 Clinical Features of Inflammatory Bowel Disease

CD can affect any area of the gut from mouth to anus, and may do so in a patchy distribution, frequently sparing the rectum. It is characterised by inflammation spanning the entire thickness of the bowel wall (transmural) with a resultant tendency to form fistulae (abnormal connections between bowel and other structures) and strictures (bowel narrowing). The classical triad of presenting symptoms are diarrhoea, weight loss and abdominal pain, although presentation with all three is not universal. Other frequent clinical features include rectal bleeding, anorexia, nausea, anaemia, fever, fatigue and generalised systemic malaise. Systemic upset is commoner in CD than UC. IBD is accompanied by extraintestinal manifestations, at some point in the individual's lifetime, in at least 25% of cases, and these occur with slightly higher frequency in CD than UC [12] [13]. These include arthritis, erythema nodosum and pyoderma gangrenosum (painful inflammatory conditions of the skin), sacroileitis and ankylosing spondylitis (back pain and immobility), primary sclerosing cholangitis (bile duct disease), uveitis, iridocyclitis, episcleritis (inflammatory eye diseases) and aphthous stomatitis (ulceration and inflammation in the mouth) [14]. Predisposing factors to CD include family history of the condition [15], residence in an industrialised country [8], Caucasian race, Jewish ethnicity [13] and smoking [16].

UC is limited to the large bowel and characterised by mucosal, rather than transmural, inflammation, without fibrosis or fistula formation. Presentation may

be with the classical triad; however the predominant symptom is usually severe diarrhoea with high blood and mucus content. In contrast to CD which is typified by skip lesions (patchy areas of disease), inflammation in UC occurs in a continuous pattern from distal (rectal) variably through to proximal colon. A similar range of extraintestinal manifestations are seen, which in UC occur with a slightly lower overall frequency of around 21% [17], although the incidence specifically of primary sclerosing cholangitis is higher (4% compared to 2% in CD) [18]. Like CD, UC is also predisposed by family history (although less strongly so), industrialised living, Caucasian race and Jewish ethnicity, but conversely smoking [16] and prior appendicectomy are protective [19].

1.1.2   Diagnosis of Inflammatory Bowel Disease

The diagnosis is suggested by a combination of the above clinical features, together with indirect blood test markers of inflammation, the finding of typical mucosal changes at colonoscopy (Figure 1.1), and, in the case of small intestinal disease, a characteristic irregular appearance of the bowel when outlined on contrast (barium) Xray. The definitive diagnosis is made on histological examination of intestinal mucosal biopsies or surgical resection specimens in association with the patient's clinical features. In CD the inflammation is transmural and characterised in 50% of cases by the presence of granulomas (aggregates of macrophage-derived giant cells), the histological hallmark of the condition. In UC the inflammation is limited

to the mucosa, with irregularity and shallow ulceration. There is a chronic inflammatory cellular infiltrate in the lamina propria, depletion of goblet (mucus producing) cells and distortion of glandular architecture.

Figure 1.1   Crohn's ulcer at colonoscopy

The two less common forms of IBD are indeterminate colitis (sometimes referred to as IBD of indefinite type) and orofacial granulomatosis (OFG). The former is an ill-defined entity, sharing some aspects of CD and UC, lacking histological features which allow definitive categorisation, and perhaps representing an entirely separate type of IBD [20]. OFG is rare and is characterised by swelling, ulceration and induration of the mouth, cheeks, gums and buccal surfaces, with a granulomatous histology similar to CD. The relationship between OFG and CD is unclear. Many patients, particularly children, with OFG have endoscopic and/or histological features of CD in the gut, and these may be clinically silent in terms of gastro-intestinal symptoms [21, 22]. OFG can be challenging to treat and may have an allergic basis, sometimes responding well to strict dietary exclusions of additives

such as cinnamon and benzoates [23]. Conversely, it is not known if there is a significant group of patients with CD who have sub-clinical disease changes in the mouth, but aphthous ulceration is certainly common in CD, and there is an anecdotal perception that this may be the case. It is possible that OFG and CD represent a disease continuum, with more patients on the CD side of the spectrum, and a degree of overlap.

1.1.3   Management of Inflammatory Bowel Disease

Current treatments for IBD are dietary, pharmacological or surgical. Newly diagnosed or relapsed CD, particularly involving the small bowel, responds well to a period of dietary (enteral) therapy, which brings about remission in around 80% of patients [24] [25]. This option is used particularly in children and pre-pubertal adolescents, in whom avoiding steroids, with their negative effects on growth and bone development, is especially important. The treatment involves six to eight weeks of taking a nutritionally complete elemental or polymeric liquid feed, to the exclusion of all other solids or liquids except water. Its mechanism of action remains obscure, although theories include reduction of dietary antigen load or alteration in gut bacterial flora [26]. This is discussed further in Chapter 1.3.0. A number of anti-inflammatory drugs are currently in use, including 5-aminosalicylic acid (5-ASA) preparations such as mesalazine, steroids such as prednisolone (both of which may be given orally or rectally) and immunosuppressants such as

azathioprine, methotrexate or cyclosporine. Other drugs used include antibiotics such as metronidazole, and in the last decade biological agents have been developed, the most commonly used of which is infliximab, a monoclonal antibody against human tumour necrosis factor alpha (TNF)-α, which is given as a series of intravenous (iv) infusions and is most effective in CD [27]. Colectomy is performed in refractory UC which fails to respond to medical therapy or threatens life (fulminant colitis). It is curative but leaves the patient with an ileostomy (stoma), which in some cases may be reversible by ileoanal pouch formation at a later date. Surgery is also employed in CD, particularly where disease is localised to the ileo-caecal region, in which case right hemi-colectomy can be temporarily or occasionally permanently effective. None of these treatments (except colectomy for UC) are curative and many are associated with significant side effects.

IBD, particularly UC, carries a long term risk of cancer. Overall prevalence of colorectal cancer in all individuals with UC is estimated at around 3.7% [28]. The risk increases in relation to extent of colonic involvement and disease duration, and as such may apply equally to Crohn's colitis [29]. Thus the probability of developing cancer is cumulative over a life time of colonic inflammation, a fact of clear relevance to people diagnosed in childhood. In the first decade after diagnosis of UC the risk is estimated at 2% and this rises to 18% by the third decade [28]. This necessitates regular surveillance colonoscopies and some individuals opt for prophylactic colectomy. There is evidence that long term use of 5-ASA medications reduces the life time cancer risk [30].

1.2   Aetiology of Inflammatory Bowel Disease

1.2.0   Genetics of Inflammatory Bowel Disease

The cause of IBD is unknown but is likely to be complex and multi-factorial, with interplay between genetic predisposition, defective mucosal immune regulation and trigger factors in the luminal and/or external environment. 10-15% of cases of IBD in adults occur with a positive family history. 75% of these are concordant for disease type, and in the remaining 25% UC and CD occur in one family [31]. This suggests that some susceptibility genes are common to both CD and UC, whilst others are specific to one form. Compared to adults with IBD, children with IBD more commonly have a positive family history [6], and this history is more often concordant for disease type [32]. CD has a higher genetic penetrance, with a monozygotic twin concordance rate of 42-58%, compared to UC with 6-17%.

The first IBD susceptibility gene to be discovered was the c-terminal caspase recruitment domain 15 (CARD15) gene on chromosome 16 [33] [34]. Originally called nucleotide-binding oligomerisation domain containing 2 (NOD2), it encodes an intracellular receptor, which, on ligation by bacterial cell wall muramyl dipeptide (MDP) [35] causes release of nuclear factor (NF)-κB and transcription of inflammatory cytokines. It is found in macrophages, dendritic cells, intestinal epithelial cells and Paneth cells. The commonest mutations of CARD15 involve

the leucine-rich repeat (LRR) region which encodes the protein's bacterial (MDP) recognition site and cause increased NF-κB production [36]. Although this is the most significant genetic association known in CD to date, evidently heterogeneous mechanisms operate, as evidenced by the relatively low incidence of the mutation in CD patients (27-39%) and significant incidence in the normal population (14-16%). In the paediatric CD population the CARD15 mutation occurs with greater frequency than in the adult CD population [37], further consistent with the observation that genetic factors are very important in childhood onset IBD. In UC CARD15 does not appear to be implicated in aetiology; mutation occurs with an incidence of 12-14%.

Subsequently other IBD susceptibility genes have been discovered, such as those encoding two organic cation transporters, OCT1 and OCT2, on chromosome 5q31 [38]. Mutation in one of the two transporters (for example transversion in the SLC22A5 promoter) occurs in 53% of individuals with CD, compared to 23% of normal controls. These variants appear to interact with variants in CARD15 to increase susceptibility to CD, although similarly they do not play a role in the pathogenesis of UC. Several genome-wide association studies have been performed in IBD; a powerful tool which, by examining a large number of single nucleotide polymorphisms (SNP's) from many individuals, can measure the relative contributions of different genes to common diseases. A series of IBD susceptibility regions are now recognised throughout the genome at 12 different loci (Table 1.1). These susceptibility genes encode a number of products including

those involved in both the innate and adaptive immune systems. Genes involved in the innate immune system such as the autophagy related 16-like protein (ATG16L)1, the immunity-related GTPase family (IRG) M and CARD15 are specific to CD, whereas those involved in the interleukin (IL)-23 pathway, such as the IL-23 receptor (IL23R), IL12B (which encodes the p40 subunit common to both IL-12 and IL-23) and STAT3 are implicated in both CD and UC [39]. The role of innate and adaptive immunity, including the IL-23 pathway, in the immunopathology of IBD is further addressed in Chapter 1.2.1.

Chromosome	Gene	Also associated with UC?
1p31.1	IL23R	Yes
2q37.1	ATG16L1	No
3p21	Macrophage stimulating (MST)1	Yes
5p13	Prostaglandin receptor EP4	No
5q31	Solute carrier family 22 member 5 (SLC22A5)	Unclear
5q33.1	IRGM	No
5q33	IL12B (IL-12β (p40))	Yes
10q21	Zinc-finger protein 365	Unclear
10q24	NK2 transcription factor related locus 3	Yes
16q12	CARD15	No
17q21	Signal transducer and activator of	Yes

	transcription (STAT) 3	
18p11	Protein tyrosine phosphatase non-receptor type 2	Unclear
12q12	LRRK2	Unclear
6p21.33	BTNL2	Yes
7q22	MUC3A	Yes
7q21.1	ABCB1	Unclear
7q32	IRF5	Yes
9q32	TNFSF15	Unclear
1q32.1	IL-10	Yes
20q13	TNFRSF6B	Yes
21q22	PSMG1	Yes

Table 1.1 Genetic susceptibility loci in inflammatory bowel disease

1.2.1 Pro-inflammatory Responses to Bacterial Flora in Inflammatory Bowel Disease

Uniquely the gut mucosal immune system is faced with the challenge of constant exposure to a heavy load of bacterial, nutritional and other potentially pro-inflammatory luminal antigens. By necessity for nutrient absorption, this surface contact is intimate and extensive.   The mucosa must simultaneously achieve

efficient extraction of nutrition and water, exclusion of bacteria and toxins, defence against potentially invasive organisms and, crucially, tolerance of non-pathogenic bacterial flora. The mucosal immune system constantly samples, processes and reacts to luminal bacterial antigens, sending pro- or anti-inflammatory signals and promoting the differentiation of naïve T cells into effector or regulatory T cells [40].

In CD there is aberrant immune reactivity to normally non-pathogenic (commensal) bacteria. In general these immune responses can be divided into innate and adaptive. Cells of the innate immune system include natural killer cells, mast cells, macrophages, neutrophils and dendritic cells; which together mount a non-specific, generic host defence against potential pathogens. The adaptive immune system conversely is characterised by memory. It is highly specialised and directed against specific pathogens which are recognised by T and B lymphocytes. The susceptibility genes for CD encode proteins which contribute to both pathways (Chapter 1.2.0 and Table 1.1).

There are two important bacterial recognition receptors in the mucosa which comprise part of the innate immune system; intracellular CARD15 receptors and membrane bound toll-like receptors (TLR's), of which ten have been identified to date [41]. TLR's are expressed on epithelial cells, dendritic cells, macrophages and other immune cells and recognise pathogen-associated molecular patterns (PAMP's), a group of ligands common to many luminal microbes. Evidence from

animal studies suggests that TLR4 is required for the development of T helper cell type 1 (Th1)-mediated colitis [42]. In normal gut, there is low level TLR expression and an environment in which inflammation is well controlled, with high levels of the anti-inflammatory cytokine IL-10, and low levels of pro-inflammatory mediators [43]. In IBD there is high expression of TLR2 and TLR4 and dendritic cells have an activated phenotype, sampling luminal bacteria and mediating pro-inflammatory consequences. The CARD15 receptor is extremely sensitive to muramyl dipeptide (MDP), a bacterial product ubiquitously present in Gram positive and negative bacteria [44]. Upon ligation by MDP, CARD15 leads to the activation of inflammatory signalling pathways including NF-κB and MAPK [35] [45].

The discovery of an association between CD and mutations in CARD15 [33] [34] supports the observation that in CD there is inappropriate innate immune reactivity to normally non-pathogenic bacteria. However the precise relationship between CARD15 and the immunopathology of CD is incompletely understood and investigation of this relationship has yielded some inconsistencies. For example MDP stimulation of primary human mononuclear cells possessing CARD15 mutations leads paradoxically to decreased NF-κB activation, in contrast to the increased NF-κB activation seen with *in vivo* disease [46]. To further evidence the complexity of the relationship, CARD15 deficient mice do not develop intestinal inflammation [45], and the presence of a CARD15 genetic variant alone is insufficient to cause disease (see Chapter 1.2.0).

Also strengthening support for aberrant innate immunity in CD is the discovery of genetic mutations encoding proteins involved in autophagy; ATG16L1 [47] and IRGM [48] (Chapter 1.2.0 and Table 1.1). Autophagy is a process by which intracellular pathogens are destroyed and antigens presented, regulating T cell responses. The precise mechanistic relationship by which these mutations lead to increased susceptibility to CD is not as yet understood. However the discovery that these mutations are relevant to CD and not UC imply that a defect in intracellular processing of bacteria is important specifically to the pathogenesis of CD.

In most people with CD there is no specifically identified genetic mutation and the reason for the generalised aberrant immune responsiveness to bacterial flora is unknown. This in turn leads to an adaptive (specific) immune response. According to the classical dogma, in CD this takes the form of a Th1 (cell-mediated) response [49] [50], with IL-2 and interferon gamma (IFN-γ) activating macrophages and driving the release of IL-1, IL-6 and TNF-α. In UC the cytokine profile is said more closely to resemble a Th2 (humoral) type response, with activation of natural killer (NK) cells, production of IL-4, IL-5 and IL-13 and antibody-mediated immunity [51] [52]. In reality, overlap between the two conditions complicates this simplified view of their respective immunopathologies.

More recently, other cytokines have been recognised as important in IBD. IL-17 is a potent pro-inflammatory cytokine which is elevated in the serum and colonic mucosa of individuals with IBD, particularly CD, in apparent correlation with

disease activity [380]. IL-17 is produced by a subset of CD4+ T cells called Th17 cells under stimulation by IL-23. Th17 cells express the IL-23 receptor at high levels [53]. Blocking IL-17 attenuates intestinal inflammation in animal models [381]. Both IL-23 and the structurally related IL-12 appear to drive T cell subsets towards Th1 responses, and blockade of both ameliorates colitis in animal models [382]. Anti-IL-12 antibodies have shown promise in the treatment of CD in humans [383]. Furthermore, as described in Chapter 1.2.0 and shown in Table 1.1, mutations in genes which encode products in the IL-23 pathway are associated with both CD and UC [39, 54], as well as with other chronic inflammatory diseases such as psoriasis (skin disease) [55] and ankylosing spondylitis (inflammatory disease of the spine) [56]. The IL-23 receptor comprises two subunits; IL-23R and IL-12Rβ1. Multiple different SNP's in the IL-23R region have been identified in association with IBD [39] and mutations of genes in the IL-23 pathway are some of the strongest genome-wide associations identified in IBD [57].

A later consequence of activation of these inflammatory cascades is recruitment of further leukocytes from the circulation into the gut wall. This occurs by two different mechanisms. Firstly the upregulation of cellular adhesion molecules such as mucosal addressin cell adhesion molecule 1 (MadCAM-1) and vascular cell adhesion molecule 1 (VCAM-1), which, when expressed on mucosal vascular endothelial surfaces, interact with circulating leukocytes possessing cell surface markers α4β7 and α4β1 [384]. By this means, activated leukocytes adhere and are recruited into the inflamed gut. Secondly, chemokine (C-C motif) ligand (CCL) 25,

a chemokine expressed by intestinal epithelial cells, is chemotactic to lymphocytes expressing chemokine (C-C motif) receptor (CCR) 9 [385]. Both of these mechanisms provide potential new therapeutic targets to prevent leukocyte recruitment into inflamed gut, and are currently under study. Natalizumab, an anti-α4 integrin, has now been licensed for CD in the United States [58] and Vedolizumab, an anti-α4β7 integrin, is currently undergoing phase III trials in CD and UC [ClinicalTrials.gov number NCT00783692]. CCX282-B, an anti-CCR9 is presently in phase II trials in CD [ClinicalTrials.gov number NCT00306215].

1.2.2   p38 Mitogen Activated Protein Kinase

The bacterial components and pro-inflammatory cytokines described above activate inflammatory signalling cascades including NF-κB and mitogen-activated protein kinases (MAPK). The MAPK's are a family of enzymes which, when activated by signals from the external environment, initiate phosphorylation cascades, culminating in cellular events such as transcription, differentiation and apoptosis. They are central to the coordination of inflammatory, cell death, growth and other responses and are highly conserved, suggesting critical functions for survival. By their most basic classification they fall into three different families; extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. Within the MAPK family, the stress-activated protein kinases are a sub-group which are activated by stimuli that cause cell stress, including lymphocyte

receptors, TLR's, cytokine receptors and physical or chemical alterations in the cellular environment. Whilst ERK pathways are largely activated by growth factors, JNK and p38 MAPK comprise the stress-activated group of kinases (figure 1.2).

Cytokines,
Cell Stress,
DNA damage,

MAPKKKs

MKK4/MKK7

MKK3/MKK6

JNK1, JNK2, JNK3

p38α, p38β, p38γ, p38δ

Immune Response
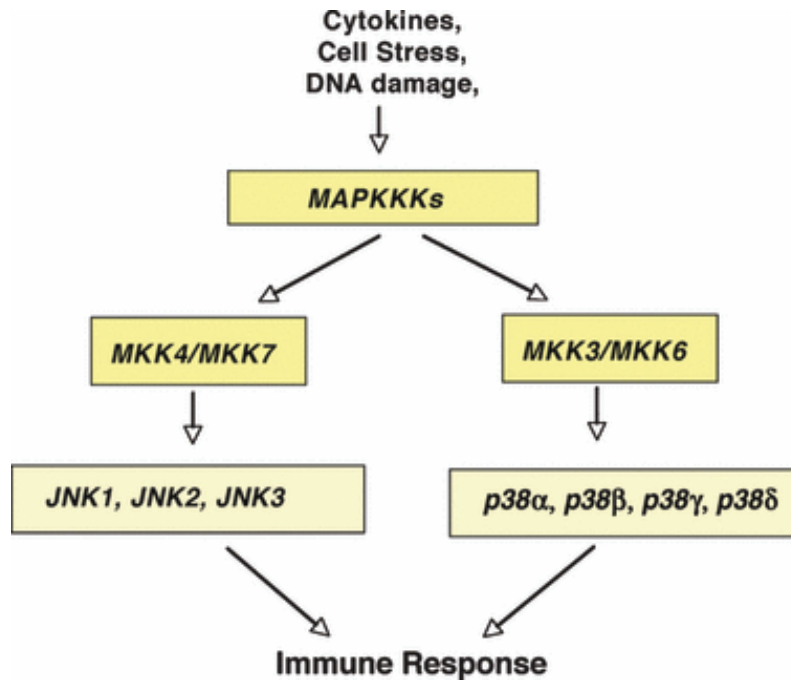
Figure 1.2   The c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signalling pathways [59]

The p38 group is further sub-classified into p38α, β, δ and γ. Each isoform is encoded by a distinct gene, although there is high amino acid homology between the isoforms, with   more than 60% overall homology and over 90% within the kinase domains of the enzyme [60]. The isoforms are expressed in a tissue-selective manner, with p38α and β the most ubiquitously expressed and p38α the

major form involved in the inflammatory response [61]. p38 MAPK was originally discovered in 1994, and this was only after its inhibitor SB2035820, which was identified in the course of a search for compounds inhibitory to TNF-α [62]. p38 MAPK, as well as regulating the production of inflammatory enzymes such as COX2 [63], plays a central role in regulating the expression of a number of key inflammatory cytokines including TNF-α, IL-1, IL-8 and interferon (IFN)-γ [64] [65].

The MAPK cascade is activated by a large number of different types of receptor, including cytokine receptors, TLR's and receptors sensitive to environmental stressors. The precise mechanisms of activation are incompletely understood. p38 MAPK becomes activated on phosphorylation by MAPK kinase (MKK) 3 and 6. This process is tightly regulated and dynamic, involving a balance between phosphorylation by upstream MKKs and dephosphorylation by MKK phosphatases [66]. Downstream, various MAPK substrates have been identified, including several different transcription factors. Final inflammatory and other cellular consequences of MAPK activation are probably mediated through multiple different mechanisms [67], and again these have not been fully elucidated.

The MAPK's and molecules in their signalling pathways therefore present interesting therapeutic targets in inflammatory disease. p38 MAPK is the most markedly elevated of the MAPK's in IBD [68] but an early trial of its pharmacological inhibition in CD has so far been disappointing [69]. This might be

because the very wide field of p38 MAPK-regulated cellular activities makes it too imprecise as a therapeutic target in IBD. Alternatively, this trial used an oral formulation and lack of clinical efficacy could also have been due to drug delivery issues. For the reasons outlined above, p38 MAPK is a highly relevant pathway in the study of inflammation in IBD.

1.2.3 Nuclear Factor-κB

The nuclear transcription factor (NF)-κB proteins are a family of five members which exist in dimers: p65 (relA), c-Rel, RelB, p50 and p52 (Figure 1.3). The first three members possess the ability to activate gene transcription directly; the latter two requiring dimerisation with the former for activation [70]. Like the MAPK family, NF-κB pathways play a central and ubiquitous role in relaying various inflammatory stimuli into transcriptional events. Under normal non-inflamed circumstances NF-κB dimers are held inactive in the cytoplasm by small inhibitory proteins IκB, of which three isoforms exist; α, β and ε [71]. There are two separate pathways by which NF-κB dimers may be activated and released from cytoplasmic hold into the nucleus; the classic and alternative pathways [72]. Classical (or canonical) activation occurs with a wide range of external and host-derived stimuli including bacterial lipopolysaccharide (LPS), viruses, TNF-α and IL-1. These trigger signalling cascades leading to activation of the IκB kinase (IKK)/NF-κB essential modulator (NEMO) complex. IKK/NEMO catalyses the phosphorylation

and proteasomic degradation of IκB, releasing NF-κB for nuclear localisation and transcriptional activity [73]. Gene targets of NF-κB include some of the same products which themselves activate NF-κB, such as TNF-α and IL-1. Thus a feed-forward loop is set up and undergoes self-amplification.

More recently alternative (or non-canonical) NF-κB activation pathways have been elucidated, which do not involve agonist stimulation but post-translational processing and cleavage of the p52 precursor, p100, to produce p52/RelB dimmers which move into the nucleus [74]. This is dependent on IKK-α homodimers, and occurs in response to B cell activating factor (BAFF), CD40 ligand (CD40L) and lymphotoxin β receptor (LTβR). These pathways are as yet less clearly delineated. It is thought that the alternative pathway plays an important role in the maintenance of secondary lymphoid organs. By either route of activation, the gene targets of NF-κB may be divided functionally into inflammatory, cell-cycle regulating and anti-apoptotic genes. Thus NF-κB sits at a crossroads between inflammation and apoptosis. NF-κB also targets genes which encode auto-inhibitory feedback loop proteins to negatively regulate its own activation [75].
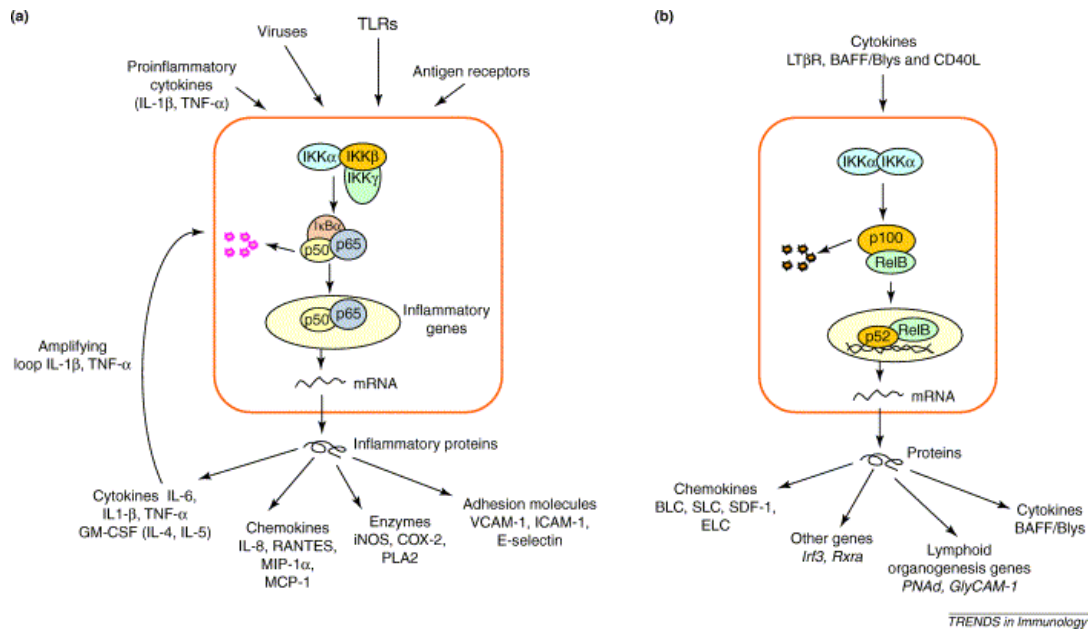
Figure 1.3 **(a) Classical and (b) alternative NF-κB activation pathways** [Bonizzi G, Karin M. *Trends in immunology* 2004;25:280-8]

NF-κB (particularly the p65 subunit) plays an important role in IBD, as evidenced by its increased expression and activation in inflamed gut tissues, which appear to correlate with disease severity [76], and by the finding that its abrogation is a highly effective treatment for experimental colitis of different types [77]. These data come from macrophages and monocytes of the mucosa and lamina propria, where the pro-inflammatory role of NF-κB is well established. However more recent animal work suggests that NF-κB may function as a master regulator of immune responses, with different, perhaps protective, effects in other cell types in IBD. For example conditional ablation of NEMO in mouse intestinal epithelial cells (which prevents NF-κB activation) causes spontaneous colitis and sensitises epithelial cells to TNF-α-induced apoptosis [78]. This suggests that NF-κB

improves epithelial integrity and barrier function, and thus in the epithelium has a homeostatic role guarding against immune dysregulation and the development of inflammation. Others have shown pro-inflammatory consequences of NF-κB activation in the epithelium, where IL-6 activates NF-κB in CaCo2 cells, leading to the induction of ICAM-1 which is known to further recruit immune cells into the inflamed gut [79]. Thus normal NF-κB functioning is crucial for intestinal homeostasis, but its functions are complex and include both pro-inflammatory and stabilising effects.

1.2.4   Anti-inflammatory Mechanisms

Much work to date has concentrated on proinflammatory pathways such as those described above, which are excessively active in the gut wall in IBD. The intestine of normal individuals, despite its intimate contact with a lumen teeming with potential inflammatory antigens, does not suffer pathological inflammation. Likewise, in individuals with IBD, spontaneous remission and periods of sustained quiescence occasionally occur in the absence of pharmacological treatment [80] [81]. These observations suggest that natural anti-inflammatory, healing mechanisms operate [82] and provide a new avenue for research and potential new lines of therapy. The immune system exerts negative control over inflammation at a number of different levels, including anergy (lack of immune reactivity in a lymphocyte), apoptosis of active lymphocytes (programmed cell death) and through

the actions of regulatory T cells, the most important group of which are understood to be the CD4+ regulatory T cells. This population can suppress immune responses and are important for maintenance of tolerance, both to self antigens and to normal bacterial gut flora. By inference this group of cells does not function sufficiently in IBD to suppress inflammation but no specific defect has as yet been identified. There is evidence that defective T cell apoptosis plays a role in maintaining inflammation in CD [83].

There are two major cytokines which inhibit inflammation; transforming growth factor beta (TGF-β) [84] and IL-10 [85], and the current work builds on the recently growing understanding of the ways in which they are abnormally regulated in IBD.

1.2.5　Transforming Growth Factor-β
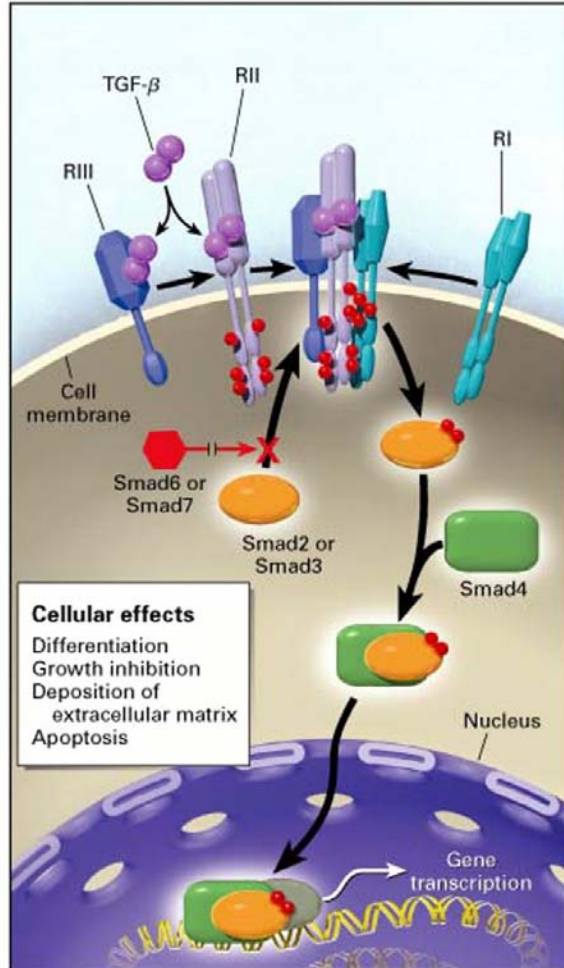
Figure 1.4　　　**Mechanism of signal transduction mediated by transforming growth factor β [Blobe GC, Schiemann WP, Lodish HF.** *The New England journal of medicine* **2000;342:1350-8]**

TGF-β was originally characterised in 1983 [87]. Nearly all cell types produce it and have receptors for it. There are three subtypes; TGF-β1 which is produced by endothelial, haematopoietic, connective tissue and importantly regulatory T cells

[82]; TGF-β2, produced by epithelial and neuronal cells and TGF-β3, produced by mesenchymal cells. TGF-β is highly conserved in mammals, suggesting critical functions for survival [86]. In support of this, TGF-β1 knock-out mice die of severe early multi-organ inflammation [88]. There is evidence to suggest that TGF-β1 produced by regulatory T cells prevents intestinal inflammation [89]. TGF-β1 is consistently associated with significant improvement in colitis in animal studies, both in trinitrobenzene sulfonic acid (TNBS)-induced colitis; a model of CD, and in oxazolone-induced disease; a model of UC.

Extracellular TGF-β binds either directly to a type II TGF-β transmembrane receptor (RII), or to a type III TGF-β receptor (RIII) which presents it to a RII. This leads to the binding of a type I TGF-β receptor (RI), and with the formation of this complex there is phosphorylation of RI (red sphere in Figure 1.4). This in turn activates the RI protein kinase, and this initiates an intracellular signal by phosphorylation of Smad proteins 2 and 3. The Smads are a family of polypeptides in the TGF-β signalling cascade. The name is an amalgam from their structural homology to the *Drosophila* MAD protein (mothers against decapentaplegic; so named because its mutation in the *Drosophila* mother insect causes its repression in the embryo) and the *C. elegans* protein SMA. Smad 3 deficient mice have impaired T cell and mucosal immunity [90]. After TGF-β binding, phosphorylated Smads 2 and 3 complex with Smad 4 and move into the nucleus where target effects are exerted. Once in the nucleus the Smad complex interacts with other transcription factors (in grey in Figure 1.4) in a cell-specific manner to mediate

ultimate TGF-β cellular effects. Inhibitory Smads 6 and 7 lack the region normally phosphorylated by RI for TGF-β signal transduction and thus interfere with normal TGF-β signalling by blocking the phosphorylation of Smad 2 or 3 by RI. This is examined in detail in Chapter 1.2.8.

The ultimate biological effects of TGF-β are complex, varied and depend on tissue type. Its immunosuppressive effects include promotion of leukocyte differentiation and inhibition of leukocyte proliferation, macrophage activation and dendritic cell maturation [91]. It inhibits growth, promotes apoptosis and causes deposition of extracellular matrix. Through this latter effect it can thus be involved in the development of tissue fibrosis; paradoxically contributing to the potential clinical problem of stricture formation in CD. It stimulates IL-10 production and inhibits NF-κB activation in some cell types.

1.2.6   Interleukin-10

IL-10 was originally described in 1989 as cytokine synthesis inhibitory factor [92]. It is released by T helper cells following antigen-specific and polyclonal activation, by B cells and by monocytes in response to TNF-α, IFN-γ and bacterial LPS [85]. It is synthesised late after a stimulus in comparison to other cytokines [93] and its key role is to inhibit the production of proinflammatory cytokines including TNF-α, IL-1, IL-2, IL-6, IL-12 and IFN-γ [94]. It also downregulates major

histocompatibility complex type II (MHC II) molecules thus inhibiting antigen presentation [95] and induces the production of various cytokine inhibitors such as IL-1 receptor antagonist [96] [97]. It directly inhibits differentiation and proliferation of T cell clones [98] and has a probable role in the generation and activation of regulatory CD4+ T cells [99] [100] [101]. It inhibits matrix metalloproteinase (MMP) activity thus limiting tissue damage [102]. The IL-10 knock out mouse is one of the few animal models in IBD with inflammation affecting the small intestine as well as the colon [103] [104]. This is mediated by CD4+ T helper cells and is entirely dependent on exposure to luminal bacteria, with an exaggerated TNF-α response to endotoxin. Thus IL-10 is important in maintaining tolerance to intestinal flora.

Recent work indicates that TGF-β and IL-10 pathways are interdependent. IL-10-deficient mice lack TGF-β signalling, with consequent failure to inhibit inflammatory responses of the gut epithelium to commensal bacteria [105]. Evidence is accumulating to suggest that both TGF-β and IL-10 are downregulated by acetylation of certain regulatory proteins, and this will now be described in more detail.

1.2.7 Acetylation
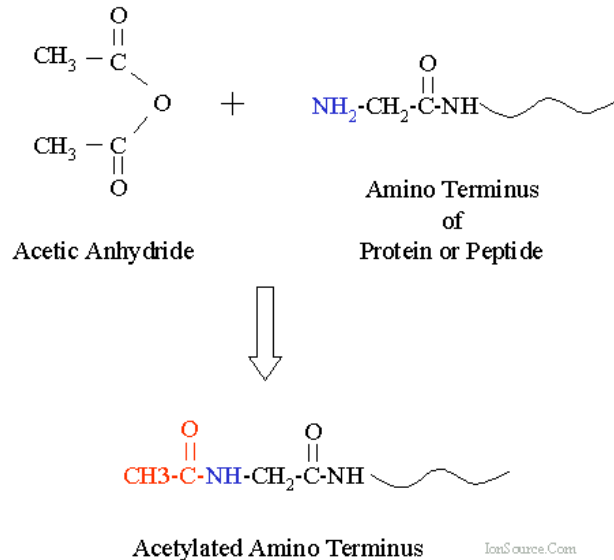
Figure 1.5   **Acetylation**

Acetylation is a chemical modification of a protein in which an acetyl group binds to the terminal lysine residue of the protein (Figure 1.5). This alters the protein's shape, charge and biological fate in the cell. Traditionally the study of acetylation has centred on acetylation of histones. Histones form a key component of chromatin, the scaffold in which deoxyribonucleic acid (DNA) is packaged into the nucleosome. When histones undergo acetylation, this changes their conformation and electrical charge, loosens their interactions with DNA and thus opens out the nucleosome, exposing DNA for gene transcription [106] [107]. The short chain fatty acid (SCFA) butyrate is a product of bacterial fermentation in the intestine and an inhibitor of the enzyme histone deacetylase (HDAC), thus it acts to increase the

acetylation status of the cell. Butyrate and its actions are discussed in greater detail in Chapter 7.4.3. As expected due to its acetylation of histones, butyrate upregulates gene expression [108] [109] [110] [111]. However, as will be introduced below, further work has shown that other (non-histone) proteins within the cell are also subject to acetylation and that this initiates separate events which affect gene expression.

1.2.8   Smad 7

As described in Chapter 1.2.5 an inhibitory Smad, Smad 7, is known to block TGF-β signalling [112]. Smad 7 interacts with the activated TGF-β receptor and prevents the docking of Smads 2 and 3 [113], which under normal circumstances would be phosphorylated on activation, starting a TGF-β cellular signalling cascade (Figure 1.4). In normal individuals Smad 7 is transcribed and transported out of the nucleus in complex with Smad ubiquitin regulatory factor (Smurf) 1 or 2 [114]. Smurf and Arkadia, which are ubiquitin ligases, in combination with the ubiquitin-binding Jun activation domain-binding protein (JAB) 1, quickly induce ubiquitination of Smad 7 at specific lysine residues. Ubiquitination is a covalent modification of a protein and an important regulatory mechanism by which cellular proteins are degraded, trafficked and quality controlled. Thus ubiquitination causes the Smad 7 to be degraded by proteasomes and removed from biological activity within the cell.

When Smad 7 is kept highly acetylated as it is in IBD, the acetylation of lysine residues on Smad 7 prevents its ubiquitiniation and thus potentiates its activity in the cell (Figure 1.6). In IBD, despite the abundant presence of TGF-β, there is a failure of TGF-β-mediated anti-inflammatory effects, including failed negative regulation of both NF-κB and proinflammatory cytokine production [115] [116]. In IBD there is reduced phosphorylation of Smad 3 and increased amounts of Smad 7 (levels of which are controlled by its acetylation status as described above).

Exogenously administered TGF-β, in cells from normal individuals, prevents TNF-α-induced NF-κB activation, but in IBD exogenous TGF-β has no effect [117]. This data is somewhat discrepant with the earlier description of TGF-β-associated improvement in animal models of colitis. A possible explanation is that very high levels of TGF-β effectively bypass the Smad 7 block, a theory supported by the observation that human CD lamina propria mononuclear cells (LPMC), whilst unresponsive to low dose TGF-β, do exhibit a partial response to high doses, in which signalling is restored to a certain extent [115]. Specific inhibition of Smad 7 in cells and tissues from patients with IBD restores Smad 3 phosphorylation and TGF-β-mediated suppression of proinflammatory cytokine production [117]. In recent animal studies, oral administration of Smad 7 antisense oligonucleotide restored TGF-β signalling and improved experimentally induced colitis by clinical and histological parameters, in both TNBS- and oxazolone-induced disease [118]. This may potentially yield a promising new line of therapy in IBD.
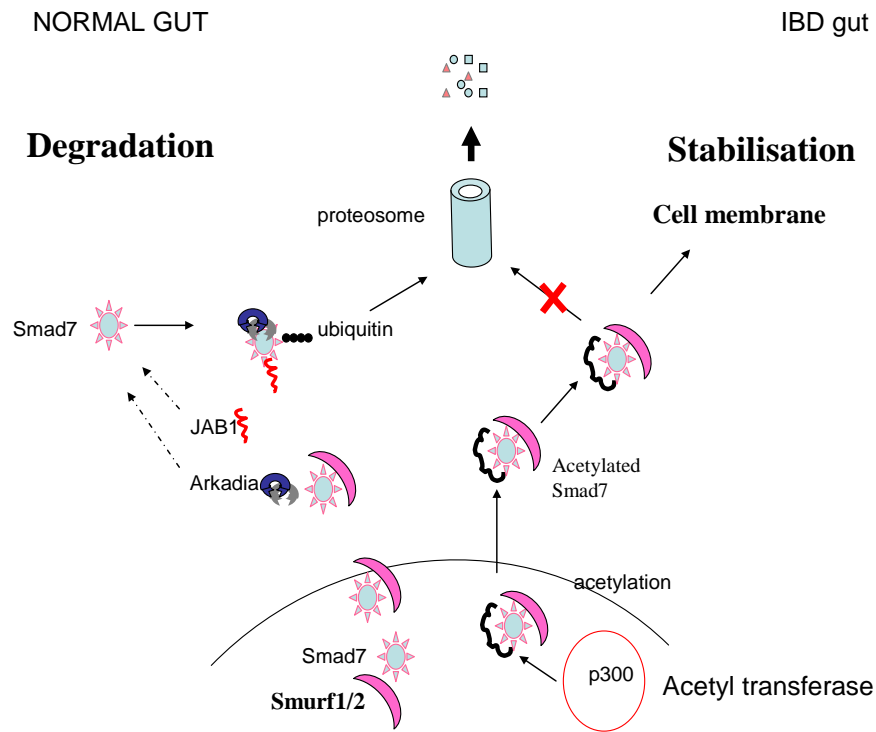
Figure 1.6   **Fate of Smad 7 in normal and inflamed gut**

The mechanisms underlying Smad 7 upregulation in IBD are not yet clear. Smad 7 manifestly undergoes post-transcriptional stabilisation in inflamed tissues, but the fundamental molecular explanation for why this is so remains obscure. Why the acetylation status in IBD is higher is not known. It has been proposed that TGF-β/Smad 3 signalling induces Smad 7 in a negative feedback loop. Against this theory is the observation that phosphorylated Smad 3 levels are reduced when Smad 7 is high [118]. It has been shown that Smad 7 is inducible by IFN-γ in monocytes (via STAT1) [119] or by TNF-α in fibroblasts (via NF-κB) [120]. However, as causative pathways these are made less likely by the observation that

blocking IFN-γ, STAT1, TNF-α and NF-κB in inflamed LPMC's leaves Smad 7 expression unchanged [115].

1.2.9 Acetyl Transferase p300

Acetyl transferase enzymes are classified into families based on their structural similarities. These include the GNAT family (Gcn5-related N-acetyl transferase), in which one example is PCAF (p300/CBP-associated factor), the MYST family (so named because it includes MOZ, YBF2/SAS3 and TIP60 groups) and the p300/cAMP response element binding protein (p300/CBP) family. In the experiments described in Chapter 1.2.8 it was demonstrated that the acetylation of Smad 7 was associated with an increase in p300 acetyl transferase. p300 acetyl transferase is a global transcriptional co-activator and, through its potent histone acetyl transferase activity, plays a role in a wide variety of gene transcription events. As previously mentioned, other non-histone proteins in the cell are subject to acetylation, and p300 also catalyses these events. An important such non-histone example is the tumour suppressor gene p53, 'the guardian of the genome', whose capacity to activate transcription and therefore DNA repair is altered by p300 status [121] [122], and indeed mutations in p300 have been found in several different types of cancer specimen, particularly in gut cancers [123]. Blocking p300 inhibits Smad 7 acetylation, enhances its ubiquitination and leads to reduced amounts of

Smad 7 in the cell [117], reflecting the situation seen in normal cells in contrast to that in IBD.

1.2.10 Transcription Factor Sp3
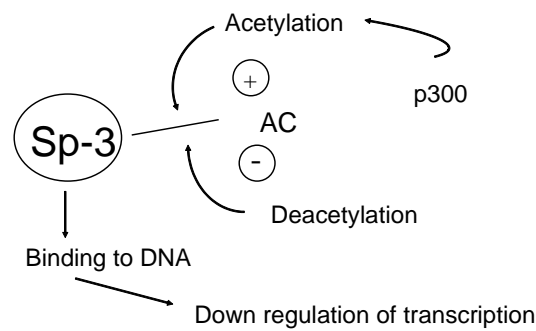
Figure 1.7　　　　　**Transcription factor Sp3**

Sp3 is a negative transcription factor which, on binding to the promoter region of insulin-like growth factor binding protein 3 (IGFBP-3), causes its expression to be downregulated [124]. This may occur by preventing the binding of Sp1, a positive transcription factor, from binding to the promoter. IGF binding proteins hold insulin-like growth factors (IGF) inactive in the circulation, limiting their bioavailability [125] and are thought to reduce T cell proliferation by preventing access of IGF's to receptors expressed by activated T cells [126], thus effecting an anti-inflammatory action. In addition, IGF binding proteins have themselves been shown to exert direct anti-proliferative effects in the gut [127]. When Sp3 is

acetylated, it binds more avidly to the IGFBP-3 promoter, further downregulating its expression, and this effect is dependent on the acetylation activity of p300 [128] (Figure 1.7). IL-10 shares the same Sp3/Sp1 binding sequence in its promoter as IGFBP-3 [129] and indeed the anti-inflammatory role of IL-10 is more clearly documented than that of IGFBP-3. This work will investigate the hypothesis that expression of IL-10 can be altered by manipulation of the acetylation status of Sp3.

1.3 Environmental Factors in Inflammatory Bowel Disease

1.3.0 Diet and Inflammatory Bowel Disease

As discussed above, the aetiology of IBD is complex and multi-factorial. As well as the genetic influences and disorders of immune regulation already addressed, environmental factors, including diet and other changes in the luminal environment, are of clear relevance. There is a robust geographic gradient in incidence of IBD from south to north, and individuals who move from a country of low incidence to one of high incidence take on the risk of the new country within the first generation born there [11]. This strongly suggests that environmental factors such as diet and other features of modern living are important causative influences. (See Chapter 1.1.0 for more on the epidemiological issues in IBD.) The rising global incidence

of IBD, especially CD, in both Western and developing countries, coincides with increasing hygiene and industrialisation, and a move away from traditional local cooking methods and ingredients, towards more 'fast food' and globalisation of the diet. Such considerations are particularly relevant to the study of a dietary agent such as curcumin, which is found in high natural concentrations in the food of India, China and other parts of Asia; regions where the incidence of IBD is low; in contrast to Europe and the United States, where dietary curcumin ingestion is low and IBD incidence much higher.

Which particular facet of Westernisation of lifestyle is to blame, if indeed it is responsible for the increasing incidence of IBD, remains a matter for debate. A recent review of seven case-control studies of patterns of food consumption in IBD concluded that certain dietary components were associated with greater odds ratios of having IBD (Table 1.2) [130]. Foods associated with both CD and UC broadly coincided, and included meat, fish, fats, sweets and 'fast food'. The finding that fish has no protective effect for IBD is consistent with a recent Cochrane review which concluded that supplementation of the diet with ω-3 polyunsaturated fatty acids (fish oil), although safe, was ineffective at maintaining remission in CD [131]. With perhaps the exception of fish, this list of foods is generally typical of a modern Western diet, whilst fresh fruit and grain-based foods like rice and bread, typical components of more traditional diets, were associated with reduced odds ratios of IBD [130]. The capacity for confounding variables in epidemiological studies of diet and disease is high, and cause or effect difficult to infer. However

the case-control design of the studies included here was rigorous and the findings do suggest that elements in the luminal environment are relevant to the disease process in IBD.
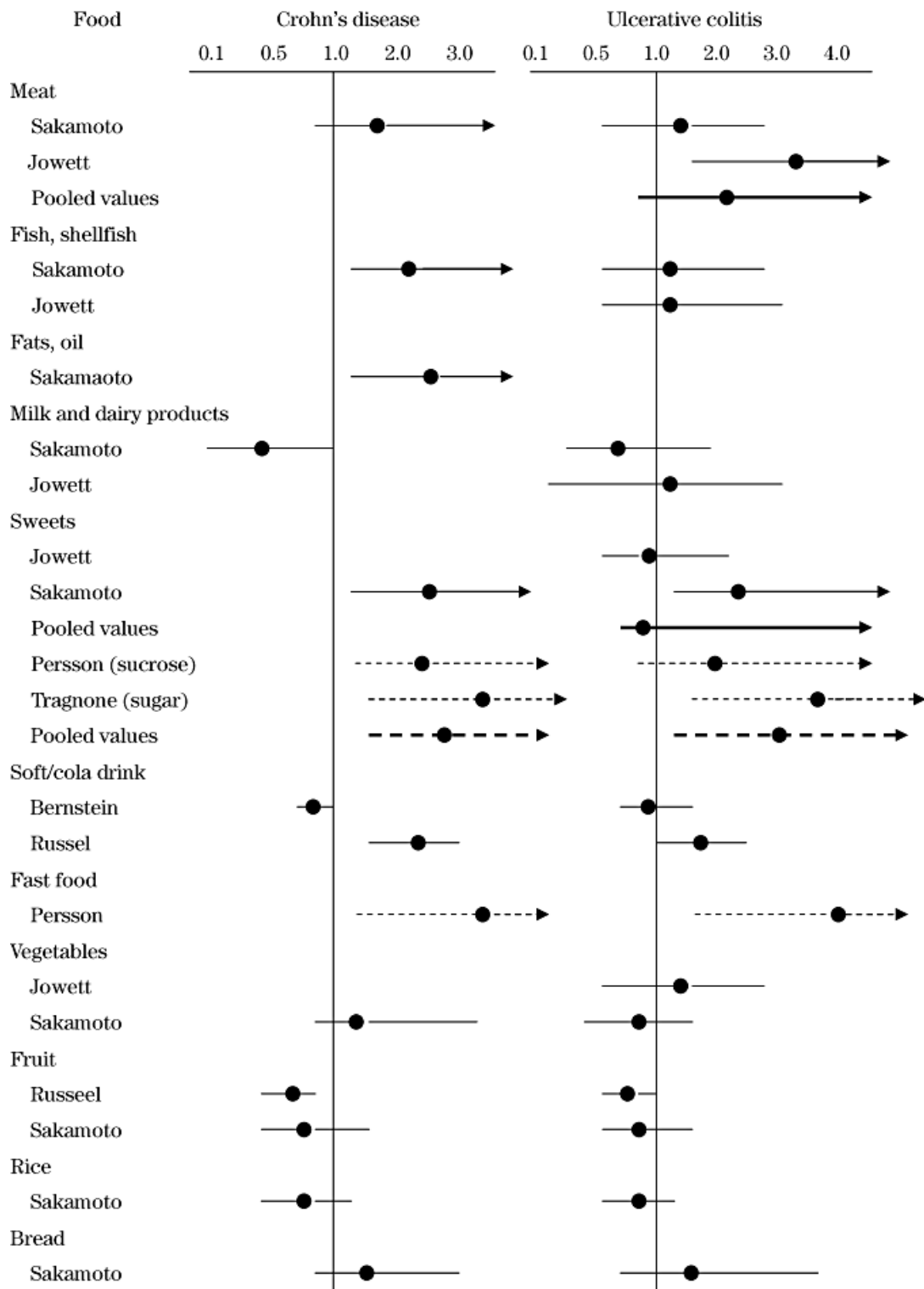
Table 1.2 Odds ratios and relative risks of foods against the occurrence of Crohn's disease and ulcerative colitis [130]

Elemental or polymeric nutrition is highly effective in bringing about remission in small bowel CD [24] [25]. The mechanism of action remains unknown. An early theory stated that the reduced antigen load somehow triggered resolution of inflammation, however subsequent trials clearly showed that polymeric formulas were equally as effective at inducing remission as elemental formulas [132]. An alternative theory proposes that alteration in bacterial flora is the important mechanism of action [26]. In either case, the efficacy of enteral nutrition therapy further demonstrates that a change in the luminal contents alters the inflammatory process in the gut in CD.

Surgical diversion of the faecal stream has been used as a treatment for Crohn's colitis for several decades [133]. Although this approach may be effective in the short term, outcomes are inconsistent and long term remission rates generally disappointing [134]. Conversely, diversion colitis occurs in the distal defunctioned rectal stump in about one third of patients who undergo ileostomy for any indication. This suggests that elements lacking in the normal faecal stream predispose to inflammation in the colon. There is evidence that this missing factor is short chain fatty acids (SCFA), and administration of a SCFA solution into the distal colon remnant can induce resolution of the diversion colitis [135]. Faecal diversion as a treatment for IBD versus faecal diversion as a cause of colitis, are clearly somewhat in contradiction with each other. Yet both observations further support the understanding that dietary factors modulate inflammatory processes in

the colon, and that the influences of luminal nutrients upon colonic inflammation and homeostasis are complex and presently far from fully understood.

1.3.1   Bacteria and Inflammatory Bowel Disease

As well as antigens of dietary origin, the bacterial population in the lumen also presents a multitude of potential antigenic stimuli to the host and is crucially involved in intestinal inflammatory responses in health and disease. As presented in Chapters 1.2.0 and 1.2.1, many of the known IBD susceptibility genes encode proteins involved in the innate and adaptive immune systems which alter the way the host processes signals from commensal gut bacteria. Furthermore strong evidence exists that IBD requires the presence of (normally harmless) bacteria in order to develop, and that individuals with IBD mount an inappropriate immune reactivity to their gut microflora (see Chapter 1.2.1).

The human gut microbiota has been evolving to its environment, and the human gut evolving to its microbiota, over millennia. New appreciation of the important interplay between the gut microbiota and human health and disease, as well as new molecular technologies for the study of microbiotic composition, architecture and function, have led to the launch of the Human Microbiome Project by the National Institutes of Health [136]. The healthy human gastrointestinal tract is colonised by up to 1000 different bacterial species, the vast majority of which have yet to be

cultured [137]. The concentration of bacteria increases greatly from proximal to distal small bowel, and the greatest concentrations are found in the colon. This coincides with the observation that the commonest sites for IBD are the colon and terminal ileum (distal small bowel).

Does the harbouring of such a large microbial biomass within the mammalian host impart mutual biological advantage? The advantage to bacteria of a homeostatically regulated environment is clear, but the symbiotic nature of the relationship may not be immediately apparent. Normal mice raised in germ-free conditions consume more food and yet have 40% less body fat than those raised in a normal environment, and conventionalisation of germ-free mice by transfer of normal microbiota completely reverses this [138]. This suggests that the microbiota greatly improves the efficiency of host nutrient handling; an enormous survival advantage during mammalian evolution. Aside from nutritional advantages, colonisation with commensal bacteria appears to confer immunological advantage on the host as well, playing a vital role in host defence by stimulating normal tolerogenic immune responses and competing with potential pathogenic species [139]. Normal mice raised in a germ-free environment fail to develop IgA-secreting plasma cells; a key element in the specific adaptive immune response of the intestinal mucosa to luminal bacteria [140].

Changing global patterns of human diet, with increased consumption of animal fats and refined sugars, appear to have an impact on the bacterial populations of the gut.

Differences in microflora are seen between individuals living in urban compared to rural areas of Japan, where rural lifestyles are associated with traditional Japanese diets and urban with more Westernised food influences [141]. Microbiotic composition also differs in health and disease, including IBD. Analysis of bacterial flora from inflamed and healthy gut reveals differences in species composition; specifically reduced species diversity in IBD [142]. Individuals with IBD have more bacteria directly attached to the colonic mucosa than do healthy controls [143]. This increases in proportion to disease severity, but is seen equally in inflamed and non-inflamed areas of gut. In patients with IBD and high concentrations of bacteria, the investigators also found characteristic bacterial inclusions in enterocytes near the lamina propria; cells which are not in contact with the faecal stream. These findings suggest that the changes in microflora seen in IBD are not merely secondary to inflammation but indicate an altered host response and different host-bacterial 'cross-talk'.

Mice ordinarily used as models of IBD either fail to develop, or develop greatly attenuated, intestinal inflammation when raised in germ-free conditions [144] [103]. This crucially implicates bacteria in the pathogenesis of IBD. Meta-analysis of ten randomised controlled trials concluded that antibiotics as an adjunct therapy are of clear benefit at inducing remission in UC [145]. Meta-analysis of six randomised controlled trials of antibiotic therapy in active CD suggested improved clinical outcomes, particularly if the therapy included ciprofloxacin [146]. These data are consistent with the observation that IBD is characterised by aberrant

bacterial processing by the host, and that manipulation of the bacterial composition can alter the inflammatory process.

Perhaps as a natural extrapolation of these observations, there has been an interest in the use of probiotics in IBD. Probiotics are living viable microorganisms which when ingested have demonstrable positive health benefits. One of the issues that has made the study of probiotics more complex is the existence of a large number of different candidate organisms, and the trialling of various different species and combinations clinically. The most widely studied species are *Lactobacillus*, *Bifidobacterium* and *Saccharomyses*. To date the only evidence with sufficient strength to support clinical use is for probiotics in pouchitis. Pouchitis is inflammation of unknown cause occurring relatively commonly in the ileo-anal reconstructed pouch which substitutes the rectum following colectomy for UC. In two separate randomised controlled trials including a total of 76 patients, probiotics showed significant benefit over placebo at maintaining antibiotic-induced remission in pouchitis [147] [148]. Both trials used VSL#3, a combination probiotic containing four strains of *Lactobacillus*, three strains of *Bifidobacterium* and *Streptococcus salivarius thermophilus*. The evidence also supports the use of VSL#3 in post-operative prophylaxis of pouchitis, in which a randomised controlled trial showed significant benefit of treatment over placebo after one year [149]. Conversely there is no evidence for efficacy of probiotics in the treatment of acute pouchitis, either of *Lactobacillus rhamnosus* GG against placebo [150], or of

an open-label combination of *Lactobacillus acidophilus* and *Bifidobacterium lactis* [151]. There is no convincing evidence that probiotics are useful in CD or UC.

1.4   Curcumin

1.4.0   Background: Human Uses of Curcumin

For thousands of years humans have used plants for therapeutics. Recent years have seen the development of highly targeted biological treatments and synthetic therapies, some with serious side effects. At the same time there is renewed public interest in complementary therapies, naturally occurring treatments with minimal toxicity, and diets related to health and disease.

Figure 1.8   **Turmeric plant (*Curcuma longa*) with flowers and root**

Curcumin is a constituent of the spice turmeric, one of the principle ingredients in curry powder. Turmeric is prepared from the root of the *Curcuma longa* plant (Figure 1.8), a member of the ginger family. It is native to India and Southeast Asia, where fresh turmeric root is widely used in a similar way to ginger; in the West turmeric is much more commonly available as a dried powder. It has been used to treat a broad range of common ailments in Indian Ayurvedic medicine for at least 4000 years, as well as in Chinese, Arabic and other traditional medicines. Curcumin is in modern use worldwide as a cooking spice, flavouring agent and colorant. Dishes traditionally made with turmeric include dahls and most other curries, as well as pickles, relishes and chutneys. It is widely used to colour mustards, mayonnaises and margarines and has been designated as international food additive E100. Because of its resemblance to saffron, curcumin is sometimes referred to as 'Indian saffron' and used as a (much less expensive) substitute.

1.4.1   Chemistry of Curcumin
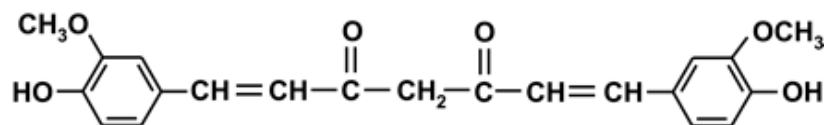
Figure 1.9   **Molecular structure of curcumin**

The active ingredient of curcumin is diferuloylmethane, a hydrophobic polyphenol with a characteristic yellow colour. In chemical terms it is a bis-α, β-unsaturated β-

diketone, a linear diarylheptanoid compound, where two oxy-substituted aryl moieties are linked together through a seven carbon chain (Figure 1.9).

1.4.2   Dose and Safety of Curcumin

The safety, tolerability and non-toxicity of curcumin at high doses are well established. Oral doses up to 12g per day are well tolerated in humans [152], although dosing regimes above 8g may be difficult to achieve due to the bulky nature of this quantity of compound [153]. However drug delivery is a problem and the bioavailability of oral curcumin is low [154, 155] due to a combination of efficient first pass metabolism, poor gastrointestinal absorption, rapid elimination and poor aqueous solubility. Elimination is largely via hepatic glucuronidation and sulfation.   Metabolites lack pharmacological activity [156], and are excreted through stool. Serum concentrations peak one to two hours after oral administration and are undetectable by 12 hours [153]. Some investigators report that serum curcumin is undetectable below oral doses of about 4g [152, 153], however others have detected curcumin not only in serum, but also urine, at much lower doses [157]. Some studies also demonstrate the presence of curcumin in colorectal tissue at oral doses of 3.6g [158], so the gut may represent a promising local clinical target for curcumin.

The apparent discrepancies in pharmacodynamics observed between different dosing regimes may relate to the differing formulations used. Curcumin constitutes about 5% of turmeric root [159] [160]; the remainder is made up of carbohydrates, proteins and essential oils. There are three natural analogues of curcumin (curcuminoids) which vary in methoxy substitution on the aromatic ring. Curcumin is the most abundant (77%), followed by demethoxycurcumin (17%), then bisdemethoxycurcumin (3%) [161]. Preparations used for human consumption are either naturally produced from purified turmeric extract, which contains varying proportions of the different curcuminoids, or are synthetically produced, containing only pure chemically synthesised curcumin. The bioactivity of these different analogues varies according to the cell type and function analysed, disease system and organism. There is no consensus as to the most effective preparation for human use. Strategies have also been employed to improve bioavailability based on changes in drug formulation, such as the use of nanoparticles to reduce particle size delivery, and micelles to counter hydrophobicity. Recently it has been reported that heat treatment improves the water solubility of curcumin [162].

In human trials only minor side effects of curcumin, namely diarrhoea [157], have been reported, and it is considered safe and well-tolerated. As a caveat however, these trials have usually examined short term outcomes. There is some evidence that long term, high dose curcumin administration in rodents can be tumorigenic [163] [164]. It has also been shown that curcumin's predominant activity switches from anti-oxidant to pro-oxidant with increasing concentration [165], which may

provide an explanation for its seemingly opposing biological effects *in vivo*. These apparent contradictory roles of curcumin, as both anti-cancer, and pro-carcinogenic agent, are as yet unexplained, and epitomise the complexity and paradoxical nature of the compound. Nevertheless there is good evidence from India, at a population level, of the safety of lifelong curcumin ingestion up to about 100mg/day [166], and it is classified 'Generally Recognised As Safe' by the United States Food and Drug Administration.

1.4.3 *In Vitro* Studies of Curcumin

A wide variety of cellular properties of curcumin have been demonstrated, including anti-oxidant, anti-inflammatory, anti-proliferative, pro-apoptotic, anti-bacterial and anti-cancer activities (Table 1.3 and Figure 1.10).

Biological Activity	Molecular Mechanism	Cell line (human unless otherwise stated)
Anti-inflammatory	↓NF-κB	Myeloid leukaemia[167] [168], B non-Hodgkin's lymphoma (NHL)[169] [170], embryonic kidney (HEK)[167], mouse macrophage[171]
	↓COX2	Intestinal microvascular endothelial[172], colonic epithelial[173], microglial[174], mouse macrophage[171]
	↓IL-1β, IL-6, IL-8	Oesophageal epithelial[175], head and neck cancer[176], mouse macrophage[171]

	↓TNF-α	Mouse macrophage[171]
	↓ICAM-1	Epithelial/umbilical vein endothelial (HUEVC) hybridoma[177]
	↓p300 acetyltransferase	Tracheal smooth muscle[178], cervical cancer[179], HEK[179], lymphoblastic T lymphoma[179], Burkitt's lymphoma[180]
	↑PPAR-γ	Colon cancer[181], rat hepatic stellate[182]
Anti-oxidant	↓Nitric oxide synthase (NOS)	Mouse macrophage[171]
	↑Glutathione	Lymphocytes[183]
	↑Haem oxygenase (HO)-1	Epithelial/HUEVC hybridoma[177], porcine renal epithelial[184]
	↑Superoxide dismutase (SOD)	Lymphocytes[183]
	↑Reactive oxygen species (ROS)	Promyelocytic leukaemia (PML)[185]
Pro-apoptotic	↓Bcl-2	B NHL[170], colon cancer[186] [187]
	↓survivin	Colon cancer[187]
	↓Akt	T leukaemia[188]
	↓c-*myc*	B NHL[170]
	↓Ornithine decarboxylase (ODC)	PML[185]
	↑Bax	Colon cancer[186] [187]
	↑caspases	PML[185], colon cancer[186] [187]
	↕JNK	T lymphocyte↓[189], breast cancer↓[189], HEK↓[189], colon cancer↑[190]
Anti-cancer	↓cyclin D1	Colon cancer[181]

	↓MMP	HUEVC[191], melanoma[191], fibrosarcoma[191, 192], breast cancer[191], blood mononuclear[193], intestinal epithelial[194]
	↓Epidermal growth factor (EGF)	Hepatocellular carcinoma (HCC)[195]
	↓STAT	Multiple myeloma[196], Hodgkin's lymphoma[197]
	↓Hypoxia-inducible factor (HIF)-1α	HCC[195]
	↓Protein kinase C (PKC)	Mouse embryonic fibroblast[198]
	↓Early growth response factor (*egr*)-1	B NHL[170], HUEVC[199], lung fibroblast[199]
	↓Activator protein (AP)-1	HEK[174], microglial[174]
	↕p38 MAPK	Intestinal microvascular endothelial↓[172], neutrophils↑[200]
	↕p53	B NHL↓[170], colon cancer↑[186]↓[201], thymocytes↓[202], myeloid leukaemia↓[202], breast cancer↑[203]

Table 1.3 Molecular targets of curcumin in cell line studies
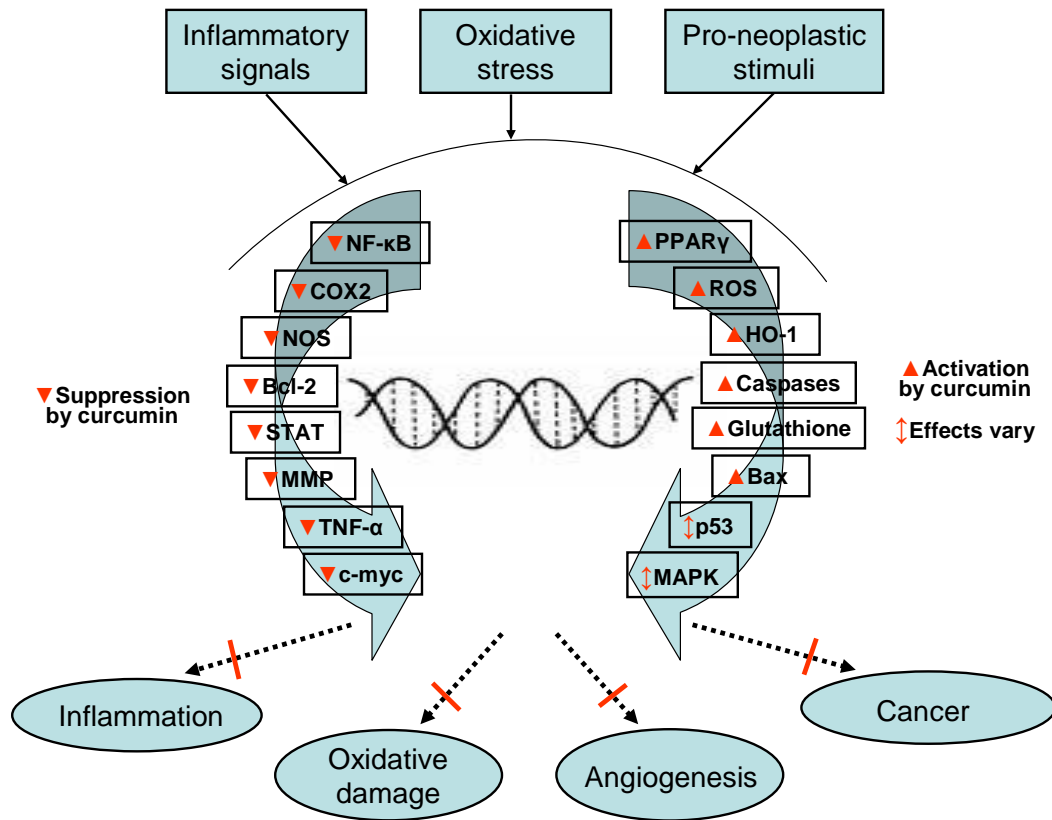
Figure 1.10   **Cellular activities of curcumin and molecular mechanisms of action**

1.4.4   Transcription Factors: *in vitro* Actions of Curcumin

Nuclear Factor (NF)-κB

General information on the role of NF-κB in inflammation and IBD is presented in

Chapter 1.2.3 and NF-κB signalling pathways are depicted in Figure 1.3. NF-κB is

one of the key transcription factors responsive to curcumin. In human myeloid

ML-1a cells, curcumin suppresses NF-κB activation induced by TNF-α, phorbol

ester and hydrogen peroxide [168]. The mechanism appears to be via reduced IκBα

phosphorylation and degradation [167], suggesting that curcumin acts at a step above IκB kinase (IKK) in the NF-κB activation pathway. Overall, inhibition of NF-κB signalling by curcumin is a consistent finding in a number of studies. For example in four different human mantle cell lymphoma lines (an aggressive non-Hodgkins B cell lymphoma), curcumin down-regulated NF-κB, inhibited IKK and reduced IκBα phosphorylation, leading to cell cycle arrest, apoptosis and suppression of proliferation [169].

Signal Transducer and Activator of Transcription (STAT) 3

STAT3 is a transcriptional activator with a ubiquitous role in tumourigenesis. It is involved in dysregulation of cell growth, invasion, angiogenesis and metastasis [204]. Curcumin inhibits STAT3 activation in human multiple myeloma cells [196], and in five different human Hodgkin and Reed-Sternberg lymphoma cell lines [197].

Peroxisome Proliferator-Activated Receptor (PPAR)-γ

PPAR-γ is a nuclear receptor and transcription factor involved in cell cycle control, proliferation and differentiation, exerting anti-inflammatory, anti-cancer and insulin-sensitising actions. It is activated by prostaglandin products of the eicosanoid cascade [205] [206], and possibly by dietary components such as linolenic and linoleic acid, although the role of these is controversial. Its activity in Moser cells (a human colon cancer cell line) is enhanced by curcumin, consequently interrupting the cell cycle through reduced expression of cyclin D1

[181]. The same group also show induction and activation of PPAR-γ by curcumin in rat hepatic stellate cells, a liver cell type responsible for fibrosis in liver injury which contributes to chronic liver damage and cirrhosis. PPAR-γ inhibited the proliferation of stellate cells, and curcumin greatly enhanced this effect [182].

1.4.5 Mitogen-Activated Protein Kinase Signalling Pathways: *in vitro* Actions of Curcumin

The MAPK cascade is activated by a large number of different types of receptor, including cytokine, growth and toll-like receptors, and receptors sensitive to environmental stressors. General background on MAPK signalling is presented in Chapter 1.2.2 and Figure 1.2. The precise mechanisms of activation are incompletely understood [207]. Curcumin modulates MAPK signalling in several different *in vitro* models, although the data are somewhat contradictory. Under some circumstances, curcumin inhibits MAPK activation, as in a recent study in primary human intestinal microvascular endothelial cells, where curcumin inhibited p38 MAPK activation in response to vascular endothelial growth factor, as well as COX-2 and prostaglandin E(2) production [172]. These anti-angiogenic properties of curcumin are of potential clinical benefit in gut inflammation and cancer. Further evidence that curcumin inhibits MAPK pathways includes its inhibition of JNK in Jurkat T cells (a human T cell line) [189].

Other investigators paradoxically show activation of MAPK by curcumin, for example of JNK in HCT116 cells, a human colon cancer cell line [190] and of p38 MAPK in primary human neutrophils [200]. Thus curcumin can activate MAPK signalling, with the biological consequence of apoptosis; and can also inhibit MAPK signalling, where the consequences are anti-inflammatory and anti-angiogenic. Whilst the mechanism for its opposing actions is unexplained, in both cases its ultimate effects are anti-neoplastic and anti-inflammatory. Where MAPK activation is seen, it is possible that this is due to bacterial contaminants such as LPS rather than a true effect of curcumin. It is likely that the primary molecular targets lie elsewhere, and the MAPK signals which are observed experimentally represent intermediary pathways by which the biological effects of curcumin are mediated.

1.4.6 Tumour Suppressor Gene p53: *in vitro* Actions of Curcumin

Mutation of the tumour suppressor gene p53 plays an important role in the evolution of many different human cancers. Once again, the role of curcumin is complex. In an early study of the effects of curcumin on BKS-2 and WEHI-231 cells (both immature B cell lymphoma mouse cell lines) proliferation was inhibited [170]. Interestingly, and with obvious potential clinical benefit in cancer chemotherapy, this inhibitory effect was much less marked on normal B cells. The investigators demonstrated (unexpected) inhibition of expression of p53 by

curcumin, as well as inhibition of various other genes involved in growth, proliferation and transcriptional activation, including early growth response factor (*egr*)-1, the proto-oncogene c-*myc* and the transmembrane anti-apoptotic *bcl*-X_L. The finding of reduced p53 activity was confirmed in RKO cells (a colon cancer cell line) where curcumin impairs the post-translational folding of p53 required for its function [201], and in myeloid leukemic cells where it induces p53 degradation [202].

Conversely, other experiments show induction of p53 by curcumin, for example in human epithelial breast cancer, prostate cancer and B cell lymphoma cell lines [203], and in HT-29 cells (a human colon adenocarcinoma cell line) [186]. In the former work, once again the authors show differential sensitivity of cancer cells compared to healthy cells to curcumin. Whilst some investigators have shown anti-proliferative effects despite inhibition of the tumour suppressor p53 [170], established precedents exist where an agent which is cancer-preventative in one system, can be carcinogenic in another, for example tamoxifen (therapeutic in breast; pro-neoplastic in uterus) [208].

1.4.7 Angiogenesis: *in vitro* Actions of Curcumin

There is strong evidence that curcumin is anti-angiogenic. Angiogenesis (the growth of new blood vessels) is required for the development of both inflammation

and cancer, where it is crucial for the survival of tumours beyond a certain size. It is also integral to the generation of diabetic eye disease, which is characterised by growth of abnormal vessels across the retina, a major cause of blindness worldwide. In an early study in both primary bovine and immortalised mouse endothelial cells, curcumin inhibited endothelial cell proliferation [209]. Curcumin inhibits the angiogenic differentiation of human umbilical vein endothelial cells (HUVECs) [210] [195]. Also in HUVECs, curcumin binds to and irreversibly inhibits aminopeptidase N [191], a membrane-bound matrix metalloproteinase (MMP) which increases tumour invasiveness and is involved in retinal neo-vascularisation and tumour angiogenesis [211]. Finally curcumin decreases hypoxia-inducible-factor (HIF)-1α, an angiogenic transcriptional activator, in human hepatocellular carcinoma cells [195]. In this work curcumin also inhibited the transcriptional action of HIF-1α, downregulating expression of vascular endothelial growth factor (VEGF), a potent hypoxia-induced angiogenic factor.

1.4.8   Inflammatory Cytokines: *in vitro* Actions of Curcumin

Several studies demonstrate the suppression of downstream pro-inflammatory and pro-neoplastic mediators by curcumin. Recent examples include reduced expression of IL-6 and IL-8 in response to acid exposure in a human oesophageal epithelial cell line [175] and reduced spontaneous expression of IL-6 and IL-8 in four different head and neck squamous carcinoma cell lines [176]. Several of the

authors suggest that these observations may be secondary to the suppression by curcumin of intermediary signalling pathways such as NF-κB, and some investigators provide evidence to this effect [176].

1.4.9   Cyclo-oxygenase 2: *in vitro* Actions of Curcumin

Cyclo-oxygenase (COX) 2 is an inducible form of prostaglandin H synthase.   It mediates inflammation through production of prostaglandins and plays an important role in colon cancer.   Over-expression of COX2 in colonic epithelium appears to promote tumour development [212] and non-steroidal anti-inflammatory drugs, which inhibit COX2, reduce the risk of colon cancer [213].   Curcumin inhibits COX2 production in a primary human intestinal microvascular endothelial cell line [172] and inhibits COX2 induction in human colonic epithelial cells [173]. In this latter work the authors note that the COX2 gene promoter contains two NF-κB binding sites and show evidence to suggest that the effect of curcumin on COX2 is due to inhibition of the NF-κB signalling pathway.

1.4.10 Matrix Metalloproteinases: *in vitro* Actions of Curcumin

In health, fibroblasts produce low levels of matrix metalloproteinases (MMP) that remain largely in latent form and mediate physiological extracellular matrix turnover. In IBD, MMP's are over-expressed and become activated in cascades causing unchecked tissue destruction, fibrosis and further increasing immune cell activation and homing [214]. MMP's also play a key role in tumour progression, since matrix dissolution is an important step in the conversion of a pre-malignant cell into a frankly malignant one, as well as in tumour growth, invasion, metastasis and angiogenesis [215]. There are over 20 different types of MMP, which are sub-classified according to the primary stromal substrate upon which they act. Curcumin down-regulates MMP production in various cell types. In human fibrosarcoma cells it decreases invasion, migration and production of MMP-2 and MMP-9 [192], and in human and rabbit peripheral blood mononuclear cells it reduces MMP-9 [193]. Recently it was shown to reduce MMP-9 in human intestinal epithelial cells [194].

1.4.11 p300 Acetyl Transferase: *in vitro* Actions of Curcumin

Lastly, curcumin is a known inhibitor of acetylation, acting on the enzyme p300 acetyl transferase [178] [179]. Acetylation modifies proteins when an acetyl group

binds to a lysine residue, altering the protein's shape, charge and biological fate in the cell. Traditionally the study of acetylation has examined how the acetylation of histones changes their conformation, loosens their interactions with deoxyribonucleic acid (DNA) and thus opens out the nucleosome, exposing DNA for gene transcription [106] [107]. However, recent work shows that other (non-histone) regulatory proteins within the cell are also subject to acetylation, initiating separate cellular events which regulate for example transforming growth factor (TGF)-β signalling [117] and insulin-like growth factor binding protein (IGFBP)-3 expression [124] [128] (Chapters 1.2.7 – 1.2.10). Such events are important in inflammation and cellular proliferation. Another important such non-histone example is the tumour suppressor gene p53, whose capacity to activate transcription and therefore DNA repair is altered by p300 status [121] [122], and indeed mutations in p300 have been found in several different types of cancer specimen, particularly in gut cancers [123].

p300 acetyl transferase, as a potent catalyst of acetylation, plays a role in a wide variety of gene transcription and other cellular events. Several effects of curcumin resulting from its p300 inhibitor activity are documented, including inhibition of inflammatory responses in human tracheal smooth muscle cells [178], suppression of human immunodeficiency viral (HIV) proliferation [179] and inhibition of proliferation of Raji cells (a non Hodgkin B cell lymphoma line) [180].

1.4.12 Curcumin in Animal Models: Inflammatory Bowel Disease

Whilst curcumin has shown benefit in a number of different models of inflammatory disease, particular interest has focused on its use in the gut. IBD is a source of considerable morbidity, and its incidence is increasing worldwide. Currently available treatments such as steroids, 5-aminosalicylic acids and immunomodulators do not offer cure, but CD responds well to polymeric or elemental feed, which brings about remission in 80% of paediatric patients [24] [25]. IBD is less common in developing countries than in the industrialised world [216], and individuals emigrating from East to West take on the Western disease risk [11, 216]. This holds further relevance to the importance of diet in inflammatory bowel disease, and there is keen interest to develop nutritional therapies.

Several studies in various rodent disease models provide strong pre-clinical evidence for the benefit of curcumin [217] [218] [219] [220]. For example in multidrug resistance gene deficient mice, which spontaneously develop colitis, the addition of curcumin to their diet significantly reduced intestinal inflammation [219]. Other investigators used 2,4 dinitrochlorobenzene colitis in rats, and showed a dose-dependent improvement in disease activity parameters with dietary curcumin, of equal potency to sulfasalazine treatment [220]. Curcumin treatment was associated with a reduction in colonic NF-κB, inducible nitric oxide synthase

(iNOS) and various measures of oxidative stress, for example myeloperoxidase and lipid peroxidation.

The efficacy of curcumin in IBD may differ according to inflammatory circumstances and dose. For example trinitrobenzene sulfonic acid (TNBS) colitis in NKT-deficient SJL/J mice exhibits a classic T helper cell (Th)1-type response, whilst BALB/c mice with TNBS colitis exhibit a mixed Th1/Th2 profile [221]. Curcumin caused improvement in all disease activity parameters only in the BALB/c mice. In simple terms Th1-type inflammation relates more closely to CD and Th2 to UC, although in real terms the situation is probably more complex with a degree of overlap. The reason for the differential efficacy of curcumin in these two models is unclear. The IL-10 knock out mouse develops spontaneous Th1-type inflammation in large and small bowel which is dependent on gut bacteria, making it a good model of CD. The protective effect of curcumin in this model (by colon morphology and colonic interferon (IFN)-γ and IL-12/23p40 mRNA) was modest [222].

This finding bears interesting relevance to the work proposed in this thesis. The effect of curcumin on the IL-10 knockout mouse may have been minimal because one of the mechanisms for the efficacy of curcumin in IBD is through enhanced IL-10 expression, and in this model curcumin was unable to increase IL-10 expression as the gene had been deleted. These investigators also found that any small protective effect of curcumin in this model occurred paradoxically only at the

lowest dietary concentration of 0.1% and was not observed at higher concentration [222]. *In vivo* NF-κB activation in the gut was unaffected by curcumin at any concentration, but curcumin acted synergistically with IL-10 on epithelial cells to decrease NF-κB activity. These data raise once again the suggestion that curcumin can have paradoxically opposing effects at different concentrations, and when clinical studies take place, a wide range of dosages is warranted.

1.4.13 Curcumin in Animal Models: Cancer

Chemoprevention

The molecular targets of curcumin include many pathways and processes involved in the generation and propagation of cancer. The observation that many common cancers (including colon, breast, prostate and lung) are commoner in the Western world than in countries such as India where there is high natural dietary curcumin consumption [166], whilst not indicative of cause and effect, is intriguing. Curcumin has been investigated as both chemotherapeutic and chemopreventive agent in many different animal (largely rodent) models of carcinogenesis. Its chemopreventive efficacy for colon cancer is particularly well established [223] [224]. Other gastrointestinal cancers against which curcumin has shown protective effects include oesophageal [225], stomach [226], liver [227] and oral [228]; all in rodent models. Curcumin also shows chemopreventive properties in rodent models

of various extra-intestinal cancers, including breast [229], lung [230], kidney [231], bladder [232], blood [229] and skin [233] (Table 1.4).

Cancer	Animal Model	Reference
Gastrointestinal System: Colon		
	Mouse	[223, 224, 234, 235]
	Rat	[236, 237, 238, 239, 240, 241, 242, 243, 244, 245]
Small intestine	Mouse	[234]
Stomach	Mouse	[246, 247, 248]
	Rat	[226]
Oesophagus	Rat	[225]
Liver	Rat	[227]
Pancreas	Mouse	[249]
Mouth	Rat	[250]
	Hamster	[228, 251]
Breast	Mouse	[229, 252]
	Rat	[238, 253, 254, 255, 256, 257]
Lung	Mouse	[230]
Skin	Mouse	[233, 246, 248, 258, 259, 260, 261, 262, 263, 264]

Blood Cancers:		
Leukaemia	Mouse	[229, 265]
Multiple myeloma	Mouse	[266]
Urinary Tract:		
Kidney	Mouse	[231, 267]
Bladder	Mouse	[232]
Brain	Mouse	[268]
Prostate	Mouse	[269]

Table 1.4 Animal models in which curcumin has chemopreventive efficacy

Chemotherapy

Curcumin inhibits tumour growth and metastasis, and has chemosensitising and radiosensitising properties. One of the earliest examples of the ability of curcumin to inhibit tumour growth is that of lymphoma cells in a mouse ascites model, when it was administered intra-peritoneally at 50mg/kg [270]. Curcumin also has anti-tumour efficacy against human melanoma cell xenografts if given intra-peritoneally [271]. Also in xenograft models, sub-cutaneous delivery of curcumin suppresses growth of head and neck squamous carcinoma cells [272], and when given orally it inhibits proliferation and angiogenesis, and induces apoptosis in prostate cancer cells [273].

Curcumin also suppresses proliferation and angiogenesis and enhances apoptosis in pancreatic cancer, both when given orally in combination with gemcitabine in an orthotopic model [274], and in a xenograft model when given intravenously in a liposomal formulation [275]. The same group have also used an intravenous liposomal curcumin preparation in luminal gastrointestinal cancers, where it has chemosensitising properties against colorectal cancer in a mouse xenograft model [276]. In this work tumour growth and angiogenesis were inhibited and apoptosis enhanced in combination with oxaliplatin. In an orthotopic implantation model of hepatocellular carcinoma, curcumin also prevented intrahepatic metastasis [277].

Finally, in recent work, oral curcumin has shown efficacy in preventing breast cancer metastasis to lung in orthotopic models, both as chemosensitiser in conjunction with paclitaxel [278] and in the prevention of its haematogenous spread in immunodeficient mice [279]. Curcumin given intraperitoneally in combination with docetaxel inhibits tumour growth and angiogenesis in an orthotopic nude mouse model of ovarian cancer [280].

1.4.14 Curcumin in Human Trials

In clinical trials, curcumin improves rheumatoid arthritis [281] [282], lowers serum cholesterol [283], treats psoriasis [284], enhances early post-transplant renal graft function [285], improves post-operative inflammation [286], reduces inflammation

in chronic anterior uveitis [287, 288] and orbital inflammatory pseudo-tumours [287, 288] and improves endothelial function in type 2 diabetes mellitus [289]. In specific relation to the gut, curcumin reduces symptoms in irritable bowel syndrome [290], improves outcomes in tropical pancreatitis [291] (a type of chronic pancreatitis involving the main pancreatic duct, occurring usually in young people and mainly in tropical countries such as southern India), normalises gall bladder and biliary motility [292, 293, 294], promotes resolution of gastric ulceration [295] and reduces polyp number and size in familial adenomatous polyposis coli [296] (Figure 1.11).
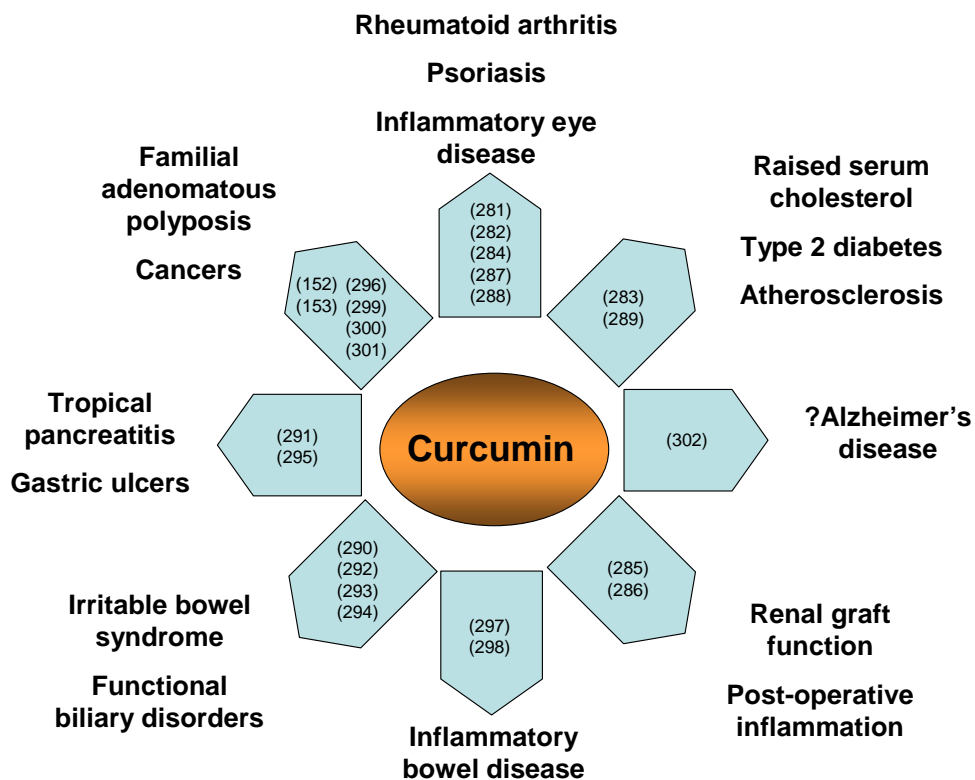
Figure 1.11　　**Clinical effects of curcumin: Results from human trials** (with references)

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Clinical Trials of Curcumin in Inflammatory Bowel Disease

Curcumin is also showing early promise as a treatment for CD and UC. In a small open-label study of five consecutive patients with CD and five with ulcerative proctitis performed in 2005 by a group in New York, improvements in clinical and laboratory parameters with reduction in need for concomitant medications were observed in nine out of ten cases [297]. The patients with ulcerative proctitis were all on concurrent 5-ASA preparations and one was also taking prednisolone and azathioprine. They received 550mg oral curcumin twice daily for one month, followed by the same dose three times daily for a further month; a relatively small dose compared to that trialled by other investigators (Chapter 1.4.2). Measurements included standard symptom questionnaires, serological markers of inflammation and sigmoidoscopy with histology, both at baseline and two months, after the treatment period had ended, as well as symptom diaries throughout the treatment period. All five patients showed global improvement and four out of five reduced their 5-ASA medications. The patient who was on steroids stopped these by the end of the trial period. The patients with CD had ileitis, colitis or ileocolitis, and all but one were taking concomitant steroid medications. They were treated with 360mg curcumin orally three times per day for the first month, followed by 1440mg four times daily for a further two months. The patients with CD were assessed in the same way as those with ulcerative colitis, except for endoscopic evaluation, which was not done. Four out of five patients successfully completed the trial; one dropped out because of lack of treatment effect. Of the four who completed CD activity index questionnaires, their scores fell in four out of four,

with percentage change -13% to -44%. The serological marker for inflammation, erythrocyte sedimentation rate, also fell in all four patients, with percentage change -17% to -71%. No side effects were reported.

Further encouraging results came from Japan in 2006, where a larger eight centre randomised double blind controlled trial of 89 patients with quiescent UC was carried out, in which 45 patients were randomised to receive curcumin at a dose of 1g twice daily alongside 5-ASA treatment; and 44 patients to receive placebo and 5-ASA [298]. Any patient receiving an immunomodulator was excluded. The treatment period was six months, and this was followed by a further six month period of ongoing 5-ASA treatment only. Disease severity was measured by both clinical activity index and endoscopic index at baseline, at the end of the six month trial period and six months after that. In addition the clinical activity index was measured every two months throughout the duration of the trial. Seven patients violated the protocol; two in the curcumin group and five in the placebo group. Disease relapse rates were evaluated on an intention to treat basis. In the curcumin treated group, 2/43 patients relapsed (4.6%), compared to 8/39 (20.5%) in the placebo group. This difference was significant at p=0.04. The investigators also showed significant clinical and endoscopic improvements in the curcumin treated group. The clinical activity index worsened in the placebo group from 1.0 to 2.2 (p=0.0003), whilst in the curcumin group it improved from 1.3 to 1.0 (p=0.04). There was no change in endoscopic appearances in the placebo group, but there was significant evidence of endoscopic healing in the curcumin group, with

endoscopic index improving from 1.3 to 0.8 (p=0.0001). During the further six month follow up period, there was no difference in number of patients relapsing in either study arm. Seven patients experienced mild transient side effects including bloating, nausea and diarrhoea, although attribution to curcumin is uncertain, particularly since the patients were also taking 5-ASA drugs. None of these patients discontinued the treatment. One patient experienced transient hypertension and curcumin was discontinued in this individual.

It is particularly encouraging to note that on ClinicalTrials.gov of the United States National Institutes of Health, a phase 1 trial of curcumin therapy in paediatric UC was recently registered at Seattle Children's Hospital (identifier NCT00889161). This will be a single centre open label dose titration study to determine the tolerability and safety of oral curcumin in children with UC. The investigators plan to recruit 20 children aged 8-18 with mild or quiescent UC, for an initial nine week study period, after which appropriate dose will be determined to proceed to double blinded placebo controlled trial. The dose will start at 500mg twice daily for three weeks, will escalate to 1g twice daily for a further three weeks and will finish at 2g twice daily for the final three weeks. The study plans to complete in May 2010.

These studies so far involve relatively small patient numbers, but the results certainly indicate that larger trials of rigorous design are warranted for curcumin therapy in IBD. It is particularly encouraging that curcumin is now being trialled in children with IBD for the first time. Its non-toxicity and normal presence in the

diet make it ideally suited to the strict ethical constraints surrounding recruitment of children into pharmaceutical trials. Many drugs are necessarily used outside their commercial licences in children because of the lack of clinical trial evidence supporting their paediatric use. The burden of IBD in childhood is increasing and with the lifetime risk of intestinal inflammation and cancer that this carries, a greater choice of long term maintenance anti-inflammatory medications with low side effect profiles is required.

Clinical Trials of Curcumin in Cancer

Lastly there is building clinical evidence that curcumin can prevent and treat cancer in humans. Results from a trial of 25 patients with various different pre-malignant or high risk lesions, suggested that oral curcumin may have chemopreventive effects in progression of these lesions [153]. Whilst two of the 25 patients progressed to frank cancer, seven regressed; a remarkably high proportion considering the high grade nature of the lesions (bladder cancer, oral leukoplakia, gastric intestinal metaplasia, cervical intraepithelial metaplasia and Bowen's disease). In another uncontrolled study of 15 patients with advanced colorectal cancer refractory to standard treatments, the lymphocytic biomarker glutathione S transferase showed a 59% reduction in activity with low dose (440mg daily) oral curcuma extract, and five patients maintained radiologically stable disease over the 2-4 month study period [299]. Once again there is a suggestion here that curcumin exhibits paradoxical efficacy at low versus high dose, since this effect was not observed at higher doses. In an interesting, but also uncontrolled, study of 62

patients with oral cancerous lesions, topical curcumin application reduced symptoms in the majority (70%) and caused tumour shrinkage in 10% [300]. Of 21 patients with advanced, normally rapidly fatal, pancreatic cancer treated with high dose oral curcumin, encouragingly four showed disease stability or regression [301]. The cancer preventive properties of curcumin would be a clearly attractive side effect if it comes into clinical use in IBD, since colitis carries a long term risk of bowel cancer.

Future Clinical Trials of Curcumin

These preliminary data hold promise, and interest in curcumin as a therapeutic agent continues to grow. There are several clinical trials currently ongoing, some involving larger numbers of patients and with a more rigorous randomised controlled design. A search on ClinicalTrials.gov currently reveals 31 human trials using curcumin, of which 14 are investigating its chemopreventive or chemotherapeutic potential in cancer or pre-malignant conditions. As in the data already reviewed, there is a preponderance of gut cancers; six are in colorectal cancer, two in familial adenomatous polyposis coli, one in ulcerative colitis and three in pancreatic cancer. A novel area of interest is in Alzheimer's disease and cognitive impairment. The first clinical trial failed to show benefit, but this may have been due to an unexpected lack of cognitive decline in the placebo group [302]. Three current ongoing trials of curcumin are further assessing its efficacy in age-related cognitive impairment. Interest also continues in systemic inflammatory

conditions and there are two ongoing trials of curcumin in arthritis and one in psoriasis.

1.4.15 Curcumin: Summary and Relevance to the Current Work

Since ancient times, curcumin has been used in a wide range of inflammatory, neoplastic and other conditions. In recent years the molecular basis for its efficacy has been extensively investigated. Many cellular and molecular targets have been identified and many questions still remain. In complex multi-factorial illnesses such as systemic inflammatory diseases and cancer, an agent that acts at a number of different cellular levels offers perhaps a better chance of effective prophylaxis or treatment. Its non-toxicity and good tolerability in humans, in combination with strong promising results from cell line, animal and early human clinical studies, support the ongoing research and development of curcumin as a preventive and disease modifying agent.

The current work will explore the effects of this biologically complex compound in inflamed human gut. We will examine the effects of curcumin on both TGF-β and IL-10 pathways, as well as on the expression of pro-inflammatory cytokines IL-1β and IFN-γ. We will study *in vitro* models of IBD, using cell and tissue culture systems derived directly from patients with active IBD. These will include colonic lamina propria mononuclear cells (LPMC's) and colonic mucosal biopsies. We

will also examine the actions of curcumin on key gut mucosal stromal effector cells in IBD, colonic myofibroblasts (CMF), also isolated from patients with active IBD. We will measure the effects of curcumin on matrix metalloproteinase (MMP) expression by CMF. We will explore potential mechanisms behind these actions, including p38 MAPK signalling, NF-κB activation, and acetylation status.

1.5   Anacardic Acid
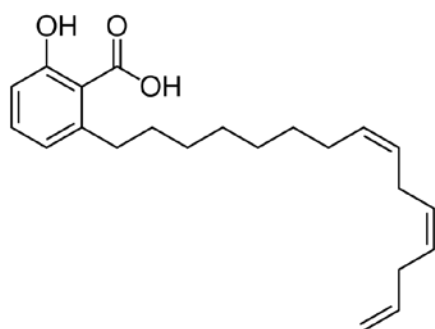
Figure 1.12   **Molecular structure of anacardic acid**

Anacardic acid (AA) is the only other known naturally occurring inhibitor of acetyl transferase p300 [303]. It is a component of cashew nut shell liquid and thus, if found to offer therapeutic potential, has the advantage of human non-toxicity. Chemically it is salicylic acid substituted with an alkyl chain which can vary slightly in composition (Figure 1.12). The alkyl chain may contain 15 or 17 carbon atoms and may be saturated or unsaturated. Naturally occurring anacardic acid contains a mixture of these closely related molecules, in varying proportion

depending on the subspecies of the plant. It is antibacterial, against both methicillin-resistant *Streptococcus aureus* (MRSA) [304] and *Streptococcus mutans*, the primary causative organism for human tooth decay [305]. In Ayurvedic and Aboriginal traditional medicine it is used to treat a wide range of ailments, including the chewing of its bark or leaves for gum and tooth ache. It is a potent non-specific inhibitor of p300, and also inhibits PCAF, a member of one of the other families of acetyl transferases as mentioned above. Inhibition of p300 is the only known molecular mechanism of action of anacardic acid, and this is the only known shared property of curcumin and anacardic acid.

1.6 Hypotheses and Objectives

We have discussed how IBD confers lifelong morbidity on its sufferers, and that the disease is becoming more common especially amongst young people. There is no cure and many available treatments have undesirable side effects. The disease is complex with a multi-factorial aetiology including genetic susceptibility, immune dysregulation and environmental factors. In IBD normal anti-inflammatory mechanisms fail. Protein acetylation is important in the regulation of these pathways, with excessive acetylation status leading to failure of normal anti-inflammatory signalling. The dietary compound curcumin is an inhibitor of acetylation, is non-toxic even at high dose and has anti-inflammatory properties.

We hypothesised that curcumin would have direct anti-inflammatory effects in the gut mucosa in IBD. To study this, we obtained mucosal tissue biopsies and lamina propria mononuclear cells from children and adults with active IBD and treated them *in vitro* with curcumin. We measured cytokine output and assessed p38 MAPK and NF-κB activation.

We hypothesised that the anti-inflammatory effects of curcumin in IBD are at least in part due to its activity as an inhibitor of acetylation. To examine this, we aimed to study the effect of curcumin on pathways known or suspected to be controlled by acetylation status. We hypothesised that curcumin, as an inhibitor of acetylation, can release TGF-β signalling from Smad 7 suppression, release IL-10 transcription from Sp3 suppression, and thus restore anti-inflammatory pathways in IBD. We measured *in vitro* Smad 7 levels in intestinal mucosal cells and tissues from patients with active IBD, cultured with curcumin. We measured *in vitro* IL-10 production by intestinal mucosal cells and tissues from patients with active IBD, in response to curcumin, and compared this with production of other cytokines.

We hypothesised that curcumin would decrease MMP-3 production, a key contributor to tissue destruction in IBD, and that this effect was at least in part acetylation-dependent. We measured *in vitro* MMP-3 production by colonic myofibroblasts (CMF) from patients with active IBD, cultured with curcumin. We examined candidate intermediary signalling pathways p38 MAPK and NF-κB to assess whether activation of these pathways was responsible for changes in MMP-3

production by CMF. We compared MMP-3 responses to curcumin with those to anacardic acid, another naturally occurring inhibitor of acetylation. This is the only known biological property of anacardic acid and the only shared property of the two compounds. We measured *in vitro* MMP-3 production by CMF in response to trichostatin A, a pro-acetylating agent.

2.0   Materials and Methods

2.1   Human Cell and Tissue Culture

2.1.0   CaCo2 Cells

CaCo2 cells (a human colon cancer epithelial cell line) (ECACC, Salisbury, UK) were cultured for 24 hours in Dulbecco's modified Eagle's medium (DMEM) supplemented with non-essential amino acids (both Invitrogen, Paisley, UK), penicillin/streptomycin and Hepes buffer (both Sigma, Gillingham, UK), stimulated with bacterial lipoprotein (BLP) (EMC, Tubingen, Germany) at 20µg/ml or unstimulated. In other experiments CaCo2 cells were cultured in the presence or absence of butyrate (Sigma) for 24 hours, and then stimulated with IL-1β (Firstlink, Brierley Hill, UK) to a concentration of 1ng/ml for a further 24 hours.

All experiments were performed on exclusively human cells and tissues, as relevant to the study of inflammatory bowel disease and its potential treatment in humans. CaCo2 cells have been used extensively to model intestinal epithelial function *in vitro* for over 20 years. The parental cell line was originally obtained from a human colon adenocarcinoma. Caco2 cells are immortalised and differentiate spontaneously in long term culture to form a monolayer. They have some characteristics of mature small intestinal enterocytes including apical microvilli and

intercellular tight junctions. They also express small intestinal hydrolases including sucrase-isomaltase and lactase. These enzymes are expressed transiently in the human foetal colon but are not found in the adult colon. Thus enterocytic and colonocytic characteristics co-exist in CaCo2 cells. Characteristics of CaCo2 cells including function, metabolism and rate of differentiation change with number of passages, including expression of enterocyte differentiation markers such as the glucose transporter (GLUT)-5 which increases with passage number [306]. The parental CaCo2 cells which generated the current CaCo2 population were intrinsically heterogeneous, and this may explain the reason for changing characteristics of the cells with rising passage, since different sub-populations are selected [307]. We addressed this potentially confounding factor by performing all experiments on CaCo2 cells between passages 20 and 29.

Other factors which influence the behaviour of CaCo2 cells relate to culture conditions, including seeding density, time of culture, composition and age of the medium and frequency of medium change. Since all of these variables can affect cell replication, senescence and differentiation, we kept these factors as strictly standardised as possible to achieve robust reproducibility of results.

The use of this cell type allowed our familiarisation with basic human cell culture techniques including maintenance, passaging, counting, stimulation and storage. We were able to achieve reproducibility of results using a cell line of relative uniformity and ready availability before progressing to the use of precious and

delicate material from patients, containing cell and tissue types of greater heterogeneity.

2.1.1   Peripheral Blood Mononuclear Cells

Blood samples from healthy volunteers with consent were collected onto lithium-heparin, diluted in equal volume phosphate buffered saline (PBS) (Invitrogen), layered onto Ficoll (GE Healthcare, Little Chalfont, UK) and centrifuged. The mononuclear cell layer was withdrawn, washed in PBS, centrifuged and the cell pellet re-suspended, counted and their viability checked by trypan blue exclusion. Cells were stimulated with lipopolysaccharide (LPS) 1µg/ml and cultured for 24 hours in the presence of graded doses of curcumin (Sigma) or anacardic acid (Merck, Nottingham, UK).

We planned to study lamina propria mononuclear cells (LPMC), but because we were reliant on unpredictable clinical factors for supply, we considered PBMC's a valuable related cell type upon which to perform experiments. They share many functional and behavioural characteristics with intestinal mononuclear cells, but have the advantage of ready availability and are easy to collect from healthy volunteers. PBMC's are a mixture of circulating lymphocytes and monocytes. Their precise composition and characteristics, as well as a comparative discussion between PBMC's and LPMC's can be found in Table 2.1 and Chapter 2.1.3.

There is inconsistent data in the literature as to whether PBMC's from normal individuals behave differently to those from individuals with IBD. Several investigators note wide inter-individual variability in cytokine production [308]. *Ex vivo* TNF-α production by PBMC's from patients with CD or UC was no different to that from healthy controls in one study [308], was increased in CD and UC in another [309], and was only increased in UC upon pokeweed mitogen stimulation in a third study [310]. The production of other pro-inflammatory cytokines by PBMC's in IBD including IL-1β, IL-6 and IFN-γ, is equally inconsistent in different studies. The only investigation to our knowledge of *ex vivo* IL-10 production by PBMC's in IBD shows no difference between PBMC's derived from healthy controls or patients with CD or UC [311]. In the present work, we studied PBMC's partly in order to refine cell separation, purification and handling techniques as well as to optimise culture conditions before applying these to LPMC's obtained from precious patient samples.

2.1.2 Intestinal Mucosal Biopsies

Colonic mucosal biopsies were obtained from children and adolescents with CD or UC undergoing ileo-colonoscopy at the Royal London Hospital. Ethics approval for the study was granted from East London and The City Health Authority Research Ethics Committee (approval numbers P/97/330; Professor Ian Sanderson

and Dr Nick Croft, and P/01/023; Professor Tom MacDonald). Consent was obtained and biopsies collected personally by us. In this way we ensured quality, uniformity and optimal treatment of samples from the outset. Biopsies were taken from areas showing clear macroscopic disease changes, and histopathological diagnosis and inflammation subsequently confirmed. Biopsies were collected into ice cold Roswell Park Memorial Institute (RPMI) medium 1640 + Glutamax (Invitrogen) supplemented with foetal bovine serum (Sigma), penicillin/streptomycin and gentamicin. Biopsies were immediately placed into wells in a 12 well plate and cultured overnight in 500μl HL-1 medium (Lonza, Wokingham, UK) supplemented with L-glutamine (Invitrogen), penicillin/streptomycin and gentamicin, with graded doses of curcumin. All efforts were made to orientate each biopsy so that the mucosal side faced up and interior side down, to mimic the *in vivo* situation, where luminal contents interact with the apical side of the epithelium. The plate size and medium volume was carefully calculated so that the mucosal side was partially exposed to air, as it would be *in vivo* (Figure 2.1). By culturing the biopsies in this way we also ensured longer tissue survival times, as we found that excessively submerged biopsies underwent more rapid disintegration.
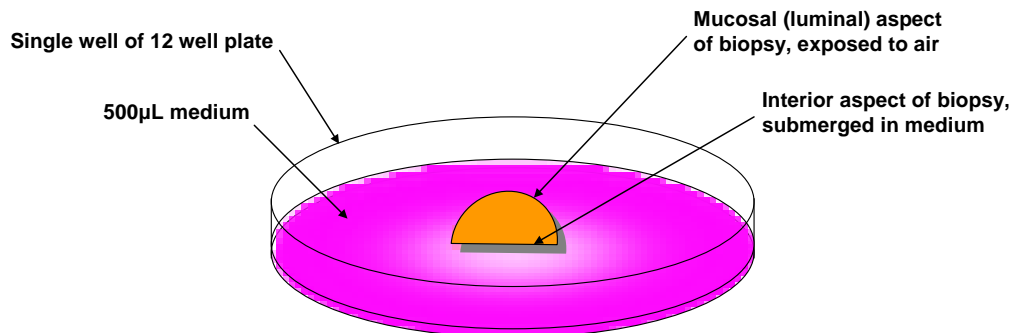
Figure 2.1   **Orientation of intestinal mucosal biopsy in culture**

As we wished to study the potential of a therapeutic agent to reduce inflammation in IBD, we chose to study tissue taken directly from areas of actively inflamed colonic mucosa in children with clinically and histologically confirmed active IBD. These were used immediately and unaltered in *ex vivo* culture. In this way we obtained a disease model which was very near to the true disease situation *in vivo*. Every experiment was carried out alongside a negative control. This negative control was a similar inflamed biopsy from the same individual placed in medium containing vehicle Dimethyl sulfoxide (DMSO) (Sigma) only, at the same concentration required to carry the curcumin. In this way we were able to discount any confounding effects of the DMSO, however minimal. Because clinically we do not wish to use curcumin in children who have no disease, our studies were carried out upon diseased tissue only. We did not study biopsies from non-diseased areas of gut or from healthy controls for this reason.

An advantage of this model is its proximity to the *in vivo* disease state. Equally the complexity of *in vivo* disease is reflected in this model, and can render the results difficult to interpret. A biopsy contains most or all of the cell types found in the intestinal mucosa, in differing and variable proportions. Thus some biopsies may contain by chance a high proportion of lymphoid tissue or immune cells, whilst others contain more fibrous tissue. Such differing collections of cells may be expected to respond to the same culture conditions in a non-uniform way, and this will result in a wide inter-experiment variability. These issues are encountered more commonly when working with biopsy cultures compared to cell lines. What is gained in terms of close resemblance to *in vivo* disease may be lost in an inability to isolate specific pathways and responses, as can be possible with a more reductionist model. To counteract the inter-individual variation and heterogeneity of biopsy work, we repeated all experiments using samples from multiple individuals with active IBD and included all results in the analysis. In this way trends could be observed, and their significance quantified statistically.

2.1.3   Intestinal Lamina Propria Mononuclear Cells

Intestinal resection specimens were obtained from children and adults undergoing surgery for CD or UC at the Royal London Hospital or the Homerton Hospital, which also forms part of Barts and The London School of Medicine and Denstistry. Once again ethics approval for the study was granted from East London and The

City Health Authority Research Ethics Committee (approval numbers P/97/330 and P/01/023). Patient consent was obtained and tissue collected from the operating theatre personally by us. In this way we ensured maximal freshness and optimal treatment of the gut tissue from patient to laboratory bench. Tissue was collected onto ice cold complete RPMI medium as above. The mucosal layer was removed, washed in Hanks balanced salt solution (HBSS) (Sigma) and incubated in ethylene diamine tetra-acetic acid (EDTA) (Sigma) to remove mucus and epithelial cells. This was followed by incubation in collagenase (Sigma), a proteolytic enzyme which breaks down intercellular connective tissues. The resultant suspension was then passed through a cell strainer, washed in complete RPMI and centrifuged. The pellet was re-suspended in complete RPMI and further purified by Ficoll density gradient separation.

Finally the cells were washed once again, centrifuged and re-suspended in complete RPMI. Any debris was allowed to settle standing on ice, then cells were counted and their viability checked by trypan blue exclusion. This is done by suspending a sample of cells 1:10 in sterile filtered trypan blue solution and immediately examining them under the microscope in a haemocytometer. Those cells which are alive and healthy are seen to be white or yellow as their cell membranes have successfully excluded the trypan blue solution. These are the cells which are counted. Cells were cultured for 24 hours in the presence of curcumin in graded doses, alongside DMSO vehicle control. For cytokine experiments cells were seeded at 4 x 10^5 cells per well for every experiment; for Smad 7 and p38 MAPK

experiments (requiring a greater quantity of protein for immunoblotting) cells were seeded at 4 x 10^6 cells per well.

The LPMC isolation method described above is adapted only minimally from its original description by a group in Harvard in 1977 [312]. This marked an important step forward in the study of gut mucosal immune processes, which prior to that had relied on PBMC's for research questions. With no guarantee that circulating mononuclear cells exhibit similar responses in health and disease to tissue-fixed lymphoid cells, results from such work may not truly reflect gut mucosal immunological events. For this reason we chose to concentrate our study upon LPMC's rather than PBMC's. The treatment processes used to isolate LPMC's, if similarly applied to PBMC's, cause no change in morphology or viability [312]. The cells obtained are 97% mononuclear and 3% polymorphonuclear (mainly eosinophils), with no contaminating epithelial, muscle, fibrous or other cell types.

The original investigators also compared the properties of PBMC's to LPMC's (Table 2.1). The differences point to a generalised increased activity of intestinal compared to circulating leukocytes; larger in size, more irregular in shape, with nucleoli and basophilic stippling suggesting active immunoglobulin synthesis. They also showed greater mobility, plate adherence and phagocytic activity, fewer null cells, more activated cells and greater intercellular associations; a likely reflection of antigen processing. The investigators also compared membrane

antibody distribution by class, and found a greatly increased proportion of cells positive for IgA and IgE, and fewer IgM-positive cells in intestinal versus circulating populations, suggesting that intestinal B-lymphocyte cells have undergone maturation. As expected the commonest immunoglobulin class found was IgA, whose vital role in intestinal secretory immunity is well documented.

Properties of Intestinal Mucosal and Circulating
*Lymphoid Cells**

	Intestinal mucosa	Peripheral blood
Lymphocytes		
A. Total mononuclear cells, %	90±4	84±4
B. Size, μm	8–17‡	7–12
C. Nuclear-cytoplasmic ratio	0.3–0.9‡	0.8–0.9
D. Cells having nucleoli, basophilic cytoplasm, %	>90‡	<5
E. Association with monocytes/macrophages	Marked	Absent
F. Total T cells (18-h E rosettes), %	58±5	62±4
G. Activated T cells (1-h E rosettes), %	55±10‡	30±3
H. Total B cells (EAC rosettes), %	32±5‡	12±4
I. Membrane immuno-globulin-positive cells, %	37±8‡	15±4
J. Null cells: E rosette-negative membrane Ig negative; [A − (F +I)],%	0	7
Monocytes/macrophages		
A. Total mononuclear cells, %	10±4‡	16±4
B. Size, μm	14–25‡	12–15
C. Granularity	+++	0
D. Motility (pseudopod formation)	++	+
E. Sustained glass adherence	+++	+
F. Phagocytic cells, %	80‡	60
G. Phagocytic activity (latex spheres/cell)	8–25‡	1–10

* All percentages are with reference to the total mononuclear cell population and are expressed as the mean±1 SD.
‡ Significantly different from peripheral blood at the 0.01 level.

Table 2.1 Properties of Intestinal Mucosal and Circulating Lymphoid Cells [312]

Our choice to use leukocytes isolated directly from areas of active inflammation in

patients with IBD was again because we wished to examine the effects of curcumin

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upon the active disease state in IBD. These cells possess an intrinsic degree of activation, and unlike PBMC's produce cytokines spontaneously. This means that we can culture them with curcumin and observe effects directly without the need for extrinsic stimulation, keeping our cell model system near to the *in vivo* situation. We wished to produce results which could as far as possible be extrapolated to understand the *in vivo* actions of curcumin in the human gut.

Although we attempted to isolate LPMC's from intestinal mucosal biopsies, we found that the cell yields from these were consistently too low to be usable for our experiments (less than $1x10^5$ cells were typically yielded from 4 biopsies). Thus the requirement for actively inflamed surgically resected IBD tissue naturally limited our progress due to its relatively low availability. However we were consistently able to isolate over $5x10^6$ cells from a 1-2cm^2 piece of tissue, which was sufficient to carry out our experiments.

2.1.4   Colonic Myofibroblasts

Colonic resection specimens were obtained from children and adults undergoing surgery for CD or UC at the Royal London Hospital or the Homerton Hospital. Once again ethics approval for the study was granted from East London and The City Health Authority Research Ethics Committee (approval numbers P/97/330 and P/01/023). Tissue was collected and mucosal cells isolated as described above. At

the final stage after washing, cells were re-suspended in DMEM supplemented as before. The adherent colonic myofibroblasts (CMF) were grown in successive passages until sufficient numbers resulted for experimentation (usually to passage 4). Cells were then plated into wells and when confluent, harvested for 24 hours in serum-free DMEM, then stimulated with TNF-α (R&D Systems, Abingdon, UK) and cultured in the presence of curcumin or anacardic acid in graded doses for either 30 minutes or 24 hours, or with trichostatin A (Sigma) for 24 hours. All experiments were performed alongside vehicle (DMSO) as a negative control.

There are two types of myofibroblasts in the intestine, the sub-epithelial fibroblasts which we isolate for this work (and refer to as CMF), and the deeper interstitial cells of Cajal which are located in the submucosa and muscularis propria in close association with the smooth muscle layers. The interstitial cells of Cajal are involved in coordination of gut motility. These fibroblasts were not included in our studies, as the initial step in our isolation technique entailed dissection and retention of the mucosa; all deeper tissues were discarded. CMF are activated and proliferate in response to various growth factors, and are able to do so *in vitro*. They play an important role in embryological evolution of the gut and are key cells in tissue injury, promoting wound healing through epithelial restitution and proliferation. They form a mucosal barrier to sodium diffusion, which may account for the ability of the gut to absorb water against an osmotic gradient [313]. Finally they are a key effector cell type in IBD, secreting matrix metalloproteinases in excess, which are

responsible for much of the tissue damage and fibrosis. The roles of CMF and MMP in IBD are described in more detail in Chapters 1.4.10 and 7.1.

As well as being a highly relevant cell type for study in IBD, CMF have a practical advantage over LPMC in experimental use. Because they can be grown in culture, sufficient numbers of CMF may be obtained from much smaller resected gut specimens than those required to provide LPMC. Therefore clinical supply was a much less restrictive issue. Again this is a primary cell type obtained exclusively from actively inflamed mucosa. Successive *in vitro* passages are required, although the number of these was minimised in order to retain as far as possible the primary phenotype, characteristics and behaviour of the cells. Nonetheless these cells do require stimulation in order to produce MMP-3, and whilst another choice of cytokine could equally have been made, we successfully achieved this with TNF-α. All cell handling and culture techniques were carefully standardised across experiments, for example cell seeding density and time period between seeding and harvesting. In this way we aimed to minimise potential confounding variable factors which could affect cell behaviour, for example senescence, proximity of cells to their neighbours, time between medium changes, and many other facets of cell handling technique.

As a possible alternative cell type for study we considered an immortalised intestinal myofibroblast cell line such as CCD-18. This would have had the advantage of ready availability and perhaps greater uniformity between individual

samples. However throughout all of our *ex vivo* experiments we aimed to focus on disease models as little removed from the *in vivo* disease state as possible, in order to maximise clinical relevance. Use of an immortalised cell line would have constituted a significant step away from this principle. In the case of intestinal epithelial cells we were obliged to use the CaCo2 cell line, since no primary isolatable alternative exists. However in the case of myofibroblasts, we were able to reject this option in favour of primary CMF.

2.2 Enzyme-Linked Immunosorbent Assay for Cytokines

Supernatants from cell or tissue cultures were subjected to enzyme-linked immunosorbent assay (ELISA) for IL-1β, IFN-γ (both R&D Systems) or IL-10 (Immunotools, Friesoythe, Germany). Each sample was tested in duplicate against the appropriate standard and optical densities measured by Microplate reader (Biorad, Hemel Hempstead, UK). Results were analysed and presented using Microsoft Excel and Prism software.

ELISA is an immunological technique used to detect and quantify the amount of antigen in a sample. The type of ELISA used here is a sandwich ELISA, where the antigen (cytokine) to be measured is sandwiched between two layers of antibodies

(Figure 2.2). Therefore this type of ELISA requires the measured protein to be multivalent; it must have at least two antigenic sites capable of binding to antibody. Thus it is useful for the detection of proteins and polysaccharides, and it can quantitate these even at low concentrations. It is also highly specific, and can be used even when the test substance is in solution with other contaminating proteins. Therefore it has found many applications ranging from diagnostics (for example measurement of serum HIV antibody concentration, or urine β-human chorionic gonadotrophin (HCG) in home pregnancy tests), toxicology (screening body fluids for drugs) and the food industry for seeking traces of potential allergens such as milk, nuts and eggs.
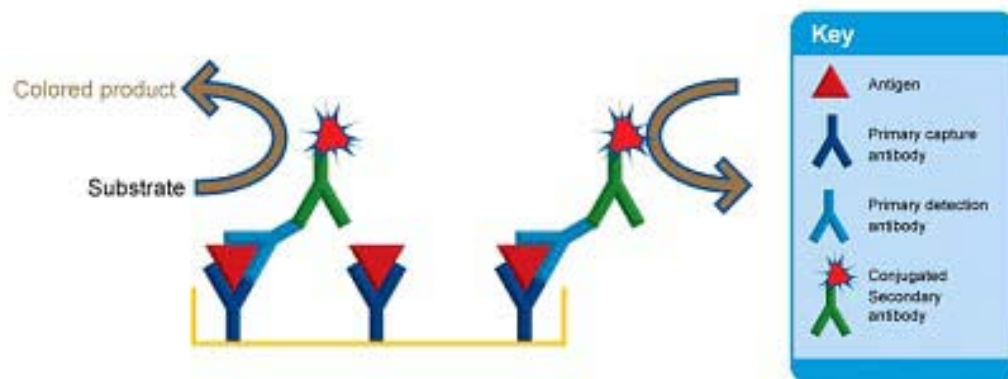
Figure 2.2   **Sandwich enzyme-linked immunosorbent assay.   Diagram from Abcam**
http://www.abcam.com/index.html?pageconfig=resource&rid=12064

A capture antibody is first applied to the plate in known concentration. After this has coated the plate and bound to it, excess is washed off and non-specific antibody binding sites are blocked with a high concentration protein solution; here we used

bovine serum albumin (BSA). After washing again the test solutions are applied in equal volume and in each sample in duplicate. A standard is also applied in serial dilutions at known concentrations. The excess is again washed off and a detection antibody solution applied in known concentration. Once this has bound to the antigen the excess is again removed and an enzyme-linked secondary antibody applied in known concentration; here this was streptavidin-horse radish peroxidase (HRP). Streptavidin is derived from *Streptomyces avidinii* and reacts specifically with biotin (in the detection antibody). Finally a substrate is applied which is converted by the HRP into a colour signal. This process is illustrated diagrammatically in Figure 2.2. The density of colour is thus proportional to the concentration of cytokine in the plate, and this is measured by a plate reader which converts the optical absorbance of the plate at a specified wavelength into a number. This number is then translated into a cytokine concentration by comparison against the known standard curve obtained in the same ELISA experiment.

The ELISA technique was well suited to our requirements for the reasons outlined above. The important steps to ensure accuracy and reproducibility in this technique are careful preparation of all reagents at known concentrations, ensuring solutions are always used at room temperature, and washing and drying plates thoroughly between each step of the procedure. We achieved consistent results with very low variability between duplicates, of less than 5% in the great majority of experiments.

Where we occasionally obtained significantly wider variation between duplicates these experiments were repeated.

2.3   Immunoblotting

2.3.0   Western Blot for Smad 7 and p38 Mitogen-Activated Protein Kinase

Cells or tissues were snap frozen and subjected to physical disruption and protein lysis with ice cold RIPA lysis buffer containing protease and phosphatase inhibitors, to prevent the digestion of the sample by its own enzymes. Protein estimation was performed using bicinchonic acid (BCA)/copper sulphate assay against bovine serum albumin (BSA) standard (all Sigma). Protein samples were resolved on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS/PAGE), transferred onto nitrocellulose membrane and probed overnight with primary antibody against Smad 7 (molecular weight 47 kDa) (Imgenex, San Diego, CA), β-actin loading control (molecular weight 42 kDa) (Abcam, Cambridge, UK); phosphorylated p38 MAPK (molecular weight 43 kDa) (R&D Systems) or non-phosphorylated p38 MAPK loading control (Cell Signalling Technology, Danvers, MA). Membranes were then re-probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (both Dako, Ely, UK) and

chemiluminescent substrate applied for photographic visualisation. Membranes were then stripped and re-probed as appropriate.

In order to quantify the signal as far as possible, the photographically developed films were scanned into electronic format. Using Adobe Photoshop software, the intensity of each band was measured under uniform conditions, giving a numerical result which in arbitrary units directly reflected the signal intensity, and thus the corresponding amount of protein. The loading control signals were similarly converted into arbitrary units of intensity, and a final result was obtained by dividing the test substance signal by its loading control, to obtain the final standardised densitometry figure.

The term immunoblotting refers to the transfer of biological samples from a gel to a membrane for their subsequent detection on the membrane, using an antibody. The procedure was originally described in 1979 [314] and since that time has had a wide impact in molecular biology, facilitating protein analysis in multiple settings. It is highly specific to its target protein, which it is able to detect in a complex mixture of other biological substances. However it provides only semi-quantitative data.

The cells are first treated with detergents, salts and buffers in order to lyse the cell membranes and solubilise the proteins. The protein mixture is then separated using gel electrophoresis. It is important to measure the protein concentrations accurately

and to load equal amounts of each sample onto the gel for meaningful comparison between samples. Each sample is combined with loading buffer containing reducing agents which removes secondary and tertiary protein bonds to maintain the polypeptides in a denatured state. The proteins, coated in negatively charged SDS, move through the gel towards the positive electrode. The acrylamide in the gel creates a mesh and the percentage of acrylamide in the gel can be adapted to the approximate size of the proteins being separated. Proteins migrate at different speeds according to their charge and size. In this case we wished to identify proteins with molecular weights in the range of 42- 47 kDa; in the middle range and therefore a 10% gel was used. A ladder containing a mixture of coloured proteins of differing molecular weights is run in one lane alongside the samples, to monitor migration of the samples and mark molecular weight positions.

The proteins are then transferred (blotted) onto a nitrocellulose membrane. The membrane avidly binds proteins of all types, therefore non-specific antibody binding sites must then be blocked using a protein solution (we used non-fat dry milk in detergent solution). The membrane is probed by applying the primary antibody, followed by a secondary antibody directed against the primary. In this case the primary antibody was derived from rabbit, therefore the secondary antibody was a goat anti-rabbit antibody. The secondary antibody is linked to the enzyme HRP. At the final stage, a chemiluminescent substrate is applied which reacts with the HRP to produce a visual signal. This can be captured on film when developed photographically. The intensity of the signal correlates with the

abundance of the antigen on the membrane and in this way the protein can be measured in a semi-quantitative fashion. In order to ensure differences in signal intensity are truly due to biological differences in protein production, equal loading must be ascertained. In the case of NF-κB this was by co-measurement of the ubiquitous cytoplasmic protein, β-actin. Against phosphorylated p38 MAPK, the appropriate load control was the total form of p38 MAPK.

We chose this technique for the measurement of p38 MAPK and Smad 7 because we wished to detect the production and activation of these proteins (rather than for example messenger (m)RNA), since changes in their production could equally be due to post-translational events. For the reasons described above Western blotting is a well-suited technique for the detection of such proteins, and semi-quantitative results were sufficient for our purposes. It is a technique which requires careful optimisation since it contains multiple critical steps, each of which must be carried out at the correct concentration, temperature and pH, for the correct duration and under the correct conditions, in order to produce a result. If the final signal is not obtained, or is inadequate, troubleshooting must be systematic and comprehensive, and can be challenging since it may need to take into account every contributory step. During the course of optimising these experiments, we gained experience in correlating specific sub-optimal results with likely problems and causes, ultimately to obtain clean and reliable signals.

2.3.1 Western Blot for Matrix Metalloproteinase-3

Equal volumes of supernatant (15µl) were resolved on 10% SDS/PAGE, transferred onto nitrocellulose membrane and probed overnight with primary antibody against MMP-3 (molecular weight 54 kDa) (The Binding Site, Birmingham, UK). Membranes were then re-probed with HRP-conjugated rabbit anti-sheep secondary antibody (Dako) and chemiluminescent substrate applied for photographic visualisation.

MMP-3 was the only protein in this work which was detected in the supernatant, rather than in cell lysates. Because each well was seeded with equal numbers of confluent cells, and contained an identical volume of medium, by loading identical volumes (15µl) of supernatant into each lane of the gel, we ensured equal loading of sample and thus valid comparison between lanes. Thus a load control signal was not indicated in these experiments unlike in all of the other Western blots in this work.

2.3.2 Western Blot for NF-κB and IκBα

Cells or tissues were fractionated using a commercial fractionation kit (Biovision, Mountain View, CA). Cells or tissues were disrupted, centrifuged and vortexed on

ice with cytosol extraction buffer containing protease inhibitors. This was centrifuged at four degrees centigrade and the supernatant (cytosolic extract) removed and stored on ice. The resulting nuclear pellet was re-suspended in ice-cold nuclear extraction buffer and after repeated intermittent vortexing was centrifuged. The resulting supernatant (nuclear extract) was removed and stored on ice. Protein estimation was performed using BCA/copper sulphate assay against BSA standard. Nuclear fractions were resolved on 10% SDS/PAGE, transferred onto nitrocellulose membrane and probed overnight with primary antibody against NF-κB p65 subunit (molecular weight 65 kDa) (Santa Cruz Biotechnology) or histone H1 loading control (molecular weight 32 kDa) (AbD Serotec, Oxford, UK). Cytosolic fractions were similarly resolved on 10% SDS/PAGE, transferred onto nitrocellulose membrane and probed overnight with primary antibody against phosphorylated IκBα (molecular weight 35 kDa) or non-phosphorylated IκBα loading control (both Santa Cruz Biotechnology). Membranes were then re-probed with HRP-conjugated goat anti-rabbit or anti-mouse secondary antibody as appropriate (Dako) and chemiluminescent substrate applied for photographic visualisation. Membranes were stripped and re-probed as required.

We used a very similar principle for the detection of proteins within the NF-κB pathway. The p65 subunit (when activated) is a nuclear protein, and therefore to detect it we separated the nuclear from the cytoplasmic cellular fractions. With p65 we used histone H1, a ubiquitous nuclear protein, as an appropriate nuclear loading

control. The load control for phosphorylated IκB was total IκB, both of which are cytosolic proteins.

2.3.3   Western Blot for p300 Acetyl Transferase

Cells or tissues were snap frozen and subjected to physical disruption and protein lysis with ice cold RIPA lysis buffer containing protease and phosphatase inhibitors. Protein estimation was performed using BCA/copper sulphate assay against BSA standard. Protein samples were resolved on 6% SDS/PAGE, transferred onto nitrocellulose membrane and probed overnight with primary antibody against p300 (Santa Cruz Biotechnology). Membranes were then re-probed with HRP-conjugated goat anti-rabbit secondary antibody and chemiluminescent substrate applied for photographic visualisation.

p300 acetyl transferase is, as its name suggests, a protein of relatively large molecular weight at 300 kDa. In order to optimise its electrophoretic separation we reduced the concentration of acrylamide in the gel to 6%, creating a wider mesh. We also increased the running time of the electrophoresis, to maximise separation of the largest (and therefore slowest moving) proteins. We encountered challenges optimising the p300 signal. After making the above adjustments and altering the chemiluminescent and photographic signal sensitivities, we ultimately obtained a signal for p300 only after greatly increasing the amount of protein loaded on the

gel. (A faint signal was obtained on loading 200μg per lane; compared to the 30μg per lane required for all other Western blot antibodies we used.) The requirement for this quantity of protein meant that this technique was unsuitable for use in our experiments, since growing, maintaining and culturing such high numbers of fibroblasts would be unfeasible, particularly as we wished to maintain standardisation of experimental conditions and low passage numbers.

2.4   Immunofluorescent staining for NF-κB

At the end of the experiments as described above, CMF were fixed in 4% paraformaldehyde, permeabilised with 0.1% Triton, washed then blocked in 10% donkey serum (Sigma). The cells were next incubated with a rabbit polyclonal antibody against NF-κB p65 subunit (Santa Cruz), washed again and then incubated with a secondary donkey anti-rabbit antibody conjugated to Alexa488 (Invitrogen). Nuclei were counter-stained with 4,6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Invitrogen) and slides mounted with ProLong antifade reagent (Invitrogen) and observed under a Leica DM5000 epi-fluorescence microscope with an attached digital camera using x63 magnification. We performed these experiments under the kind supervision of Dr Olivier Marches, whose generous help we appreciate greatly.

Since our results examining the role of NF-κB in mediating the effects of curcumin in both biopsies and CMF were negative by Western blot, we wished to verify these results using an alternative method. Immunofluorescence is another antibody-based detection method, this time using antibodies linked to fluorescent dyes. The principle is related to immunoblotting in that a primary anti-p65 rabbit antibody is recognised by a secondary donkey anti-rabbit antibody. However the secondary antibody is dye-coupled. Unlike Western blotting this technique allows visualisation of the precise location of the antigen within different cellular or histological compartments. This is particularly useful for measurement of elements in the NF-κB activation pathway, since nuclear translocation is a key event in activation. The position of the nuclei is visualised by DAPI counter-stain. In this way the relative position of the p65 NF-κB subunit can be assessed. In the quiescent state p65 is seen largely in the cytoplasmic compartment, where it is held inactive in complex with IκB; when activated it is seen more abundantly in the nucleus. By staining the nuclear proteins and the p65 subunit with opposing colours, their positions can be pinpointed and any changes noted.

The advantage of this technique is its specificity, and the opportunity to observe molecular events, albeit indirectly, on a sub-cellular level. A disadvantage can be the qualitative nature of the observations. This can be minimised by repeating experiments for example in triplicate, ensuring that the entire microscopic field is examined in every slide, and a random selection of cells photographed.

2.5   Statistics

Each patient donated four inflamed biopsies, which were cultured in four separate wells with curcumin concentrations ranging from 0-30μM. LPMC experiments were performed under similar conditions. No clear dose-response relationship was observed against the curcumin doses tested (5-30μM). Therefore the question arose as to which dose to select for the purposes of statistical analysis. We elected to express our results as two straightforward numbers for each patient; negative internal control and curcumin-treated. Curcumin response was given as a single figure for each individual patient, which was calculated as the average response of that patient to curcumin across all three concentrations tested. In the absence of a dose-response relationship this was chosen as the most representative way to express our data.

In retrospect it would have been equally valid simply to culture three biopsies from each individual in a single concentration of curcumin, for example 20μM. At the outset however we wished to test more than one dose, expecting a dose response. Concentrations over about 50μM were associated with decreased cell viability so we elected to avoid these. In previously published work concentrations in the range 2-50μM are largely studied. If other concentrations had been tested in the current work, it may have been useful to test a much lower dose, for example 0.1μM. This would have broadened our dosing range and might have brought surprising results, as there are some reports of curcumin's paradoxical efficacy at low dose.

The inter-individual variability was generally wide and Gaussian distribution could not be assumed. Therefore data were analysed as non-parametric paired differences using the Wilcoxon signed rank test, 95% confidence intervals applied and two-tailed p values calculated. The Wilcoxon test was chosen because (a) it was unsafe to assume that our data were normally distributed, and (b) we wished to test a statistical hypothesis concerning a series of related pairs of measurements. It is a good alternative to the paired Student's t-test when normal distribution cannot be assumed. Our null hypothesis stated that any difference between our pairs of results (control versus curcumin) was no greater than would be expected to arise by chance alone.

The choice of 95% confidence intervals is in part based on accepted statistical convention. Confidence intervals give an estimate of the amount of error involved in the data. They are based on the value of the statistic, the standard error of the measurements, and the desired width of the confidence interval. As we are seeking a difference between a set of two pairs (A and B), we are asking the question, does the 95% confidence interval for median A include median B? If so, then the medians are not statistically different from each other. If not, then the medians are different from each other, at p=0.05. p is the probability of the result arising if the null hypothesis is true (ie by chance alone). Conventionally data is often regarded as significant if the p value is less than 0.05 (that is, a 5% probability that the difference arose by chance alone), and this is the p value that we have taken as significant. The application of two-tailed p values is more stringent, as it tests

whether deviation in either direction from the null hypothesis can be ascribed to chance alone.

Power calculations were not performed prior to the experiments. This was because we were planning novel experiments, in which it was not possible to estimate the size of any effect of curcumin. Furthermore the relevance of power calculations to clinical or epidemiological studies, in which recruitment numbers are critical to study design at the outset, is not applicable in the same way to basic experimental science.

Data were analysed and presented using Prism statistical and graphical software.

3.0 Effect of Curcumin on Smad 7 Levels in Intestinal Mucosal Tissue and Mononuclear Cells

3.1 Smad 7 Western Blot Optimisation

As described in Chapter 2.3.0, a Western blot entails multiple steps, the conditions for each of which must be optimised in order to obtain a good signal. One of the key choices early in the experimental protocol is the quantity of protein loaded on the gel. Our choice between 50, 75 and 100μg was based on the practice of experienced investigators working in Rome who were previous collaborators of our department and experts in Smad 7 immunoblotting [117]. After repeating this experiment in duplicate, we established that for an optimal Smad 7 signal, the quantity of protein load per lane was 75μg (Figure 3.1). This was therefore the amount of protein we loaded onto the gel in all future Smad 7 experiments. In this way we obtained consistent and reliable protein bands, which were sufficiently prominent to read clearly, and not excessively dense so as to obscure differences in signal strength.

The purpose of normalising the Smad 7 signal against a housekeeping protein (in this case β-actin) is to correct for any differences in sample loading and for factors

such as transfer efficiency when blotting the protein bands from gel to membrane. Thus the housekeeping gene acts as an internal standard and is chosen for its ubiquitous, stable and constitutive expression in every tissue. Others which are commonly used include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-tubulin and phospholipase A (PLA)2. Actins occur in three main isoforms; α, β and γ. They are the most abundant proteins in eukaryotic cells, playing key roles in cell motility and cytoskeletal maintenance. They are expressed constitutively; β- and γ-actin being found in practically all cells; α-actin being confined to smooth muscle cells [315]. Recent evidence suggests that under some circumstances β-actin, and other housekeeping genes in common use, are subject to more variation than was previously thought, and this therefore may call into question the validity of their use as internal controls. Such variation may occur in response to for example drug treatments, disease states, cell cycle phase, differentiation, age and sex of the individual and source of tissue [315, 316]. It seems however that such variability may affect RNA-based studies (polymerase chain reaction (PCR)) more than protein-based (Western blot), perhaps due to the higher sensitivity of PCR, and the amplification involved in this technique.

In specific relation to Smad 7, the loading control used by investigators who have previously published is consistently β-actin. There is no published comparison of housekeeping proteins in this precise context, therefore unambiguous identification of a 'gold standard' protein is not possible. However there is some work on choice of housekeeping genes for PCR in the gut [317]. These investigators mainly

126

focused on normal differentiating enterocytes versus gut adenocarcinomas. They examined the expression of a number of classical and novel candidate housekeeping genes in enterocytes at varying stages in differentiation and in adenocarcinoma cells. They analysed their respective standard deviations by a variety of statistical methods and concluded that β-2-microglobulin was the most robust normalising gene overall, although β-actin was an acceptable alternative. The investigators commented that due to non-uniformity of expression in different cell types, it was not possible to select an overall gold standard.

For the reasons outlined above, we chose β-actin as our loading control for Smad 7 protein. Because protein blotting is a less sensitive and less quantitative tool than PCR, and since the existing evidence from PCR work suggests that β-actin performs acceptably well as a loading control, we accepted this choice in our immunoblots. We found that our β-actin signals were clear, consistent and of similar intensity throughout all experiments in intestinal tissues and LPMC's, reinforcing the validity of this choice.
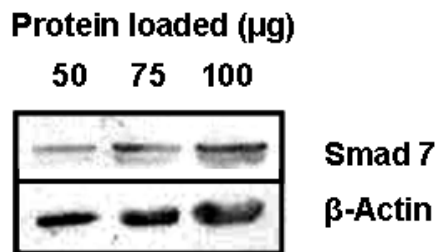
Figure 3.1   **W estern blot for Smad 7 with β-actin loading control showing optimal protein load 75µg**

3.2   Curcumin Does Not Reduce Smad 7 in Intestinal Mucosal Tissue or Mononuclear Cells

We hypothesised that curcumin, as an inhibitor of acetylation, can release TGF-β signalling from Smad 7 suppression, and thus restore this anti-inflammatory pathway in IBD. We measured *in vitro* Smad 7 levels in intestinal mucosal tissue biopsies and LPMC's from patients with active IBD, cultured *ex vivo* with curcumin. Precise methods are detailed in Chapters 2.1.2, 2.1.3 and 2.3.0. Our choice of curcumin concentrations was largely influenced by concentrations used by other investigators who have previously published in the field. The doses used in cell culture work largely range from 2-50μM. We found that cell viability (as assessed by trypan blue exclusion) dropped below 80% at concentrations above about 50μM and therefore to avoid the risk of contaminating our results in this way, we only tested concentrations below this.

Such *in vitro* concentrations are probably considerably higher than blood or tissue concentrations that can be achieved *in vivo* through oral dosing in humans, due to drug delivery and bioavailability issues as described in Chapter 1.4.2. In one study 3.6g of oral curcumin achieved plasma levels of 11.1nmol/L one hour post dose in humans [157]; a concentration a factor of 1000 lower than those we have used *in vitro*. Other investigators found peak human plasma levels of 0.41-1.75μM one hour after oral dosing of 4-8g of curcumin [153]; these are only a factor of 10 lower than the concentrations we have used. Due to the wide variability in

pharmacokinetic data from different studies it is difficult to ascertain for certain the

relationship between *in vitro* and *in vivo* curcumin dosing. In retrospect we would

have liked to study some much lower curcumin concentrations in our *in vitro* work,

since these would probably hold relevance to the human clinical situation and there

is some evidence from *in vitro* and animal work that curcumin can have paradoxical

high efficacy at low doses.

We found that Smad 7 levels did not alter in any consistent pattern with the

addition of curcumin in *ex vivo* intestinal mucosal tissue or mononuclear cell

cultures (figure 3.2). All patient details and individual results can be found in the

Appendix.

A

Curcumin µM

- 5 10 20

Smad 7

β-Actin

B

Smad 7/β-actin densitometry (percentage change)

Curcumin µM

- ▲ CD biopsies
- ● CD LPMC's
- △ UC biopsies
- ○ UC LPMC's
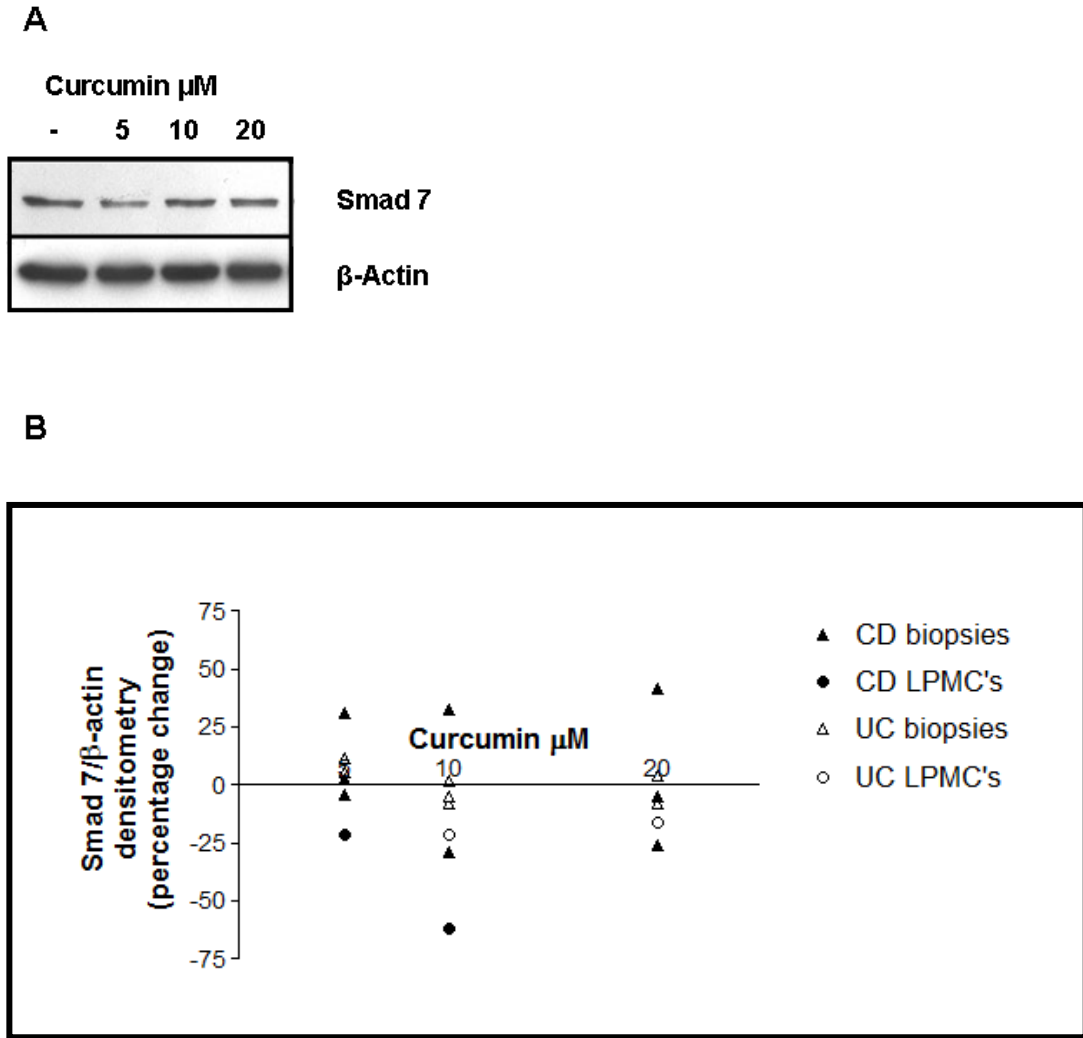
Figure 3.2 **Curcumin causes no consistent change in Smad 7 in inflamed intestinal mucosal tissue or mononuclear cells. (A) Western blot from single representative experiment showing Smad 7 with β-actin loading control in CD biopsies with varying concentrations of curcumin. (B) Smad 7/β-actin densitometry as percentage change from baseline (vehicle control) with varying concentrations of curcumin. Each point represents a single experiment. Data from 8 individuals [5CD + 3UC].**

As discussed in detail in Chapters 1.2.5 and 1.2.8, Smad 7 is a negative regulator of TGF-β signalling, an important anti-inflammatory pathway. This is the first time to

our knowledge that the response of Smad 7 to curcumin has been investigated in any animal or cell type. Although we know Smad 7 levels are increased by acetylation, that curcumin is an inhibitor of acetyl transferase p300, and that curcumin does indeed show positive clinical benefit in animal and human IBD studies, we found no consistent response of Smad 7 to curcumin in our *ex vivo* experimental models, either in biopsies or mononuclear cells, and this data is presented together in figure 3.2. This could be due to the lower anti-acetylating potency of curcumin compared to other inhibitory strategies previously employed experimentally, namely short interfering ribonucleic acid (siRNA) against p300 [117]. Alternatively, it may be that curcumin exerts some of its clinical effects in IBD through mechanisms unrelated to acetylation, or through acetylation of other non-Smad elements.

Thus our hypothesis that curcumin restores TGF-β signalling by reducing Smad 7 acetylation, is rejected. However it is possible that acetylation of different proteins is dependent on different acetylation pathways. Whilst Smad 7 is not affected by curcumin, other proteins relevant to inflammation in IBD may be regulated by acetylation-dependent mechanisms and these may be susceptible to manipulation by curcumin.

4.0 Effects of Curcumin on Cytokine Expression in *ex vivo* Intestinal Mucosal Tissue

4.1 Curcumin Increases Interleukin-10 Production in Intestinal Mucosal Tissue

We hypothesised that curcumin, as an inhibitor of acetylation, can release IL-10 transcription from Sp3 suppression, and thus restore anti-inflammatory pathways in IBD. We measured *in vitro* IL-10 production by intestinal mucosal tissue biopsies from children and adolescents with active IBD, in response to curcumin, and compared this with production of other cytokines.

With curcumin there was an overall increase in IL-10 production by inflamed intestinal tissue from children with active IBD (Figure 4.1A). No clear dose-response relationship was evident over the curcumin dosing range tested (5-20μM). Therefore for the purposes of statistical analysis, these data were expressed as a single mean response to curcumin across all doses (5-20μM) for each patient (Figure 4.1B and Table 4.1). This rationale is explained in more detail in chapter 2.5. Thus Figure 4.1B displays the same data as figure 4.1A, only expressed as a matched pair of results for each individual patient; negative (vehicle) control and curcumin. Curcumin caused a median rise of +265% (+283pg/ml) in IL-10

expression in *ex vivo* intestinal mucosal tissue culture from patients with active IBD

(Figure 4.1B), and this increase was significant (p=0.0015) (table 4.1).

When analysed by separate disease groups, CD biopsies showed a 92% increase in

IL-10 expression with curcumin, which was significant (p=0.016) (Figure 4.2A),

and UC samples a large 498% increase, which did not however reach statistical

significance (p=0.13) (Figure 4.2B). All patient details and individual results can

be found in the Appendix.
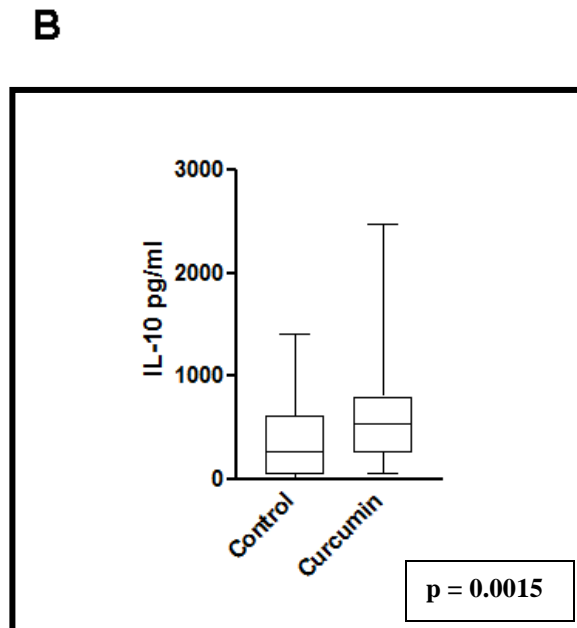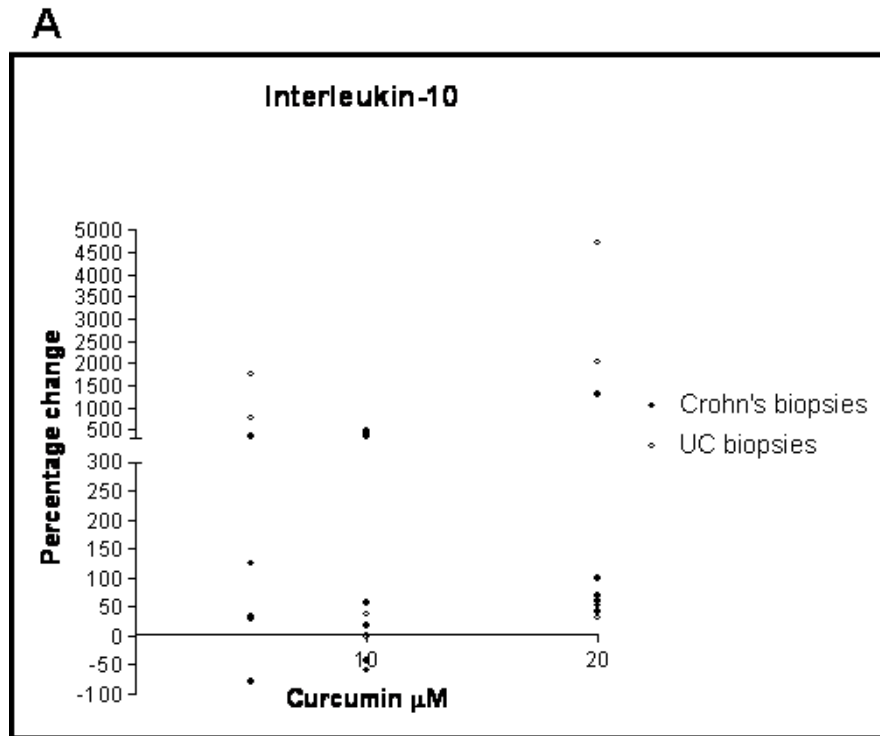
Figure 4.1 Curcumin enhances interleukin-10 production in inflamed intestinal mucosal tissue. (A) IL-10 production by intestinal mucosal biopsies as percentage change from baseline (vehicle control) with varying concentrations of curcumin. Each point represents a single experiment. Data from 12 individuals (8 CD + 4 UC). (B) The same data expressed as mean IL-10 response by each patient to all curcumin concentrations 5-20μM.

	Median IL-10 (pg/ml)	Inter-quartile range	p value
Vehicle control	273	41-617	0.0015
Curcumin	536	253-804	

Table 4.1 Statistics describing figure 4.1B. Wilcoxon matched pairs test shows significant difference between the two groups.
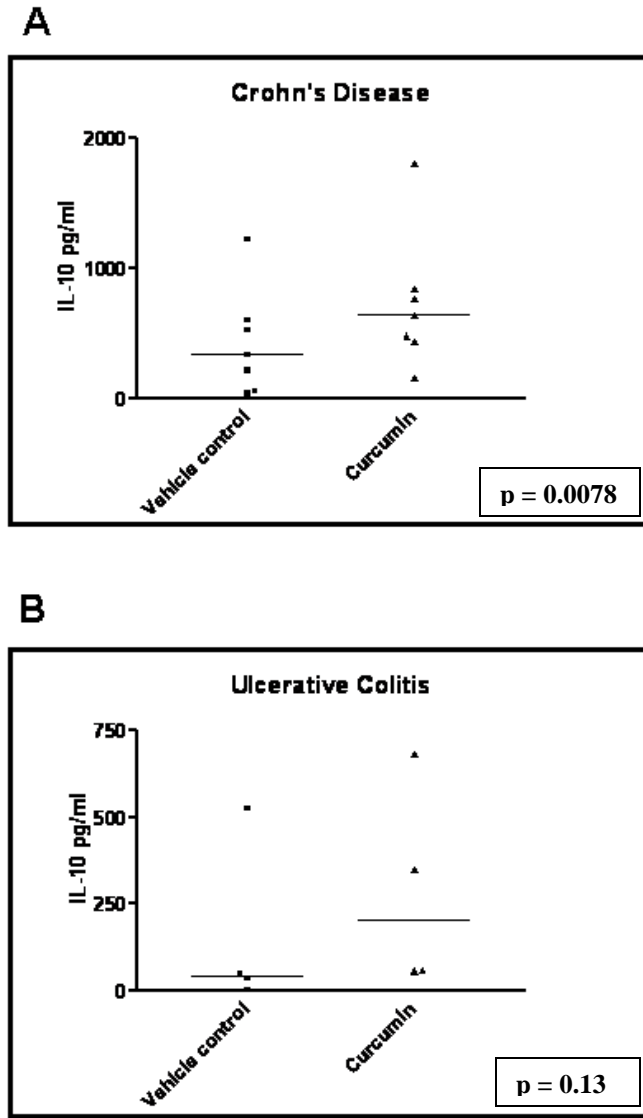
Figure 4.2 **Interleukin-10 responses of inflamed intestinal mucosal tissue to curcumin by disease group. (A) CD: median difference +276pg/ml (+65%) (p=0.0078). (B) UC: median difference +163pg/ml (+498%) (p=0.13).**

Published data demonstrate that curcumin increases IL-10 production in various cell types, including human T cells [318] and in the colonic mucosa in animal studies of experimentally induced colitis [319]. To our knowledge this is the first

study of curcumin in human *ex vivo* intestinal mucosal tissue. We show a marked, consistent and significant overall increase in IL-10 expression of 265% with curcumin. This increase in IL-10 was observed in both CD and UC groups, however it only attained statistical significance in CD, and overall in both disease groups. As expected, generally higher levels of cytokine expression were observed in CD than UC, both spontaneously and after culture with curcumin. Consistent with known epidemiological patterns (Chapter 1.1.0), there were lower numbers of children with UC available from whom to obtain biopsies. This, coupled with the lower levels of cytokine expression in UC, explains why in the UC group, response to curcumin did not achieve statistical significance. Nonetheless a convincing trend towards increased IL-10 expression with curcumin was observed in UC.

We observed a wide variability in IL-10 expression, both between individuals, between different biopsies from the same individual and between differing concentrations of curcumin. To explain this we consider the random composition of biopsies, the nature of *ex vivo* disease models and the heterogeneous and sometimes patchy disease phenotype in IBD. Despite measures to standardise, including taking a single biopsy only at each pass of the forceps, taking samples from a small disease area, and uniformity of handling and culture, four biopsies taken from one individual will inevitably be of variable composition in terms of cellularity and size. For example a biopsy which contains by chance a high proportion of immune cells can be expected to produce large amounts of cytokines, whilst one which contains a high proportion of fibrous tissue may produce less. To

minimise the confounding effect of these factors, we examined a relatively high sample size of 12 individuals with active IBD, and simplified our data analysis by presenting it as a matched pair of results for each patient. In this way we were able to quantify the overall direction of effect of curcumin upon heterogeneous samples, and to measure its statistical significance.

This rise in IL-10 is consistent with our hypothesis (discussed in greater detail in Chapter 1.2.10 and depicted in Figure 1.7) that curcumin inhibits acetylation of Sp3, reducing its binding to the IL-10 promoter and releasing suppression of IL-10 gene transcription. To further strengthen support for our hypothesis, we wished to investigate whether the actions of curcumin on IL-10 expression were specific to IL-10 and different to any effects on other cytokines. We therefore next examined the expression of key pro-inflammatory cytokines in the same experimental system.

4.2 Curcumin Decreases Interleukin-1β Production in Intestinal Mucosal Tissue

We wished to show that the effect of curcumin to enhance IL-10 production was specific to that cytokine and not shared by its effects on other, pro-inflammatory cytokines. Here we show that the overall IL-1β response to curcumin is to decrease production. Once again, a dose-response relationship was not clearly observed

(Figure 4.3A). Therefore for statistical analysis these data were treated exactly as before, and expressed as a single matched pair of results for each patient; control versus curcumin. Curcumin response was given as the individual's mean response across all curcumin doses tested (5-20μM) (Figure 4.3B and Table 4.2).

Curcumin caused a median reduction of -17% (-79pg/ml) in IL-1β expression in *ex vivo* tissue cultures from children and adolescents with active IBD, and this reduction was significant (p=0.0098) (Table 4.2). When analysed by separate disease groups, CD samples showed a 13% decrease in IL-1β expression with curcumin, which was significant (p=0.031) (Figure 4.4A), and UC samples a 15% decrease, which did not reach statistical significance (p=0.25) (Figure 4.4B).
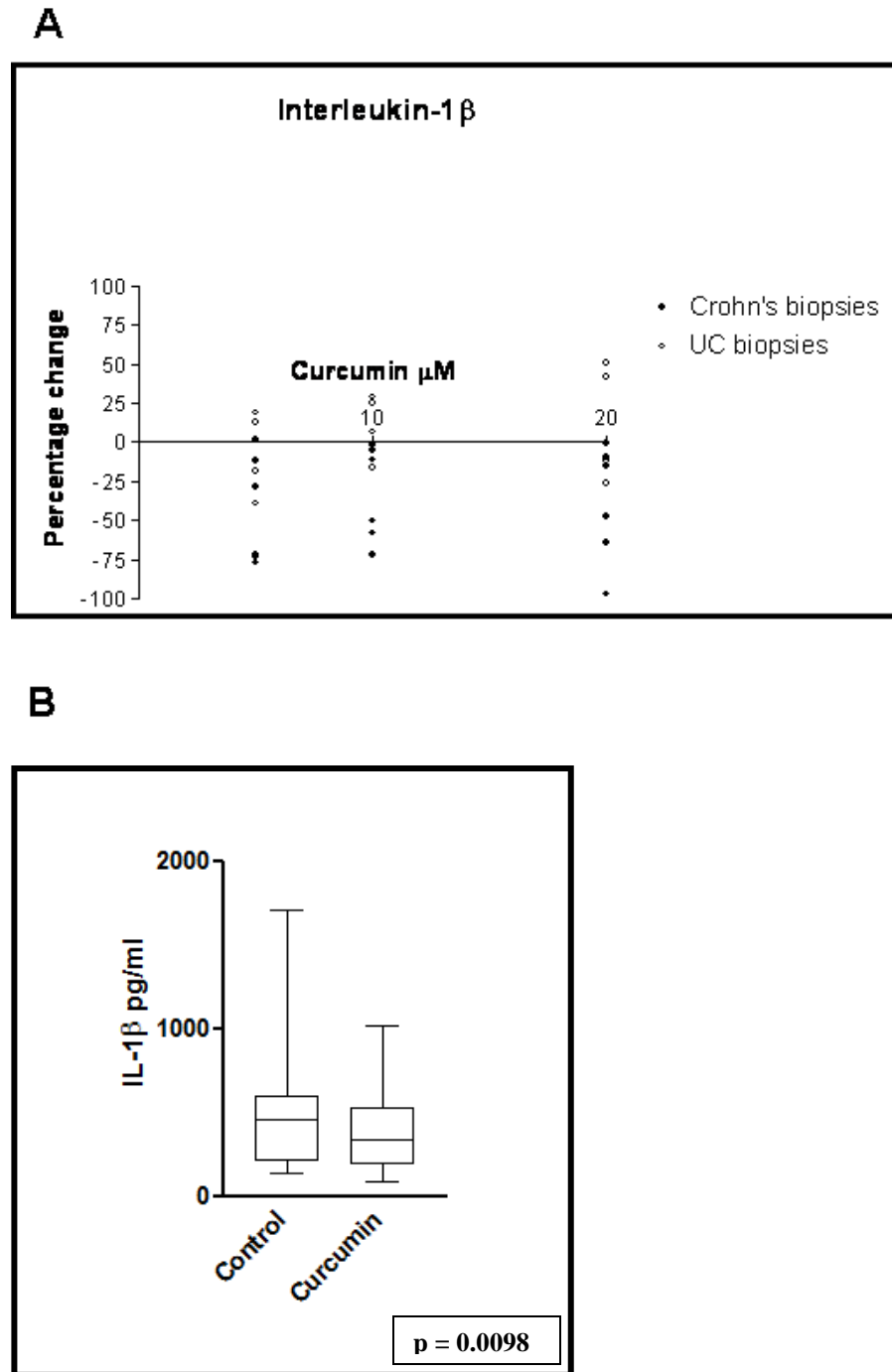
Figure 4.3 **Curcumin reduces interleukin (IL)-1β production in inflamed intestinal mucosal tissue. (A) IL-1β production by intestinal tissue as percentage change from baseline (vehicle control) with varying concentrations of curcumin. Each point represents a single experiment. Data from 10 individuals (6CD + 4UC). (B) The same data expressed as mean IL-1β response by each patient to all curcumin concentrations 5-20μM.**

	Median IL-1β (pg/ml)	Inter-quartile range	p value
Vehicle control	463	210-913	0.0098
Curcumin	384	201-789	

Table 4.2 Statistics describing figure 4.2B. Wilcoxon matched pairs test shows significant difference between the two groups.

A

Crohn's Disease

IL-1β pg/ml

2000

1000

0

Vehicle control

Curcumin

p = 0.031

B

Ulcerative Colitis

IL-1β pg/ml

750

500

250

0

Vehicle control
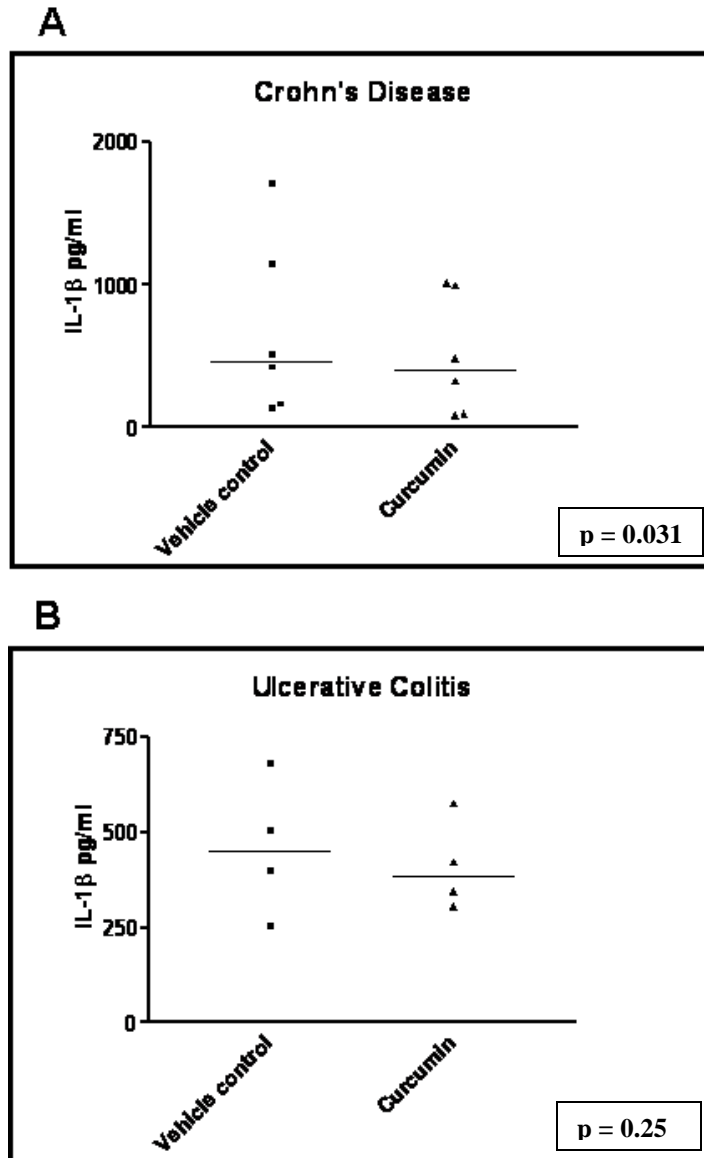
Curcumin

p = 0.25

Figure 4.4 **Interleukin-1β responses to curcumin by disease group. (A) CD: median difference -59pg/ml (-13%) (p=0.031). (B) UC: median difference -68pg/ml (-15%) (p=0.25).**

Our results show for the first time the suppression by curcumin of IL-1β expression in *ex vivo* human intestinal mucosal tissue culture from patients with active IBD. This reached statistical significance in the case of CD samples and in overall data from both disease types. Furthermore, although not statistically significant, in UC

samples a trend towards decreased IL-1β with curcumin was also seen. This reduction in IL-1β, although relatively modest at -17% overall, holds likely biological and future clinical importance in the treatment of IBD. Importantly the effect of curcumin on IL-1β is to decrease its production; in contrast to its enhancing effect upon IL-10. This indicates that IL-10 is subject to a particular action by curcumin, which directly opposes its effect on pro-inflammatory cytokines such as IL-1β. As with our IL-10 data, we observed a wide inter-individual variability in IL-1β production, and the reasons for this have previously been discussed (Chapter 4.1). To counteract this we once again included samples from a relatively large number of individuals in our analysis. For reference, the raw individual patient data can be found in the Appendix.

IL-1β is one of the most central and important effectors of the inflammatory response. It is produced by monocytes, macrophages, dendritic cells and other immune cells in response to all types of inflammatory stimuli and mediates a wide range of pro-inflammatory consequences. It is transcribed in the form of its 35kDa precursor, pro-IL-1β, cleaved to its 17kDa active form by caspase-1 (also known as interleukin converting enzyme (ICE)), then secreted, the precise mechanisms by which are incompletely understood [320]. It exerts its effects by binding to the IL-1 receptor IL-1RI [321], leading to recruitment of myeloid differentiation marker Myd88 [322], Toll interacting protein Tollip [323] and IL-1 receptor associated kinase IRAK [324]. This launches an activation cascade starting with

phosphorylation of IRAK [325], and leading ultimately to the activation of NF-κB. It is characteristically raised in the serum and tissues of patients with IBD.

Curcumin is known to suppress IL-1β production in numerous different cell types [326] [327] [328], including in the intestinal mucosa in models of colitis [319]. Various putative mechanisms have been proposed, including inhibition of NF-κB and MAPK [329] and prevention of recruitment of IRAK to IL-1RI [330]. Once again it seems likely that curcumin has a complex mode of action, possibly involving more than one mechanism. Whilst our hypothesis for the mechanism of action of curcumin upon IL-10 is via the acetylation-sensitive transcription factor Sp3, the question remains as to the mechanism(s) of action of curcumin on IL-1β. Putative candidate mechanisms include inhibition of elements in the receptor and signalling cascades, inhibition of cleavage of its inactive precursor, and inhibition at, or prior to, the transcriptional level, for example through NF-κB or MAPK signalling. These latter possibilities in particular are further to be explored in this work.

4.3   Interferon-γ Production in Intestinal Mucosal Tissue is Not Significantly Affected by Curcumin

Although in the majority of individuals (9/13) we observed that IFN-γ expression decreased with curcumin (Figure 4.5A), overall no significant change in IFN-γ expression was identified in response to curcumin in *ex vivo* intestinal mucosal tissue (Figure 4.5B). Similarly when examined by separate disease groups, changes did not reach statistical significance in either CD or UC (Figure 4.6). We conclude that curcumin does not significantly affect IFN-γ in intestinal mucosal tissue from children with active IBD. Once again and for the reasons discussed above variability was wide. All patient details and individual results can be found in the Appendix.

A

B
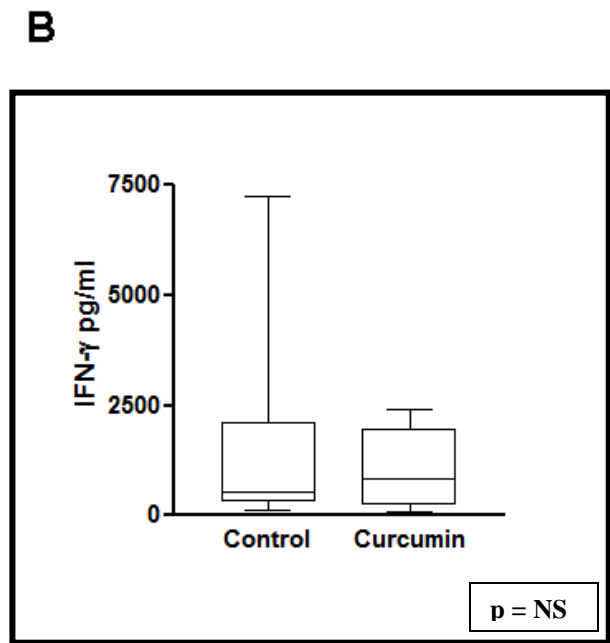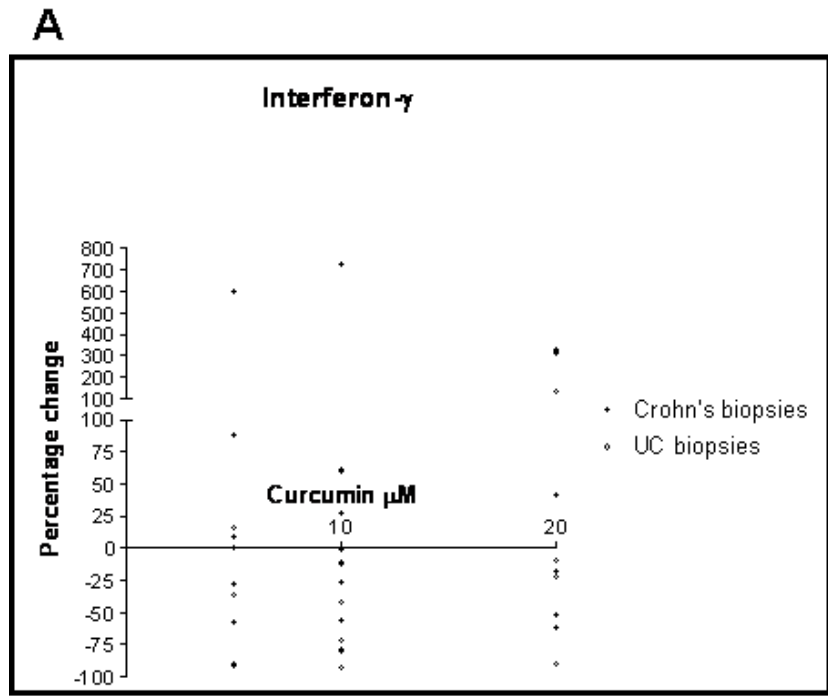
Figure 4.5   **Curcumin causes no significant change in interferon (IFN)-γ production in inflamed intestinal mucosal tissue. (A) IFN-γ production by intestinal tissue as percentage change from baseline (vehicle control) with varying concentrations of curcumin. Each point represents a single experiment. Data from 13 individuals (8 CD + 5 UC). (B) The same data expressed as mean IFN-γ response by each patient to all curcumin concentrations 5-20μM.**
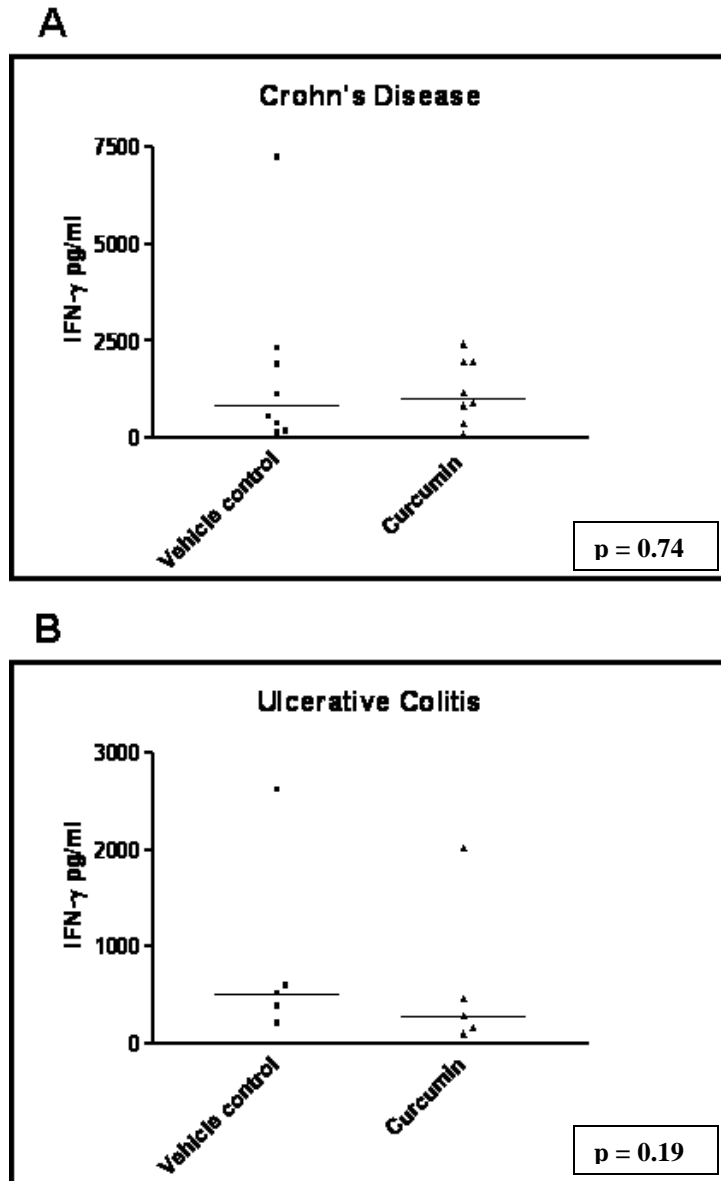
Figure 4.6   **Interferon-γ responses to curcumin by disease group. (A) CD: Median difference +187pg/ml (+22%) (p=0.74). (B) UC: Median difference -232pg/ml (-45%) (p=0.19).**

Ours is the first investigation, to our knowledge, of the effect of curcumin on IFN-γ expression in human *ex vivo* intestinal mucosal tissue.   We observed that spontaneous IFN-γ production by intestinal mucosal biopsies was higher in CD

than UC. This is an expected finding for a cytokine of Th1 type such as IFN-γ and is consistent with the findings of other groups [331]. We found no statistically significant overall or disease-specific change in IFN-γ levels with curcumin. Nonetheless a key aspect of our findings is that the responses to curcumin of both pro-inflammatory cytokines IFN-γ and IL-1β did not mirror that of IL-10.

IFN-γ is a pro-inflammatory cytokine produced in response to a wide range of host invaders including viruses, bacteria, parasites and cancer cells. It is a member of the type II group of interferons and binds to the type II IFN transmembrane receptor. This leads to activation of Janus family Kinases Jak 1 and Jak 2 and phosphorylation of STAT1, homodimers of which then translocate to the nucleus and initiate transcriptional events. By a separate pathway IFN-γ also activates p42 MAPK. Its downstream effects are wide ranging and include activation of lymphocytes, macrophages and other immune cells, and upregulation of expression of MHC II on antigen presenting cells (APC) [332]. Serum and tissue levels of IFN-γ are raised in IBD. Like IL-1β, IFN-γ is classically considered a cytokine of Th1 type, although the concept of CD as a Th1 disease and UC as Th2 is evidently an over-simplification, and our data corresponds with other published studies in which IL-1β and IFN-γ are observed to be raised in UC as well as CD [333]. IFN-γ is a novel target for biological therapies in IBD; a humanised monoclonal antibody fontolizumab is currently showing promise in early phase human trials [334] [335].

There is some published evidence that curcumin decreases IFN-γ production in T cells *in vitro* [327] [336]. Various potential mechanistic explanations are given for this effect, including suppression of IL-12 [327] and inhibition of NF-κB [336], but these are largely speculative. From a separate angle, in brain microglial cells, studies show suppression of IFN-γ signalling by curcumin, via inhibition of the Jak/STAT1 pathway. It seems likely that the effects of curcumin differ according to cell type, and occur through complex and possibly multiple mechanisms.

To summarise our cytokine findings in the inflamed intestinal mucosa, we observed significantly enhanced IL-10 production, significantly decreased IL-1β and unaltered IFN-γ expression, in *ex vivo* culture with curcumin. Although they do not provide conclusive proof, these findings are consistent with our original hypothesis that a specific, acetylation-sensitive element exists in the IL-10 promoter region, leading to enhanced IL-10 production in response to curcumin (Chapter 1.2.10 and Figure 1.7); an effect which is separate from the action of curcumin upon other cytokines. Furthermore they show favourable modulation of the cytokine profile in the human intestinal mucosa in response to curcumin, an observation which holds clear potential therapeutic relevance for curcumin in IBD. The mechanism(s) of action of curcumin upon cytokine production remains unknown, but candidate mechanisms include intermediary signalling cascades such as NF-κB and p38 MAPK. As previously mentioned, some investigators provide evidence suggesting that these pathways are involved in the modulation of IL-1β and IFN-γ by curcumin [329, 336]. We will proceed to investigate these possibilities in the current work.

5.0   Cytokine Production by Mononuclear Cells

5.1   There is a Trend Towards Decreased Interleukin-1β and Interferon-γ in Lamina Propria Mononuclear Cells With Curcumin

From our work on cytokine expression in intestinal mucosal biopsies with curcumin (Chapter 4.0), we concluded that there was a modest but significant decrease in IL-1β and a non-significant trend towards decreased IFN-γ production. Importantly we found that neither of these pro-inflammatory cytokines mirrored the enhanced production of IL-10 which we observed with curcumin. The wide inter-sample variability which we observed in these tissue experiments was ascribed to the use of biopsy culture as a model of disease, containing heterogeneous mixtures of cell types. Thus we wished to refine our study to a single cell type from the inflamed gut mucosa. A natural choice was LPMC's, since mononuclear cells are activated in IBD and are a major source of cytokine production.

During the time period in which we were collecting surgical resection specimens for cytokine analysis, we encountered a prolonged shortage of clinical supply due to reduced numbers of patients undergoing surgery for active inflammation in IBD. The data we were able to collect strongly suggest that curcumin causes decreased

production of both IL-1β and IFN-γ (Figure 5.1). Furthermore they appear to point to a more consistent and less variable response, with every one of the six individual patients showing a reduction in pro-inflammatory cytokine production with curcumin. Unfortunately patient numbers were insufficient to reach statistical significance. All patient details and individual results can be found in the Appendix.

A
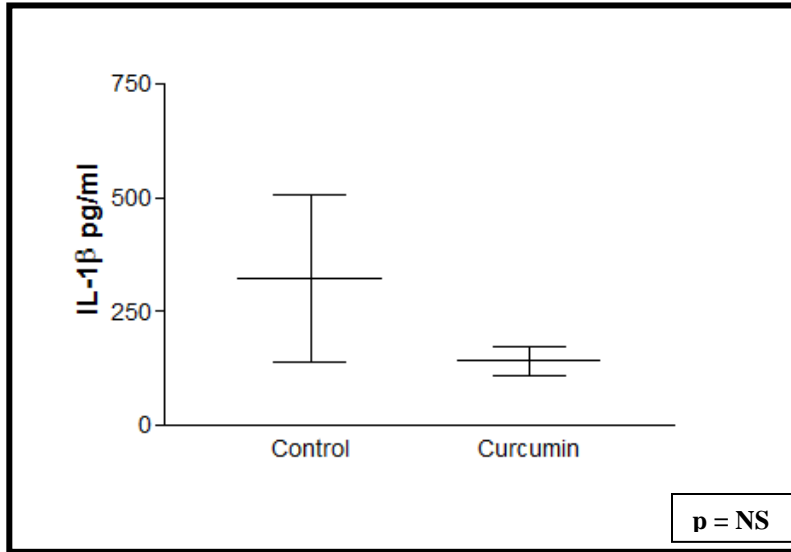
B
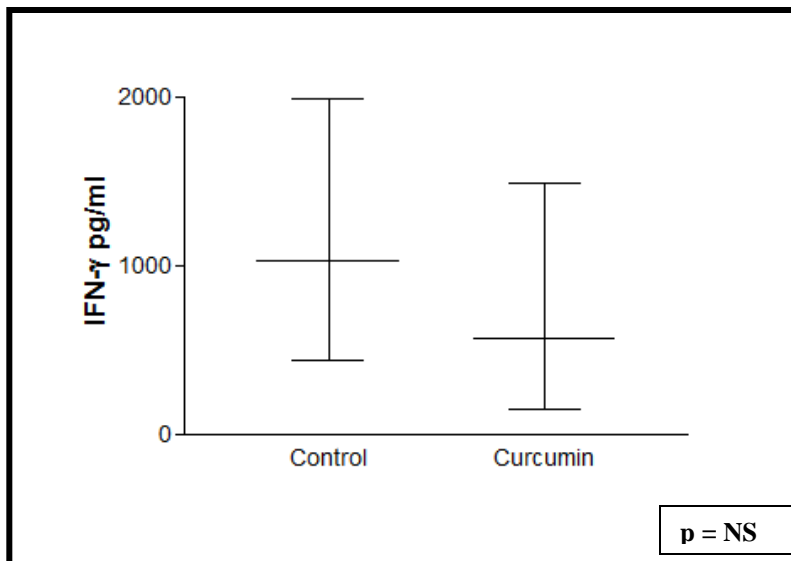
Figure 5.1   **Curcumin may reduce interleukin (IL)-1β and interferon (IFN)-γ production in lamina propria mononuclear cells (LPMC) from patients with active IBD. (A) IL-1β production by LPMC's cultured *ex vivo* with curcumin. Each point represents a single experiment.   Data from 2 individuals (1CD + 1UC).   (B) IFN-γ production by LPMC's cultured with curcumin. Data from 4 individuals (3CD + 1UC).**

The data shown complement our intestinal mucosal biopsy findings, and provide evidence that the most part of cytokine production measured in tissue culture comes from mononuclear cells. LPMC's are described in more detail in Chapter 2.1.3. They are gut mucosal lymphocytes and monocytes, comprising a large proportion of T cells and macrophages and have a generally activated phenotype. Therefore they are an excellent model of disease and do not require *in vitro* stimulation. Cytokine production by LPMC's from actively diseased tissue is spontaneous, and as we show here amenable to inhibition by curcumin. We would have liked to obtain LPMC's from a greater number of individuals to further examine these effects, including upon IL-10 production, but unfortunately this was not possible so we considered alternative strategies. Firstly we studied readily available peripheral blood mononuclear cells, and later we will isolate another gut mucosal cell type of key relevance to IBD, myofibroblasts.

5.2 Interleukin-10 Production by Peripheral Blood Mononuclear Cells Increases with Lipopolysaccharide

Ideally we would study IL-10 production in gut mononuclear cells, but because of clinical supply interruption, we instead elected to examine IL-10 production in PBMC's from healthy donors. There are some differences in composition,

phenotype, morphology and immunology between PBMC's and LPMC's, which are largely attributed to the increased reactivity of mononuclear cells in the gut compared to peripheral blood (Chapter 2.1.3) [312]. For these reasons, healthy PBMC's differ from LPMC's taken from inflamed gut in that PBMC's require stimulation in order to induce significant cytokine production. Nonetheless these two groups of leukocytes behave similarly *in vitro* and share many similarities; both immunologically active cells comprising predominantly T lymphocytes, with lesser proportions of B cells and natural killer cells, as well as monocytes which may differentiate into macrophages or dendritic cells.

Spontaneous IL-10 production by normal PBMC's was generally low, but a wide variation between individual donors was seen. Stimulation with LPS caused a dose-dependent increase in IL-10 production (Figure 5.2).
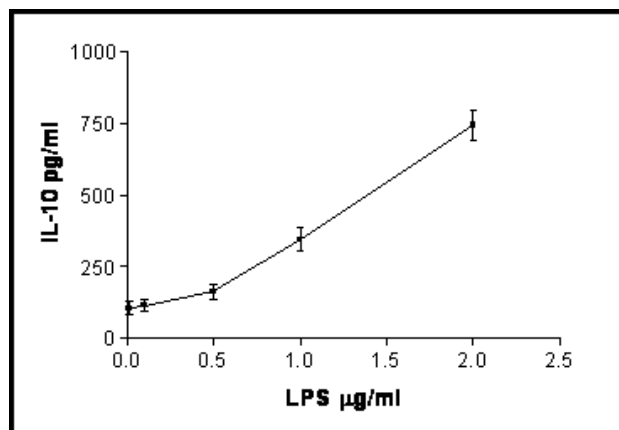
Figure 5.2 **Interleukin-10 production by peripheral blood mononuclear cells after 24 hours of culture with varying concentrations of lipopolysaccharide. Error bars represent standard errors of the mean.**

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We confirm successful activation of PBMC's with LPS in a dose-dependent fashion (Figure 5.2). LPS is a structural component of the outer membrane of Gram negative bacteria. Structural motifs expressed by bacteria, viruses and fungi, known as pathogen-associated molecular patterns (PAMP's), stimulate the host innate immune system via toll-like receptors (TLR's). The main PAMP of LPS is lipid A; also known as endotoxin [337]. Lipid A is the hydrophobic domain of LPS, and it is flanked by two hydrophilic components, a core oligosaccharide and a distal polysaccharide, or O antigen. LPS is largely sensed by TLR4 [338] and the transfer of LPS onto TLR4 is mediated by interaction with several other proteins including LPS binding protein (LPB) and the co-receptor cluster of differentiation (CD)14 [339]. Upon TLR4 ligation by LPS, TLR4 recruits downstream adapter proteins and its subsequent signal transduction can be divided into myeloid differentiation primary response gene (MyD)88-dependent and MyD88-independent pathways. The MyD88-dependent pathway is responsible for the expression of pro-inflammatory cytokines and the MyD88-independent pathway for the induction of type I interferon genes. NF-κB and MAPK activation can occur through both pathways [340].

Although other stimulants could have been used such as TNF-α or IL-1, we felt that LPS was a suitable choice in this case. With relevance to intestinal inflammatory signalling, there is a high presence of Gram negative bacteria in the gut, many species of which are potentially pathogenic, and toll-like receptor signalling plays a pivotal role in the generation of appropriate and inappropriate inflammation in the

intestine [42]. This is addressed in more detail in chapter 1.2.1. Our dose-response experiments allowed us to select an optimal LPS concentration of 1µg/ml for further experiments. At this concentration IL-10 production by PBMC's doubled; a clear upregulation potentially amenable to inhibition by a test agent such as curcumin.

5.3 Curcumin and Anacardic Acid Induce a Bell-Shaped Response in Interleukin-10 Production by Peripheral Blood Mononuclear Cells

With our human PBMC culture model established, we proceeded to investigate the responses of these cells to curcumin. At low concentrations, there was an initial rise in IL-10 production by normal PBMC's stimulated with LPS. With increasing doses of curcumin, IL-10 production diminished, creating a bell-shaped dose-response curve (Figure 5.3). This response was mirrored when experiments were repeated with anacardic acid, another known inhibitor of acetylation. Cell viability was confirmed at the end of all experiments by trypan blue exclusion, and remained above 80%. There was as expected a wide variability in both spontaneous and post-stimulation IL-10 production between donors, as noted by other investigators [308]. This can be appreciated by comparing Figures 5.3A and 5.3B; showing quite different levels of IL-10 production in PBMC's donated by two healthy

donors. Although basal IL-10 production varied, the finding of a bell-shaped pattern of response to curcumin and anacardic acid was consistent in all samples tested. These experiments were repeated at least in triplicate.

Choice of curcumin and anacardic acid concentrations was based upon concentrations studied by previous investigators, on cytokine responses as measured here and on cell viability assays in our laboratory. By trypan blue exclusion testing we found that PBMC's remained above 80% viable in curcumin concentrations of up to 50μM, and in anacardic acid concentrations of up to 20μM, after 24 hours of incubation. As discussed earlier (Chapter 3.2) in the case of curcumin this probably corresponds to concentrations considerably higher than those achievable by standard oral dosing in humans. In the case of anacardic acid such factors are unknown as it has never been studied as a therapeutic agent *in vivo*.

A

B
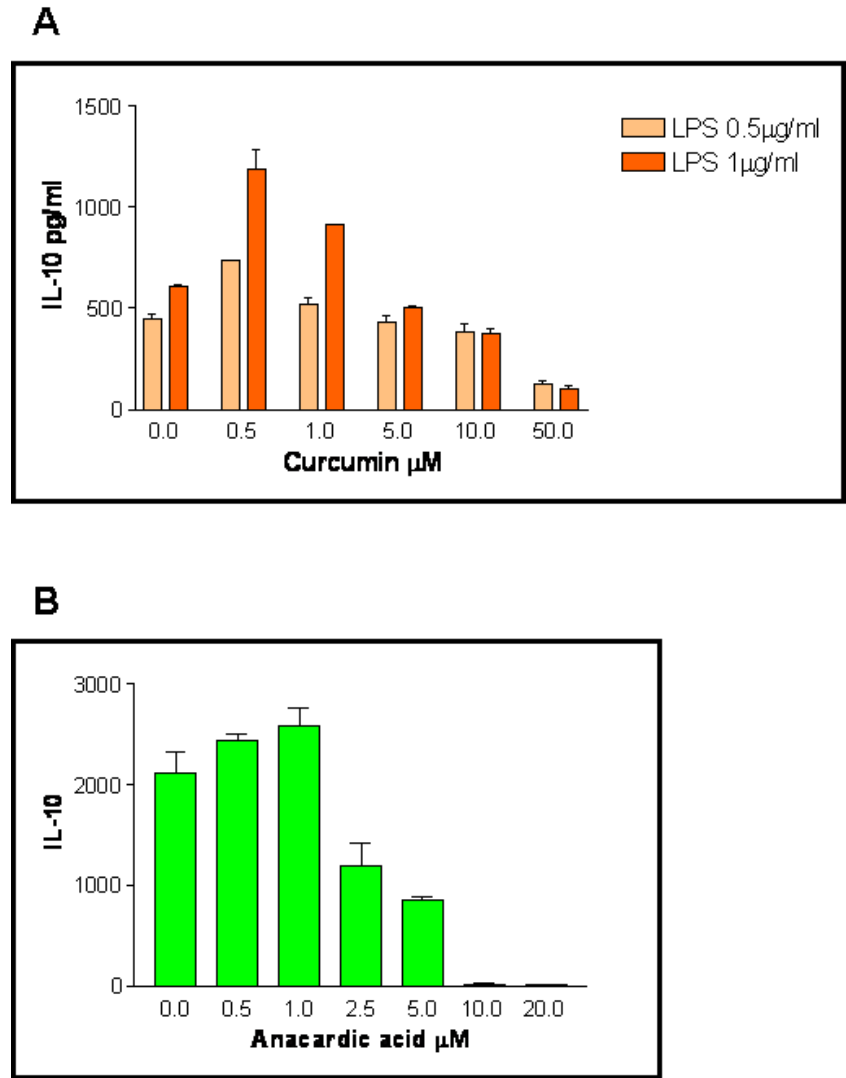
Figure 5.3 **Curcumin and anacardic acid induce a bell-shaped interleukin (IL)-10 response in peripheral blood mononuclear cells (PBMC's). (A) IL-10 production by lipopolysaccharide (LPS)-stimulated PBMC's with increasing curcumin dose. (B) IL-10 production by PBMC's stimulated with 1μg/ml LPS with increasing anacardic acid dose.**

The interleukins are cytokines which are secreted by leukocytes and communicate messages between them. As discussed in Chapter 1.2.6, IL-10 is an important anti-inflammatory cytokine in the gut. Curcumin has previously been shown to decrease expression of pro-inflammatory cytokines in a number of different cell

types including normal human PBMC's [341]. Some published data also exist showing upregulation of IL-10 by curcumin, including in experimentally transformed human T cell blasts [318]. To our knowledge there is no published work on the effect of anacardic acid on mammalian cytokine profiles.

In the current study, normal human PBMC's exhibited a bell-shaped IL-10 response to increasing doses of curcumin (Figure 5.3A). Anacardic acid, like curcumin, is an inhibitor of acetylation (Chapter 1.5). This is the only known biological property of anacardic acid, and the only known shared property of the two agents [303]. The finding of a similar bell-shaped IL-10 response by PBMC's to anacardic acid (Figure 5.3B), whilst not conclusive for mechanism, suggests that both agents are acting via an acetylation-dependent mechanism. These data are compatible with our hypothesis that curcumin acts to decrease the acetylation of Sp3, reducing its binding to the IL-10 gene promoter and releasing its suppression of IL-10 transcription (presented in detail in Chapter 1.2.10). Why IL-10 production is only enhanced at low concentrations of curcumin is unknown, and the current hypothesis does not explain the bell-shaped nature of the response. Intriguingly, this finding is in accordance with a number of reports of curcumin having paradoxically opposing biological actions at differing doses [342], and this lends weight to the notion that curcumin exerts its effects through more than one molecular mechanism. It may be that at higher concentrations, the anti-acetylating effect of curcumin upon histones predominates, blocking access of positive transcription factors and thus reducing IL-10 expression.

Whilst our PBMC data demonstrate that curcumin has a biological impact on IL-10 expression in human mononuclear cells, this bell-shaped pattern was not reproduced in LPMC's for IL-1β or IFN-γ, nor in intestinal biopsies for IL-10, and we did not consider the extrapolation of data from circulating mononuclear cells a valid strategy in relation to the behaviour of intestinal mononuclear cells. However, by employing these techniques and studying the responses we were able to ascertain optimal culture conditions, including cell isolation techniques, handling procedures, culture time periods and dosing ranges, and apply these to experiments on LPMC's. These data also support the need for trialling of a wide range of doses in clinical studies, including low dose.

5.4 Interleukin-10 Production by Peripheral Blood Mononuclear Cells Occurs Largely During Hours 12 to 24 of Culture

Time course experiments revealed that the greatest rate of IL-10 production by PBMC's occurs during the second half of the 24 hour incubation period (Figure 5.4).
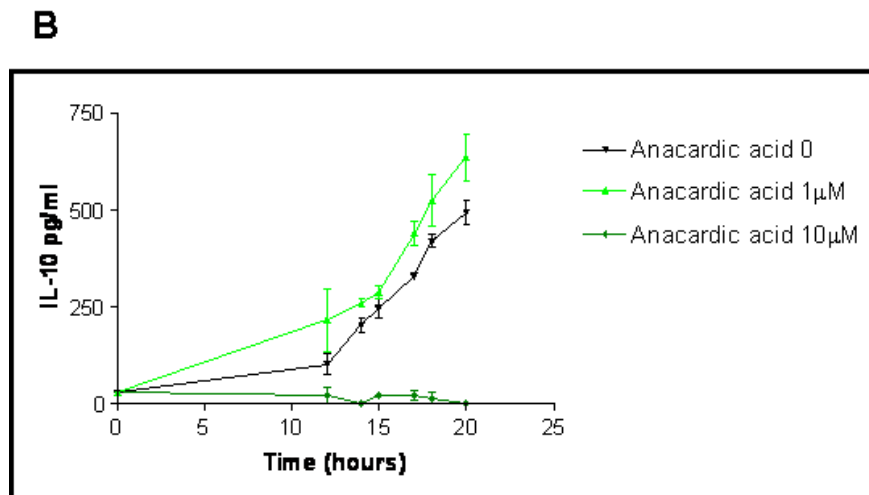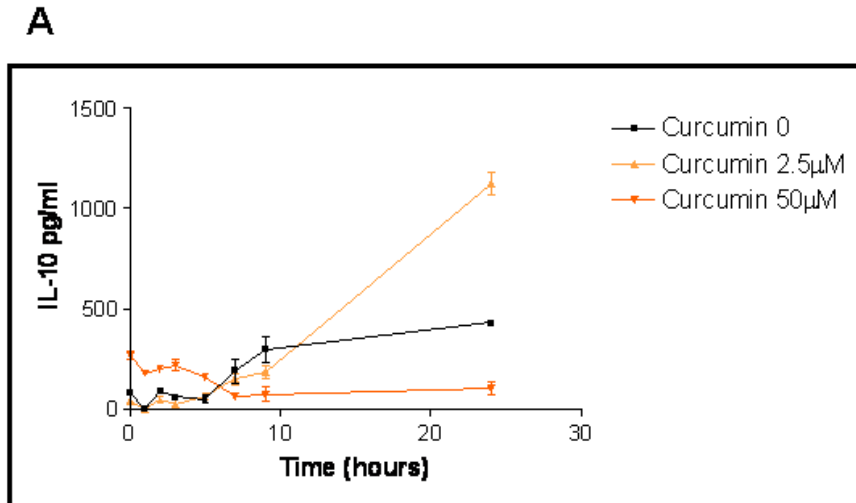
Figure 5.4 **Interleukin (IL)-10 is produced by peripheral blood mononuclear cells (PBMC's) largely during hours 12-24 of culture. (A) IL-10 production by lipopolysaccharide (LPS)-stimulated PBMC's with different doses of curcumin. (B) IL-10 production by LPS-stimulated PBMC's with different doses of anacardic acid.**

In vivo, IL-10 is released later than the pro-inflammatory cytokines after a stimulus such as LPS, and this has been shown in both animal and human models [343, 344]. Thus IL-10 plays an important biological role in limiting unchecked inflammation

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(Chapter 1.2.6). It is likely that, had we measured the time courses for release of inflammatory cytokines such as TNF-α, IL-1, IFN-γ or IL-8, these would have peaked earlier. However, such experiments have previously been published. Our time course experiments allowed us to ascertain the ideal culture time period of 24 hours; a balance between maximal cellular activity versus risk of infection or cell death – a risk which is higher in mononuclear cells isolated from (particularly diseased) intestinal specimens than from peripheral blood.

6.0 p38 Mitogen-Activated Protein Kinase and Nuclear Factor-κB Signalling in *ex vivo* Intestinal Mucosal Tissue

6.1 p38 Mitogen-Activated Protein Kinase is Spontaneously Activated in Inflammatory Bowel Disease and this is Completely Abrogated by the Inhibitor SB203580

p38 MAPK activation (phosphorylation) is characteristically greatly increased in mucosal biopsies from patients with active CD and UC, compared with normal mucosal biopsies taken from subjects without disease (Figure 6.1A). The addition of specific p38 MAPK inhibitor SB203580 in 24 hour culture with biopsies from patients with active IBD, almost completely abrogates this spontaneous p38 MAPK activation (Figure 6.1B).
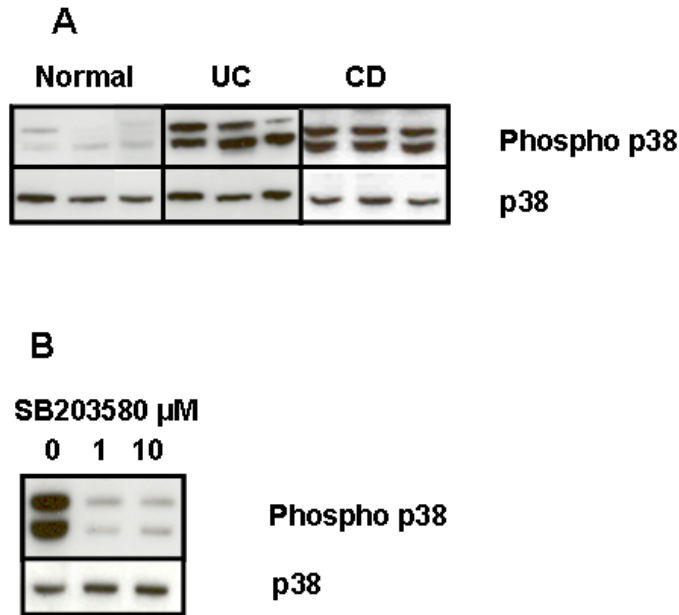
Figure 6.1 **p38 mitogen-activated protein kinase (MAPK) is activated in inflammatory bowel disease. (A) Phospho p38 MAPK and p38 MAPK loading control in normal, ulcerative colitis (UC) and Crohn's disease (CD) biopsies. 3 individuals in each group. (B) UC biopsies cultured with p38 MAPK inhibitor SB203580. The experiments in figure 6.1 were performed by Dr Guillermo Docena who kindly shared his results with us.**

p38 MAPK becomes activated when it is phosphorylated (Figure 1.2 and Chapter 1.2.2). Total p38 MAPK serves as a loading control for its phosphorylated form. This is a more specific and appropriate standardiser for phospho-p38 MAPK than the more generic cytosolic housekeeping gene β-actin, since it controls not only for equal protein loading but also for the proportion of p38 MAPK which is activated out of the total potential p38 MAPK in that particular tissue. This is especially useful for biopsy work as opposed to work on a single cell type, because it helps to

correct for the variable proportions of cell types within the tissue, only some of which may be MAPK-producing cells.

We confirm here as expected the greatly increased p38 MAPK activation in both CD and UC, as well as the minimal detectable activation in normal healthy gut. This illustrates the reason we have chosen to use only tissue from patients with active disease, upon which to study the effects of curcumin as a potential therapeutic agent in IBD. In actively diseased tissue inflammatory mediators are activated and this provides a good *ex vivo* model to examine for suppression by curcumin. As can be seen in Figure 6.1A these experiments would not be relevant or possible in healthy gut tissue.

The specific p38 MAPK inhibitor SB203580 is a member of the pyridinyl imidazole compounds, and is known to block the pathway by competing for adenosine triphosphate (ATP) in the active pocket of the enzyme [345]. This compound was being tested as part of work funded by Glaxo Smith Kline, and is included here to illustrate the near total inhibition which occurs with directed inhibition of this kind. We will compare with this the effects of curcumin as a potential p38 MAPK inhibitor.

6.2 Curcumin Decreases p38 Mitogen-Activated Protein Kinase Activation in Inflamed Intestinal Mucosal Tissue

We hypothesised that curcumin would have direct anti-inflammatory effects in the gut mucosa in IBD. In Chapters 4.0 and 5.0 we studied mucosal tissue biopsies and mononuclear cells from children and adults with active IBD and treated them *in vitro* with curcumin. We showed favourable modulation of the cytokine profile with enhanced IL-10 production and reduced IL-1β. We wished to seek a mechanistic explanation for the reduced expression of IL-1β observed with curcumin in intestinal mucosal tissue and LPMC's. In pursuit of this we first assessed the p38 MAPK signalling pathway in the inflamed intestinal mucosa in IBD.

Overall, curcumin caused a median reduction of 42% in phosphorylated (activated) p38 MAPK in *ex vivo* tissue cultures from patients with active IBD, and this reduction was significant (p=0.023). Once again data from all curcumin concentrations (Figure 6.2B) were combined for the purpose of statistical analysis (Figure 6.2C and Table 6.1). Total p38 MAPK was used as a loading control for phosphorylated p38 (Figure 6.2A) and all p38 MAPK data is expressed as a densitometry ratio of phosphorylated p38/total p38 MAPK. The slight difference in band morphology seen in Figure 6.2 compared to Figure 6.1 is due to the fact that

those in Figure 6.1 were run on pre-cast gels whereas those in Figure 6.2 were run on prepared gels. All patient details and individual results can be found in the Appendix.
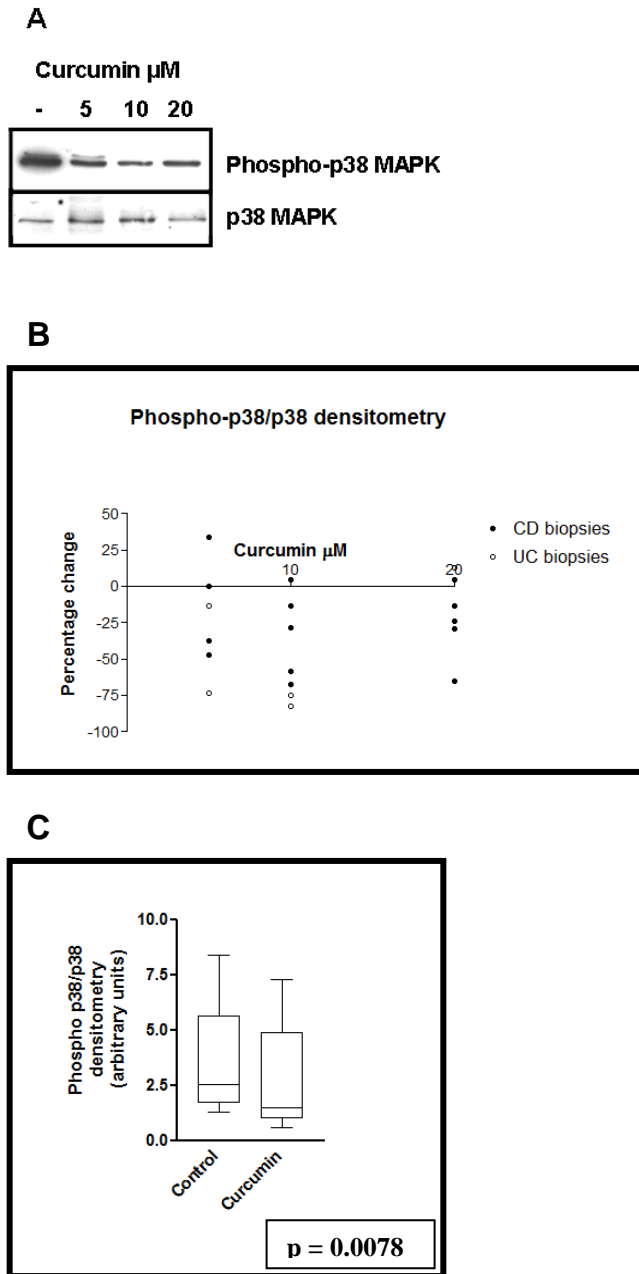
Figure 6.2 Curcumin reduces p38 mitogen-activated protein kinase (MAPK) activation in inflamed intestinal mucosal tissue. (A) Western blot from single representative experiment showing phosphorylated p38 MAPK and p38 loading control in CD biopsies with varying concentrations of curcumin. (B) Phospho-p38/total p38 densitometry as percentage change from baseline (vehicle control) with varying concentrations of curcumin. Each point represents a single experiment. Data from 8 individuals [5CD + 3UC]. (C) Phospho-p38/p38 as average response by each patient to all curcumin concentrations 5-20μM.

	Median phospho-p38/p38 densitometry	Inter-quartile range	p value
Vehicle control	2.55	1.7-5.7	0.0078
Curcumin	1.50	1.0-4.9	

Table 6.1 Statistics describing figure 6.1C. Wilcoxon matched pairs test shows significant difference between the two groups.

p38 MAPK plays a central role in inflammatory signalling, and is known to be inhibited by curcumin in various cell types [346] [347] [348]. It was therefore a candidate mechanism for the IL-1β suppression we observed in intestinal mucosal tissue and mononuclear cells (Chapters 4.0 and 5.0). We hypothesised that curcumin may suppress inflammatory cytokine release by inhibition of p38 MAPK. To our knowledge this is the first work to examine the effect of curcumin on p38 MAPK in *ex vivo* intestinal mucosal tissue in IBD.

Because of the high spontaneous activation of p38 MAPK in active IBD, and since we are investigating curcumin as a potential therapy for IBD, we chose to study material from patients with active disease, placing a similarly inflamed specimen in vehicle control conditions alongside every experiment as an internal negative control. We found a marked and significant reduction in activation (phosphorylation) of p38 MAPK with curcumin, of 42% overall in intestinal tissue from patients with active IBD. This is a potential mechanism by which curcumin

reduces the expression of downstream pro-inflammatory cytokines such as IL-1β in the inflamed gut mucosa.

The specific p38 MAPK inhibitor SB203580 blocks the pathway by competing for adenosine triphosphate (ATP) in the active pocket of the enzyme [345]. The inhibition of p38 MAPK phosphorylation seen with curcumin in intestinal mucosal biopsies occurs by a mechanism remaining as yet obscure, and may involve upstream elements in the p38 MAPK signalling pathway. In contrast to the near total inhibition seen with SB203580 in figure 6.0B, the inhibition of p38 MAPK which we observed with curcumin was incomplete, and we therefore conclude that the effects of curcumin in the human intestinal mucosa in IBD are in part p38 MAPK-dependent. Curcumin is a biologically complex molecule, and it is likely that its anti-inflammatory effects occur through multiple mechanisms.

6.3 Effect of Curcumin on Nuclear Factor-κB Activation in *ex vivo* Intestinal Mucosal Tissue

In Chapters 4.0 and 5.0 we studied mucosal tissue biopsies and mononuclear cells from children and adults with active IBD and treated them *in vitro* with curcumin. We showed favourable modulation of the cytokine profile with enhanced IL-10 production and reduced IL-1β. Seeking a mechanistic explanation for the reduced expression of IL-1β observed with curcumin in intestinal mucosal tissue and

LPMC's we first assessed the p38 MAPK signalling pathway in the inflamed intestinal mucosa in IBD (Chapters 6.1 and 6.2). We showed that curcumin inhibited p38 MAPK activation and concluded that the IL-1β suppression is in part p38-MAPK dependent.

As curcumin has been shown in some cell types to suppress the NF-κB signalling pathway [167] [168], we next proceeded to examine whether NF-κB suppression also played a role in the downstream anti-inflammatory effects of curcumin upon cytokines which we observed in the inflamed human gut mucosa. We hypothesised that both the enhanced IL-10 and the reduced IL-1β observed with curcumin may be mediated via a NF-κB-dependent mechanism. We found no evidence to suggest a change in either the nuclear p65 subunit or cytosolic IκB in *ex vivo* tissue cultures from paediatric patients with active IBD (Figure 6.3). All patient details and individual results can be found in the Appendix.

A

Curcumin µM
0   20

Nuclear p65
Nuclear histone H1
Cytosolic IκB
Cytosolic β-Actin

B

p65/H1 densitometry (arbitrary units)

Vehicle control   Curcumin 20µM

p = NS
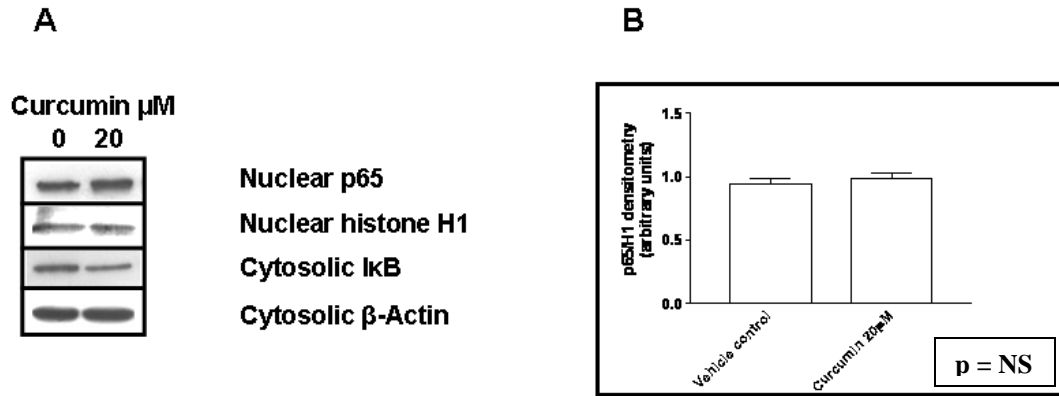
Figure 6.3 There is no evidence of reduced NF-κB activation with curcumin in intestinal mucosal biopsies from children with active inflammatory bowel disease. (A) Western blot from a single representative experiment showing nuclear p65 subunit and histone H1 loading control with corresponding cytosolic IκB and β-actin loading control in a Crohn's disease (CD) biopsy. (B) Data from all nuclear p65 experiments [n=4 (3CD + 1UC)]. Error bars represent standard errors of the mean.

The NF-κB signalling pathways and their importance in inflammation and IBD are described in detail in Chapter 1.2.3, and illustrated in Figure 1.3. As explored in Chapter 1.4.4, curcumin inhibits NF-κB activation in a number of different models. This is the first study to our knowledge in *ex vivo* human intestinal mucosal tissue culture. We show no clear difference in NF-κB activation in response to curcumin. It may be that in inflamed human gut, NF-κB signalling is not a relevant mechanism by which curcumin exerts its anti-inflammatory effects.

Alternatively we consider the possibility that in our experimental model we were unable to show effectively the NF-κB inhibition which would occur with curcumin in the *in vivo* state. Immunoblotting may be a technique of insufficient quantitative sensitivity to demonstrate partial changes in elements within the NF-κB pathway.

Or it is possible that, in a disease model which relies on complex tissue samples such as an intestinal mucosal biopsy, and in the presence of multiple potential bacterial and other inflammatory mediators in culture, we were unable to isolate or observe a true effect of curcumin upon NF-κB signalling. This was one of the reasons for which, in the absence of a steady clinical supply of LPMC's, we decided to proceed to a different reductionist experimental model to further examine the effects of curcumin in IBD.

7.0 Effects of Curcumin on Colonic Myofibroblasts from Patients with Active Inflammatory Bowel Disease

We hypothesised that curcumin would decrease MMP-3 production; a key contributor to tissue destruction in IBD; and that this effect may be acetylation-dependent. We first measured *in vitro* MMP-3 production by colonic myofibroblasts (CMF) from patients with active IBD, cultured with curcumin.

7.1 Curcumin reduces Matrix Metalloproteinase-3 Production by Colonic Myofibroblasts in a Dose-Dependent Manner

Whilst an intestinal biopsy is a good model of true disease, it consists of many different cell types, in variable and different proportions in each biopsy. We wished to study the effect of curcumin on a single key component, whilst still retaining the active IBD phenotype and not reverting to more distant cell lines. Following the issues with clinical supply of LPMC's as described in chapter 5.0, we sought another alternative relevant cell type in IBD for *in vitro* study. We therefore next chose to examine colonic myofibroblasts (CMF). These are key

stromal effector cells in IBD, and are amenable to *in vitro* culture directly from a patient with active disease. Furthermore, because this cell type divides *in vitro* it is possible to culture primary CMF from much smaller pieces of surgically resected intestinal tissue than the minimum size required to obtain LPMC's. This solved the supply problem associated with running LPMC experiments. Myofibroblasts do not express IL-1β or IL-10, but play an active role in IBD, expressing MMP-3 on stimulation with TNF-α.

We found that curcumin suppressed MMP-3 production in TNF-α-stimulated CMF from patients with active IBD and the response was clearly dose-dependent (Figure 7.1). All patient details and individual results can be found in the Appendix.
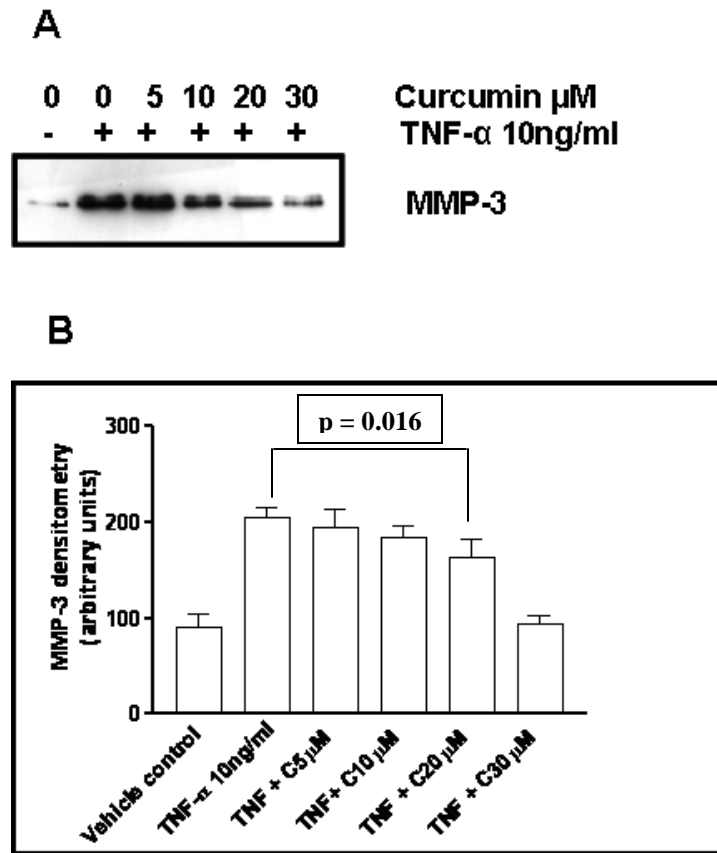
Figure 7.1　　　Curcumin decreases matrix metalloproteinase (MMP)-3 production in colonic myofibroblasts (CMF) from patients with active inflammatory bowel disease. (A) Western blot from a single representative experiment in Crohn's disease (CD) CMF. (B) Data from all experiments [n=7 (5CD + 2UC)]. Error bars represent standard errors of the mean. Because MMP-3 is measured in CMF supernatants, equal loading is controlled through the seeding of equal cell numbers in each culture well (3×10^5) and equal volume supernatant (15μl) in each electrophoresis well.

This work shows for the first time in human intestinal stromal cells a dose-dependent suppression of MMP-3 with curcumin (Figure 7.1). This finding holds

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clear clinical relevance for the therapeutic potential of curcumin in limiting tissue damage and disease progression in IBD.

CMF are intestinal stromal cells which, in health, produce low levels of matrix metalloproteinases (MMP) that remain largely in latent form. They effect physiological cell turnover and promote wound healing. In IBD, CMF over-express MMP's, which become activated in cascades causing unchecked tissue destruction and fibrosis. In addition to this well documented role, it is increasingly recognised that CMF are also immunologically active cells. They respond directly to bacterial products by up-regulating expression of TLR and CARD15 [349], they over-express TNF-α in CD [350] and the MMP's produced by CMF increase immune cell activation and homing to the gut [214, 351]. MMP-3 enhances chemotaxis by intestinal epithelial cells to neutrophils in the gut via the chemokine CXCL7 (neutrophil activating peptide 2) [352]. CXCL7 is a potent neutrophil chemokine and is released by intestinal epithelial cells when MMP-3 proteolytically cleaves platelet basic protein. Furthermore myofibroblasts are required to maximise this intestinal epithelial chemokine signalling pathway, as demonstrated by activated CCD-18 (fibroblast) and CaCo2 (epithelial cell) co-culture experiments [352].

There are over 20 different types of MMP, which are sub-classified according to the primary stromal substrate upon which they act (eg collagenases: MMP-1, -8, -13, 18; elastases: MMP-12; and stromelysins: MMP-3, -7, -10, -11). They are zinc-

dependent endopeptidases which as a family are capable of degrading all types of extracellular matrix proteins [353]. In addition to this function, as mentioned above they also activate or degrade a number of other non-mesenchymal substrates including immunologically active proteins such as chemokines and cytokines [352] [351]. Their activity is closely regulated at multiple levels, including by 1:1 inactive complex formation with endogenous tissue inhibitors of MMP (TIMP's). Many MMP's are over-expressed both in animal models of colitis and in human IBD, in seeming proportion to disease activity [354]. MMP-3 and -9 are the most consistently raised in different models of IBD, and are perhaps the most extensively studied to date.

Thus CMF are an important cell type in IBD, involved in multiple aspects of disease evolution. Furthermore unlike LPMC's, CMF may be grown in primary culture in from patients with IBD, making them an ideal model for further study.

CMF, although responsive to cytokines [355], do not themselves produce IL-1β or IL-10, as indicated by an absence of reports in the literature and confirmed in our laboratory. We measured IL-1β and IL-10 in CMF supernatants, both TNF-α-stimulated and unstimulated, and found minimal or no cytokine production. Instead we examined MMP-3 (stromelysin-1) as a measure of CMF activation. Curcumin has been shown to down-regulate MMP production in various cell types [192] [193], including recently MMP-9 in CaCo2 cells [356]. This latter work shows a dose-dependent response similar to our present results. The effects of curcumin on

MMP's are explored in greater detail in chapter 1.3.10. Here we have concentrated on MMP-3 (stromelysin-1), whose importance in IBD has been clearly demonstrated. MMP-3 is over-expressed in LPMC's [354] and biopsies [357] from patients with CD, and a polymorphism in the MMP-3 gene promoter (causing increased MMP-3 production) is associated with increased susceptibility to CD [358].

The effect of curcumin to decrease MMP production in the gut, as well as limiting tissue destruction, could reduce influx of activated leukocytes into inflamed gut tissue. Theoretical evidence for this comes from cell line studies as described above, showing that MMP's cause production of chemotactic factors and tissue-specific homing of activated leukocytes into various tissues [214, 351, 352]. Specific evidence that curcumin can indeed reduce the homing of leukocytes into inflamed gut comes from a recent study in which curcumin suppressed TNF-α- and LPS-induced vascular cell adhesion molecule (VCAM)-1 expression in human intestinal microvascular endothelial cells (HIMEC), and attenuated leukocyte adhesion to stimulated HIMEC's [359]. These data support that the present finding of reduced MMP-3 production by CMF represents an important therapeutic role for curcumin in IBD; one which can result both in limitation of tissue damage and dampening of the excessive inflammatory and chemotactic response *in vivo*.

7.2 p38 Mitogen-Activated Protein Kinase is Not Significantly Affected by Curcumin in Colonic Myofibroblasts

To seek a mechanistic explanation for the MMP-3 suppression observed with curcumin (Figure 7.1), we examined early (30 minute) and late (24 hour) p38 MAPK activation in the CMF. There was no difference between early and late samples. Although we did observe a tendency towards decreased p38 MAPK activation with curcumin, the pattern was not clear or consistent (Figure 7.2). We therefore conclude that curcumin does not significantly reduce p38 MAPK activation in TNF-α-stimulated *ex vivo* CMF from patients with active IBD. This suggests that, in contrast to our mucosal tissue biopsy model (Chapter 6.2), p38 MAPK signalling pathways are not of primary importance in the action of curcumin on stromal cells in IBD. All patient details and individual results can be found in the Appendix.

A

| - | - | 5 | 20 | 30 | Curcumin µM |
| - | + | + | + | + | TNF-α 10ng/ml |

Phospho p38

p38

B
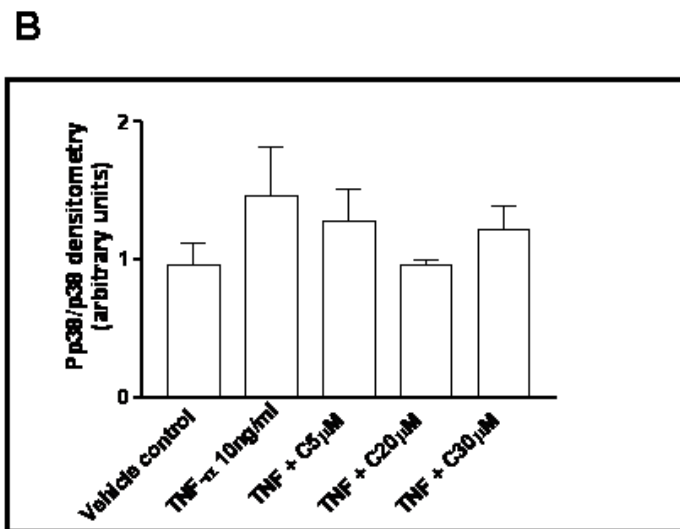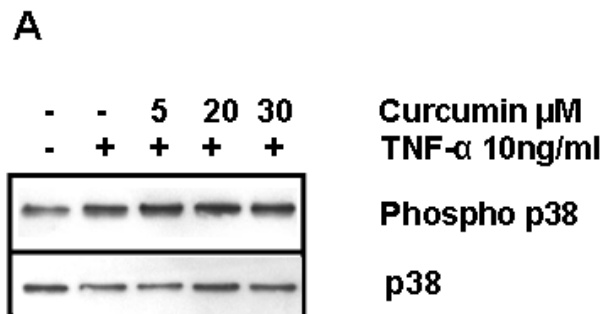
Figure 7.2 **p38 mitogen-activated protein kinase is not significantly affected by curcumin in colonic myofibroblasts (CMF) from patients with active inflammatory bowel disease. (A) Western blot from a single representative experiment in ulcerative colitis (UC) CMF. (B) Data from all experiments [n=5 (4CD + 1UC)]. Error bars represent standard errors of the mean.**

Unlike in our mucosal whole tissue system, in CMF curcumin had no clear effect on p38 MAPK activation. Therefore the suppression of MMP-3 production by curcumin occurs largely through a p38 MAPK-independent mechanism. To explain this discrepancy between biopsies and CMF, we postulate that the

inhibition of p38 MAPK signalling by curcumin in IBD occurs largely in cells of the immune system such as lymphocytes, macrophages, monocytes and dendritic cells, rather than in fibroblasts. These cell types (which cannot without transformation be grown in successive passages *in vitro*) are richly found in intestinal mucosal biopsies.

Indeed the contradictory effects of curcumin, on MAPK and other targets, are well documented in the literature and are addressed in detail in chapter 1.3.5. Under some circumstances curcumin inhibits MAPK activation, as in a recent study using a primary human intestinal microvascular endothelial cell line, where curcumin is shown to inhibit p38 MAPK activation [172]; similarly curcumin is shown to inhibit JNK in Jurkat T cells (a human T cell line) [189]. Paradoxically other investigators show activation of MAPK by curcumin, for example of JNK in HCT116 cells, a human colon cancer cell line [190] and of p38 MAPK in primary human neutrophils [200]. Whilst curcumin has manifestly opposing effects on MAPK signaling pathways, in both cases the ultimate biologic outcomes are anti-neoplastic and anti-inflammatory, and it appears that mechanisms vary according to cell and disease system, and even dose. Thus curcumin is shown in some environments to activate MAPK signalling, with the biologic consequence of apoptosis; and elsewhere to inhibit MAPK signalling, where the consequences are anti-inflammatory and anti-angiogenic.

We also note that the p38 MAPK response by CMF to TNF-α stimulation (Figure 7.2) was less pronounced than the MMP-3 response after the same stimulus (Figure 7.1). This further suggests that p38 MAPK pathways are not of primary relevance in gut mucosal fibroblasts as they are in cells of the mucosal immune system such as LPMC's, and may explain why p38 MAPK signalling was affected by curcumin in these classic inflammatory cells and tissues but not in CMF.

7.3 Nuclear Factor-κB Signalling is Not Significantly Inhibited by Curcumin in Colonic Myofibroblasts

In light of previous reports on the mechanism of action of curcumin in other cell types [167] [168], we next proceeded to investigate whether the response of CMF to curcumin was NF-κB-dependent. To this end we employed two separate methods; immunofluorescent staining and Western blotting. By immunostaining we confirm successful *ex vivo* activation of CMF using TNF-α, with translocation of NF-κB p65 from the cytoplasm into the nuclei (Figure 7.3A). Similarly to our earlier data on NF-κB in biopsies (Figure 6.3), we found no clear difference in NF-κB nuclear translocation with curcumin in CMF (Figure 7.3A). By Western blot we show only a small decrease in nuclear p65 with curcumin (Figures 7.3B and C). We conclude that while NF-κB signalling may be marginally inhibited by curcumin, this is not the primary mechanism through which it inhibits MMP-3

expression in CMF. All patient details and individual results can be found in the Appendix.
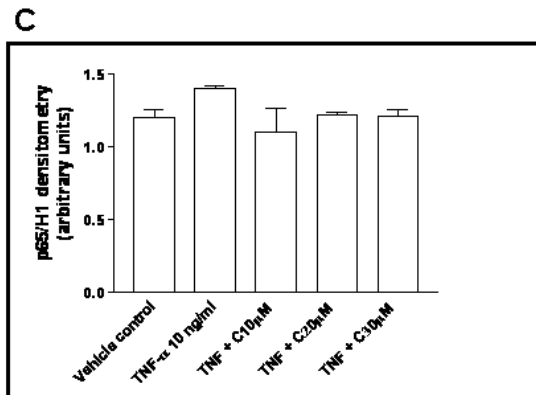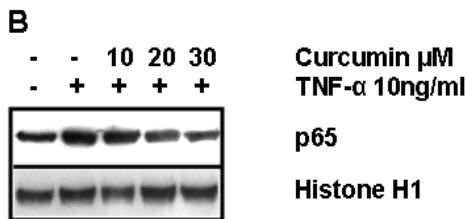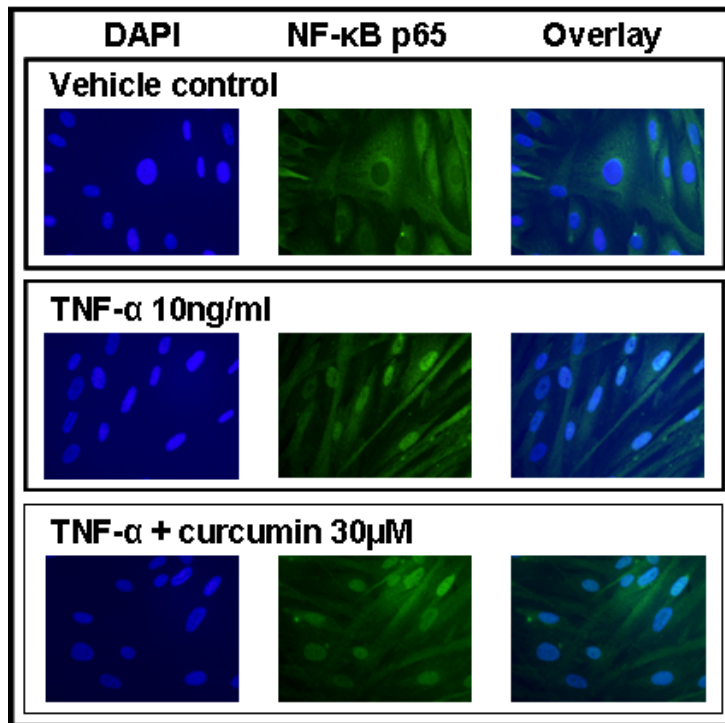
Figure 7.3 NF-κB signalling is not significantly affected by curcumin in colonic myofibroblasts (CMF) from patients with active inflammatory bowel disease. (A) Immunofluorescent staining of Crohn's disease (CD) CMF for DAPI (nuclei) and NF-κB p65 subunit; single representative experiment shown. (B) Western blot from a single representative experiment showing nuclear p65 and histone H1 loading control. (C) Western blot data from all experiments [n=3 (2CD + 1UC)]. Error bars represent standard errors of the mean.

Curcumin did not significantly affect NF-κB signalling either in colonic mucosal biopsies (Figure 6.3) or CMF (Figure 7.3) from patients with active IBD. Therefore in the inflamed gut mucosa the actions of curcumin do not appear to be primarily NF-κB-dependent. This finding is consistent and reproducible in both biopsy and CMF disease models. Whilst it could be a reflection of the limitations of *ex vivo* models of disease, it is verified by two different experimental methods, Western blot and immunofluorescence. It does suggest that neither the cytokine modulation nor the suppression of MMP-3 production in the inflamed gut mucosa by curcumin depend on NF-κB inhibition, and therefore a separate mechanism is in operation.

7.4 The Suppression of Matrix Metalloproteinase-3 Production in Colonic Myofibroblasts by Curcumin May Be Acetylation-Dependent

7.4.0 Immunoblotting for p300 Acetyl Transferase

In further pursuit of a mechanistic explanation for the MMP-3 suppression observed with curcumin (Figure 7.1), we next considered curcumin's known potency as an inhibitor of acetylation. We wished to demonstrate changes in the acetylation status of p300 acetyl transferase, a ubiquitous catalyst of acetylation, an

enzyme known to be involved in MMP production and an established target of curcumin (explored in more detail in Chapters 1.2.9 and 1.4.11). Due to the large molecular weight of the protein (300kDa) and technical aspects concerning the sensitivity of the primary antibody, during optimisation of the Western blot it became apparent that in our experimental system the technique would be unfeasible (Chapter 2.3.3). In order to obtain sufficient protein to yield a usable signal for p300, we were obliged to use several million CMF in each culture condition, a factor of ten higher than in all other experiments. To grow this quantity of cells, multiple passages were required, producing unreliable yields of CMF which were further removed from the active IBD phenotype. As well as the technical problems associated with attempts to obtain such numbers of cells, it has been shown by other investigators in our lab, and published by other groups, that CMF display significantly altered features of senescence at high passages, and this impacts greatly on their behaviour and function [360]. For this reason we decided that other avenues of investigation would be more fruitful.

7.4.1 The Acetylation Inhibitor Anacardic Acid Suppresses Matrix Metalloproteinase-3 in a Dose-Dependent Fashion which Mirrors that Seen with Curcumin

We then employed another method to address the possibility that curcumin's effect on MMP-3 was due to its properties as an acetylation inhibitor. We treated TNF-α-

stimulated CMF with a different inhibitor of acetylation, anacardic acid. Anacardic acid is, like curcumin, a naturally occurring plant-based substance, in this case found in cashew nut shell liquid. Like curcumin, it is a non-competitive inhibitor of p300 acetyl transferase, a ubiquitous catalyst of acetylation [303] with no other reported regulatory effects such as MAPK or NF-κB modulation. Anacardic acid suppressed MMP-3 production in TNF-α-stimulated CMF from patients with active IBD, in a dose-dependent manner (figures 7.4A and B) which closely mirrored that seen with curcumin. This suggests that in CMF both compounds are acting via a mechanism dependent on their ability to inhibit acetylation. All patient details and individual results can be found in the Appendix.

A

0	0	2	5	10	20	Anacardic acid µM
-	+	+	+	+	+	TNF-α 10ng/ml

MMP-3

B
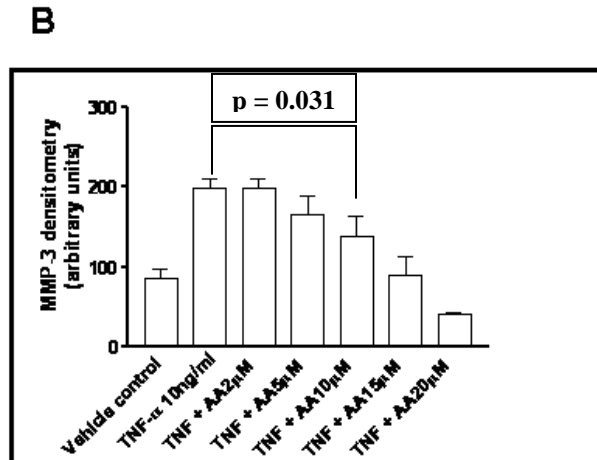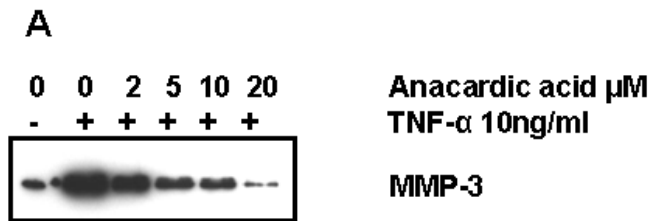
Figure 7.4   **Anacardic acid (AA) suppresses MMP-3 production in colonic myofibroblasts (CMF) from patients with active inflammatory bowel disease. (A) Western blot from a single representative experiment with AA in Crohn's disease (CD) CMF. (B) AA data from all experiments [n=7 (5CD + 2UC)]. Error bars represent standard errors of the mean.**

There is some published evidence that MMP production is p300 acetyl transferase-dependent. This is shown (for MMP-9) in a rat astrocyte model [361] and in mouse macrophages [362]. The substrate for acetylation remains obscure but in this latter model the authors show evidence of chromatin modification (acetylation of histones). Further evidence for TNF-dependent MMP-9 production requiring histone acetylation comes from a human tracheal smooth muscle cell model [363], a process which interestingly in this work is indeed blocked by curcumin.

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Furthermore, our group has previously shown upregulation of MMP-3 in human fetal intestinal mesenchymal cells by butyrate, a histone deacetylase (HDAC) inhibitor [108].

To examine this possibility we tested another known inhibitor of acetylation anacardic acid in the same experimental system. Anacardic acid has a very similar mode of action to curcumin, in that both compounds are reversible non-competitive inhibitors of p300 acetyl transferase, acting at a site remote from the active site of the enzyme [303]; this is the only known biological property of anacardic acid and the only known shared property of the two substances. There is no previously published work on anacardic acid in the human gut. The finding of a dose-dependent pattern of MMP-3 suppression with anacardic acid in CMF, which parallels that seen with curcumin, supports the hypothesis that the effect is acetylation-dependent.

7.4.2 The Pro-acetylating Agent Trichostatin A Enhances Matrix Metalloproteinase-3 Production and this is Abrogated by Both Curcumin and Anacardic Acid

To examine the role of acetylation further, we used the well-established inhibitor of histone deacetylase (HDAC) trichostatin A (TSA), which is therefore a pro-

acetylating agent [364]. In agreement with our earlier data (figure 7.1), we confirmed the upregulation of MMP-3 with TNF-α, and the suppression of this effect by curcumin; we also demonstrated that TSA up-regulated MMP-3 (figure 7.5). This upregulation was almost totally abrogated by both inhibitors of acetylation curcumin and anacardic acid (figure 7.5).
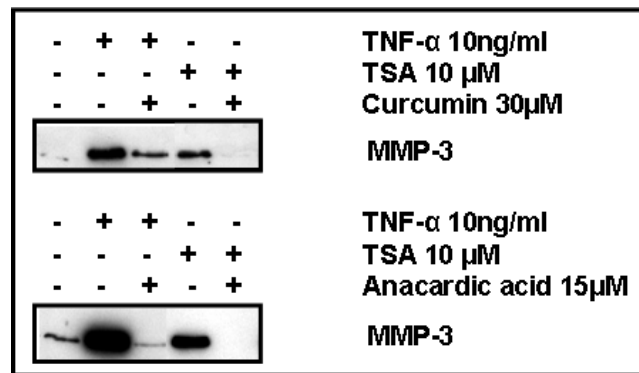
Figure 7.5 **Responses of Crohn's disease (CD) colonic myofibroblasts (CMF) to tumour necrosis factor (TNF)-α, trichostatin (TS) A, curcumin and anacardic acid. TSA enhances matrix metalloprotein (MMP)-3 production in CMF from patients with active CD and this is abrogated by both curcumin and anacardic acid.**

The HDAC's are a family of enzymes which, in balance with the histone acetyl transferases, regulate the acetylation status of histone proteins within chromatin, playing an important epigenetic role in gene transcription, and thus have a ubiquitous role in cellular processes in health and disease. Just as other non-histone proteins within the cell are now known to be subject to (reversible) acetylation, it is also becoming clear that the substrates of HDAC enzymes are not limited to histones either, but include a number of transcription factors, signal transducers and other proteins [365]. TSA was originally discovered as a product of

microorganisms. It is antifungal and inhibits the eukaryotic cell cycle, and it is a potent and selective inhibitor of HDAC's. TSA is not stable *in vivo* and therefore its use has been primarily as an important tool in the understanding of epigenetic regulation and protein acetylation.

Taken together, the findings displayed in figures 7.4 and 7.5 suggest that MMP-3 production in CMF occurs at least in part through an acetylation-dependent mechanism, and that its suppression by curcumin relates to curcumin's known potency as an inhibitor of p300 acetyl transferase [178] [179].

7.4.3 Butyrate Enhances Matrix Metalloproteinase 3 Production and this is Abrogated by Anacardic Acid but not Curcumin

TSA is a strong HDAC inhibitor, and as a pro-acetylating agent it a useful experimental tool. Butyrate is a naturally occurring HDAC inhibitor, present in significant concentrations in the human gut lumen. Butyrate, acetate and propionate are the main short chain fatty acids (SCFA) produced by bacteria in the colon. In light of the results obtained with TSA (Chapter 7.4.2) we wished finally to examine the response of fibroblasts to butyrate. Because of the clinical relevance of butyrate and its relationship to TSA, this was a logical final step within this work.

We again confirmed successful upregulation of MMP-3 production by TNF-α, and we showed that butyrate, like TSA, also enhanced MMP-3 expression (Figure 7.6). There was no difference observed between the two butyrate doses applied, 5 and 20mM. The butyrate concentrations were chosen on the basis of *in vitro* work previously published in the field and also upon the evidence that usual clinical butyrate concentrations in the faecal stream range from 0 to approximately 20mM [366, 367]. The upregulation of MMP-3 by butyrate was partially abrogated by the acetylation inhibitor anacardic acid, in a similar (yet less complete) fashion to its abrogation of TSA-mediated MMP-3 production (Figure 7.5). Conversely the addition of curcumin alongside butyrate caused MMP-3 production to increase yet further (Figure 7.6).

-	+	-	-	-	-	TNF-α 10ng/ml
-	-	5	20	5	20	Butyrate (mM)
-	-	-	-	+	+	Curcumin 30µM
						MMP-3

-	+	-	-	-	-	TNF-α 10ng/ml
-	-	5	20	5	20	Butyrate (mM)
-	-	-	-	+	+	Anacardic acid 15µM
						MMP-3
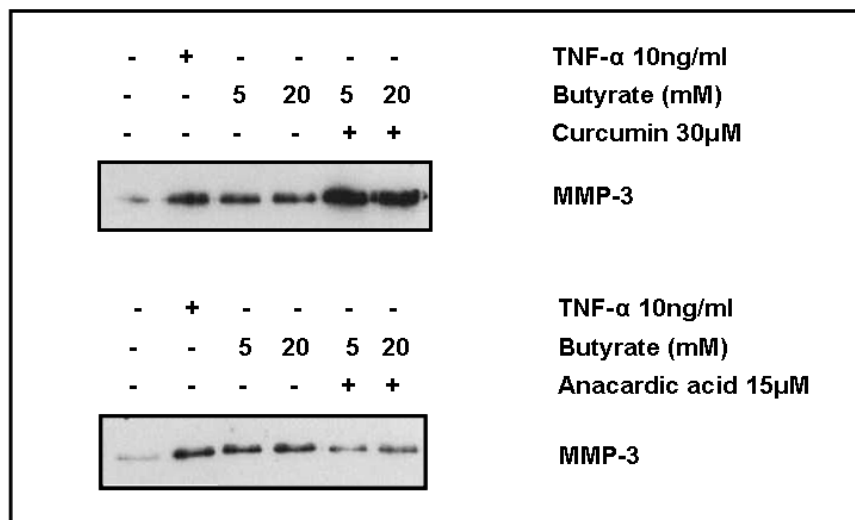
Figure 7.6 **Responses of Crohn's disease (CD) colonic myofibroblasts (CMF) to tumour necrosis factor (TNF)-α, butyrate, curcumin and anacardic acid. Butyrate enhances matrix metalloprotein (MMP)-3 production in CMF from patients with active CD and this is patially abrogated by anacardic acid but not by curcumin.**

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In agreement with previously published work showing increased histone acetylation and upregulation of MMP-3 production in human foetal small intestinal mucosal mesenchymal cells by both butyrate and TSA [108], we showed that TSA enhanced MMP-3 production by CMF (Figure 7.5), and butyrate reproduced this effect (Figure 7.6). Butyrate and TSA are pro-acetylating agents. They both increased MMP-3 production and this effect directly opposed that of the two inhibitors of acetylation curcumin and anacardic acid, which decreased MMP-3 production (Figures 7.1 and 7.4). This is therefore suggestive of, but not conclusive for, an acetylation-dependent mechanism of action upon MMP-3 production by CMF. Furthermore the acetylation potency of TSA is approximately 1000 fold greater than that of butyrate [108], a fact consistent with the observation that TSA at micromolar concentration appeared to enhance MMP-3 production more strongly than butyrate at milimolar concentration, even allowing for the semi-quantitative nature of Western blot protein estimation.

Why anacardic acid abrogated the butyrate-mediated increase in MMP-3 and curcumin did not, remains unexplained. Whilst the only known biochemical properties of TSA and anacardic acid are pro- and anti-acetylation respectively, both butyrate and curcumin are known to be much more complex molecules with many and varied biological actions. The complexity of curcumin has already largely been discussed. It may be that where curcumin and butyrate interact, some of these wider mechanisms come into play and the situation no longer depends upon straightforward acetylation alone, or perhaps depends on acetylation of different

nuclear or cellular targets in changing relative contribution. This could explain why simple abrogation is not seen, but the mechanism(s) behind the interaction remain at present a matter for surmise.

Butyrate is a short chain fatty acid (SCFA) and a major fermentation product of the metabolism of dietary (largely soluble) fibre by colonic bacteria. It is therefore an important component of the luminal environment, and is the direct product of a key interaction between nutrition and the host bacterial flora. As well as being the most potent HDAC inhibitor of all the colonic SCFA's [111], it is a nutrient in its own right and has varied biological effects. Its interactions with the host are complex and its mechanisms not fully elucidated, but there is evidence that it modulates inflammation in the human gut and reprograms colonic epithelial cells, inducing differentiation, cell cycle arrest and apoptosis [368]. Whether some or all of these actions are a result of its HDAC inhibiting activity is not clear. Butyrate levels vary considerably throughout life and alter in response to bacterial flora and substrate composition. Levels are very low at birth and rise to adult levels by the age of two years [369]. Bottle fed babies have much higher butyrate levels in the gut than breast fed babies, for unknown reasons [369].

Butyrate increases IL-8 secretion and decreases mast cell protein (MCP)-1 in intestinal epithelial cells, and these effects are much more pronounced in LPS- or IL-1β-stimulated cells [370]. Butyrate therapy shows anti-inflammatory effects in

preclinical models [371] and when administered topically in some clinical trials in UC [372, 373]; but not in CD, where luminal butyrate concentrations are raised [374]. In UC its beneficial effects appear to be limited to the initial six months of disease duration, during the early phase of epithelial cell disruption when it seems to improve mucosal barrier function [373]. In other clinical trials in UC, including well conducted controlled trials on patients with more established disease, butyrate therapy did not show benefit [375, 376].

The mechanisms behind the actions of butyrate are incompletely understood. One group has studied its effects on cytokine production in PBMC's. In *Staphylococcus aureus-* or anti-CD3-stimulated human PBMC's it enhanced IL-4 and IL-10 production and decreased IL-12 and IFN-γ [377]. The effects on IL-12 were unchanged by the addition of anti-IL-10 antibodies, suggesting that its inhibition of IL-12 is independent of IL-10 suppression. It is difficult to explain these differing effects on cytokine expression through acetylation alone, unless it is acting on promoters or repressors as well as upon histones. In porcine concanavalin A-stimulated PBMC's butyrate increased IL-2 and IFN-γ secretion and mRNA levels [378]. Interestingly in this work, low dose butyrate enhanced IL-10 production whilst high dose butyrate paradoxically decreased it. The investigators also show that butyrate increased production of suppressor of cytokine signalling (SOCS)3, a negative regulator of cytokine signalling, induced by, among other factors, IL-10. Addition of an adenyl cyclase inhibitor abolished some of the effects of butyrate, suggesting that these may be cyclic adenosine monophosphate (cAMP)-dependent.

As well as these mixed effects on cytokines there is evidence that butyrate inhibits carcinogenesis, reduces oxidative stress and strengthens the colonic mucosal defence barrier, possibly by improving the mucous layer [379]. The mechanisms underlying these effects remain unclear. Most of this work comes from animal and cell culture work. Human interventional trials of butyrate have to date centred on its use in various types of intestinal inflammation, some of which have been described above.

Using microarray technology one group has compared the genetic reprogramming which takes place in an *in vitro* model of the colonic epithelium in response to butyrate, TSA and curcumin [368]. They showed that responses to butyrate were highly complex, involving recruitment of multiple different sequences of genes which show transient or more sustained increases or decreases over different time periods. The responsive genes included those involved in signal transduction, cell cycling, cell maturation and apoptotic pathways. The large number of genes recruited, their wide variation in function and their altered responsiveness over time imply that colonic epithelial reprogramming in response to butyrate is a complex and highly orchestrated sequence of events. In contrast the changes induced by TSA were more limited and transient with no expansion or decrease with time. There were small clusters of genes which exhibited similarity of response to all three compounds; with greater overlap between butyrate and TSA, implying that these genes were regulated by their shared mechanism of HDAC inhibition.

However the more striking finding was how different the responses were; even between butyrate and TSA.

Thus to view curcumin and butyrate as simple opposites, or butyrate and TSA as wholly analogous, is clearly an over-simplification, as acetylation is not their only property, and the multiple biological actions of both compounds are not yet fully understood. This may explain the discrepancy within the results in Figure 7.6 and the differing actions of curcumin and anacardic acid observed with butyrate.

8.0   Discussion

We have shown in the *in vitro* setting that curcumin favourably modulates the intestinal mucosal cytokine profile, with enhanced IL-10 production and reduced IL-1β. These data were derived predominantly from colonic mucosal tissue biopsy work, using exclusively tissue taken from children with active IBD. As such a major strength is the proximity of the experimental model to the true clinical disease situation. Equally similar to the *in vivo* reality, this approach yielded a model with natural intrinsic complexity. The inflammatory pathways at play in IBD are multiple and interconnected, and our tissue explant disease model provided a true echo of this. Therefore we experienced wide inter-individual variability in cytokine production and in response to curcumin as a potential therapeutic agent. Once again these outcomes mirror the clinical experience of variability of response to treatments between individuals. To overcome these factors we tested samples from a relatively large number of children and demonstrated a statistically significant increase in IL-10, a significant decrease in IL-1β and no significant change in IFN-γ.

These findings are in keeping with our original hypothesis that curcumin, as an inhibitor of acetylation, enhances binding of the transcription factor Sp3 to the IL-10 gene promoter, decreasing IL-10 transcription. We demonstrated that the rise in IL-10 with curcumin was an effect particular to that cytokine, and not shared by its effects upon IL-1β or IFN-γ. This neither proves nor disproves our hypothesis in

mechanistic terms; however as well as being consistent with our hypothesis it provides strong evidence that curcumin exerts beneficial effects in the intestinal mucosa in IBD.

Also in *ex vivo* intestinal mucosal tissue from children with active IBD we have shown that curcumin reduces p38 MAPK activation. Thus we have shown that curcumin inhibits p38 MAPK signalling and we have shown that curcumin decreases IL-1β expression. This suggests that its downstream suppression of IL-1β expression is at least in part p38 MAPK dependent. The ability of curcumin to inhibit p38 MAPK is additionally relevant to the study of a potential therapeutic agent in IBD, since p38 MAPK is a key orchestrator of the inflammatory response, is consistently elevated in IBD and is of central importance to the pathogenesis of the disease.

The experiments which we performed on LPMC's from patients with active IBD yielded data in accordance with that derived from our work on intestinal mucosal biopsies; we showed a trend towards decreased production of both inflammatory cytokines IL-1β and IFN-γ. If we had had access to a greater clinical supply of LPMC's we would have hoped to be able to demonstrate statistically significant results in parallel with our tissue culture findings, and perhaps with greater consistency and less inter-individual variability. In the absence of LPMC availability we examined the IL-10 responses of PBMC's to curcumin. We showed a consistent and reproducible bell-shaped response to curcumin, with enhanced IL-

10 production at low curcumin concentrations and decreased production at high concentrations. The paradoxical effects of curcumin at low versus high dose have been described by other groups [342] [222] and remain unexplained. We propose the hypothesis that at low dose, inhibition of acetylation of Sp3 predominates, with consequent release of the IL-10 promoter to specific gene transcription; whilst at high dose, generalised inhibition of histone acetylation predominates, with widespread reduction in access to DNA for generalised gene transcription.

The applicability of PBMC data to intestinal inflammation is uncertain and we did not attempt to extrapolate our PBMC findings in this way. Without further access to supplies of LMPC's we refined the isolation of colonic mucosal myofibroblasts (CMF) from surgically resected specimens of actively inflamed gut from patients with IBD. In this way we obtained a dependable supply of activated primary intestinal cells which play an important role in the pathogenesis of IBD, and are amenable to *in vitro* growth. In CMF we have demonstrated inhibition of matrix metalloproteinase (MMP)-3, an important contributor to tissue destruction, immune activation and leukocyte recruitment in IBD, by curcumin. Perhaps because this disease model employed a single cell type only, we obtained results of much smaller variability, greater reproducibility and clear dose-responsiveness.

We demonstrated that the inhibition of MMP-3 production by curcumin in CMF occurs via NF-κB- and p38 MAPK-independent pathways. Thus our data show that curcumin inhibits p38 MAPK in intestinal mucosal tissue, but not in intestinal

mucosal fibroblasts. In explanation we propose that MAPK signalling is a critical mechanism in mediating IL-1β production and other cytokine cascades, occurring largely in classic immunologically active cells such as lymphoctyes, which are richly found in mucosal biopsies. On the other hand, regulation of MMP production in myofibroblasts is not yet well understood, and we suggest that MAPK signalling as an intermediary mechanism is relevant to a much lesser extent in stromal cells. In support of this hypothesis, we show a less complete activation of p38 MAPK upon TNF-α stimulation of CMF (Figure 7.2), compared with the strong MMP-3 response seen upon TNF-α stimulation of CMF (Figure 7.1). Furthermore, p38 MAPK activation is very clearly relevant as an inflammatory signal in intestinal mucosal biopsies, where we show a strong degree of activation in IBD samples compared with healthy controls (Figure 6.1A).

We have shown evidence that these effects of curcumin are at least partially dependent on its power to inhibit protein acetylation (p300 acetyl transferase) in the intestinal mucosa. The inhibitor of acetylation anacardic acid produced a dose-dependent MMP-3 response in CMF which closely mirrored that seen with curcumin. Anacardic acid and curcumin are both plant-based non-competitive inhibitors of p300 acetyl transferase. This is the only known biological property of anacardic acid and the only known shared property of the two compounds. The pro-acetylating agents trichostatin A (TSA) and butyrate produced the opposite effect, increasing MMP-3 production by CMF. TSA is a more potent acetylator than butyrate by approximately 1000 fold, and TSA enhanced MMP-3 more

strongly than butyrate, at a 1000 fold lower concentration. The TSA-mediated increase in MMP-3 was almost entirely abrogated by both curcumin and anacardic acid. These findings do not constitute conclusive proof, but provide evidence strongly in support of the hypothesis that the action of curcumin upon CMF in the inflamed intestine is acetylation dependent.

Our hypothesis that curcumin inhibits the acetylation of Smad 7 was rejected, since we showed no consistent alteration in Smad 7 levels (known to be controlled by its acetylation status) in intestinal mucosal tissue or LPMC's with curcumin. Therefore we conclude that curcumin does not exert its effects in IBD through a Smad 7 dependent mechanism. However this does not preclude the possibility that curcumin's potency as an inhibitor of acetylation is relevant to its other actions in IBD. Above we describe the evidence for its effects upon CMF being acetylation dependent. Furthermore its effect to enhance IL-10 production in inflamed gut, and its effects upon IL-1β and IFN-γ which oppose this, are consistent with our original hypothesis that curcumin enhances IL-10 expression through a specific acetylation-sensitive element in the IL-10 gene promoter. Finally in PBMC's we show once again a close mirroring of the actions of curcumin upon IL-10 by anacardic acid, suggesting that these occur through the only shared property of these two compounds, inhibition of acetylation.

All tissues and cells used in this work were primary, human, and almost exclusively derived from children and adults with active IBD. Thus our findings hold good

translational relevance and provide a strong basis for the development of clinical trials of curcumin in IBD.

The curcumin concentrations used in this and other published work correspond to doses many fold higher than those found in the diet, even in Asian populations. Therefore whilst the potential benefit of curcumin at therapeutic dose is clear, this may not extend to dietary or population relevance. The safety, tolerability and non-toxicity of curcumin at doses many fold higher than dietary have been well established, and it is classified 'Generally Recognized as Safe' by the United States Food and Drug Administration. Oral doses up to 12g per day have been well tolerated in humans [152]. There is also good evidence at a population level of the safety of lifelong curcumin ingestion up to about 100mg/day from India, where there is a very high natural dietary curcumin content [166]. The question of dose is especially relevant to the study of curcumin, which appears to be capable of paradoxical efficacy at low dose under some circumstances [342] [165] [222].

In vitro concentrations such as those used in this work are probably considerably higher than serum or tissue concentrations that can be achieved *in vivo* through oral dosing in humans, due to the drug delivery and bioavailability issues previously described. We have already highlighted some of the issues which must be considered when assessing the translational relevance of *in vitro* work to the *in vivo* situation. The question of curcumin concentration in cell culture in relation to that achievable clinically is central to this and other preclinical studies of curcumin. In

one human trial 3.6g of oral curcumin achieved plasma levels of 11.1nmol/L in humans [157]; a concentration a factor of 1000 lower than those we have used *in vitro*. Other investigators however found peak human plasma levels of 0.41-1.75µM after oral dosing of 4-8g of curcumin [153]; these are only a factor of ten lower than the concentrations we have used. Due to the wide variability in pharmacokinetic data from different studies it is difficult to ascertain for certain the relationship between *in vitro* and *in vivo* curcumin dosing. In retrospect we would have liked to study some much lower curcumin concentrations in our *in vitro* work. These would hold relevance to the human clinical situation and would also be interesting because of the evidence to which we have already alluded from *in vitro* and animal work that curcumin can have paradoxical high efficacy at low doses.

There is no study to date of the use of curcumin in topical rectal formulation. It may be that a rectal delivery would surpass many of the drug delivery, tissue concentration and hydrophobicity challenges described, and could certainly achieve curcumin concentrations in the colonic lumen equivalent to those studied in supernatants in our *in vitro* work.

We conclude that curcumin holds promise as a novel therapy for children, adolescents and adults with IBD. Curcumin is a complex molecule whose precise modes of action remain obscure, and it seems likely that its molecular targets differ according to cell and disease system. However we conclude that its actions in the inflamed human gut in IBD are at least in part acetylation dependent.

9.0   Future Directions

We have demonstrated that curcumin beneficially modulates the cytokine profile and reduces MMP-3 production in the inflamed human gut mucosa in IBD. Curcumin is a known inhibitor of acetylation and we have shown evidence to suggest that its effects in the human gut are at least partially acetylation-dependent. We would like to elucidate clear mechanism(s) for the effects of curcumin in IBD.

Our attempts to measure p300 acetyl transferase (Chapter 7.4.0) have not so far been productive. As a next step in this direction we would like to examine p300 acetyl transferase expression in gut mucosal biopsies, from which a higher yield of tissue can be obtained. This may require the use of a large electrophoresis system allowing much higher protein loading. If this technique is successful, we would aim to demonstrate changes in p300 with curcumin. This could be done alongside TSA as a positive (pro-acetylating) control.

Such lines of study would seek to prove that curcumin is acting through its potency as an inhibitor of acetylation. The substrate(s) for acetylation remain unknown. A natural question would be whether histones are the substrate. To answer this, nuclear histones could be extracted and separated electrophoretically on a Triton X acetic acid-urea gel. After Coomassie blue staining, the histones could be visualised with a marker, and relative quantities of acetylated forms assessed. This is similar to a method previously used by our group [108].

As in our original hypothesis, non-histone proteins are also known to be subject to acetylation, and are involved in signalling processes relevant to inflammation (Chapter 1.2.7). Since our results clearly indicate that curcumin increases production of IL-10, we would like to turn our attention to the mechanism behind this. We have shown that curcumin's effect on IL-10 is opposite to its effects on pro-inflammatory cytokines IL-1β and IFN-γ. This result is consistent with our hypothesis that curcumin acetylates the transcription factor Sp3, which in turn binds to a specific site in the promoter region of the IL-10 gene, causing down-regulation of IL-10 transcription (Chapter 1.2.10). We would like to undertake further work to prove this hypothesis.

Firstly we would examine whether this is due to a true increase in gene transcription, by measuring IL-10 mRNA by reverse transcriptase polymerase chain reaction (RT-PCR). If the mRNA increases in parallel to the IL-10 protein it would confirm that the increase in IL-10 expression we observed is not due to post-translational events. We would then wish to construct luciferase reporter plasmids containing the IL-10 promoter and transfect them into an appropriate cell line such as a macrophage cell line (eg RAW 264), similarly to a previously published technique [129]. Using electrophoretic mobility shift assay (EMSA) we would aim to determine the binding of transcription factors to the IL-10 promoter. Specifically we would seek to demonstrate the binding of Sp3 to the IL-10 promoter using an anti-Sp3 antibody, and to detect changes in this binding in the presence of curcumin. A comparison using short interfering (si) RNA against p300 acetyl

transferase would be a valid strategy in such experiments, to prove that any effect of curcumin on Sp3 binding to DNA is due to its inhibition of p300. Unfortunately such work is outside the capacities of the present fellowship, but would be an interesting direction in which to proceed given available time and resources.

Finally it would be useful to build on our *in vitro* work with *in vivo* studies. Considerable evidence already exists in support of the efficacy of curcumin in preclinical animal models of IBD. The clinical data however are much less complete at present. Future clinical trials should include wide curcumin dosing ranges, including low dose, as there is evidence from several sources (including our work) that the efficacy of curcumin in relation to dosage may be paradoxical. Other clinical issues such as drug delivery should also be addressed in future patient trials. Curcumin achieves particularly good local penetrance and tissue concentrations in the gut, and due to its hydrophobicity and rapid elimination, oral administration is associated with problems. Therefore alternative formulations or routes of administration can be considered, such as rectal. To date, fewer than 100 patients with IBD have been involved in published trials of curcumin therapy. None of these are children. In light of strong early evidence of benefit and good data on human safety, further clinical trials of curcumin in IBD are certainly warranted.

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Appendix 1

In Appendix 1 we include for reference some key demographic and clinical data on the patients who kindly donated clinical material to our study. The (anonymised) origins of all of the biopsies and cells used in this work can be found below. Appendix 2 includes tables relating which patients' materials were used in which experiments. Our patient base reflects our clinical and epidemiological knowledge about IBD in childhood and adulthood. We selected our patients based on (a) known or suspected, and subsequently histologically confirmed, active IBD; (b) ability and willingness to give informed consent; and (c) undergoing colonoscopy at the Royal London Hospital or bowel surgery at the Royal London or Homerton Hospitals. Other than these criteria we were unselective, and approached all children and their families or adults presenting consecutively to our clinical unit, during the time periods that particular samples were required in the laboratory.

We used in this work clinical specimens from 30 individuals. 21 of our patients were children (16 years of age or under; average age 12.6 years). We used biopsies only from children since numbers of children undergoing colonoscopy were sufficient to achieve this (15/15). However only six of the 15 surgical samples came from children, since the throughput of patients having bowel resections for IBD was relatively small and we were obliged to use samples from patients of all ages in order to obtain sufficient numbers. The average age of the patients who donated surgical specimens of bowel was 26.4 years. Overall ten patients had UC

and 20 CD. This is consistent with known differences in the two disease incidences, and with the currently rising incidence of CD, especially in childhood and adolescence [2]. 13 of the 30 patients were female (8 CD and 5 UC). Of the 14 children with CD, 5 of them were female. This once again is consistent with the knowledge that whilst overall CD is commoner amongst women, in childhood it affects more boys than girls [1, 5].

Nine of the 10 patients with UC had pancolitis, reflecting both the more severe phenotype seen in childhood [1], as well as our obligate selection of adult patients whose disease necessitated surgery. The commonest affected sites in CD were as expected the terminal ileum and caecum. The overall average age of our UC patients was 16 years; average age of CD patients was 20.9 years. Of our paediatric patients, the average age of those with CD was 14.1 years, and the average age of those with UC was 9.6 years. This in consistent with the epidemiological observation that UC can occur in younger children than CD. Average C reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were 18.3mg/l and 23.8mm/hr respectively in patients with UC and 53.9mg/l and 34.3mm/hr in CD. This coincides with the expected higher systemic inflammatory state in CD compared with the relatively more localised inflammation limited to the colon in UC.

Patient Characteristics

Patient Number	Disease type	Disease site	Age	Sex	Blood results	Drugs	Notes
6315	UC	Pancolitis	14	M	CRP <5 Hb 7.2 Plt 253 ESR 25	-	New diagnosis
6316	CD	Terminal ileum, caecum	15	M	CRP 10 Hb 12.4 Plt 454 ESR 18	-	New diagnosis
6317	CD	Terminal ileum, caecum	15	F	CRP 19 Hb 12.4 Plt 415 ESR 15	Pentasa Azathioprine	
6319	CD	Terminal ileum, colon	15	M	Hb 9.2 Plt 242	Mesalazine Azathioprine	
6322	UC	Pancolitis	4	F	CRP <5 Hb 10.3 Plt 521	-	New diagnosis
6323	UC	Rectum to proximal transverse colon	13	M	CRP 11 Hb 6.9 Plt 366 ESR 54	-	New diagnosis
6327	UC	Pancolitis	8	F	CRP <5 Hb 14 Plt 311 ESR 4	Asacol Prednisolone	
6328	CD	Terminal	12	M	CRP 15	-	New

		ileum, caecum			Hb 12.4 Plt 448 ESR 27		diagnosis
6329	CD	Terminal ileum, colon, oesophagus, stomach	14	M	CRP 58 Hb 11.8 Plt 490 ESR 55	-	New diagnosis
6330	CD	Terminal ileum, colon	13	M	CRP 71 Hb 9.5 Plt 664 ESR 7	-	New diagnosis
6331	UC	Pancolitis	6	F	CRP <5 Hb 13.6 Plt 387	-	New diagnosis
6332	CD	Terminal ileum, caecum	15	M	CRP 111 Hb 9.8 Plt 742 ESR 54	-	New diagnosis
6336	CD	Terminal ileum, colon	15	F	CRP 55 Hb 9.4 Plt 633 ESR 53	-	New diagnosis
6338	CD	Terminal ileum, caecum	16	M	CRP 32 Hb 9.3 Plt 613 ESR 34	Sulfasalazine Azathioprine	
6341	UC	Pancolitis	6	F	CRP <5 Hb 11.1 Plt 513 ESR 12	-	New diagnosis

JE29	CD	Ileum, colon, stomach	15	M	CRP 108 Hb 12 Plt 482 ESR 52	Mesalazine Azathioprine Prednisolone Infliximab Metronidazole	Fistula Abscess
JE31	CD	Terminal ileum, caecum	35	F	CRP 233 Hb 9 Plt 194	-	Stricture Right hemi-colectomy
JE32	CD	Left colon	15	F	CRP 13 Hb 12.6 Plt 337 ESR 22	Mesalazine Azathioprine	
JE38	UC	Pancolitis	34	M	Unavailable	Azathioprine CyclosporinA	Subtotal colectomy
JE43	CD	Terminal ileum, colon, perianal	29	F	CRP 178 Hb 11.3 Plt 705	Asacol Methotrexate Budesonide Ciprofloxacin	Subtotal colectomy Completion proctectomy
JE45	UC	Pancolitis	25	F	CRP 117 Hb 10.6 Plt 788	Asacol	Subtotal colectomy
JE47	CD	Terminal ileum, caecum	63	M	CRP <5 Hb 12.1 Plt 482	Sulfasalazine Prednisolone	Right hemi-colectomy
JE49	CD	Colon	35	M	CRP 13 Hb 11.4 Plt 427 ESR 59	Asacol	Subtotal colectomy
JE51	CD	Terminal ileum, colon	8	F	CRP <5 Hb 11.4 Plt 588	Asacol Prednisolone	

1f	CD	Colon	26	F	CRP 47 Hb 9.1 Plt 159	Azathioprine	Right hemi-colectomy Small bowel resection
200	UC	Pancolitis	34	M	Unavailable	Azathioprine Prednisolone CyclosporinA	Subtotal colectomy
226	UC	Pancolitis	16	M	Unavailable	Mesalazine Azathioprine	Subtotal colectomy
227	CD	Colon	32	M	Unavailable	-	Colonic stricture
249	CD	Left colon	14	M	CRP <5 Hb 13.4 Plt 282	-	Colonic stricture
PB1	CD	Ileum, colon	15	F	CRP 8 Hb 13.4 Plt 344 ESR 16	Asacol	Subtotal colectomy Abscess Fistulae

Appendix 2

Every patient who donated material used in this thesis is anonymously identified above by number in Appendix 1. Appendix 2 describes which of these individual patients, and where relevant which types of cells or tissues, contributed to each group of experiments. Each table in Appendix 2 is referenced to its corresponding Figure in the text of the thesis.

Figure 3.1: Patient Identification

Disease type	Tissue or cell type	Patient number
CD	LPMC's	JE29

Figure 3.2A: Smad 7/β-actin Densitometry (percentage change from baseline)

Disease type	Tissue or cell type	Patient number	Negative control	Curcumin 5μM (Δ%)	Curcumin 10μM (Δ%)	Curcumin 20μM (Δ%)
CD	Biopsies	6317	0	-4	-29	-5

Figure 3.2B: Smad 7/β-actin Densitometry (percentage change from baseline)

Disease type	Tissue or cell type	Patient number	Negative control	Curcumin 5μM (Δ%)	Curcumin 10μM (Δ%)	Curcumin 20μM (Δ%)
CD	Biopsies	6316	0	+3	-	-26
CD	Biopsies	6317	0	-4	-29	-5
CD	Biopsies	6330	0	+31	+33	+42
CD	LPMC's	JE31	0	-21	-62	-
CD	Biopsies	JE32	0	+12	+2	-
UC	Biopsies	6315	0	+5.8	-5	+4.1
UC	Biopsies	6323	0	-	-7.5	-7.5
UC	LPMC's	JE38	0	-	-21	-16

Figures 4.1 and 4.2: Interleukin-10 (pg/ml)

Disease type	Patient number	Negative control	Curcumin 5µM	Curcumin 10µM	Curcumin 20µM
CD	6316	596	114	1229	959
CD	6319	35	152	161	-
CD	6328	1408	2859	2432	2130
CD	6329	520	665	208	1041
CD	6336	57	246	248	801
CD	6330	332	746	188	532
CD	6332	213	1015	1202	305
CD	JE47	1227	1618	1930	1863
UC	6315	35	306	193	746
UC	6322	47	56	68	48
UC	6323	638	411	683	685
UC	6331	2.6	48	120	130

Figures 4.3 and 4.4: Interleukin-1β (pg/ml)

Disease type	Patient number	Negative control	Curcumin 5μM	Curcumin 10μM	Curcumin 20μM
CD	6316	1711	485	864	1700
CD	6319	513	485	492	492
CD	6329	138	98	135	57
CD	6336	166	45	69	151
CD	6330	1146	1008	1021	974
CD	JE47	421	429	398	150
UC	6315	398	244	499	293
UC	6322	460	551	433	411
UC	6327	680	556	573	600
UC	6331	253	286	270	360

Figures 4.5 and 4.6: Interferon-γ (pg/ml)

Disease type	Patient number	Negative control	Curcumin 5µM	Curcumin 10µM	Curcumin 20µM
CD	JE32	7246	2226	1476	4688
CD	6316	2307	209	1014	1099
CD	6319	119	129	104	0
CD	6329	2311	2311	1688	1888
CD	6336	724	681	461	1524
CD	6330	541	391	477	411
CD	6332	179	1249	1479	725
CD	JE47	1882	2049	3004	2652
UC	6315	213	248	124	484
UC	6322	574	659	339	79
UC	6323	2619	2005	-	2035
UC	6331	389	245	29	39

Figure 5.1: Interleukin-1β and Interferon-γ (pg/ml)

Disease type	Patient number	Cytokine	Negative control	Curcumin 5μM	Curcumin 10μM	Curcumin 20μM
CD	JE43	IL-1β	139	122	95	97
UC	JE38	IL-1β	506	261	157	95
CD	JE31	IFN-γ	1988	788	576	-
CD	JE43	IFN-γ	1608	1524	1547	1411
CD	JE47	IFN-γ	444	657	357	357
UC	JE45	IFN-γ	459	257	52	-

Figure 6.2A: Phosphorylated p38 MAPK/p38 MAPK Densitometry (arbitrary units)

Disease type	Patient number	Negative control	Curcumin 5μM	Curcumin 10μM	Curcumin 20μM
CD	6316	2.1	1.1	0.7	1.5

Figure 6.2B: Phosphorylated p38 MAPK/p38 MAPK Densitometry (arbitrary units)

Disease type	Patient number	Negative control	Curcumin 5μM	Curcumin 10μM	Curcumin 20μM
CD	6316	2.1	1.1	0.7	1.5
CD	6317	1.3	0.8	0.5	0.5
CD	6330	8.0	8.0	7.0	7.0
CD	JE31	3.0	4.0	2.0	2.0
CD	JE47	1.9	1.2	2.0	2.0
UC	6322	3.3	0.9	0.6	-
UC	6323	1.5	1.3	-	1.3
UC	6331	8.4	8.0	2.0	9.0

Figure 6.3: Nuclear Factor-κB p65 Subunit/Histone H1 Densitometry (arbitrary units)

Disease type	Patient number	Negative control	Curcumin 20μM
CD	6338	1	1
CD	6341	0.8	0.8
CD	JE49	0.9	1
UC	JE51	1	1.1

Figure 7.1: Matrix metalloproteinase-3 Densitometry (arbitrary units)

Disease type	Patient number	Negative control	TNF-α	Curcumin 5µM	Curcumin 10µM	Curcumin 20µM	Curcumin 30µM
CD	1f	44	163	88	139	71	-
CD	227	51	228	211	201	212	170
CD	249	121	233	235	235	181	-
CD	PB1	128	212	223	184	208	103
CD	JE43	96	186	198	176	-	163
UC	200	53	199	194	149	122	86
UC	226	137	217	218	206	187	157

Figure 7.2: Phosphorylated p38 MAPK/p38 MAPK Densitometry (arbitrary units)

Disease type	Patient number	Negative control	TNF-α	Curcumin 5µM	Curcumin 20µM	Curcumin 30µM
CD	1f	1.1	1.1	-	1.1	1.2
CD	227	1	1	1	0.9	0.9
CD	249	1.4	2.8	-	1.7	1.5
CD	JE43	0.7	1.6	1.3	1	-
UC	200	0.6	0.8	0.9	1	0.9

Figure 7.3: Nuclear Factor-κB p65 Subunit/Histone H1 Densitometry

(arbitrary units)

Disease type	Patient number	Negative control	TNF-α	Curcumin 10μM	Curcumin 20μM	Curcumin 30μM
CD	1f	130	145	131	139	138
CD	227	172	188	183	169	161
UC	226	91	110	75	97	93

Figure 7.4: Matrix metalloproteinase Densitometry (arbitrary units)

Disease type	Patient number	Negative control	TNF-α	Anacardic acid 2μM	Anacardic acid 5μM	Anacardic acid 10μM	Anacardic acid 15μM	Anacardic acid 20μM
CD	1f	74	194	207	186	150	90	-
CD	227	65	222	223	231	201	195	154
CD	249	144	236	217	164	140	70	38
CD	PB1	58	154	139	-	54	-	41
CD	JE43	108	183	191	202	210	203	43
UC	200	96	186	-	144	63	-	32
UC	226	49	217	143	187	176	178	45

Figure 7.5 and 7.6: Patient Identification

Disease type	Patient number
CD	227