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Matrix degrading enzymes in IBD – dependence on TNFα and indentification of IgG plasma cells as a novel source of MMP-3

by

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ABSTRACT

DIVISION OF INFECTION, INFLAMMATION, AND REPAIR
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MATRIX DEGRADING ENZYMES IN IBD – DEPENDENCE ON TNFα AND INDENTIFICATION OF IgG PLASMA CELLS AS A NOVEL SOURCE OF MMP-3

Dr John N Gordon

Crohn's disease and ulcerative disease are chronic inflammatory disorders of the gastrointestinal tract in which TNFα is now known to be central to disease pathogenesis. Recent work has shown that one of the major methods by which TNFα causes mucosal damage is to increase matrix metalloproteinase-3 (MMP-3), which leads to matrix destruction and ulcer formation. In this thesis I have undertaken two separate but related studies looking at the control and the source of MMP-3 in IBD tissues.

Thalidomide is an immunomodulatory drug that inhibits TNFα production by peripheral blood mononuclear cells (PBMC) and has been used in the treatment of resistant CD. However its mode of action is unknown and its clinical effectiveness remains unclear. Recently, experimental thalidomide derivatives have been developed that are reportedly more potent inhibitors of TNFα, though they have not been tested in IBD. In this study I investigated the effect of thalidomide and derivatives on lamina propria mononuclear cell (LPMC) TNFα and MMP-3 production. Though thalidomide inhibits PBMC TNFα production, it does not inhibit LPMC TNFα and MMP-3 production. In contrast, the thalidomide derivative CC-10004 inhibits production of both PBMC and LPMC TNFα, and downregulates LPMC MMP-3 production from subjects with IBD. Accordingly, CC-10004 may be a new and effective therapy for the treatment of IBD.

One of the most striking features in IBD is the vast influx of IgG plasma cells into the diseased mucosa. However these cells are difficult to isolate, and consequently little is known about the immune mediators they produce. In this study I have developed a novel method of isolating a pure population of functionally active plasma cell from the gut. I then showed that IgG plasma cells from patients with IBD are long-lived in-vitro and produce MMP-3. Depleting plasma cells may therefore represent a novel strategy to help treat IBD.

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Gordon JN, Thornton C, Pender SL, Goggin PM, MacDonald TT. The thalidomide analogue CC-10004 inhibits stromelysin-1 (MMP-3) production from lamina propria mononuclear cells isolated from subjects with inflammatory bowel disease. Manuscript in preparation for *Alimentary Pharmacology and Therapeutics*

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List of abbreviations

APC	Antigen presenting cell
BCR	B-cell receptor
CD	Crohn's disease
C_T	Cycle threshold
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
GALT	Gut associated lymphoid tissue
GAPDH	Gylceraldehyde-3-phosphate dehydrogenase
HBSS	Hank's balanced salt solution
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IκB	Inhibitory kappa
IL	Interleukin
IMiD	Immunomodulatory derivative
INF	Interferon
LPMC	Lamina propria mononuclear cell
LPS	Lipopolysaccharide
MAdCAM	Mucosal addressin cell adhesion molecule
MDP	Muramyl dipeptide
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase

NFκB	Nuclear factor kappa B
Nod	Nucleotide oligomerisation domain
PBMC	Peripheral blood mononuclear cell
PC	Plasma cell
PCR	Polymerase chain reaction
PDE4	Phosphodiesterase E4
PP	Peyer's patch
PWM	Pokeweed mitogen
RPMI	Roswell Park Memorial Institute Media
RT	Reverse transcriptase
SelCID	Selective cytokine inhibitory drug
SFU	Spot forming unit
STAT	Signal transducer and activator of transcription
TCR	T-cell receptor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TNBS	Trinitobenzene sulfonate
TNF	Tumour necrosis factor
TLR	Toll-like receptor
UC	Ulcerative colitis
VCAM	Vascular adhesion molecule

Chapter 1

1 Introduction

1.1 The scale of disease

Crohn's disease (CD) and ulcerative colitis (UC) are chronic, idiopathic, inflammatory disorders of the gastrointestinal tract. Although they can occur at any age, disease onset is predominantly in adolescents and young adults, and followed by a life-long relapsing and remitting course. This results in considerable morbidity and occasional mortality, with affected subjects often shouldering the burden of regular time off work, expensive and potentially toxic therapy, recurrent surgery, and an increased cancer risk. Although classically a disease associated with North American and Northern European populations, in recent years a progressive rise in incidence and prevalence of inflammatory bowel disease (IBD) has also become apparent in developing countries whish appears to parallel the increasing westernization of their economies. IBD now therefore constitutes a growing worldwide healthcare burden.

In the last 10-15 years there has been a vast increase in our understanding of the complex immunological and genetic mechanisms underlying these disorders. This, in turn, has uncovered many novel therapeutic targets. Simultaneous progress in molecular biology means it is now possible to develop immunological agents that can block or manipulate these pathways. The first of these immunological therapies, infliximab, is now in routine clinical practice and has revolutionized the treatment of CD. This has also provided proof-of-concept that immunologically directed therapy can profoundly modify disease activity, with many other therapies currently undergoing clinical trials. However, these are not a panacea and we are currently only at the start of the immunological revolution in IBD. This is witnessed by the serendipitous nature of the success of infliximab as it is now becoming clear its effectiveness in CD bears little relation to its postulated mechanism of action. In addition, progress with UC has been considerably slower and its aetiology remains unclear. However, this remains an extremely exciting time for research in IBD, as the immunological basis of UC and CD continues to be unraveled, and safe and effective therapies developed. This will ultimately benefit patients as in the coming

years these advances lead to a paradigm shift in the treatment of IBD, away from the empirical towards a more logical immuno-pathologically driven approach.

1.2 Epidemiology of Inflammatory Bowel Disease

1.2.1 Geographical, racial incidence, and prevalence of IBD

The highest reported incidence and prevalence rates for CD and UC are from the United Kingdom, Scandanavia, and North America. However, over the last twenty years epidemiological data has emerged from Asia, Africa, and South America showing that the incidence and prevalence of IBD is increasing in these areas. Thus IBD is no longer predominantly a Western disease and represents a much more fluid and dynamic disorder dependent on a complex interplay of genetic and environmental factors (Table 1.1).

In North America, incidence rates range from 2.2-14.3 cases per 100,000 for UC, and from 3.1 to 14.6 cases per 100,000 for CD. Prevalence ranges from 37 to 246 cases per 100,000 for UC and from 26 to 199 cases per 100,000 for CD.[1] In Europe incidence ranges from 1.5 to 20.3 cases per 100,000 for UC, and from 0.7 to 9.8 cases per 100,000 for CD .[1] Probably the most robust European data comes from a collaborative study involving twenty centres throughout Europe in which data was collected on a cohort of 2201 patients newly diagnosed with IBD between 1991 and 1993.[2] In this study the mean reported incidence was 10.4 per 100,000 for UC and 5.6 per 100,000 for CD. There was a North-South divide with the mean incidence for UC 40% higher, and CD 80% higher, in Northern Europe. In developed countries, the incidence of UC now appears to have peaked while CD continues to rise, save in some high-incidence areas such as Minnesota and Scandinavia. Despite the plateau in incidence in Minnesota, the prevalence of CD continues to rise, which likely reflects longer-life expectancy in CD in recent years. Overall, the changing epidemiology of IBD means that in developed countries the incidence and prevalence of CD now often matches that of UC and in some studies surpasses it.[3]

In contrast, the overall incidence and prevalence of IBD in the developing world is considerably lower than in the West.[4] This in part reflects diagnostic difficulties in countries with poor access to health care and endemic infectious colitides, although these factors alone cannot explain the entire difference. As societies become more 'Westernised' first ulcerative colitis and subsequently CD emerge and incidence rates increase. This is clearly demonstrated in Seoul, Korea, where the incidence of UC has increased over 10-fold in the space of a decade.[5] The reason for this remains unclear but it is likely that changing environmental factors including diet and lifestyle play a significant role.

It is also likely that some of the historical differences in the incidence of IBD in racial groups can be explained predominantly by environmental rather than genetic effects. IBD was previously thought to occur more frequently in the Jewish than non-Jewish population and less frequently in African-Americans than whites. However, in a series of studies from Baltimore in the 1960s and 1970s, the incidence gap between blacks and whites narrowed until the incidence of both UC and CD was equal or higher in black women compared with white men or women.[6] Likewise, in Israel the incidence of IBD in Jews born in Europe is higher than that of Jews born in Asia, but lower than that of Jews that reside in America.[7] Similarly, although IBD is rare in India, a study from Leicester revealed that the incidence of UC in second generation Asian immigrants was over double that of whites (17.2 versus 7.0 per 100,000 cases).[8] Comparable results were seen in study of Bangladeshis living in East London where the incidence of IBD has risen sharply over the last decade while that of abdominal TB has fallen.[9] In short, migrant populations appear to acquire a similar incidence of IBD to the indigenous population, indicating the importance of environmental features in shaping disease penetrance.

Both CD and UC have a peak in incidence in late adolescence and early adulthood although the diagnosis can occur at any age. Mean and median age at diagnosis of UC is in general 5 to 10 years later than with CD. The classical bimodal distribution found in early studies with a second smaller peak in incidence in later decades has not been found in more recent epidemiological studies.[10] Although an increased incidence rate has been

seen in some recent paediatric studies this appears to mirror the adult increase in IBD incidence, and the overall percentage of paediatric cases has not changed. A recent study of paediatric IBD in Scotland found a North-South variation for CD with increased incidence in the North, though no variation for UC.[11] In this study CD was also associated with increasing affluence as measured by postcode indexes of material deprivation. Finally, there is also a slight gender variation in incidence of both UC and CD. UC is more common in men than women and the gap appears to be widening as the incidence continues to rise in men but fall in females. In contrast in CD there is a female predominance in the region of 1.5-1.[10]

TABLE 1.1 REPORTED INCIDENCE AND PREVALENCE RATES FOR UC AND CD IN DIFFERENT GEOGRAPHICAL REGIONS

Geographical Location	Incidence / 100,000		Prevalence / 100,000	
	UC	CD	UC	CD
United Kingdom	13.9	5.6-9.8	122-243	144-214
European Mean	10.4	5.6	-	-
Northern Europe	11.8	7.0	-	-
Southern Europe	8.7	3.9	-	-
North America	2.2-14.3	3.1-14.6	37-246	26-199
Japan	1.9	0.5	18.1	5.8
Latin America	1.2-2.2	0-0.03	-	-

(Modified from Loftus EV, Gastroenterology 2004)

1.2.2 Environmental Factors

It is evident from the rapidly changing incidence of IBD in both migrants and developing countries that environmental factors play a crucial role in the development of IBD. Thus susceptible individuals only develop overt disease once subjected to the correct environmental triggers. Though the majority of these are unknown, certain risk factors such as smoking and appendectomy are now widely accepted to influence the course of IBD, while others such as *Mycobacterium paratuberculosis* remain speculative (Table 1.2).

TABLE 1.2 ENVIRONMENTAL FACTORS CONSIDERED TO INFLUENCE THE DEVELOPMENT OF IBD

Environmental factor
Proven
Smoking - protective for UC / Risk factor for CD
Appendectomy – protective for UC / ?Risk factor for CD
NSAIDS
Higher socioeconomic status
Putative
Oral contraceptive
High sugar diet
High fat diet
Breastfeeding – protective
Mycobacterium paratuberculosis – unlikely
Measles infection / vaccination – highly unlikely

1.2.3 Cigarette smoking

The inverse association of smoking with UC was first reported in 1983,[12] and has subsequently been widely confirmed in other studies.[13] Smokers with UC generally have a more benign disease course than non-smokers, and in ex-smokers with UC, the severity of the disease increases, with an increase in the need for hospital admission and major medical therapy.[14] In contrast, smoking is a significant risk factor for CD, and smokers with CD tend to have more severe disease, use more immunosuppressive medications, and relapse sooner following surgery.[15]

Smoking also appears protective against the development of primary sclerosing cholangitis, a disease that is strongly associated with UC. The odds of developing PSC are significantly reduced in smokers and former smokers, independent of whether the patient has underlying UC.[16]

1.2.4 Appendicectomy

Appendicectomy was first reported to protect against the development of UC in a paediatric multicentre study in which only 1 of 174 consecutive UC patients had undergone appendicectomy compared with 41/161 controls giving a highly significant odds ratio of 59.1 (95% CI, 18-189; $P < 0.001$) compared with 2.95 (95% CI, 1.69-5.17) for non-smoking.[17] Ensuing studies have confirmed this finding, and a meta-analysis of 3600 cases found appendicectomy was associated with a 69% reduction in subsequent risk of developing UC.[18] In a paediatric case-control study, Duggan et al also showed that the protective effect of appendicectomy was greatest when performed at an early age.[19] This has been replicated in a large cohort study of 212,963 patients based on the Swedish registry, in which an inverse relationship was only found in patients who underwent appendicectomy before the age of 20.[20] Interestingly, they also found that appendicectomy conferred a protective effect if performed for appendicitis or mesenteric-adentitis, but not if for non-specific abdominal pain.

Appendectomy also appears able to alter the natural history of UC. In a study from Japan, patients who developed UC following appendicectomy had lower recurrence rates and

less extensive colitis than those with an intact appendix.[21] A further two large series from France and Australia involving over 600 cases of UC both found that patients who had undergone appendectomy had significantly fewer colectomies or immunosuppressive drugs.[22, 23] However another Australian study confirmed that patients with UC who had undergone colectomy were older at diagnosis indicating that appendicectomy may delay onset of UC, but could not demonstrate any effect on disease severity.[24]

The mechanism of protection is unclear. It is possible that appendicitis itself is protective, or alternatively that removal of the appendix has a direct effect on UC. However the decrease in appendicectomies in Britain and Sweden would be expected to be associated with an increase in UC although this has not been seen.[25, 26]

1.2.5 Other environmental factors

In the 1970s and early 80s several case reports were published describing a potential association between oral contraceptive use and IBD.[27] Subsequently observational case control studies have in general found a weak positive association between oral contraceptive use and the development of IBD, although results are inconsistent.[28] This may relate to difficulties in controlling for confounding factors. Oral contraceptive users are more likely to be smokers, and smokers are more likely to use oral contraceptives. In addition, women with IBD may be more likely to use the oral contraceptive pill to prevent pregnancy, and oral contraceptives can themselves occasionally induce a colitis.[13] Overall, a meta-analysis that pooled the results of 2 cohort studies and 7 case-control studies found an overall weak positive association between oral contraceptive use and the development of IBD (RR 1.44 for CD; RR 1.29 for UC), although a non-causal association could not be excluded.[29]

Due to the fundamental relationship between the gut and dietary antigens considerable effort has been made to try to identify specific factors that may play a role in the development of IBD. Coffee, alcohol, fast-food, cereal, fibre and toothpaste have all at various times been linked with IBD, although none are particularly compelling.[1] The

most convincing data comes for refined sugar which has been consistently shown in several studies to be associated with the development of CD.[13] In one study, patients who ingested more than 55g of sucrose per day have an overall RR of 2.6 for the development of CD, which was most pronounced for ileal disease (RR 3.4).[30] However, it has been argued that subjects with CD change their diet towards more easily digested foods and that increased sugar consumption is therefore a secondary phenomenon.

NSAIDS have been demonstrated to be a risk factor for the development of IBD in several studies.[31] In one case control analysis, 74% of new cases of IBD had recent exposure to NSAIDS compared to 20% of controls.[32] Their use is also associated with higher disease activity and more frequent relapse.[33, 34] Currently there is no published data on COX-2 antagonists.

1.3 Genetics of Inflammatory Bowel Disease

1.3.1 Family history

The family history of IBD in patients with CD and UC varies from 5-30% with the majority of studies reporting rates of 10-20%. In general, subjects with CD are more likely to report a positive history of IBD than subjects with UC. The risk for IBD in first-degree relatives of CD probands is 10-20 times that age of age, sex and geographically matched controls.[35] Two large studies, one American and one European, have estimated lifetime risk for IBD in first-degree relatives of IBD probands.[36, 37] The risk for CD is higher in relatives of probands with CD and reverse in true for UC. The incidence of CD and UC existing together in the same family is also higher than that expected by chance. In a study from England the relative risk to siblings of a CD proband for CD was 36.5, for UC was 16.6, and IBD as a whole was 24.7.[38] These findings gave rise to the hypothesis that CD and UC are genetically related disorders that share some but not all genetic loci.

The strongest evidence for the importance of genetic susceptibility in CD comes from studies of IBD concordance rates in monozygotic and dizygotic twin pairs. These studies

assume that environmental influences are similar in each twin pair. A higher concordance in monozygotic than dizygotic twins is evidence for a genetic component, and lack of 100% concordance in monozygotic twins is evidence of the role of non-genetic factors. In such studies, concordance rates for IBD are consistently higher in monozygotic than dizygotic twins. In CD, concordance rates are 20-50% in monozygotic twins compared with 0-7% in dizygotic twins, and in UC are 6-16% in monozygotic pairs compared with 0-5% in dizygotic pairs.[39-41] In all three studies the substantial non-concordance rate in monozygotic twin pairs also serves to underscore the likely importance of environmental factors in disease penetrance.

1.3.2 Molecular genetics

Both CD and UC are considered complex genetic disorders, as they do not follow simple Mendelian laws of inheritance. Instead, they are currently thought to be related heterogenous and polygenic syndromes that share some susceptibility loci. The varied clinical phenotypes probably reflect the interaction of multiple allelic variants at multiple loci in tandem with environmental factors. Thus IBD can be thought of as multiple, genetically diverse diseases, that ultimately result in two predominant clinical phenotypes, either that of CD or UC.

Over the past decade huge progress has been made in our understanding of the genetics of IBD. This has resulted in the identification of multiple IBD susceptibility loci and the first specific gene for any complex disease.

Genome wide scanning can be used to identify specific disease susceptibility loci. In genome wide scanning, multiple affected families are genotyped for approximately 400 microsatellite markers which are spaced evenly across a chromosome. Sub-chromosomal areas of interest that contain higher than expected degrees of allelic sharing can then be identified. This was first used in 1996 to report the IBD1 locus on chromosome 16.[42] Since then another 7 loci have been reported that meet the specific criteria for linkage (Table 1.3).[43]

TABLE 1.3 SUMMARY OF CONFIRMED IBD SUSCEPTIBILITY LOCI

Locus	Chromosomal region	Variation identified	Comments
IBD1	16q12	Yes	CD specific NOD2/CARD15
IBD2	12p	No	Possibly UC specific
IBD3	6p	?HLA alleles	IBD HLA region
IBD4	14q11-12	No	Possibly CD specific
IBD5	5q31	Yes	CD specific cytokine cluster
IBD6	19p13	No	IBD
IBD7	1p36	No	IBD
IBD8	16p	No	IBD

(Modified from Wild GE, 2004)

1.3.3 The NOD2/CARD15 Gene

Genome-wide scans typically only map susceptibility genes to relatively large regions of approximately 30 million base pairs of DNA, which contain between 250-500 genes. Alternative methods must therefore be used to identify the specific gene or genes within each locus. In 2001 two different methods were successfully used to identify the first susceptibility gene for CD in the IBD1 region on chromosome 16, named the NOD2/CARD15 gene. The first group used positional association mapping in which they narrowed the region of linkage by using microsatellite markers to physically map the region, followed by sequencing of the genomic DNA.[44] The second group used a candidate gene approach in which they selected the NOD2 gene for mutation screening based on knowledge of its location and function, and used case control studies and transmission disequilibrium testing analysis to show NOD2/CARD15 mutations were associated with increased susceptibility to CD.[45] Three common polymorphisms of the NOD2/CARD15 gene occur, two missense mutations and a frameshift mutation (Arg702Trp, Gly908Arg, Leu1007fsinsC). Approximately 3-15% of CD patients are either homozygotes or compound heterozygotes for these alleles compared with 1% of the general population.[46] However, it is now clear that a large number of rarer disease

causing mutations also exist and that approximately 50% of CD carry at least one abnormal allele.[47] In total, 93% of all mutations are located in the leucine-rich region (LRR) of the gene which is specifically involved in bacterial recognition.

The NOD2/CARD15 protein contains two N-terminal CARD domains that are involved in protein binding, a centrally located nucleotide binding domain, and at the C terminus the LRR domains. NOD2 functions as an intracellular patter-recognition protein for muramyl dipeptide (MDP), a component of peptidoglycan in gram-positive bacterial cell walls.[48, 49] It is present in epithelial cells, myelomonocitic cells and Paneth cells.[50, 51] NOD2 activation leads to phosphorylation of IκB via recruitment of RICK/RIP, resulting in the release and nuclear translocation of the transcription factor nuclear factor kappa-B (NFκB).[52] NOD2 variants associated with CD are defective in their ability to respond to LPS and PGN.[53] Epithelial cells that express abnormal NOD2 protein also show a reduced ability to kill salmonella.[54] Thus it appears that the major disease causing mutations result in loss of function leading to a decreased ability to mount an effective immune response against gut bacteria.

NOD2 deficient mice do not develop spontaneous gut inflammation. However they show enhanced response to TLR2 ligation with increased NFκB-c-Rel production and increased IL-12.[55] Thus loss of negative control of TLR2 mediated NFκB activation may lead to the enhanced Th1 response seen in CD. However, NOD2 null mice have also been made by another group with somewhat contradictory results. In these mice activation of NOD2 with MDP enhances IL-12 production. The mice also show increased susceptibility to oral infection with *Listeria monocytogenes*, but not intravenous or peritoneal infection, indicating that NOD2 is critical in providing protective immunity against gut bacterial infection.[56] These results emphasize the complexity of NOD2/CARD15 activation and it remains unclear exactly how NOD2/CARD15 influences CD susceptibility. NOD2 may also be required for the expression of cryptdins, a subgroup of defensins, antimicrobial peptides made in Paneth cells. In human small bowel, NOD2 is highly expressed in Paneth cells and recent data suggests there are less defensin transcripts in patients with Crohn's disease who have NOD2 mutations.[57]

Activation of wildtype NOD2 by muramyl dipeptide results in the induction of human beta-defensin-2 (hBD-2), an antimicrobial peptide, whereas NOD2 containing the 3020insC frameshift-mutation results in defective production of hBD-2.[58] NOD2 mutations may therefore predispose to Crohn's disease indirectly, by reducing defensin-mediated innate anti-microbial immunity.

Very recently 2 other genes associated with CD have been identified. The first is the OCTN gene found in the IBD5 locus on 5q31 which codes for organic cation transporters in cell membranes.[59] The second is the DLG5 gene found on chromosome 10q23, which encodes a scaffolding protein involved in the maintenance of epithelial integrity.[60] Two recent papers have confirmed that the IBD5 locus containing the OCTN1/2 genes is associated with an increased risk of complicated CD including perianal, and structuring and penetrating disease.[61, 62] However, it is not clear whether the OCTN1/2 genes contain the disease-causing mutation or whether this gene is simply in strong linkage disequilibrium with another gene in the IBD5 locus. Functional studies have not yet been performed, although the suggestion that these genes may be important in epithelial integrity links in with hypothesis that an abnormal response to the enteric flora is a critical step in the development of gut inflammation.

1.4 Natural history and clinical course of IBD

1.4.1 Natural history of Crohn's disease and ulcerative colitis

CD and UC are characterised by recurrent intermittent episodes of diarrhoea. Blood loss is more common in UC reflecting the typically distal and continuous colonic inflammation, and abdominal pain more prevalent in CD.

The natural history of untreated patients with IBD is unknown as virtually all patients now undergo some sort of treatment. Furthermore, data from old studies of long-term follow-up is not representative as patients were selected from tertiary referral centres and generally revealed the worst prognosis, with a high incidence of cancer and mortality.[63, 64]

Thus, the most reliable current data comes from the placebo arms of treatment trials, despite subjects being, to a degree, selected as those with very severe disease are not included, and subjects are usually withdrawn if their symptoms deteriorate. Additionally it is well recognized that regular follow-up and administration of placebo may itself affect the natural history of IBD.

The majority of patients with UC usually have a chronic relapsing history, although some may only experience one isolated episode, while a minority follow a chronic continuous course.[65] In cohort studies, up to 23% of subjects are reported to experience a single episode of UC with no subsequent recurrence, although many of these cases may represent undiagnosed enteric infections.[66] A recent meta-analysis of placebo response rates in 38 trials found that overall remission occurs in 10% of cases and significant symptomatic improvement in a further 30%.[67] The need for surgery relates to both disease activity and extent of disease, and is highest in the first year following diagnosis, subsequently diminishing over time. Overall, approximately 10% of patients undergo colectomy within the first year and 25-33% by 10 years.[68, 69]

CD also typically follows a chronic relapsing and remitting course. Approximately 66% of patients experience years in relapse and years in remission, while 20% have relapses every year, and 1-4% suffer a chronic continuous course.[70] Results from the placebo-arm of treatment trials have revealed that within 3 months 25-50% of patients spontaneously enter remission; although after 12 months only 15-20% of patients are still in remission.[71] Following medically or surgically induced remission, 50% of patients remain in remission at 1 year, and 40% at 2 years. The cumulative probability of surgery is 50% at 15 years with age, ileal, and fistulating disease all independent risk factors.[72] The cumulative probability of colectomy was similar to that of UC.

1.4.2 Clinical course of Crohn's disease

Although CD can affect any part of the gastrointestinal tract over 90% of cases are restricted to 3 sites. Isolated small bowel involvement occurs in approximately one-third

of cases, isolated colonic involvement in a further third, with the remaining third having combined small and large bowel disease. The single most commonly affected area is the terminal ileum which is clinically inflamed in two-thirds of patients. Eosophageal and gastroduodenal disease is rare occurring in 0.2% and 1-4% of cases respectively, and then usually in conjunction with small or large bowel involvement.[73, 74] Disease location may change over time with one study reporting that a quarter of patients with Crohn's colitis developed ileal disease during follow-up.[75]

Fistulising CD occurs in 14-20% of patients, and is most frequently associated with colorectal disease. The most common sites for fistulae are perianal, enteroenteric, rectovaginal and enterocutaneous.

In an attempt to aid to accurate patient classification and stratification a new clinical classification for CD has been developed by an international consortium of IBD experts.[76] According to the *Vienna* classification patients can be divided into terminal ileal, colonic, ileocolonic, and upper gastrointestinal disease dependent on disease location, and into inflammatory, penetrating, or stricturing dependant on disease behaviour. Very recently, this has been further revised to form the *Montreal* classification which allows concomitant disease locations to be added (Table 1.4).[77] Prospective evaluation of these changes classification is currently underway.

TABLE 1.4 VIENNA AND MONTREAL CLASSIFICATION FOR CROHN'S DISEASE

	Vienna	Montreal
Age at	A1 below 40 y	A1 below 16 y
	A2 above 40 y	A2 between 17 and 40 y
		A3 above 40 y
Location	L1 ileal	L1 ileal
	L2 colonic	L2 colonic
	L3 ileocolonic	L3 ileocolonic
	L4 upper	L4 isolated upper disease*
Behaviour	B1 non-stricturing, non-	B1 non-stricturing, non-
	B2 stricturing	B2 stricturing
	B3 penetrating	B3 penetrating
		p perianal disease modifier†

*L4 is a modifier that can be added to L1–L3 when concomitant upper gastrointestinal disease is present.

†"p" is added to B1–B3 when concomitant perianal disease is present (Satsangi et al, Gut 2006).

The predominant symptoms in CD are abdominal pain and diarrhoea. Weight loss is more common and overt bleeding rarer than in UC, reflecting disease location. The transmural nature of the inflammation in CD means strictures and fistulae may develop. Inflammatory disease is more common at initial presentation though over time stricturing and subsequently penetrating disease often develops. For example, in one study 74% of patients were classified as having inflammatory disease at diagnosis, with 11% stricturing and 15% penetrating. Ten years later only 30% were still inflammatory whereas 32% now showed stricturing disease, and 37% penetrating disease.[78]

1.4.3 Clinical course of ulcerative colitis

UC is characterised by chronic mucosal inflammation that starts in the rectum and proceeds proximally. It is limited to the colon, except in extensive disease where 'backwash ileitis' may occur. Disease is categorized according to extent; ulcerative proctitis (limited to the rectum), proctosigmoiditis (does not extend beyond the sigmoid), left-sided colitis (does not extend beyond the splenic flexure), and extensive or pancolitis (disease extending beyond the splenic flexure). Features of proctitis include haematochezia, tenesmus, increased stool frequency, and passage of mucus, although occasionally constipation can occur. Systemic symptoms are usually absent. With more extensive and severe disease, bowel movements become progressively more frequent and liquid, blood loss more extensive, and abdominal pain, nausea, vomiting and weight-loss may be seen. In severe disease, complications include toxic megacolon, perforation, and haemorrhage, all of which can be potentially life threatening. Overall, mortality rates are only minimally increased in UC, with most excess risk occurring in the first year of diagnosis, although colorectal cancer may occur in later life.

Disease activity is classified clinically using the Truelove and Witt scoring system as either mild, moderate, or severe (Table 1.5).[79] In hospitalized patients with severe disease, lack of a significant decrease in bowel motion by day 3 and a CRP>45 has a predictive value of 85% for failure of medical therapy and subsequent need for colectomy.[80]

TABLE 1.5 TRUELOVE AND WITT'S CLASSIFICATION OF DISEASE SEVERITY IN UC

Mild	Moderate	Severe
< 4 bowel movements/day	Between mild and severe	>6 bowel movements/day
Small amounts of blood in stool		Large amounts of blood in stool
Fever absent		Fever >37.5°C
No tachycardia		HR > 90b/min
Hb >75% of normal		Hb <75% of normal
ESR <30		ESR >30

1.4.4 Extraintestinal manifestations of IBD

IBD is associated with a number of complications with the skin, joints, eyes, and biliary tract most commonly affected. Some complications occur at a similar frequency in both CD and UC, whereas others predominate in one disorder. Additionally, the course of some extraintestinal manifestations, such as erythema nodosum, relate to underlying IBD activity wheras others, such as primary sclerosing cholangitis (PSC), are independent of disease activity (Table 1.6).

Joint complications are the most common extraintestinal manifestation of IBD, with arthralgia, in the absence of arthritis, occurring in up to one-third of patients. Arthritis occurs in 15-20% of patients, usually as either peripheral arthritis or ankylosing spondylitis (AS).[81]

Peripheral arthritis is classified as either type 1 or type 2. Type 1 is pauciarticular affecting fewer than five joints, including a weightbearing joint, and in the majority of cases is acute, self-limiting, and reflects IBD activity. Type 2 is a polyarticular small joint

arthritis that affects the metacarpophalyngeal joints in particular, and can cause persistent problems with a median duration of 3 years. Both types are seronegative non-deforming arthropathies and can occur prior to the onset of IBD. They are approximately twice as common in CD as in UC.[81]

Ankylosing spondylitis occurs in 1-6% of IBD patients, compared to 0.25%-1% of the general population, with a 1:1 male to female ration in contrast to the male predominance in idiopathic disease. The association with HLA-B27* is also weaker in IBD associated-disease than in idiopathic disease.[82] The course of AS is not related to underlying disease activity and is indistinguishable from non-IBD related AS.[83]

Many mucocutaneous lesions have been reported in IBD, although only aphthous oral ulceration, erythema nodosum, and pyoderma gangrenosum occur commonly. Oral ulceration is very common occurring in 20-30% of subjects with CD and to a lesser degree in UC. Active ulceration is virtually always associated with active bowel disease.

Erythema nodosum is reported in 1-10% of UC, and 6-15% of CD patients, with a marked female preponderance of approximately 5:1. Lesions are typically associated with active IBD and regress when bowel disease is controlled.[84]

Pyoderma gangrenosum presents in 0.5-2% of cases of IBD and is often unrelated to disease activity. Lesions typically occur on the lower limbs, may be up to 4cm across, and reoccur in a third of patients. Treatment is difficult and patients usually need steroids and/or immunosuppressive agents. Recently, a small randomized controlled trial has shown that anti-TNFα therapy is effective in resistant cases.[85]

Eye complications occur in 5% of CD and 3% of UC patients, and include iritis, episcleritis, and uveitis. Females are 3 times more likely to be affected than males, and in 30% of patients lesions are recurrent. Uveitis is more common in patients with the HLA-B27* haplotype.[84]

Primary sclerosing cholongitis occurs in approximately 3-5% of cases of UC and is less common in CD, although in a subset of CD colitis patients, prevalence may be similar to that seen in UC. PSC affects men (70% of cases) more than women.[86] Other hepatobiliary complications are rare, and include hepatic steatosis, hepatic amyloidosis, and autoimmune hepatitis.

The pathophysiological basis of extraintestinal manifestions in IBD is poorly understood. The relationship of many of them to IBD activity suggests that increased systemic exposure to luminal antigens may occur in active gut disease due to increased barrier permeability. As the immune system is already highly activated this may then lead to significant inflammatory responses at other sites. During inflammation other tissues may express ligands such as MAdCAM-1 resulting in recruitment of $\alpha 4\beta 7$ T-cells to extraintestinal sites.[87]

In addition to the above extra-intestinal manifestations of IBD, osteoporosis is common in both CD and UC with a prevalence of approximately 15%. IBD patients have an overall fracture incidence 40% above that of age and sex matched controls.[88]

TABLE 1.6 THE FREQUENCY OF EXTRAINTESTINAL DISEASE MANIFESTATIONS IN CD AND UC

Manifestation	Association with IBD activity	% affected	
		CD	UC
Apthous oral ulceration	Yes	20-30%	10%
Erythema Nodosum	Yes	6-15%	1-10%
Pyoderma Gangrenosum	No	0.5-2%	0.5-2%
Type I pauciarticular arthritis	Yes	8%	4%
Type II polyarticular arthritis	No	5%	2.5%
Ankylosing Spondylitis	No	1-6%	1-6%
Iritis, Episcleritis, Uveitis	Yes	5%	3%
Primary Sclerosing Cholangitis	No	1%	3%

1.5 Pathology of IBD

The diagnosis of IBD and differential diagnosis between CD and UC is usually made on the basis of a combination of clinical, radiological, endoscopic, and pathological evidence (Table 1.7).[89] In most cases, a constellation of typical histological features will favour a specific pathological diagnosis of UC or CD, despite the lack of pathognomonic features for either disorder. The term indeterminate colitis (IC) has been introduced to refer to a specific subgroup of IBD cases (10-15% of colectomy specimens) where it remains impossible to distinguish between UC and CD.[90] However IC is now increasingly used clinically for cases of IBD when a firm diagnosis cannot be made on the basis of current clinicopathological evidence. Most of these patients evolve to a definitive diagnosis over time.

1.5.1 Macroscopic findings in Crohn's disease and ulcerative colitis

UC characteristically affects the rectum with disease advancing proximally in a symmetrical and continuous manner. Typically, disease is more severe distally.

Macroscopically active UC is distinguished by a red, friable, granular, oedematous mucosa with contact bleeding. In severe disease epithelial destruction and ulceration occur against a background of mucosal inflammation. Spared islands of inflamed mucosa surrounded by ulceration give rise to the appearance of 'pseudopolyps'. In contrast, CD is not symmetrical or continuous, and need not involve the rectum. Ulcers vary in size and may appear round and punched out, or serpiginous and linear. Multiple linear ulcers contiguous to normal mucosa give rise to the classical cobblestone appearance. Although the inflammation seen in UC is traditionally described as continuous, it is now increasingly recognized that patchiness with or without rectal sparing often occurs in treated cases. However, true segmental disease is nearly always CD.[91] In long-standing UC and CD the colon gradually becomes foreshortened, tubular, and featureless.

1.5.2 Microscopic findings in Crohn's disease

The pathological hallmark of CD is focal intestinal inflammation. Apthous ulcers, which are the earliest lesion seen in CD, arise over focal areas of lymphoid aggregates with destruction of the overlying M cells. In more extensive disease the inflammatory infiltrate becomes more widespread leading to alteration and destruction of the mucosal architecture. Distortion of the mucosal architecture is indicated by changes in crypt density and appearance. Normal colorectal crypts are straight, parallel, and extend from just above the muscularis mucosae to the surface. In both CD and UC there is distortion of this appearance, with a reduced number of small irregular, branched crypts with an increased distance between crypt bases and the muscularis mucosae. In CD, the distribution and density of this infiltrate varies widely dependent on duration and severity of disease. Though CD is widely believed to result in transmural inflammation it more commonly results in disease confined to the mucosa or submucosa. However, when transmural inflammation does occur it is highly consistent with a diagnosis of CD. In addition, the presence of lymphoid aggregates in both the submucosa and external to the muscularis propria is highly specific for CD.

Non-caseating epitheliod granulomas are found in 15-70% of cases of CD depending on disease location and the maount of tissue sampled.[92] They can be found in involved and

uninvolved bowel, in any layer of the gut, and in mesenteric lymph nodes. They consist of a discrete collection of at least five epitheliod cells, with or without accompanying multinucleate giant cells. They are usually diffusely scattered and not well formed. Occasionally, histiocytic reaction around a ruptured crypt or a 'foreign body' reaction can mimic epitheliod granuloma formation.

1.5.3 Microscopic findings in UC

Lamina propria cellularity increases in IBD due to an influx of mononuclear cells predominantly comprising plasma cells, lymphocytes, macrophages, mast cells and eosinophils. In UC this increase in cellularity is typically continuous. Neutrophil polymorph infiltration of the crypt epithelium (cryptitis), and crypt lumen (crypt abscess) is common. Inflammation is usually confined to the mucosa and submucosa, and granuloma formation is rare. Pathological features that favour a diagnosis of either UC or CD are shown in table 1.7. Diagnosis may also be hindered by medical treatment of IBD which can have a profound affect on specific morphological features.[93]

TABLE 1.7 PATHOLOGICAL FEATURES THAT FAVOUR DIAGNOSIS OF UC OR CD

Clinical Feature	Ulcerative Colitis	Crohn's Disease
Disease distribution	Continuous colonic involvement from rectum	Segmental involvement from mouth to anus
Fistulae	V Rare	Common
Strictures/Abscesses	Rare	Common
Pseudopolyps	Common	Rare
Cobblestoning	Absent	Common
Mucosal bridging	Common	Rare
Inflammation	Confined to mucosa	Transmural
Severe crypt distortion and decreased crypt density	Common	Rare
Severe mucin depletion	Common	Rare
Crypt abscesses	Common	Rare
Lamina propria cellularity	Heavy, diffuse transmucosal infiltrate	Discontinuous infiltrate
Epitheliod granulomas	Rare	30% biopsies 50% surgical specimens

1.6 The basis of the mucosal immune system and the immunopathogenesis of IBD

1.6.1 The mucosal immune system

In order to encompass the twin demands of absorbing nutrients whilst maintaining an effective barrier to pathogens, the gut has evolved highly sophisticated mechanisms to sense and respond to its environment. This is reflected in both its enormous surface area ($400m^2$) and its abundant lymphoid tissue. Continued exposure to a vast antigenic load in the form of dietary antigens, commensal bacteria, and pathogens results in a perpetual state of low-grade or controlled 'physiological' inflammation. Its major components are the epithelium, the gut associated lymphoid tissue (GALT), and the lamina propria, which can be thought of respectively as the barrier, and the induction and effector sites of the gut immune system. Other important defense mechanisms extrinsic to the epithelium include mucins, secretory IgA, bicarbonate, and secreted antimicrobial peptides such as α-defensins (Figure 1.1).

1.6.2 The epithelium

The epithelium consists of a single monolayer of epithelial cells that play a crucial role as the boundary between the body's internal and external environment. Each cell is connected to its neighbour by tight junctions, forming a relatively impermeable barrier. This barrier is not, however, complete. Passive diffusion of solutes can occur either between cells by the paracellular pathway, or across cells by the transcellular pathway. The major permeation pathway is paracellular and this is controlled by the tight junctions; dynamic structures formed from a complex of occludin, claudin, and junctional adhesion molecule (JAM) transmembrane proteins that connect to the cellular cytoskeleton. Their importance has been demonstrated in the N-cadherin dominant negative mouse where forced disruption of the tight junction results in the spontaneous development of a Crohn's like phenotype.[94] Recent work has revealed that epithelial permeability can be regulated by cytokines. INFγ, TNFα, IL-4 and IL-13 all disrupt tight junction integrity, while IL-17 and TGFβ promote barrier function and are involved in epithelial restitution following injury.[95]

FIGURE 1.1 THE MUCOSAL IMMUNE SYSTEM

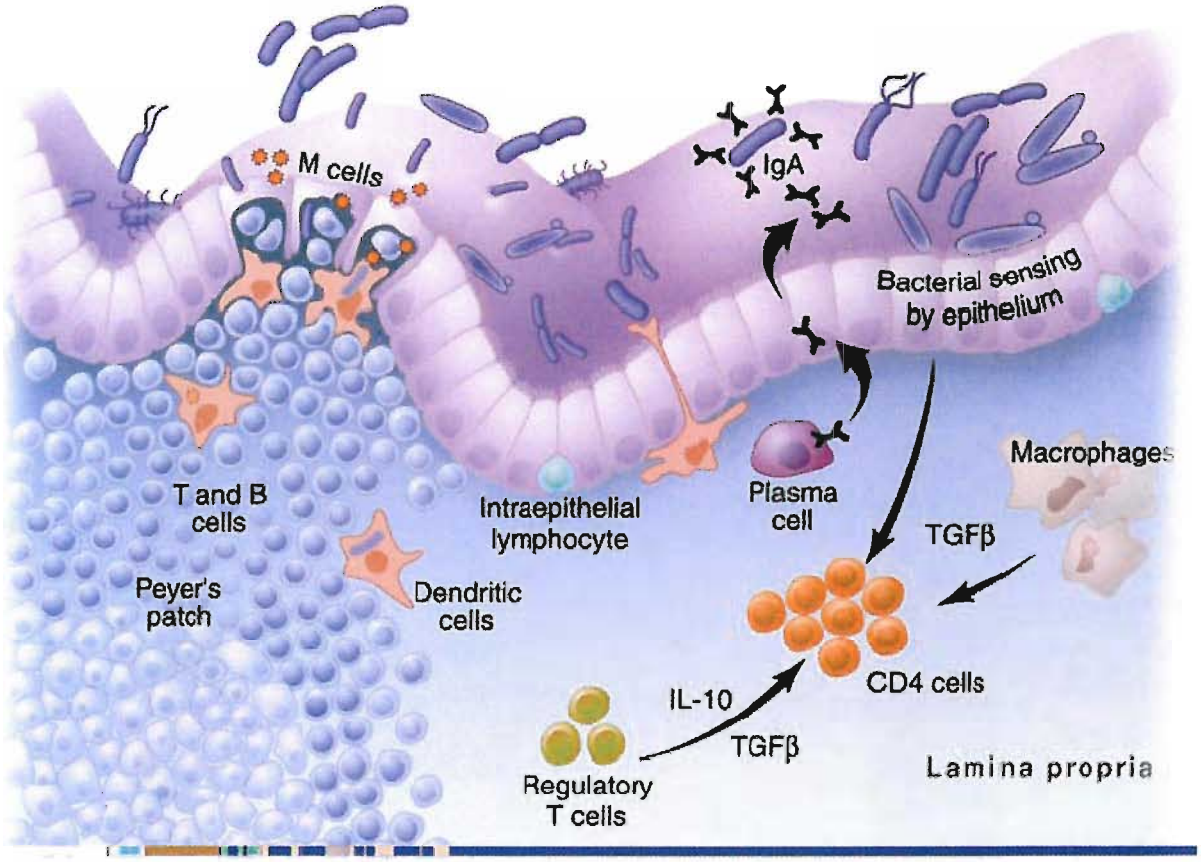


Figure 1.1 The epithelium overlying Peyer's patches contains specialised M cells that transport antigens from the gut lumen to the lymphoid tissue. Activated T cells primed by DCs in the GALT subsequently home to the lamina propria where under normal circumstances their potentially damaging responses are inhibited by immunosuppressive cytokines and regulatory cells (MacDonald TT, 2005).

It has become increasingly clear that in addition to its role as a barrier that epithelium also plays an active role in immune responses. Individual enterocytes express a variety of molecules including class I and II MHC, TLRs, and surface immunoglobulin domains.[96] They can respond to bacterial invasion by secreting a variety of pro-inflammatory cytokines and chemoattractants,[97, 98] and have also been shown to secrete IL-7 which acts as a growth factor, inducing the development of mucosal γδ T-cells and Peyer's patches.[99] In addition, as they can take up antigen by pinocytosis, it is possible they can act as APCs, processing and displaying antigens to T-cells in the context of MHC class I and II restriction, although this has not been proven in vivo.

1.6.3 Intraepithelial lymphocytes

The gut epithelium contains a specialized population of intra-epithelial lymphocytes (IEL) that develop independently of Peyer's patches. They are predominantly $CD8^+$ cells with a much larger proportion than in other tissues expressing a γδ TCR.[100] The exact role these cells play in the gut is unclear although there is increasing evidence they contribute in regulating intestinal immune responses, including protection against epithelial pathogens and the promotion of epithelial restitution.[101] They have also recently been shown to play a role in protecting against the development of colitis in a murine model of IBD.[102]

1.6.4 The gut-associated lymphoid tissue (GALT)

Gut-associated lymphoid tissue (GALT) comprises the Peyer's patches (PP), appendix, and small intestinal and colonic lymphoid follicles. An adult human has approximately 150-200 Peyer's patches in the small intestine, and thousands of smaller lymphoid follicles throughout the gut. PP are aggregates of lymphoid tissue with a follicles overlain by specialized follicle associated epithelium (FAE), which contain epithelial 'M'-cells. These cells provide a portal of entry for antigen to cross the epithelium and come into direct contact with dendritic cells within the PP. PP act as the induction site of the mucosal immune system and have two major roles. One is the generation of the secretory IgA response, and the other is the presentation of antigen to CD4 T-cells.

It is now well accepted that PP are the site of induction of the secretory IgA response.[103] Although there are no plasma cells in the lamina propria at birth, within a few weeks, PP develop germinal centres and IgA plasma cells start to appear in the gut mucosa, presumably in response to bacterial antigens.[104] IgA B-cell development depends on the antigenic stimulation and induction of germinal centres where DCs and $CD4^+$ T-cells facilitate B-cell proliferation, class-switch recombination (CSR), somatic hypermutation (SHM) and affinity maturation.[105] PP germinal centres are different to other germinal centres because of the presence of T-cell and DCs which promote isotype switching. IgA class switching requires the presence of TGFβ.[106] IgA+ve plasmablasts then migrate from PPs to the draining mesenteric lymph nodes and then to the systemic circulation. They home specifically to mucosal lamina propria through $\alpha 4\beta 7$/MAdCAM-1.[107] Preferential homing of IgA but not IgM or IgG is explained by the selective response of IgA B-cells to thymus-expressed chemokine (TECK; also called CCL25), a chemokine expressed by the small intestinal epithelium.[108] IgA blasmablasts are also selectively attracted to other mucosal surfaces by mucosal-associated epithelial chemokine (MEC; also called CCL28).[109]

The literature on T-cell responses in PP in man is limited but there is evidence to suggest that DCs produce IL-12, polarizing naïve $CD4^+$ T-cells towards a Th1 dominated profile. When measured by ELISpot PPs contain high numbers of INFγ-secreting T-cells, but few that secrete IL-4, IL-5 or IL-10.[110] Additionally, PP T-cells show a strong Th1 skewed antigen-specific recall response to milk proteins, an essentially ubiquitous dietary antigen.[111] PPs also contain abundant IL-12p40 transcripts and IL12-p70 can be seen in cells just below the dome epithelium, an area rich in DCs.[112] Thus, it appears likely that antigen presentation by DCs in PPs drives Th1 polarisation in gut $CD4^+$ T-cells.

1.6.5 The lamina propria

The lamina propria is the effector site of the gut immune system. It consists of a matrix of connective tissue between the epithelium and muscularis mucosae containing smooth muscle cells, fibroblasts, blood vessels, and lymphatics. It has a marked infiltration of immunocytes, predominantly macrophages, dendritic cells, plasma cells, and T-lymphocytes. Plasma cells comprise approximately thirty percent of the lamina propria mononuclear cells.[113] Approximately eighty percent secrete IgA, with fifteen percent secreting IgM, and three percent IgG.[114] Sixty percent of the secreted IgA is IgA2, in contrast to lymph nodes and tonsils where it is predominantly IgA1.[105] In healthy adults, gut plasma cells produce up to 3-5g of secretory IgA every day.[115] Approximately fifty to seventy percent of isolated lamina propria mononuclear cells are T-cells.[116] These are predominantly $\alpha\beta$TCR $CD4^+$ T-cells with a minority of $CD8^+$ T-cells. These appear to be derived from PP T-cell blasts which have extravasated from the blood using $\alpha4\beta7$ integrin binding to MAdCAM-1.[117] The majority of these cells secrete IFNγ with 10 fold less secreting IL-4.[118] They express the typical phenotype of activated cells being L-selectin^{lo}, $\alpha4\beta7^{+ve}$, $CD25^{lo}$, and some are DR^{+ve}.[119] The rest of the lamina propria cell population predominantly consists of B-cells, macrophages, dendritic cells, and myofibroblasts.

1.7 Immunopathogenesis of IBD

1.7.1 Crohn's disease

CD results from an excessive and persistent CD4 T helper cell type I (Th1) in the gut mucosa (Figure 1.2). Tissue from the gut of patients with CD contains abundant transcripts for IL-2 and IFNγ, and isolated mucosal T-cells secrete large amounts of INFγ. Production of IL-12, one of the key cytokines involved in Th1 polarisation and differentiation, is markedly increased in subjects with CD. CD T-cells also express the high affinity IL-12Rβ2 chain and contain phosphorylated STAT4, characteristic of Th1 cells. The increased expression of Th1 cytokines in CD is associated with T-bet, an IFN-γ inducible novel member of the T-box family of transcription factors.[120] In CD, T-bet

upregulation correlates with INFγ production, increased expression of IL12Rβ2, and with IL-12 stimulation appears essential for the development of Th1 mediated immunopathology.[121] IL-18 also drives Th1 cell differentiation, activates the transcription factors AP-1 and nuclear factor-κB (NF-κB) in T cells, and acts synergistically with IL-12 and is markedly up regulated in CD.[122, 123] IL-12 p40 chain can form a heterodimer with p19 protein to form a recently described cytokine, IL-23. Overexpression of p19 in transgenic mice leads to runting, systemic inflammation, and death.[124] In an IL-12p40 transgenic mouse model constitutive p40 promoter activity was seen in the terminal ileum alongside high expression of IL-23 p19/p40 proteins. The cellular source of IL-23 production is lamina propria dendritic cells which send processes through the epithelium to take up bacteria.[125] This links the production of IL-23 and a chronic inflammatory response with bacterial uptake by DCs in the terminal ileum, a common site for CD. However, there are no publications reporting over-expression of IL-23 in CD.

IL-21 is another newly described T cell cytokine with homology to IL-3, IL-4, and IL-15. It is upregulated in CD in comparison with UC and controls, and enhances Th1 signaling and IFNγ production.[126] In addition IL-21 acts synergistically with TNFα to increase the production of MMPs by intestinal myofibroblasts.[127]

IL-17 is a cytokine with strong proinflammatory activity. T-cell and macrophage production of IL-17 is significantly upregulated in both CD and UC but not in infective or ischaemic colitis.[128]

1.7.2 Ulcerative Colitis

The immunological basis for UC is much less clearly understood. Mucosal T-cell production of IFNγ is no higher than in controls, and although isolated T-cells from UC subjects make considerably more IL-5 than CD or control subjects, IL-4 production is reduced.[129] IL-13 is another Th2 cytokine that may be involved in UC. In the mouse model of colitis induced by intra-colonic injection of oxazolone, IL-13 produced by natural killer (NK) T cells seems to be important since IL-13 blockade prevents disease.[130] Nonclassical NK T cells isolated from UC mucosa also produce markedly

increased levels of IL-13, and are cytotoxic to epithelial cell targets.[131] IL-13 also increases epithelial permeability.[132]

IL-27 is another newly described heterodimeric cytokine related to IL-12 that consists of Epstein-Barr virus-induced gene 3 (EBI3), an IL-12p40-related protein, and p28, an IL12p35-like protein. It is produced by professional APCs, induces the proliferation of naïve T cells, and synergises with IL-12 to trigger IFNγ production.[133] The EBI3 subunit is over-expressed in ulcerative colitis and in a subset of patients with CD.[134, 135] More recently, the expression of the two subunits of IL-27, EBI3 and p28, in granulomatous disease has been investigated. Both subunits are coexpressed in epitheliod and multinucleate giant cells in granulomas from subjects with tuberculosis, sarcoidosis, or CD, and in other cells such as macrophages and plasma cells.[136]

FIGURE 1.2 THE PATHOGENESIS OF IBD

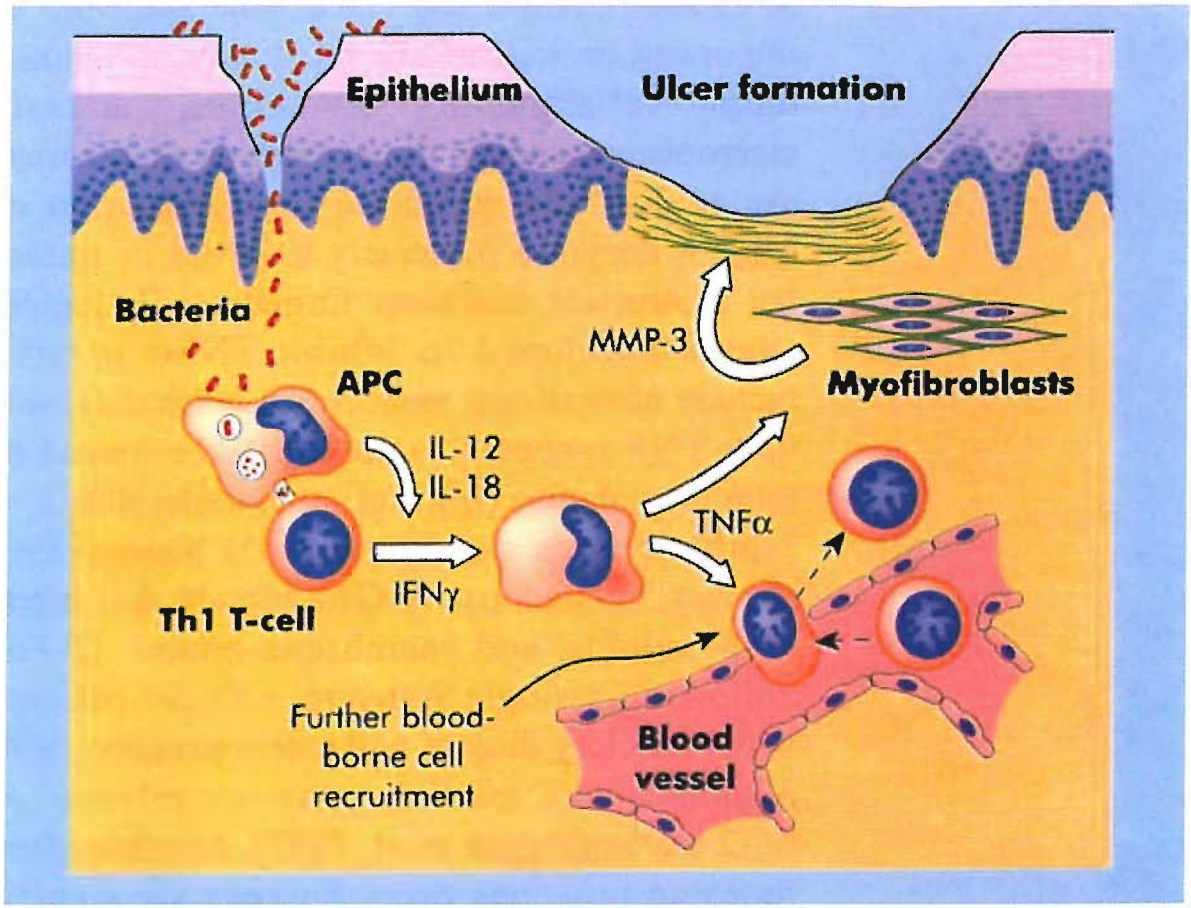


Figure 1.2 Chronic inflammation results from continued stimulation of the mucosal immune system by the commensal flora. Bacterial antigens activate DC cells resulting in an excessive and persistant Th1 response in patients with CD, or an atypical Th2 response in patients with UC. The pro-inflammatory mileau results in the recruitment of further blood-borne immune cells to the LP, and the activation of myofibroblasts to produce MMPs resulting in tissue destruction and ulcer formation (Gordon JN et al, 2003).

1.7.3 Cytokines which promote T-cell survival\apoptosis

In order to promote an effective immune response, activated T-cells proliferate in response to antigen, increasing the immune effector response. In the gut, T cell apoptosis is critical to maintaining immune homeostasis despite constant antigenic exposure. Isolated lamina propria T cells from normal subjects show high levels of activation induced Fas-mediated apoptosis, whilst in tissue sections approximately 15% of LP lymphocytes are TUNEL+, indicating a high level of apoptosis in vivo.[137] In contrast, apoptosis is greatly reduced in CD and UC.[138] In CD this is associated with defective CD2-mediated apoptosis, and an increase in the ratio of anti-apoptotic (Bcl-2, Bcl-xl) to pro-apoptotic (Bax) proteins.[139, 140] Fas-FasL ligation leads to activation of the initiator caspase 8 with subsequent downstream activation of caspase 3, and ultimately apoptosis. Activation of caspase 8 is negatively regulated by its biological inhibitor FLIP. Levels of FLIP are lower in lamina propria T-cells than peripheral blood T-cells, which may explain the susceptibility of LP T cells to apoptosis.[141]
The cytokine milieu also plays a crucial role in delivering anti-apoptotic signals in IBD. IL-2, IL-6, IL-15, IL-17, IL-18 can all prevent apoptosis, and their inhibition with specific antibodies abrogates colitis in mouse models.[142] Howevre, it is not clear why, given the multiple pathways by which mucosal T-cells can be driven to prevent apoptosis, inhibiting a single cytokine such as IL-6 has such a dramatic effect.

The p53 protein slows cell division, and thus cell proliferation.[143] CD T cells have decreased p53 levels, cycle faster, display less caspase activity, proliferate more, and ultimately are less susceptible to apoptosis than T-cells from normal controls. In contrast, UC T cells cycle slower, have normal levels of p53, and more caspase activity, are more susceptible to apoptosis and have a limited capacity to expand.[144]

Induction of apoptosis in effector T cells and macrophages would be a highly effective way of dampening gut inflammation. The effectiveness of anti-TNFα antibody in CD is not simply related to neutralization of soluble TNFα but is in part linked to induction of T-cell apoptosis. Infliximab, but not etanercept, is effective in the treatment of CD,

despite both being powerful inhibitors of TNFα. This is likely to reflect the fact that in vitro infliximab binds to activated lamina propria lymphocytes, activates caspase 3, and induces apoptosis.[145] In vivo, infliximab causes a rapid and specific increase in gut mucosa T-cell apoptosis in patients with CD through a caspase-dependent pathway.[146, 147] Adalimumab, a fully human anti-TNFα antibody, also induces apoptosis in activated monocytes.[148]

Other treatments for CD may also act through induction of apoptosis. Sulphasalazine, in contrast to 5-ASA, is a potent inducer of T-cell.[149] Azathioprine can induce apoptosis in CD-28 stimulated T-cells by specific blockade of Rac1 activation.[150] Finally, thalidomide can also induce apoptosis in human monocytes through a Fas independent pathway.[151]

1.7.4 STAT3 intracellular signaling

STAT3 is involved in a wide variety of, sometimes opposing, signaling pathways. It is the major signaling molecule for the IL-6 family of cytokines, but is also activated by other cytokines and growth factors including IL-10, G-CSF, and HGF.[152] STAT3 and phospho-STAT3 levels are markedly increased in IBD patients compared with controls.[153] Deletion of STAT3 results in early embryonic death in mice,[154] so its in vivo role has been investigated using cell or tissue specific conditional gene ablation. Myeloid-cell-specific STAT3 deletion in a mouse model makes neutrophils and macrophages unresponsive to IL-10, and produces a slow-onset chronic Th1 mediated colitis, very similar to that seen in IL-10 knockout mice.[155] In a somewhat similar fashion, IFNγ-induced somatic inactivation of STAT3 in myeloid cells triggers an aggressive and fatal colitis characterised by high production of IL-6, INFγ, IL-10, and IL-12. Blockade of IL-12 with neutralizing antibodies completely prevents disease.[156] Deletion of STAT3 in the bone marrow during haematopoiesis, with compensatory pseudoactivation of the innate immune system, also leads to the development of a rapidly fatal, Crohn's-like enteropathy.[157] Kobayashi et al used LysMCre/Stat3flox- mice to investigate how colitis develops in the absence of STAT3 and IL-10 signaling. Using double mutant mice they demonstrated that TLR-4 mediated recognition of microbial

components triggers aberrant IL-12p40 production and the subsequent development of colitis.[158]

The IL-6 cytokine family signals through the gp130-like receptor, activating both STAT3 and SHP-2/ras/Erk pathways. In addition to conventional signaling, IL-6 when in complex with the soluble IL-6 receptor can bind to cells lacking the IL-6R (trans signaling). IL-6 trans-signaling is elevated in subjects with IBD and enhances T-cell resistance to apoptosis. A neutralising antibody to IL-6R induces T-cell apoptosis and prevents TNBS colitis.[159] To determine the specific role of IL-6/STAT3 signaling in vivo, a gp130 "knock in" mouse was created in which all gp130 STAT binding sites were deleted. These gp130ΔSTAT mice developed gastrointestinal ulceration and severe joint disease due to impaired STAT3 mediated induction of suppressor of cytokine signaling (SOCS) proteins, that limit gp130 signaling, leading to sustained activation of the SHP-2/ras/Erk pathway.[160] More recently, STAT3 and SOCS3 have been shown to be constitutively activated in subjects with CD but not healthy controls.[161] The spontaneous IBD, which occurs in mice with targeted disruption of STAT3 in immune cells, is probably due to a failure of IL-10 down-regulation which signals through STAT3. At the same time in models of immune-mediated gut inflammation in animals with intact immune systems, IL-6 is overexpressed in the mucosa (as it is in IBD) and signaling through gp130 helps keep mucosal T cells alive to drive inflammation. A further layer of complexity lies in the fact that SOCS3, the endogenous intracellular inhibitor of STAT3, is not induced in STAT3 null mice, so that other signaling pathways initiated by the IL-6R proceed unchecked and cause disease.

1.7.5 Negative regulation by TGFβ1 in the gut

In health, TGFβ1 is a potent down-regulator of immune responses, and its deletion in mice results in chronic inflammatory disease and early death.[162, 163] Paradoxically however, despite being abundantly expressed it IBD tissue, TGFβ is unable to downregulate the ongoing inflammatory response.[164] This appears to result from a block in TGFβ signaling in IBD. Binding of TGFβ1 to its receptor causes activation of

TGFβ1RI, and phosphorylation of the intracellular proteins Smad2 and 3. These then associate with Smad4 and translocate to the nucleus where they regulate gene expression. In IBD, the intracellular inhibitor of Smad signaling, Smad7, is markedly increase and prevents the phosphorylation of Smad2 and 3. In LPMCs and mucosal tissue from IBD patients, blocking Smad7 with an antisense oligonucleotide restores TGFβ1 signaling and inhibits pro-inflammatory cytokine production.[165] In normal LPMCs, pretreatment with TGFβ1 also suppresses TNFα-induced activation of NFκB. In contrast, in patients with IBD, TGFβ1 is unable to suppress NF-κB due to Smad7 overexpression. Inhibition of Smad7 restores TGFβ1 signaling, increasing IκBα expression, and reduces NF-κB.[166, 167]

1.7.6 Downstream effector mechanisms of gut damage and repair

Matrix metalloproteinases (MMPs), zinc containing neutral endopeptidases, can degrade all components of the extracellular matrix and play an important role in both tissue injury and healing in the gut.[168] Collagenase-1 (MMP-1), collagenase 2 (MMP13), gelatinase A (MMP2), gelatinase B (MMP-9), stromelysin-1 (MMP-3), stromelysin 2 (MMP10) and macrophage metalloelastase (MMP-12) are highly expressed in IBD tissues as assessed by in-situ hybridization, western blotting, and gene array.[169-173] MMP-3 and -9 are also increased in fistulous tracts in CD.[174] Very high mucosal levels of MMP-3 are seen in necrotizing enterocolitis, a devastating condition of premature infants.[175] There is a very well characterized polymorphism in the promoter region of the MMP-3 gene that is functionally associated with increased MMP3 production. Individuals with this polymorphism show no increased in susceptibility to CD per se, but in Crohn's patients with any of the common Nod2 mutations, co-expression of the MMP-3 polymorphism associated with increased MMP-3 production is associated with more severe disease.[176]

1.7.7 Other pathways involved in gut damage

Proteinase-activated receptors (PARs) are a novel class of membrane G protein-coupled receptors activated by the proteolytic cleavage of their NH2-terminal domain. Molecular cloning has identified four PARs: PAR1 and PAR3, both preferentially activated by thrombin; PAR2, selectively activated by trypsin; and PAR4, activated by both thrombin

and trypsin.[177] PAR1 is highly expressed in many cell types of the gastrointestinal tract, including enterocytes, endothelial cells, enteric neurons and immune cells. PAR1 agonists increase intestinal permeability by a mechanism involving the induction of epithelial cell apoptosis.[178] PAR1 is also markedly increased in the colon of inflammatory bowel disease patients compared with controls. Intracolonic administration of PAR1 agonists to mice induces an intestinal inflammatory reaction, while antagonising PAR1 decreases the severity of TNBS-induced colitis. These findings, together with the evidence that PAR1 agonists are not effective in PAR1-deficient mice, support the notion that PAR1 signaling pathway is important in inflammatory bowel disease.[179] Blockers of PAR1 activation have not been tested in IBD patients.

In complete contrast to PAR1, PAR2 activation protects against colitis in experimental models, leading to an increased survival rate, improved macroscopic and histologic damage, and a decrease in mucosal T helper cell type 1 cytokines.[180] At the same time other studies suggest a proinflammatory role for PAR2 in the gastrointestinal tract. Acute intracolonic administration of a selective PAR2 agonist promotes intestinal inflammation with increased wall thickness, erythema, impaired function and visceral hyperalgesia in rats.[181] Moreover, PAR2 activation is accompanied by increased epithelial permeability and resulting bacterial translocation in the colon of mice.[182] Further studies are needed to clarify the role of PAR2 in colitis. Recently, PAR2 has been shown to be involved in the mast-cell tryptase-activated signaling pathway leading to fibroblast proliferation. PAR2 activation increases the expression of cyclooxygenase 2 (COX2), thus enhancing prostaglandin synthesis which induces fibroblast proliferation via the nuclear peroxisome proliferator-activated receptor γ (PPARγ). Drugs targeting tryptase, PAR2, COX2 and PPARγ are being developed for the treatment of inflammatory conditions and as antifibrotic agents.[183] In particular, the use of selective PPARγ agonists has received some attention in the treatment of inflammatory bowel disease, since synthetic PPARγ agonists, such as thiazolidinediones, have been shown to ameliorate murine experimental colitis.[184] Additionally, PPARγ heterozygous mice show increased susceptibility to colonic inflammation,[185] and activation of PPARγ by conjugated linoleic acid contributes to the maintenance of intestinal homeostasis and prevention of experimental colitis in mice.[186]

Activins, members of the TGF-β superfamily, and their natural antagonist, follistatin, appear to be important in intestinal inflammation and repair in murine experimental colitis by modulating the epithelial cell regeneration. Activins are increased in the colon during the acute phase of TNBS colitis, and are probably made by lamina propria mononuclear cells and epithelial cells. Neutralization of activins with follistatin effectively ameliorates inflammation in both TNBS and DSS colitis, and IL-10 gene-deficient (IL-$10^{-/-}$) mice, suggesting a new treatment modality for intestinal inflammation.[187]

Another signaling pathway involved in modulating the balance between tissue damage and repair in intestinal inflammation is represented by kinase suppressor of Ras-1 (KSR1), an essential regulatory kinase that protects epithelial cells from TNF-mediated apoptosis and is required for TNF activation of cell survival signal transduction pathways, including ERK1/2 MAPK, Akt/PKB and NF-κB.[188] Increased activity of KSR1 has a protective role in colonic epithelial cells exposed to cytokines during inflammation by promoting intestinal homeostasis, while partial loss of KSR1 expression increases epithelial ulceration and apoptosis.[189]

Recently, Massa et al. demonstrated a role for the endogenous cannabinoid system in the development of murine experimental colitis, as indicated by the abnormal inflammatory response of mice lacking cannabinoid receptors type 1 (CB1) or fatty acid amide hydrolase (FAAH), the enzyme that degrades the endogenous cannabinoid agonist anandamide.[190] Genetic ablation of CB1 receptors renders mice more sensitive to inflammatory insults, indicating a protective role of the CB1 receptors during inflammation, whilst pharmacological blockade of CB1 with the specific antagonist SR141716A leads to a worsening of colitis that is similar to that observed in CB1-deficient mice. Finally, FAAH-deficient mice show significant protection against DNBS treatment.[191] Taken together, these findings provide evidence that endogenous cannabinoid system is physiologically involved in the protection against excessive inflammation in the colon, and indicate that restoring this protective pathway by preventing the breakdown of anandamide with a FAAH inhibitor might represent a promising therapeutic target for the treatment of inflammatory bowel disease.[192]

1.8 Treatment of Inflammatory Bowel Disease

Over the last thirty years IBD has conventionally been treated using corticosteroids, aminosalicylates, and immunosuppressive agents such as azathioprine and methotrexate, with surgery reserved for refractory cases. However, since the mid-1990s advances in our understanding of the immuno-pathogenesis of inflammatory bowel disease has led to the development of targeted biological therapies. This is most apparent in CD where the clinical introduction of the anti-TNF antibody infliximab, has changed the way the disease in managed. In contrast, progress in both unraveling the molecular basis and the development of new therapies in UC has been slower although this is now beginning to change.

1.8.1 Steroids

Corticosteroids have been used in the treatment of inflammatory bowel disease for over half a century. Patients with CD who receive prednisolone 40mg/day (or 0.5-0.75mg/kg/day) have a 50-70% remission rate at 8-12 weeks.[193] However more than half of steroid treated patients either do not respond to steroids (steroid refractory), or flare when steroids are tapered (steroid dependent).[194] Risk factors for non-response include smoking and colonic disease. Steroids are not safe or effective in the maintenance of remission and should not be continued long-term.[195] Steroids are also effective for the induction of remission in patients with UC. In the first controlled trial by Truelove and Witts in 1955 40% of patients treated with oral cortisone achieved remission.[79] Subsequent studies have shown a variation in response according to disease severity; 84% of patients with mild, 80% with moderate, and 47% with severe disease enter remission with 40-80mg/day of oral prednisolone.[196] More recently rapidly, metabolised steroids such as budesonide have been used in the treatment of IBD. Budesonide is a non-systemic steroid that undergoes extensive first-pass metabolism in the liver, decreasing systemic absorption and the adverse events associated with traditional steroids. In mild-moderate CD budesonide 9mg/day is more effective than mesalazine 4g/day in inducing remission (69% vs 45%) and associated with a lower withdrawal rate.[197] However it is not as effective as conventional systemic steroids although it is associated with

significantly fewer steroid related adverse events.[198] Budesonide has not been extensively studied in the treatment of active extensive ulcerative colitis though in one reported study in was not as effective as prednisolone in inducing remission.[199] More recently interest has centred round its application in foam enemas for both induction and maintenance of remission in distal disease where it appears safe and effective.[200, 201]

1.8.2 Aminosalicylates

Sulphasalazine and the newer aminosalicylates are currently used as first-line therapy in the treatment of mild-moderate UC. Sulphasalazine consists of a 5-aminosalicylic acid (5-ASA) moiety linked to sulfapyridine by an azo bond. This is delivered intact to the colon where bacterial azo-reductase cleaves the azo bond releasing the two components. Sulfapyridine is absorbed systemically and is responsible for most of the drug's toxicity while the 5-ASA component is considered the anti-inflammatory component. Accordingly newer, better tolerated, 5-ASA preparations lack the sulfapyridine moiety and are classified according to the type of delivery system or presence of an azo bond.[202] Interestingly however, recent research has shown that sulfasalzine promotes T-lymphocyte apoptosis in patients with CD, whereas 5-ASA products themselves do not induce apoptosis.[149] Thus it is possible that the parent compound has some advantages over the newer derivatives.

Aminosalicylates are effective in the induction and maintenance of remission in patients with mild to moderate UC. Sulfasalazine induces remission in approximately 70% of patients with UC,[203] and similar results have been achieved with the newer derivatives. Mesalazine contains 5-ASA and can be delivered by a delayed release mechanism (pH dependent) in the ileum to colon (Asacol, Salofalk) or by a sustained release mechanism (time dependent) from the stomach to colon (Pentasa). A dose response relationship exists with recent research indicating that supra-maximal doses of up to 4.8g/day may prolong remission and reduce relapse rates.[204] The combined use of oral and enema treatment is superior to oral therapy alone.[205] The newer 5-ASA preparations balsalazide

and olsalazine contain an azo bond and are delivered solely in the colon. These appear to be more effective than delayed-release mesalazine, with recent trials showing Balsalazide to be superior to mesalazine at both inducing and maintaining remission.[206, 207] However, the lack of willingness of drug companies to undertake head-head trials and issues of cost-effectiveness means that considerable debate remains over which 5-ASA should be used.[208] The advent of generic mesalazine in the coming years will likely further muddy the water.

In contast with UC the role of 5-ASA products in CD is much less clearly defined. Some patients with mild disease respond to high-dose mesalazine (40-50%) and it may be useful in weaning patients off steroids following treatment for a flare.[209, 210] However, its value in maintaining remission is controversial. Some 5-ASA formulations show prophylactic activity after mesalamine induced remissions and for patients with disease of the ileum who have undergone surgical resection.[211] However the trials are conflicting, benefit appears modest at best, and considerable debate remains as to whether 5-ASAs should be used in CD.[212]

1.8.3 Immune modulators

Immunomodulatory drugs such as azathioprine and methotrextae can potentiate the therapeutic effect of corticosteroids and exert a steroid-sparing effect in patients with IBD. Their role is now well established in the treatment of both CD and UC, though there has been more of a reluctance to use such agents in UC due to concerns over toxicity and since cure is possible with colectomy.

1.8.3.1 Azathioprine

Azathioprine is a pro-drug which is rapidly converted to 6-mercaptopurine (6-MP) following oral absorption. Its precise mechanism of action is unknown although 6-MP is then metabolised through the liver to its active metabolite 6-thioguanine (6-TG) along with other inactive metabolites. 6-TG then accumulates in tissues where it is thought to

exert its effect by inhibition of purine sythesis and thus DNA and RNA synthesis. 6-MP also inhibits the proliferation of T- and B-lymphocytes and thus cytokine production.

The efficacy of azathioprine in CD has been confirmed in both clinical trials and meta-analysis.[213, 214] It is effective both in induction and maintenance of remission and in the treatment of fistulating disease with an overall response rate of approximately 50%. Although there is less published data, its effectiveness in induction and maintenance of remission in UC has also been clearly shown with similar response rates to CD.[215-217] More recently the Oxford group reviewed their experience of using azathioprine in the treatment of IBD over the last 30 years.[218] Overall remission rates were 58% for UC and 45% for CD. Optimal length of treatment is unknown with expert opinion suggesting that for patients in remission for over 4 years, azathioprine could be withdrawn without increasing the relapse rate. However a recently published randomized, placebo-controlled study has now shown that in this group azathioprine withdrawal increased relapse rates by a factor of three over those continued on maintenance therapy.[219] The major drawback of azathioprine treatment is its toxicity. Approximately 10-15% of patients suffer an adverse reaction necessitating withdrawal of therapy, with the most serious side-effects of leucopenia and pancreatitis according in approximately 2% of patients respectively.[220] An approximately 4 fold increased risk of lymphoma in IBD patients treated with azathioprine/6-MP has also been reported.[221] However it is unclear whether the increased risk results from the severity of the underlying disease or the medication itself. Overall, compared with the other known risks of immunosuppression, such as myelosuppression and infection, the risk of developing lymphoma is likely to be of minor clinical significance, and to be outweighed by the potential benefit of these treatments in patients with IBD.[222]

1.8.3.2 Methotrexate

Methotrexate is a structural analogue of folic acid that inhibits the reduction of dihydrofolic acid to folinic acid. Its mechanism of action in IBD is unclear although it is possible it results in apoptosis of activated T-cells, or enhances production of the anti-

inflammatory compound adenosine.[223] In CD there is a considerable amount of data indicating that it is effective in the induction and maintenance of remission. In the largest placebo controlled trial, intramuscular methotrexate at a dose of 25mg/week induced remission in 40% of patients with chronic active CD compared with 19% in the placebo group.[224] However it does not appear effective when given at a lower oral dose,[225] and controlled data on higher dose oral administration is lacking. In patients with CD who enter remission after treatment with methotrexate, a low dose of methotrexate effectively maintains remission.[226]

In contrast to CD, the data supporting the use of methotrexate in UC is not so robust. In the only controlled trial methotrexate was no more effective than placebo, although this study has been criticized for using a sub-therapeutic oral dose.[227] However, in uncontrolled trials the results have more encouraging, with a recent audit published this year achieving results similar to those reported in CD, with 42% of treated subjects entering remission.[228]

Side-effects of methotrexate include opportunistic infections, bone marrow depression, pneumonitis, and dose related hepatotoxicity. Folic acid should routinely be given to patients receiving methotrexate as it appears to reduce toxicity.

1.8.3.3 Cyclosporine

Intravenous cyclosporine has consistently been shown to be effective in the short-term management of severe, steroid-refractory UC. It is not effective in the treatment of CD.[229] In pooled data from controlled and uncontrolled series, 50-80% of patients respond to treatment, with 40-50% of responders achieving long-term benefit.[230] Successful therapy also leads to improved quality of life compared with patients who undergo immediate colectomy.[231] However, its usefulness is limited by its unfavourable side-effect profile, in particular that of serious nephrotoxicity and the risk of opportunistic infections such as *Pneumocystis carinii*. Furthermore, is role is likely to be further eroded by the recent success of infliximab in the treatment of severe refractory UC patients in the ACT I and II studies.

1.8.4 Biological therapy

Over the last ten years biological therapies (predominantly monoclonal antibodies) that target specific steps in the immune cascade have been an area of intense research in the ongoing quest for more effective treatments for CD and UC. Infliximab, the first biological to be licensed for the treatment of CD has already revolutionized its treatment and has now been granted a license for the treatment of UC. Adalimumab, a fully humanized anti-TNFα antibody, is likely to become the second biological to be licensed for the treatment of CD, and more are likely to follow (Table 1.8). This is currently an extremely exciting area of translational research in IBD and will be discussed in more detail below.

1.8.5 Anti-cytokine therapy

Anti-TNFα therapy is the most successful biological therapy in CD. TNFα is an attractive target, as it is markedly elevated in CD and plays a central role in orchestrating the inflammatory response due to its wide spectrum of biological effects. Anti-TNFα agents include chimeric and humanised monoclonal antibodies, soluble TNFα receptor fusion proteins, and small molecules such as thalidomide and MAP kinase inhibitors (Table 1.8).

TABLE 1.8 BIOLOGICAL THERAPY IN IBD

TARGET	THERAPEUTIC STRATEGY
Inflammatory Cytokine	
TNFα IL-6 IL-12 IFNγ IL-2	Chimeric or humanised monoclonal antibodies Soluble receptors Small molecular weight inhibitors
Adhesion Molecule	
ICAM α4β7 integrin	Anti-sense Humanised monoclonal antibodies
Inflammatory cell	
Neutrophils	Leukocyte plasmapheresis
Immune deviation	
Th1➔ Th2 switch	*Trichuris suis* infection
Therapeutic cytokine	
IL-10 IL-11 GM-CSF	Administration of recombinant cytokine

1.8.5.1 Infliximab

Infliximab is a chimeric monoclonal IgG1 antibody that binds soluble and membrane bound TNFα. It is effective in the treatment of refractory CD with 65% of patients entering remission following a single infusion.[232] In responders, maintenance therapy, consisting of 8-weekly infusions, reduces likelihood of relapse, reduces steroid usage and improves quality of life over 1 year.[233] Fistulating CD is also effectively treated with infliximab,[234] with maintenance therapy proving more effective than episodic.[235] The development of human antichimeric antibodies is a major drawback of episodic therapy with high pre-infusion antibody titres associated with an increased risk of transfusion reactions and a reduced duration of response to treatment. Concomitant immunosuppressive therapy reduces the magnitude of the immunogenic response.[236] The specific mechanism of action of infliximab in CD remains unclear. In addition to neutralizing soluble TNFα, it can also induce antibody and complement dependant cell lysis of immune cells expressing membrane bound TNFα, and induce apoptosis and thus clonal deletion of activated T cells.[146, 147, 237]

In contrast to CD, the response of UC to treatment with infliximab has been more difficult to elucidate. Initial uncontrolled studies were encouraging with an 88% response rate in one trial,[238] and 44% remission rate in another.[239] However a subsequent randomized controlled trial involving 43 patients with moderately severe steroid resistant UC showed no difference in response or remission rates between the treatment and control groups.[240] However, three further larger randomized placebo controlled trials have recently been published. These all found infliximab to be effective in the treatment of both moderate and severe refractory UC. Janerot et al studied 45 patients with severe refractory UC. 7/24 patients in the infliximab group compared with 14/21 in the placebo group required colectomy ($p=0.17$) and no serious side-effects occurred.[241] Furthermore, two larger studies (ACT I and II), which have as yet only been reported in abstract form, have also shown infliximab to be effective in the treatment of UC.

1.8.5.2 CDP571

CDP571 is humanized IgG4 anti-TNFα monoclonal antibody. It was initially shown to be more effective than placebo in inducing a clinical response (45% vs 27%).[242] However in a larger, 28 week, placebo controlled study, long-term therapy was no better than placebo, although post hoc sub-group analysis suggested it may be more effective in patients with an elevated CRP.[243] These relatively disappointing results may relate to the inability of IgG4 antibodies to fix complement and induce complement dependant cell lysis.

1.8.5.3 CDP870

CDP870 is a pegylated humanized monoclonal anti-TNFα antibody Fab fragment. In an initial randomized controlled trial of 92 patients with moderate to severe CD, no significant difference in response rates was seen between treatment and control groups although the placebo response rate of 56% was high. The remission rate was significantly greater in the group that received 10mg/kg CDP870 compared with placebo (47.1% vs 16%).[244] More recently, a large phase two randomised controlled trial of 292 pateints has been reported in which patients received subcutaneous certolizumab 100, 200 or 400mg or placebo at weeks 0, 4, and 8. All certolizumab doses produced significant clinical benefit over placebo at week 2, and at week 10 the highest dose (400mg) resulted in a 52.8% response rate compared with 30.1% in the placebo group (p=0.006). Elevated CRP acted as an independent predictor of response.[245]

1.8.5.4 Adalimumab (Humira)

Adalimumab is a fully human recombinant IgG1 anti-TNF monoclonal antibody. In an open label study of 17 patients with CDAI>220, who had lost response to, or were intolerant of infliximab, 59% had responded, and 29% were in remission at 12 weeks.[246] In a subsequent randomized placebo controlled trial of 299 patients with moderate to severely active CD, the highest dose of 160mg at baseline followed by 80mg at week 2 was significantly more effective than placebo at inducing remission (36% vs 12%).[247]

1.8.5.5 Soluble TNF receptor fusion proteins

An alternative strategy is the use of genetically engineered fusion proteins that combine a TNF receptor with an immunoglobulin tail. Etanercept is a completely human fusion protein that combines two chains of the p75 TNF receptor with an IgG1 tail. Although effective in the treatment of rheumatoid arthritis it is ineffective in the treatment of CD.[248] Onercept is recombinant human p55 TNF receptor. In an uncontrolled pilot study of 12 patients it appeared effective in inducing remission in active CD and a larger scale study is now underway.[249]

1.8.6 Small molecule TNFα antagonists

1.8.6.1 Thalidomide

Thalidomide is a unique drug with complex immunomodulatory and anti-inflammatory properties. It can down-regulate TNFα production from monocytes by enhancing TNFα mRNA degradation.[250, 251] In addition it enhances levels of IL-4 and IL-5, thus affecting a shift from a Th1 to Th2 cytokine profile, inhibits the transcription factor nuclear factor kappa-B (NFκB), and downregulates IL-12 production from lamina propria mononuclear cells.[252, 253]

In 1999 two open label pilot studies were simultaneously published in which refractory Crohn's disease was successfully treated with oral thalidomide.[254, 255] Subsequently, two further case series have been published which when combined with the original studies give a total of 84 patients.[253, 256] Overall 85% of patients who completed the studies responded to treatment although there was a high (27%) attrition rate due to adverse events.[257] Several new thalidomide analogues have now been developed that are reported to be both more effective and less toxigenic than the parent compound, although their effect in CD has not yet been reported.

1.8.6.2 CNI-1493

CNI-1493 is a small molecule that can inhibit mitogen activated protein kinase (MAPK) signal-transducing pathways, which links, among others, TNFα binding to its membrane receptor and transcription of pro-inflammatory molecules. In an uncontrolled study inhibition of JNK and p38 MAPK activation resulted in a clinical response in 67% of subjects at 4 weeks, and 58% at 8 weeks, with endoscopic improvement in all but one patient.[258]

1.8.7 Inhibition of other cytokines

1.8.7.1 Anti-IL-12 Antibody

IL-12 is central to the induction and maintenance of a Th1 cytokine response. In a recently reported study, an exclusively human IgG1 anti-IL-12p40 antibody was effective in the treatment of active CD. In this randomized double-blind placebo controlled trial, subjects receiving the highest dose had a significantly higher response rate of 75% compared with 25% in the placebo group. This was accompanied by a decrease in secretion of IL-12, INFγ, and TNFα by colonic lamina propria mononuclear cells.[259] However, it has subsequently become apparent that the anti-IL-12 p40 antibody also blocks the activity of IL-23, another cytokine which is upregulated in CD, which also signals through p40. Thus, it currently remains unclear whether the beneficial reported effect is due to inhibition of IL-12 or IL-23.

1.8.7.2 Anti-IL-6 Antibody

IL-6 is a pleiotropic cytokine which occupies a central role in the regulation of immune responses in inflammation, and in the inhibition of apoptosis. MRA is a humanised IgG1 monoclonal antibody that binds to both membrane and soluble forms of the human IL-6R. In a pilot placebo controlled study involving 36 patients, 80% of patients receiving biweekly infusions responded compared with 31% in the placebo group, with 20% entering remission compared with 0% of placebos.[260]

1.8.7.3 Anti-Interferon γAntibody

Fontolizumab is a humanised monoclonal antibody against INFγ. In a dose finding phase II study involving 133 patients with moderate to severely active CD, there was no overall significant difference in response rates between placebo and treatment groups. However, subgroup analysis revealed that subjects with a raised CRP who received two infusions of the drug had a significantly higher response rate than the placebo group at 4 weeks.[261]

1.8.8 Immunoregulatory cytokines

The administration of counter-regulatory Th2 cytokines to downregulate the Th1 immune response is an alternative approach for the treatment of CD. IL-10 is a Th2 cytokine that inhibits effector functions of activated immune cells and downregulates the production of pro-inflammatory cytokines by macrophages, and inhibits antigen presentation. Though initially successful in animal models, rHuIL-10 (Tenovil) was ineffective in the treatment of active CD, or prevention of post-operative recurrence following surgery, in large scale placebo controlled trials.[262, 263] It has since been suggested that this may be related to the inability to attain high local levels of IL-10 with conventional delivery methods. Alternative delivery methods including gene therapy and oral administration in lactobacilli are currently under investigation.[264] However a recent ex-vivo analysis of stimulated leucocytes from trial patients revealed that IFNγ production was increased in those that received the highest dose of IL-10. It has been proposed that this may explain the lack of efficacy of IL-10 in CD.[265]

IL-11 is another Th2 cytokine that downregulates the pro-inflammatory immune response, along with enhancing epithelial barrier function. An initial placebo-controlled pilot study suggested recombinant higher dose IL-11 was more effective than placebo at inducing remission in active CD (37% vs 16%).[266] However a further large scale trial was subsequently abandoned when interim analysis revealed no benefit.

1.8.9 Inhibitors of lymphocyte tracking

Integrins are a family of transmembrane molecules that mediate migration of inflammatory cells from the blood streams to sites of inflammation by interacting with specific ligands such as VCAM-1 ($\alpha4\beta1$) or MAdCAM-1 ($\alpha4\beta7$) on endothelial cells. Antibody blockade of $\alpha4$ gut integrin binding interrupts lymphocyte homing to the lamina propria and therefore presents an attractive alternative method for decreasing mucosal inflammation.

1.8.9.1 Natalizumab

Natalizumab is a chimeric IgG4 monoclonal antibody directed against the human $\alpha4$-integrin. It decreases lymphocyte trafficking both into the gut and across the blood-brain barrier. In a phase II trial of 248 patients with active CD, clinical response rates were significantly higher than placebo at all three doses used. However remission rate at the highest dose at week 6, the primary endpoint, was not significantly different from placebo (31% vs 27%).[267] A larger phase III study (ENACT-1) involving 905 patients was then undertaken. Its primary endpoint of clinical response at week 10 was not met, although response and remission rates at week 12 were significantly higher than placebo (62% vs 53% and 40% vs 31% respectively).[268] Subgroup analysis showed that patients with active inflammation evidenced by a raised CRP and platelet count were more likely to respond. The ENACT-2 study enrolled 339 responders from ENACT-1 to assess maintenance response. It demonstrated that monthly intravenous natalizumab was effective in maintaing response rates over 6 months and was steroid sparing.[268] However a major concern was the development of JC virus infection in several subjects who received the drug as treatment for multiple sclerosis. This has halted its use in CD at the present time.

Natalizumab has been reported in one small uncontrolled study to show efficacy in the short-term treatment of active UC, although no larger studies have been undertaken.[269]

1.8.9.2 Alicaforsen

Alicaforsen (ISIS-2302) is anti-sense oligonucleotide that reduces ICAM-1 message and protein production. There is conflicting data on its efficacy in active CD. In an initial pilot study 47% in the treatment group entered remission compared with 20% in the placebo group,[270] although a subsequent multicentre study of 75 patients failed to demonstrate efficacy.[271] In distal ulcerative colitis alicaforsen ememas were significantly more effective than placebo in reducing disease activity (70% vs 28%) [272].

MLN-02 is a humanized IgG1 monoclonal antibody against the gut specific $\alpha 4\beta 7$ integrin. Pilot studies have indicating its potential efficacy in both UC and CD, although at present these have only been presented in abstract form.

1.8.10 Other emerging therapies

1.8.10.1 Human GM-CSF

GM-CSF stimulates the production of immune cells from the bone marrow and boosts immune function. In an uncontrolled pilot study it was effective in inducing remission in active CD.[273] Phase III trials are currently underway.

1.8.10.2 Trichuris suis therapy

It has been proposed that the reduced incidence of CD in the developing world may in part be explained by the increased incidence of helminth infestation. Helminths induce a Th2 immune response in the gut which may be beneficial in preventing the Th1 response seen in CD. In a recent trial, 29 patients with moderate to severely active CD were infected with *Trichuris suis*, the pork whipworm, which causes a self-limiting infection in humans. At 24 weeks 79% of patients had responded to treatment, and 72% were in remission.[274] In a similar study 54 patients with active UC were also infected with *Trichuris suis* ova. 43% of patients in the treatment group improved compared with 16.4% in the placebo group ($p=0.04$).[275] No adverse effects were reported in either study.

1.8.10.3 Selective leukopheresis in UC

Recently several studies have been reported where adsorption apheresis has been successfully used in the treatment of UC. Selective apheresis removes peripheral circulating granulocytes and monocytes, and may reduce the capability of granulocytes to migrate to sites of inflammation. In a prospective study of 69 patients with severe refractory UC 83% were in remission at week 12 and steroid dosage was significantly less than controls who received steroid alone.[276] Similar results were found in two other uncontrolled pilot studies of severe UC,[277, 278] and in an uncontrolled study involving 30 patients with active distal UC.[279]

1.8.10.4 Basiliximab

In-vitro studies suggest IL-2 confers steroid resistance to CD4 T-cells. Basiliximab is a monoclonal antibody against CD25, the interleukin 2 receptor. It is used as an immunosuppressive agent in transplantation and has been shown to inhibit T cell proliferation, and to increase steroid sensitivity. In an open label study, 10 patients with steroid resistant UC received a single 40mg bolus of basiliximab plus steroid treatment. Nine out of ten patients were in remission within 8 weeks, with all rendered steroid sensitive following treatment.[280]

1.9 Conclusions

Biological therapies are revolutionizing the treatment of CD. It is now clear that manipulation of specific aspects of the immune response can result in mucosal healing in CD and in long-term clinical remission. Of all therapies tested so far anti-TNFα appears to be the most effective, although all agents are not equal. Among alternative strategies newer anti-integrin and anti-IL-12 antibodies are likely to eventually become licensed. Optimal positioning and treatment strategies remain to be determined. Treatment options lag behind in UC although it is hopeful that strategies such as the use of anti-ICAM antibodies will translate to clinical practice. Ultimately further progress in understanding the immunological basis of these disorders should lead to a shift from the empirical to a

more direct immunophysiological approach to the treatment of inflammatory bowel disease.

1.10 Part II

1.11 The matrix metalloproteinases

The matrix metalloproteinases (MMPs) are a family of zinc containing, Ca^{2+}-dependent neutral endopeptidases that can degrade all components of the extracellular matrix.[168] They play a crucial role in regulating tissue degradation and repair in both physiological and pathological conditions. Thus, they are highly expressed in human reproductive organs such as the ovary, uterus, and breast where extensive tissue remodeling occurs, and in pathological condition such as rheumatoid arthritis, periodontal disease, and inflammatory bowel disease where tissue destruction predominates.

1.11.1 MMP nomenclature, structure and function

Over 24 human MMPs have been identified to date that can be broadly split into five main groups depending on their primary substrate specificity: collagenases (MMP-1, -8, -13 and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -7, -10, and -11), elastase (MMP-12), and membrane types (MMP -14, -15, -16, -17, -24 and -25) (Table 1.9). They are named either by their common names or by sequential nomenclature. The majority of MMPs are secreted except for the six membrane-types that contain transmembrane domains and are expressed as cell surface enzymes. All MMPs contain an N-terminal signal sequence (predomain) that is removed after it directs synthesis to the endoplasmic reticulum. The predomain is followed by a prodomain that contains the cysteine switch which chelates the active zinc in the catalytic site, maintaining latency until the pro-domain is removed. The majority of MMPs also contain a hemopexin domain which is connected to the catalytic domain by a hinge region. The hemopexin domain influences TIMP and substrate binding and membrane activation of the MMP.

1.12 MMP regulation

Since MMPS have the fundamental ability to degrade virtually all types of matrix components, under normal physiological conditions their activities are precisely controlled by a combination of transcriptional regulation, post-translational modification, activation of the precursor zymogens, interaction with specific ECM components, and inhibition by endogenous inhibitors.

1.12.1 Transcription and translational regulation

Most MMPs are transcriptionally regulated except for MMP-2 which is constitutively expressed by many cell types and whose activation is controlled by MT1-MMP. MMP expression is regulated by many cytokines and growth factors such as LPS, IL-4, TNFα, TGF-β, and PDGF.[281] These factors operate by triggering transcription factors such as MAPK and NF-κB which in turn activate the activating protein-1 (AP-1) family members c-Jun which and c-Fos to drive transcription of multiple MMP genes.[282] Cytokines such as TGFβ can also post transcriptionally regulate MMP expression by stabilizing or destabilizing MMP mRNA.[282]

1.12.2 Activation of MMPs

MMPs are initially synthesized as zymogens (latent or pro- enzymes). They are secreted into the extracellular space by a variety of cell types including mesenchymal cells, monocytes, macrophages, neutrophils, T-cells, and tumour cells. All, except for membrane types, require proteolytic cleavage for activation. In vitro chemical agents such as glutathione, SDS and low pH and heat treatment can also lead to activation. In vivo plasmin produced by the action of tissue plasminogen activator (TPA) is an important pathway of activation.[283] Following activation, MMPs can in turn activate other zymogens creating a powerful catalytic cascade.

1.12.3 The Tissue Inhibitors of metalloproteinases (TIMPs)

Tissue inhibitors of metalloproteinase (TIMPs) are the endogenous local inhibitors of MMPs. Four TIMPs are recognized which under physiological conditions tightly regulate MMP activity through 1:1 complexing with the activated enzyme. TIMP-1 and TIMP-2 inhibit the active forms of all MMPS except for MT1-MMP which TIMP-1 fails to inhibit.[281] In pathological situations changes in TIMP levels are important as alterations in the ratio of MMPs to TIMPs can directly affect MMP activity leading to excessive degradation or formation of ECM. Serum-borne inhibitors include the macroglobulin family which has a potent ability to inhibit a broad range of proteinases including MMPs.

TABLE 1.9 MAIN MAMMALIAN MMPS ALONGSIDE THEIR TYPICAL MATRIX SUBSTRATES

Name	Matrix Substrate
Collagenases	
MMP-1 (Collagenase-1)	Collagen I/II/III/VII/X, gelatin, entactin, aggrecan, tenascin
MMP-8 (Collagenase-2)	
MMP-13 (Collagenase-3)	
Stromelysins	
MMP-3 (Stromelysin-1)	Proteoglycans, laminin, fibronectin, gelatin, collagen III/IV/V/IX/X/XI, fibrin/fibrinogen, entactin, tenascin, vitronectin
MMP-7 (Matrilysin)	
MMP-10 (Stromelysin-2)	
MMP-11 (Stromelysin-3)	
Gelatinases	
MMP-2 (Gelatinase A)	Gelatin, elastin, fibronectin, collagen I/IV/V/VII/X/XI, laminin, aggrecan, Vitronectin
MMP-9 (Gelatinase B)	
Membrane associated	
MMP-14, -15, -16, -24	Gelatin, fibronectin, vitronectin, collagen, aggregan
MMP-17, -25	Gelatin, collagen IV, fibrin, fibronectin
Others	
MMP-12 (Metalloelastase)	Elastin, fibronectin, fibrin/fibrinogen, laminin, proteoglycan

1.13 MMPs in IBD

1.13.1 MMP expression in IBD

Increased MMP expression in IBD was initially reported by Bailey et al who, using immuno-histochemistry, demonstrated marked MMP-9 staining in infiltrating neutrophils, and MMP-3 staining in areas of mucosal degradation.[284] Subsequent in-situ hybridization studies found high levels of transcripts for MMP-1 and MMP-3 in the granulation tissue, and MMP-7 in the bordering epithelium, of IBD ulcers.[285] This suggests that matrilysin plays a significant role in epithelial remodelling in contrast to collagenase and stromelysin-1, which are involved in the destructive process in the ulcer bed. Further work by the same group demonstrated abundant transcripts for MMP-13 and TIMP-3 in fibroblasts, and MMP-12 in macrophages, in and around ulcer beds. MMP-10 mRNA was also found in the epithelium that bordered the ulcers.[170]

High levels of MMPs can also found in tissue homogenates and conditioned media from patients with IBD by western blotting and zymography. Although there is some variability between studies it is now clear that MMP-1, MMP-3, and MMP-9 are particularly highly expressed in both active UC and CD tissue.[171-173, 286, 287] MMP-1, MMP-3, and MMP-9, along with MMP-10 and MMP-12 have also been demonstrated using gene array analysis to be markedly upregulated in a fetal gut explant model of T-cell mediated gut injury.[288] MMP-3 and -9 expression is also increased in fistulous tracts in CD, and very high levels of MMP-3 can be found in infants with necrotizing enterocolitis, a condition that causes extensive mucosal destruction.[174, 175]

1.13.2 TIMP expression in IBD

TIMP expression has also been investigated in IBD although study results are heterogeneous. Overall it appears that TIMP-1 and TIMP-3 production is moderately increased in active IBD although to a lesser extent than MMPs. It probably represents a simple physiological response to counteract the increase in MMP production.[172, 173, 285] No increase in TIMP-1, TIMP-2, or TIMP-3 was seen in fistulating CD.[174]

1.13.3 Functional role of MMPs in IBD

1.13.3.1 Tissue destruction

The functional role of MMPs has been investigated using a T-cell mediated fetal gut explant model of gut injury. Activation of T-cells by PWM results in severe mucosal destruction, which is accompanied by a 3-fold increase in MMP-1 and a 10-fold increase in MMP-3, while TIMP-1 and -2 levels remain unchanged. The addition of nanomolar amounts of recombinant MMP-3 directly to the explants also produces rapid severe tissue injury.[289] Tissue injury can be inhibited by the addition of a p55 TNF receptor- human IgG fusion protein, or IL-10 which both downregulated MMP-3 production.[290, 291] IL-17, a T-cell specific cytokine that is upregulated in IBD can also, in conjunction with TNFα or IL-1β, increase secretion of MMP-3 by colonic myofibroblasts.[292] Pretreatment with MMP inhibitors has been demonstrated to ameliorate colitis in murine TNBS models, although their ability to therapeutically heal the gut has not been reported.[293, 294]

1.13.3.2 Tissue repair

Fibroblasts and matrix metalloproteinases are intimately involved in wound healing. Fibroblasts isolated from patients with UC and CD have a reduced migratory potential compared to fibroblasts isolated from normal controls, and co-culture of fibroblasts from normal patients with either INFγ or TNFα reduces their migratory potential.[295] Mucosal repair following the resolution of inflammation is accompanied by the synthesis and deposition of components of the extracellular matrix by myofibroblasts. In CD chronic inflammation is often accompanied by excessive collagen deposition, fibrosis, and stricture formation. Myofibroblasts isolated from fibrotic CD tissue express high levels of TIMP-1, which inhibits MMP-mediated ECM degradation.[296] TGFβ2, but not TGFβ3, can induce expression of TIMP-1, thereby providing a mechanism by which differential expression of TGFβ isoforms can lead to excessive matrix deposition and stricture formation.[297] An increase in collagen and TIMP-1 is also seen in collagenous colitis and colonic diverticular disease,[298, 299] again suggesting that fibrosis may be due to inhibition of matrix degradation by MMP's as well as increased matrix production per se. In a murine model of chronic inflammation, fibrosis is associated with an increase in TIMP-

1.[300] Ex-vivo reversal of abnormal collagen production in human CD tissue has also been demonstrated using a novel family of regenerating agents based on heparin sulphate.[301]

Migrating epithelial cells bordering areas of ulceration express collagenase-1 (MMP-1), matrilysin-1 (MMP-7), and stromelysin-2 (MMP-10), which are upregulated in vitro by cytokines involved in wound repair.[302] Epithelial expression of MMPs may be necessary to allow enterocytes to migrate across the surface of granulation tissue. Matrilysin expression is related to the degree of inflammation.[303] In small intestinal epithelial cells peptide YY and neuropeptide Y induce differentiation, and enhance migration through downregulation of CD63 expression alongside upregulation of MMP-3 expression.[304]

MMP-3 also plays an unexpected role in T cell migration into the gut. Mice infected with the bacterial pathogen Citrobacter rodentium develop Th1 mediated colonic inflammation similar to mouse IBD. MMP3-/- mice showed impaired immunity to infection with delayed clearance of bacteria and delayed appearance of CD4+ T cells into the gut lamina propria.[305]

MMP inhibitors are widely available and are effective in ameliorating mouse colitis,[306] but there are no reports of efficacy in patients.

1.13.4 MMP polymorphisms

Finally, there is a very well characterized polymorphism in the promoter region of the MMP-3 gene which is functionally associated with increased MMP3 production. Individuals with this polymorphism show no increased in susceptibility to CD, but in patients with CD that have any of the common Nod2 mutations, co-expression of the MMP3 polymorphism associated with increased MMP3 production is associated with more severe disease.[176]

1.13.5 Summary

There is now considerable evidence that MMPs play a fundamental role in the tissue damage that occurs in IBD. In particular, MMP-3 upregulation driven by pro-inflammatory cytokines such as TNFα and IL-1β, is critical to the development of mucosal destruction. The efficacy of the p55 TNFα receptor fusion protein in preventing mucosal damage in the fetal gut explant model suggest an important pathway through which anti-TNFα therapies may act in IBD, although it has yet to be reported if anti-TNF agents can down regulate human MMP-3 production ex-vivo. Finally, it is also clear that MMPs play an extremely complex role in tissue remodeling over and above simple tissue destruction. Thus, although the use of MMP inhibitors as a therapeutic option in IBD is an attractive potential strategy, their potential to alter wound healing and immunity would need to be addressed.

1.14 Thalidomide

Thalidomide was first introduced in West Germany in 1956 and in the rest of Europe, Australia, Canada, and South Africa in 1957. Fortuitously, its release in the United States was delayed by the FDA pending clarification of concerns regarding the results of neuro-toxicological studies in animals. It was initially marketed as a sedative, with its rapid speed of onset, lack of hangover effect, and apparent safety following overdose making it an attractive alternative to barbiturates. In addition it was a powerful anti-emetic, and was widely taken by pregnant women for the treatment of morning sickness. However, soon after its release, there followed a rapid rise in reported cases of a previously rare birth defect, phocomelia (congenital limb foreshortening). In 1961, following reports linking this to in-utero thalidomide exposure, the drug was withdrawn leaving a legacy of between six and ten thousand affected children.[307, 308]

However, a few years later, the serendipitous discovery of thalidomide's anti-inflammatory potential ensured it was never entirely forgotten. In the course of treating a patient with mania and leprosy, Sheskin - an Israeli physician, administered some old supplies of thalidomide for its sedative effect. This resulted in the dramatic and virtually

complete resolution of the patient's cutaneous symptoms, and was the first indication of the drug's potential.[309] However, it was not until the discovery of thalidomide's anti-TNFα activity in 1991 that interest intensified.[251] Since then, a significant body of work has now been published that has helped to gradually elucidate the mode of action, and potential uses of this unique drug.

1.14.1 Pharmacokinetics

Thalidomide, a derivative of glutamic acid, is administered clinically as a 1:1 racaemic mixture of its S and R isomers. It is well absorbed following oral administration with maximal plasma levels of around 1-4μg/ml reached within 2-4 hours. There is some evidence to suggest that individual isomers have different biological properties with the S-isomer responsible for the immunomodulatory effects, while the R-isomer accounts for the sedative effects.[310, 311] In animal studies, both isomers caused foetal malformations when administered to the New Zealand rabbit (a species known to be sensitive to its teratogenic effects), whereas in rodents (a less sensitive species), malformations were only observed in those who received the S-isomer.[312, 313] However, in humans, the rapid chiral interconversion that occurs between the two isomers in vivo limits any potential benefit from administering a specific isomer.[314] Thalidomide is eliminated by pH-dependant spontaneous hydrolysis to multiple chemically inactive metabolites, and has a half-life of approximately 5 hours. As there is virtually no excretion via the liver or renal pathways the risk of drug interactions is low. Specifically, it has been shown that thalidomide does not affect the metabolism of oral contraceptive pills containing norethindrone or ethinyl estradiol.[315] Thalidomide has recently been shown to be present in the semen following oral dosing.[316]

1.14.2 Immunological properties

Thalidomide's immunomodulatory properties are complex and incompletely understood. Multiple mechanisms of action have been reported with the best recognised its ability to inhibit the production of TNFα. This was initially shown by Sampaio and colleagues in 1991, when they demonstrated that thalidomide at the physiological dose of 1μg/ml

resulted in a selective 40% inhibition of TNFα production from LPS-activated monocytes, without altering IL-1β, IL-6 or GM-CSF production.[251] In a subsequent study it was shown to selectively inhibit TNFα and IL-6 production from PHA-stimulated PBMCs without inhibiting IL-2, IL-4, or IL-10, in contrast to dexamethasone which markedly inhibited the expression of all cytokines.[317] Thalidomide has since been shown to down-regulate TNFα production across a broad range of cell types including T-lymphocytes, alveolar macrophages, lamina propria mononuclear cells, and microglial cells.[318, 319 253, 320] Maximal inhibition is in the order of approximately 50-70% with some variation between cell types.[321] The mechanism by which thalidomide suppresses TNFα production remains unclear. It appears to interfere with gene transcription or translation, and has been demonstrated to enhance TNFα mRNA degradation.[250] More recently it has been shown to inhibit NF-kappaB activation, a ubiquitous transcription factor that promotes the production of inflammatory cytokines, through suppressing I-kappaB kinase activity.[322] In contrast, there is also evidence that in certain circumstances thalidomide is able to augment TNFα production, potentially indicating cell specific effects.[323] In addition to its effects on TNFα production thalidomide can influence other cytokine production. Concurrently with suppressing TNFα production, thalidomide has been shown to simultaneously enhance production of IL-4 and IL-5, in effect promoting a shift from a Th1 to Th2 cytokine pattern.[324] Additionally, thalidomide has a bi-directional effect on IL-12 production, a cytokine that is central to the development of an effective cellular immune response. Thalidomide suppresses IL-12 production by mitogen-activated monocytes and lamina propria cells, while it enhances production when stimulated through the T-cell receptor.[253, 325, 326] These dichotomous results may in part be explained by the recently recognized ability of thalidomide to act as a T-cell co-stimulant under certain circumstances. The addition of thalidomide to cultures of T-cells activated through the T-cell receptor, results in an enhanced Th1 response with augmented production of IL-2 and IFNγ along with an increase in cell proliferation. The co-stimulatory effect was greater on the CD8+ T-cell than the CD4+ T-cell subset. Interestingly, in these experiments thalidomide did not inhibit TNFα production by anti-CD3 stimulated purified T-cells.[327] The co-stimulatory activity of thalidomide is worthy of note as the ability to provoke a marked Th1 response is potentially beneficial in

enhancing anti-tumour activity. In one recent study a thalidomide derivative (CC-4047) with marked co-stimulatory activity was able to prime protective, long-lasting, tumour-specific responses in a mouse model.[328]

Finally, a further potentially important property of thalidomide is its anti-angiogenic effects. In vitro studies have shown thalidomide to be capable of inhibiting angiogenesis induced by vascular endothelial derived growth factor (VEGF), and basic fibroblast growth factor (bFGF) (Table 1.10).[329, 330]

TABLE 1.10 REPORTED IMMUNOLOGICAL EFFECTS OF THALIDOMIDE

Immunological effects of thalidomide
Inhibit TNFα synthesis
Inhibit IL-6 synthesis
Inhibit IL-12 synthesis
Inhibit or augment IFN-γ sythesis
Augment IL-2 synthesis
Augment IL-4 synthesis
Augment IL-10 synthesis
Switch cytokine production from a Th1 to Th2 profile
Act as a T-cell co-stimulant
Reduce expression of ICAM-1
Reduce expression of VCAM-1
Reduce expression of bFGF
Reduce expression of VEDG

1.14.3 Thalidomide derivatives

Over the last few years the re-emergence of thalidomide has awakened interest in developing structural analogues that possess its immunomodulatory properties without the associated side-effects. Several such compounds have recently been developed that are up to 50,000 fold more potent than thalidomide at inhibiting TNFα on a molar basis.[321] They are broadly split into two main groups, dependent on their biological effects. The first class, ImiDs (Immunomodulatory drugs), strongly inhibit TNFα along with IL-1β, IL-6, and IL-12 whilst augmenting IL-10 production. They are also potent co-stimulators of T-cells when activated through the TCR and dramatically increase T-cell proliferation. They do not inhibit PDE4. The second class, SelCIDs (Selective cytokine inhibitory drugs) again potently inhibit TNFα although much more selectively, having considerably less effect on other inflammatory cytokines. They have little effect on T cell activation, causing only a slight inhibition in T cell proliferation. These compounds do markedly inhibit PDE4, although it is currently unclear how much this contributes to their biological effects.[331]

Importantly, preliminary results from animal studies have shown at least some of these compounds to be clinically effective, and non-toxic and non-mutagenic.[332, 333] Several are now undergoing phase I and phase II clinical trials although none are currently in routine clinical use.

1.14.4 Clinical studies on thalidomide in IBD

Thalidomide's potential as a treatment for inflammatory bowel disease was first reported in 1979 following its successful use in a patient with severe unresponsive ulcerative colitis.[334] However it is only recently, following the discovery of the central role of TNFα in the pathogenesis of CD that interest in the use of thalidomide in IBD has reawakened. In 1999 two papers were published simultaneously, in which patients with steroid resistant or steroid refractory Crohn's disease were successfully treated with thalidomide. In the study by Ehrenpreis et al, 22 patients with refractory CD and a CDAI>200 were started on thalidomide 200/300mg daily and assessed at 0, 4 and 12 weeks. 16/22 patients completed 4 weeks of treatment and 14/22 completed 12 weeks. All patients completing

at least 4 weeks of treatment met the criteria for a clinical response with 9/14 in remission at 12 weeks.[255] In the study by Vasiliauskas et al, 12 patients with chronic active steroid-dependent CD and a CDAI of between 250 and 500 were started on 50-100mg of thalidomide at night. By week 12 70% of patients had responded with 20% achieving remission.[254] Subsequently, two other open label trials have now been published with similar results.[253, 256] In total in the four trials 49 patients have been treated. Overall 85% of patients who completed the trials responded to treatment, although a relatively high number (27%) withdrew due to the development of adverse events. A preliminary report has also found thalidomide to be effective as maintenance therapy following induction of thalidomide with infliximab.[335]

1.14.5 Laboratory studies on thalidomide in IBD

In comparison to the number of clinical studies and reviews of thalidomide in the treatment of IBD, there is a remarkable paucity of published research on its mechanism of action. Although it has been hypothesized that it acts through inhibition of TNFα, thalidomide possesses a wide range of other complex immunomodulatory, and anti-angiogenic effects, all of which could potentially contribute to its efficacy in IBD. However few of these have been investigated in any detail.

It is clear that in rodents thalidomide is effective in attenuating the subsequent development of hapten-induced colitis. Lienenluke *et al* demonstrated that oral thalidomide given six hours prior to the induction of colitis, and then daily for one week, significantly reduced the severity of TNBS-induced colitis compared with controls.[336]It also resulted in a reduction in colonic expression of VCAM-1 and IL-8 suggesting that part of its beneficial effect was due to interruption of endothelial cell-leucocyte interaction. In a more recent study the extent and severity of DNBS-induced colitis, measured both histologically and by myeloperoxidase (MPO) activity, was significantly lower in mice pretreated with high dose oral thalidomide (200mg/kg) than in controls.[337] However no studies have been reported on the effectiveness of thalidomide in the treatment of established colitis in rodents or of its relative efficacy in Th1 versus Th2 disease models.

There is also only a limited body of research investigating the efficacy of thalidomide in human tissue. Prehn and colleagues explored the effect of thalidomide and three newer analogues (SelCIDs A, B, and C) on cytokine production from gut lamina propria mononuclear cells (LPMCs) and peripheral blood mononuclear cells (PBMCs) from healthy volunteers.[338] Thalidomide did not inhibit TNFα or IFNγ production from anti-CD3 stimulated LPMCs, whilst SelCIDs A and B were potent inhibitors of TNFα, IFNγ and IL-10 production. LPMCs were 10-100 fold less sensitive to cytokine inhibition than PBMCs. In contrast, Bauditz et al investigated 12 patients with steroid refractory CD. In this study thalidomide produced a dose-dependent inhibition of TNFα and IL-12 production from gut LPMCs isolated from subjects with CD. Additionally, paired colonic biopsies, taken from thalidomide responders at the start and end of treatment, showed a significant reduction in overall TNFα and IL-12 production.[253] Thus the effect of thalidomide on LPMC TNFα production remains controversial.

Recent work has also suggested that induction of apoptosis may play a role in the effectiveness of the anti-TNFα antibody infliximab, and other anti-inflammatory agents such as sulfasalazine, in the treatment of IBD. Gockel et al investigated the effect of thalidomide on monocyte apoptosis. They demonstrated that thalidomide induced apoptosis in human peripheral blood monocytes in a dose and time dependent manner using a cytochrome c-dependent pathway.[151] However, to date, no studies have been reported on the effect of thalidomide on apoptosis of gut LPMCs.

1.15 Plasma cells in inflammatory bowel disease

1.15.1 Introduction

One of the most impressive immuno-histological abnormalities in IBD is the huge influx of IgG secreting plasma cells into the diseased mucosa. In UC this increase is uniform along the length of the involved gut, while in CD it is centred round areas of inflammation and ulceration. However, to date, little research has been carried out on the role of these plasma cells, mainly because they have classically been thought to represent a secondary immune response to gut inflammation, and not be of primary pathogenic significance. In addition, they are difficult to isolate, and reported to die quickly ex-vivo. However, it is now clear that historical concepts of plasma cells as simple immunoglobulin producing cells are naïve, and that plasma cells secrete a variety of immune mediators, and have a wide range of diverse functions. Accordingly, they may play a more active role in the pathognesis of IBD than previously envisaged. Further circumstantial evidence to support this concept, comes from epidemiological studies in IBD published over the last ten years. It is now well established through such studies that removal of the appendix provides significant protection against the subsequent development of UC though the reason for this remains obscure. The appendix is classically considered a vestigial organ in humans though this is not the case in other animals. There is an increasing body of evidence, from both animal and human studies, that the appendix may play a role in immune responses, and that it can act as a priming site for the colonic humoral immune systemin, influencing the development of the B-cell repertoire in the colon. Thus, we have hypothesised that plasma cells may play a hitherto unknown pathogenic role in IBD.

1.15.2 Evidence supporting a pathological role for the appendix in IBD

Although there is now strong epidemiological evidence that appendicectomy early in life protects against the subsequent development of UC, little is known about the possible mechanisms involved. In particular, it is not clear if it is actual appendicitis that is protective or alternatively, removal of the appendix per se. However, over the last few

years several studies have been published that have begun to shed light on the immunology of the appendix and provide support for a possible immunopathological role in IBD.

1.15.3 The appendix

Though considered to be a vestigial organ of little significance in man, the appendix has been identified as an important component of the mammalian mucosal immune system. In rabbits, the appendix is known to be a central site of selection and development of the B-cell repertoire.[339] It plays a major role in seeding plasma cell precursors to the gut, and mucosal immunity is markedly impaired following neonatal appendicectomy.[340] Similarly, although functional studies cannot be performed, the young human appendix is morphologically and immunohistologically very similar to the rabbit suggesting it may have an analogous role in the early neonatal period.[341] Ileal Peyer's patch emigrants play an equivalent role in sheep.[342] In rodents the appendix is the primary site of CD4-8-B220+ $\alpha\beta$T-cells suggesting it is important in primary expansion of the $\alpha\beta$ T-cell repertoire.[343]

1.15.4 Animal studies of the appendix in IBD

From the above it is possible that aberrant appendiceal B or T-cell development may predispose to the development of ulcerative colitis, and that appendicectomy may remove this source of potentially autoreactive cells. Support for this hypothesis comes from rodent models of colitis. Mice with targeted disruption of the T cell receptor alpha gene (TCR$\alpha^{-/-}$) spontaneously develop colitis. This is associated with autoantibodies (anti-tropomyosin and anti-neutrophil cytoplasmic antibodies) and histologically resembles human ulcerative colitis. In these mice the amount of proliferation in the appendix lymphoid follicle (ALF) is over twice that seen in the Peyer's patches (PP) and in association with this there is a marked increase in the number of appendiceal IgA and IgG plasma cells. Furthermore, appendicectomy at 1 month of age suppresses the development of IBD with only 3.3% of mice developing colitis compared with 80% of controls.[344] Chronic colitis can also be induced in mice through activation of the IL-

6/STAT-3 signaling pathway by adoptive transfer of CD62L+ CD4+ T-cells.[345] Following transfer these cells preferentially migrate into the appendix of mice as compared to the colon. A high proportion of CD62L+CD4+ cells re-isolated from the appendix of colitic mice expressed $\alpha 4\beta 7$ integrin and CD40 ligand suggesting an important role for the appendix in the pathogenesis of colitis in this model.[346]

1.15.5 Human studies of the appendix in IBD

In a recent Japanese study, nine patients with mildly active UC were treated by surgical appendicectomy resulting in a transient but significant decrease in disease activity. There was a significantly higher proportion of activated CD4+CD45RO+ and CD8+CD45RO+ T-cells in the appendices of patients with UC compared with patients with acute appendicitis or normal controls.[347] Kawachita et al examined the phenotypical lymphocyte subpopulations in the appendix of subjects with UC, CD, and normal controls. They demonstrated a marked increase in the number of proliferating $CD19^{+}/CD38^{+}$ immature plasma cells in the appendices of subjects with UC compared with those of CD subjects or normal controls suggesting a primary role for the humoral immune response in UC.[348] Taken together these papers provide further support for the concept that the appendix plays an important role in the pathogenesis of ulcerative colitis.

1.16 Plasma cell biology

1.16.1 Plasma cell isotype

In normal subjects plasma cells are one of the major components of the gut, accounting for 20-30% of the total lamina propria mononuclear cell population.[349] The vast majority of these are IgA producing plasma cells which constitute the basis of the secretory IgA system, with only a small proportion (3-5%) secreting IgG.[350] However, in inflammatory bowel disease there is a striking increase in the total number of IgG secreting plasma cells.[351] Although the number of IgA secreting plasma cells in IBD also increases,[352] and they still remain numerically the largest isotype in IBD, the relative increase (>10 fold) in IgG secreting plasma cells dwarves that of any other isotype.[353] In ulcerative colitis this

increase is uniform along the length of the bowel while in Crohn's disease it is predominantly seen around ulcer beds.[114] The vast majority of IgG secreting cells are of the IgG1 subclass in UC with a relative decrease in IgG2, whereas conversely in Crohn's disease there is a relative increase in IgG2, and decrease in IgG1 subclass.[354, 355] There is also a small but significant increase in IgM secreting plasma cells in Crohn's disease, whereas levels in subjects with ulcerative colitis do not differ significantly from controls.[356]

1.16.2 Generation of the mucosal antibody repertoire

Antibody-secreting plasma cells are generated through the terminal differentiation of naïve circulating B-cells following antigen priming in the GALT (gut-associated lymphoid tissue). This primarily occurs in Peyer's patch germinal centres where T-cell and dendritic cells facilitate B-cell proliferation, class-switch recombination (CSR), somatic hypermutation (SHM) and affinity maturation. In man intestinal plasma cells are derived solely from bone marrow precursors, whereas in mice an additional precursor population (B1) exists that is derived from the peritoneal cavity.[357] These peritoneal B1 cells can generate T-cell independent IgA that is important in preventing systemic invasion of intestinal bacteria.[358] However, this pathway does not exist in man. The primary inductive site for plasma cell isotype switching in the gut is in Peyer's patches, and a clonal relationship between Peyer's patch IgA B-cells and gut IgA plasma cells has previously been demonstrated.[359] However, the finding that mice that lack GALT could still generate normal amounts of IgA-positive plasma cell in the lamina propria following transplantation with normal bone marrow suggesting that mucosal IgA may also be generated in the lamina propria.[360] Fagarasan et al subsequently demonstrated that activation induced cytidine deaminase (AID), along with α-germline 'loop' transcripts, was present in lamina propria $B220^{+}IgA^{+}$ plasma cells indicating recent CSR, and that IgA plasma cells could be generated from LP B-cells in in-situ.[361] However a more recent study has challenged this concept as class switch molecules such as AID and circle transcripts could only be found in GALT tissue and not in the diffuse intestinal lamina propria.[362]

1.16.3 Plasma cell growth and differentiation

Following antigen presentation naïve B-cells initially undergo isotype switching from $sIgD^+$ IgM^+ to $sIgD^-$ IgM^+, and then following secondary stimulation may switch again, normally to either a sIgA or sIgG isotype. The surface immunoglobulin is retained if the cell then enters a memory pathway, but is gradually lost during terminal plasma cell differentiation. During terminal differentiation other B-cell markers such CD19 and CD20 are also lost while CD38 and CD138 are strongly upregulated. This is associated with an increase in the transcriptional repressor, B-lymphocyte-induced maturation protein-1 (BLIMP-1). This blocks the expression of many other transcription factors necessary for normal B-cell functioning while only allowing the expression of a few genes such as X-box binding protein-1 (XBP-1) which are required for the generation of plasma cells.[363] It is thought that the small intestinal plasma cell population is largely derived from Peyer's patches, while the appendix and smaller lymphoid aggregates may prime for the large intestine.

TGFβ plays an important role in growth, differentiation, and maturation of B-cells, and in particular promotes IgA isotype switching. It was initially found to increase IgA secretion in LPS stimulated murine B-cell cultures.[364] Molecular studies subsequently demonstrated this resulted from the promotion of isotype switching of sIgM+ to sIgA+ cells.[365] Other cytokines such as IL-2 and IL-5 can also act synergistically to promote IgA isotype switching in cultures of LPS and TGFβ stimulated B-cells.[366] However, in contrast to TGFβ, none of these cytokines can promote IgA isotype switching when given individually. Interestingly TGF-β was also found to be able to inhibit IgA secretion from sIgA+ cells although the mechanism for this is unknown.[367]

1.16.4 Long-lived plasma cells

Plasma cells are terminally differentiated B-cells that provide specific humoral immunity through the secretion of antibodies. Long-lasted protective antibody titres against specific antigens, such as polio and tetanus, can be detected in human sera for decades.[368] Up until

recently it was assumed that the plasma cells providing this immunity were short-lived and continuously replaced by plasma cells newly generated from memory cells. However recent evidence suggests that long-lasting humoral immunity is provided by long-lived plasma cells. Antigen specific plasma cells can be detected in the bone marrow of mice one year after immunization.[369] These plasma cells have been shown to survive for more than 3 months without DNA synthesis and have an estimated half-life of over 6 months.[370] The longevity of plasma cells appears to be related to signals received from their microenvironment. In culture plasma cells die within 3 days in vitro if cultured in normal medium. Conditioned media, individual cytokines, and contact with stromal cells can all prolong plasma cell survival.[371-373] Optimal survival of plasma cells requires stimulation with a combination of factors that act synergistically.[374] Thus, plasma cell survival in vivo is dependant on the presence of specific niches in areas such as the bone marrow and gut that provide a specific combination of signals. Finally, many more plasma cells are generated during an immune response than survive to become long-lived plasma cells meaning that competition for survival niches will influence specific humoral immunity. Thus long-lived plasma cells may be extremely important to the development and persistence of auto-immune disease.

1.16.5 Plasma cell homing

Following their generation in germinal centres, newly generated plasma blasts migrate via the blood stream to specific effector sites such as the gut and bone marrow. Migration is regulated by chemokines which control the cellular trafficking of plasma cells. Recent work has started to uncover the specific chemokines involved in the trafficking of plasma cell to mucosal surfaces. The chemokine MEC/CCL28 is widely expressed on mucosal epithelial cells in the small and large intestine.[375] Correspondingly, its ligand CCR10 is expressed on IgA plasma cells isolated from these regions. Thus MEC and its ligand provide a mechanisitic basis for IgA plasma cells to disseminate widely to mucosal tissues following local immunization. In contrast, the chemokine TECK/CCL25 is only highly expressed in the small intestine, where it attracts IgA plasma cells that express CCR9.[108, 376] Interestingly IgG and IgM antibodies from the gut do not migrate towards

TECK. Thus, differential chemokine expression results in the presence of different plasma cell population in different areas of the gut.

1.16.6 Cytokine production by plasma cells

As described above plasma cells are the final products of a cascade of events following antigen-specific activation of naïve B lymphocytes. Their main role in the immune system has been assumed to be the production of antibodies to foreign antigen. In addition to this they also play an important role, through the production of antibodies against self-antigens, in the pathogenesis of a number of antibody mediated autoimmune disorders. Although it has been known for many years that B-cells are capable of producing cytokines it has only recently become clear that they are at least equipotent on a per cell basis with other cell types.[377] Furthermore, it is now apparent that plasma cells can secrete both regulatory and inflammatory cytokines. TGFβ mRNA is easily detectable in plasma cells isolated from human bone marrow.[378] In addition, Matthes et al also demonstrated that cultured B-cells continue to secrete bioactive TGFβ1 as they differentiate into plasma cells while IL-6 and IL-10 expression is gradually lost. TNFα can be detected in plasma cells from subjects with inflammatory disorders such as rheumatoid arthritis, scleritis, and polyarteritis nodosa. IgG plasma cells from these tissues show intense cytoplasmic immunoreactivity when stained for TNFα.[379] In the same study TNFα was also found in cultured EBV-B plasma cells and a human plasma cell line (ARH-77) following PMA stimulation. Secreted biologically active TNFα was identified in conditioned media by ELISA and western blotting. Intense TNFα and IL-6 immunoreactivity can also be detected in plasma cells from subjects with chronic granulomatous skin disorders.[380] Plasma cells in close proximity to blood vessels also express high levels of VPF/VEGF mRNA.[381]

Mechanistic animal studies have also shown that B-cells and plasma cells play an important role in both autoimmunity and T-cell regulation.[382, 383] It is well known that B-cells drive the development of several autoimmune disorders such as haemolytic anaemic and systemic lupus erythematosus through plasma cell antibody production. However,

more recently, it has recently become apparent that B-cells can also play a regulatory or inflammatory role in certain conditions. Experimental autoimmune encephalomyelitis (EAE) is a demyelinating and paralyzing model of model sclerosis characterised by a Th1 response associated with the production of large amounts of INFγ and TNFα. In this model B-cells downregulate the Th1 response via the production of IL-10 aiding recovery.[384] B-cells also play a role in regulating inflammation in murine models of colitis. $TCR^{-/-}$ mice develop a spontaneous Th2 mediated colitis similar to ulcerative colitis. Following the development of colitis is these mice a B-cell subset characterised by CD1d upregulation and the production of IL-10 can be identified that suppresses progression of intestinal inflammation by downregulating IL-1 regulation and STAT3 activation.[385] The $G\alpha i2^{-/-}$ mouse is another murine model that develops spontaneous colitis. In this model the absence of $G\alpha i2$ results in a relative deficit in the formation of an IL-10 secreting B- cell subpopulation which is linked to the development of intestinal inflammation.[386] These studies indicate that B-cells, by producing cytokines such as IL-10, can act as regulatory cells in immunologically mediated inflammatory disorders.
In contrast, in certain mice models B-cells exacerbate colitis. In the SAMP/YitFc mice, which develop CD like discontinuous ileitis, there is a considerable expansion in B-cell numbers that is linked to disease severity. Furthermore, adoptive transfer of B-cells exacerbated colitis indicating a pro-inflammatory role. As neutrophil infiltration was increased in SCID mice receiving SAMP1/YitFc B-cells the authors hypothesised that B-cells may exacerbate ileitis through immunoglobulin production and immune coimplex formation which then enhanced neutrophil recruitment through a Mac-1 integrin-dependent pathway.[387] In another model ectopic CD40 ligand expression on B-cells can trigger intestinal inflammation. Transgenic mice that ectopically express CD40L on B-cells develop severe transmural inflammation of the ileum and colon characterise by a massive inflammatory infiltrate of IgM-positive B-cells, and the presence of anti-colon antibodies. $CD40^{-/-}$ mice do not develop inflammation indicating the importance of CD40-CD40L interaction in this model.[388]

1.16.7 Plasma cell production of other inflammatory mediators

In addition to cytokine production, there is now clear evidence that both benign and malignant plasma cells are capable of synthesizing and secreting various MMPs. Using *in situ* hybridisation and immuno-histochemistry Di Girolamo et al demonstrated that IgG-positive plasma cells from inflamed synovial and scleral tissue expressed stromelysin (MMP-3) and gelatinase-B (MMP-9) but little or no TIMP-1 mRNA.[389] Secretion of biologically active protein was confirmed by western blotting and zymography on conditioned media from cytokine treated plasma cells. Plasma cells from both benign and malignant chronic inflammatory bone lesions also express MMP-8 and MMP-13.[390] Interestingly, although plasma cell isotype was not investigated in this study, plasma cells from bone destructive keratocysts and malignant plasmacytomas predominantly secrete IgA. More recently MMP-8 and MMP-13 have both also been identified by several separate groups in plasma cells in inflammatory periapical granulomas.[391-393] Malignant plasma cells from subjects with multiple myeloma have been shown to produce MMP-2, matrilysin (MMP-7), and gelatinase-B (MMP-9) *in vitro*.[394-397] Recent work has also shown that TIMP-1 can also promote plasma cell differentiation in B-cells.[398]

Urocortin 1 (Ucn1) is a recently characterised neuropeptide of the corticotrophin-releasing factor (CRF) family that binds to both CRF type I and type II receptors. It appears to play a role in both the brain and gastrointestinal tract in modulating response to stress. When injected into rodents Ucn1 inhibits gastric emptying and increases colonic motility.[399] Ucn1 is markedly increased in the lamina propria from subjects with UC compared with inflamed and non-inflamed controls. This increase in Ucn1 was localised to lamina propria plasma cells, with no Ucn1 seen in tissue macrophages.[400]

Osteopontin (also known as early T lymphocyte activation ETA-1) is a phosphoprotein constitutively secreted by epithelial cells and bone. In addition to its role in regulating calcium metabolism in bone, it is important in immune responses and granuloma formation.[401] It acts as a T-cell chemoattractant that increases adhesion of activated T-cells and promotes Th1 responses while inhibiting Th2 responses. Sato and colleagues recently demonstrated that osteopontin transcripts and protein are elevated in both CD

and UC.[402] This increase was primarily localised to IgG+ plasma cells. This is in keeping with another recent study in which intense osteopontin staining was demonstrated in large oval cells in the submucosa of subjects with severe UC.[403] Previously, osteopontin has also been found in terminal ileal plasma cells from subjects with CD and in normal controls.[404]

1.17 Hypothesis and Aims

1.17.1 Thalidomide

Thalidomide promotes mucosal healing in IBD through the inhibition of TNFα with subsequent downregulation of MMP-3 production. Newer derivatives of thalidomide with greater efficacy at inhibiting TNFα production may be more effective than thalidomide at inhibiting MMP-3 production.

Accordingly the aims of this study were as follows:

1. To determine if thalidomide can inhibit TNFα production by PBMCs and LPMCs from normal volunteers
2. To determine if thalidomide can inhibit TNFα production by LPMCs from patients with IBD
3. To determine whether newer thalidomide analogues are more effective than the parent compound in inhibiting TNFα production by BPMCs and LPMCs
4. To determine if thalidomide or analogues can inhibit production of MMP-3 by LPMCs
5. To determine if MMP-3 inhibition (if any) is due to inhibition of TNFα production or to a direct effect of the compounds on myofibroblasts

1.17.2 Plasma cells

The expression of inflammatory mediators by plasma cells suggests these cells may play an additional role inflammation beyond that of antibody production. We hypothesized that mucosal plasma cells could produce inflammatory mediators and play a pathogenic role in IBD.

Accordingly, the aims of this study were as follows:

1. To develop a method of extracting functional plasma cells from intestinal tissue samples
2. To investigate the life-span of isolated plasma cells ex-vivo
3. To investigate the production of inflammatory and regulatory cytokines by plasma cells
4. To investigate the production of stromelysin-1 (MMP-3) by plasma cells

Chapter 2

2 Materials and Methods

2.1 Lamina Propria Cell Isolation

Lamina propria mononuclear cells (LPMCs) were isolated according to a modification of the method of Bull et al.[405] Colonic mucosal tissue was obtained both from intestinal resection specimens and pinch biopsies taken during flexible sigmoidoscopy or colonoscopy. Approximately 2.5-5 cm^2 of tissue was obtained from surgical specimens and 7-15 biopsies from endoscopic procedures. Both resection and biopsy specimens were obtained fresh, placed in ice-cold medium (RPMI with 10%FCS) for transport, and used within 2hrs of removal. Patients give specific informed consent for the use of tissue for research purposes.

IBD biopsy specimens were taken from inflamed areas of mucosa in patients undergoing endoscopic examination as part of their usual management. IBD surgical specimens were taken from macroscopically inflamed areas not involving the resection margins of the removed tissue. In all cases the diagnosis was confirmed on the basis of typical clinical features, radiological, endoscopic, and laboratory findings with confirmatory histology. Normal colonic biopsy specimens were obtained from subjects undergoing endoscopic evaluation for IBS, polyp follow-up, or rectal bleeding. Normal surgical specimens were obtained from subjects undergoing surgery for colonic cancer or uncomplicated diverticular disease. Tissue was taken from macroscopically normal areas at least 5cm away from any visible pathological lesion (such as tumour), that did not involve the resection margins.

The mucosa from surgical specimens was dissected away from the submucosa, cut into 3-5 millimetre (mm) pieces before being rinsed three times in 50mls of ice-cold Hank's Balanced Salt Solution (HBSS, pH7.3; Invitrogen). Biopsy specimens were simply rinsed three times in 25mls of ice-cold HBSS. Tissue was then washed in HBSS containing 0.5mM dithiothreitol (DTT), penicillin, streptomycin, and gentamicin at 37^0C for 15 minutes with constant stirring to remove mucous and debris. The clean mucosa was subsequently transferred into a new sterile container and washed with a 0.1M EDTA containing chelating buffer (appendix 1) at 37^0C for 30 minutes with constant stirring. The supernatant containing epithelial cells, intraepithelial lymphocytes (IEL), debris and

dead cells were then discarded and the remaining cells resuspended in fresh medium. This step was then repeated two to four times until the supernatant was clear. The remaining tissue fragments were retrieved, minced finely with scalpel blades and transferred to a new sterile container. The tissue fragments were incubated in culture medium (appendix 1) supplemented with 0.25 to 1mg/collagenase type V (Sigma, Poole, UK) and stirred constantly at 37^0C for 15 minutes to 2 hours as required, until tissue appeared sufficiently digested. The cell suspension was then passed through a 100μm nylon filter and the cells pelleted by centrifugation (12000rpm for 10 minutes at 4^0C). The resulting cell suspension, wwas washed twice more and then resuspended in ice-cold culture medium. This suspension was termed the LPMC (lamina propria mononuclear cell) fraction. Cell count and viability was determined using Trypan blue exclusion staining. Cells isolations with less than 90% viability were discarded.

2.2 Separation of LPMC fraction by density centrifugation

For some experiments the LPMC fraction was then further separated by Percoll density centrifugation to achieve a more homogenous dispersed LPMC sample. The cell fraction was resuspended in 40% Percoll (Amersham Biosciences, UK). Discontinuous Percoll gradients were prepared with 100%, 60%, 40% and 30% Percoll (appendix 1), using 2mls of each layered in a 15ml Falcon tube taking care not to disrupt the interface between layers. Following layering, samples underwent centrifugation without brake at 1500rpm for 25 minutes. The 30%/40% interface containing low density LPMCs (predominantly macrophages, fibroblasts and epithelial cells), and the 40%/60% interface containing higher density LPMCs (predominantly lymphocytes, granulocytes, and plasma cells) were then harvested, washed with culture medium twice and resuspended. Cell count and viability was determined using Trypan blue exclusion staining. Cells isolations with less than 90% viability were discarded.

2.3 Cell culture

2.3.1 Ex-vivo short-term culture of LPMCs and fibroblasts

Cells were cultured in specific culture media according to experimental design. In some experiments RPMI (Sigma, UK) containing 10% foetal calf serum (FCS) and antibiotics (appendix 1) was used while in others requiring serum-free conditions, the growth factor concentrate HL-1 (Cambrex, UK), was substituted for FCS. Cells were resuspended at a concentration not exceeding 2 x 10^6 cells/ml in 12 well or 96 well culture plates according to experimental design. Cells were then incubated for 12-72 hrs at 37°C in a constant 5%CO_2.

2.3.2 Long-term fibroblast culture

Fibroblast cell lines were cultured in 20 or 75cm^2 flasks (BD Biosciences, UK) in Dulbecco's Modified Eagle Medium (DMEM) / Hams Nutrient F12 Mix, supplemented with 10% FCS, 100u/ml penicillin, 100μg/ml streptomycin and 2mM glutamine (all from Invitrogen). All cells were incubated at 37°C in 5%CO_2. Medium was changed every third day, and when cells reached confluence they were passaged, and split in a 1:4 ratio. Confluency was assessed by phase contrast light microscopy.

2.3.3 Cytokines, Mitogens, and Inhibitors

The cytokines used were TNFα, IL-1β, and IFNγ (R&D Systems Europe Ltd, Abingdon, UK). The mitogens used were PWM, LPS, and ConA (Sigma, Poole, UK). They were dissolved in PBS to make stock solutions that were then stored at -70°C for later use.

2.3.4 Drugs

Thalidomide and the derivatives CC-10004, CC-5013, and CC-4047 (gift of Celgene Corporation, New Jersey, USA) were all dissolved in dimethyl-sulphoxide (DMSO) to make stock solutions of 10mg/ml. Immediately prior to use these were further diluted in 100% DMSO to the appropriate concentrations to be added to culture experiments. This

resulted in a final concentration of 0.1% DMSO in all culture experiments unless otherwise stated. Hydrocortisone (Sigma, Poole, UK) was dissolved in PBS to a stock concentration of 10mg/ml and stored at -20°C for further use. Infliximab (Schering Plough Ltd, Welwyn Garden City, UK) and the p55TNFR fusion protein (gift of Dr SL Pender) were stored neat at a concentration of 100mg/ml at 4°C. Cycloheximide was diluted in PBS and stored at a concentration of 10mg/ml at -20°C.

2.4 Isolation of plasma cells from LPMCs

2.4.1 MACS cell separation

Plasma cells were isolated from LPMCs using MACS® technology based on the use of immunomagnetic MACS microbeads and MACS cell separation columns. The principal for this technology is that when MACS columns are places in a MACS separator magnet the MACS column provides a strong enough magnetic field to retain cells labeled with a small amount of magnetic material. Accordingly, with positive selection, cells of interest are labeled with a specific antibody bound to magnetic microbeads, and are retained in the column while unlabelled cells pass through. They can then be eluted following removal from the magnet. In contrast, with a negative selection technique, unwanted cells are labeled with antibody bound microbeads causing them to be retained in the column while the unlabeled cells are collected as they pass through the magnet.

2.4.2 $CD54^+$ cell isolation

$CD54^+$ cells were isolated from LPMCs by the use of a primary mouse anti-CD54 antibody followed by the addition of goat anti-mouse magnetic microbeads according to the manufacturer's instructions. Briefly, up to 10×10^7 LPMCs/ml were incubated with anti-CD54 Ab (10μg/ml) in MACS buffer (2mM EDTA 0.5% BSA in PBS) for 10-15 minutes in the dark at 4°C. After two washes in buffer cells were incubated with 20μl of goat anti-mouse IgG microbeads per 10^7 cells, and incubated for 15 minutes in the dark at 4°C. The cells were then washed and resuspended in 500μl of buffer. A LS^+ positive selection column was then placed in a Midi MACS magnet and ice-cold buffer run

through and discarded. The cell suspension was then passed through a 30μm filter and applied to the column. The column was washed three times with ice-cold buffer. Following this, the column was removed from the magnet, 5mls of buffer applied and the cells flushed through using the supplied plunger. This eluted fraction represented the positive cell fraction. In some experiments the eluted fraction was then passed through a second LS^+ column to improve purity.

2.4.3 $CD138^+$ cell isolation

$CD138^+$ cells were isolated by the use of MACS CD138 microbeads (Miltenyi Biotec) which contained a monoclonal mouse CD138 antibody that was directly conjugated to super-paramagnetic microbeads. 20μl of CD138 microbeads and 20μl of FcR blocking reagent (Miltenyi Biotec) were added for each 10^7 LPMCs, and the cells were incubated for 20 minutes at 4°C in the dark. The cells were then washed and resuspended in 500μl of buffer. A LS^+ positive selection column was then placed in a Midi MACS magnet and ice-cold buffer run through and discarded. The cell suspension was then passed through a 30μm filter and applied to the column. The column was washed three times with ice-cold buffer. Following this, the column was removed from the magnet, 5mls of buffer applied and the cells flushed through using the supplied plunger. This eluted fraction represented the positive cell fraction. In some experiments the eluted fraction was then passed through a second LS^+ column to improve purity.

2.4.4 $CD3^-$ $CD138^+$ cell isolation

$CD3^-$ $CD138^+$ cells were isolated using a two stage process. Initially, 20μl of CD3 microbeads (Miltenyi Biotec) and 20μl of FcR blocking reagent were added for each 10^7 LPMCs and then incubated in the dark at 4°C for 20 minutes. The cells were then washed and resuspended in 500μl of buffer. A LS^+ positive selection column was placed in a Midi MACS magnet and ice-cold buffer run through and discarded. The cell suspension was passed through a 30μm filter and applied to the column. The column was washed three times with ice-cold buffer and the negative cell fraction that passed through the

column was collected, and washed twice in ice-cold buffer. A positive selection using CD138 microbeads was then performed on this fraction as described above.

In some cell isolation experiments an autoMACS™ separator was used instead of LS^+ columns according to the manufacturer's instructions.

2.5 Flow cytometric analysis

For flow cytometric analysis 200μl cell fractions containing 2 x 10^5 cells were initially incubated for 20 minutes on ice in the dark to block Fc receptors. The cells were then labeled by incubating with FITC and PE conjugated antibodies for a further 20 minutes in the dark at 4°C. Cells were subsequently washed twice in PBS containing 0.25% BSA and analysed on a FACSCalibur cytometer (BD Biosciences). Intracellular staining for immunoglobulin was performed by using a fixation/permeabilisation kit (Dako) according to the manufacturer's instructions. Antibodies used were CD38-FITC (AT13/5 Dako), CD19-PE (HD37 Dako), CD54-PE (HA58 BD Biosciences), CD3-FITC (UCHT1 Dako), CD138-PE (DL-101 BD Biosciences). FITC conjugated rabbit antihuman IgA and IgG antibodies specific for $F(ab')_2$ (Dako) were used for intracellular immunoglobulin staining. Fluorochrome-conjugated isotype control Igs were used for all analyses. Cell analysis was performed using Cellquest software with a minimum of 10,000 events recorded for each experiment.

2.6 Protein Methods

2.6.1 Protein extraction and concentration measurement

Whole cellular protein was extracted from tissue pellets by homogenizing with an equal volume of lysate buffer. The homogenate was centrifuging at 13,000rpm at 4°C for 5 minutes and the supernatants removed and assayed for protein concentration. Protein concentration in cultured media was measured directly. Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) according to the manufacturer's instructions. Briefly, 2μl of protein extract was

added to 25μl of reagent A and 200μl of reagent B in a 96 well microtitre plate alongside a protein standard. After 15 minutes absorbance was read at 750nm on a spectrophotometer. A standard curve was plotted and the protein extract protein concentrations calculated from the standard curve equation.

2.6.2 Western blotting

Similar amounts of protein were denatured in reducing treatment buffer by heating to 70°C for ten minutes. Equal volumes of protein were then loaded onto 12 or 15 lane 10% SDS-PAGE gels (Novex Bis-Tris; Invitrogen, Paisley, UK) and run under reducing conditions at 150V for approximately 90-120 minutes. After electrophoresis, protein was electrotransferred onto nitrocellulose (BioRad) at 30mV in chilled transfer buffer for one hour. The membrane was then blocked in 5% non-fat dry milk in 0.1% Tween TBS. Membranes were incubated overnight at room temperature with the primary antibody. Membranes were washed three times for 15 minutes in 0.1% Tween TBS before the addition of the secondary antibody in 0.1% Tween TBS containing 5% non-fat dry milk for 1-3hrs. The membranes were then washed in 0.1% Tween TBS three times for i5 minutes. Reactive bands were identified using the ECL plus kit (Amersham Pharmacies, Amersham, UK). Photographic film (Amersham) was used for visualization Computer assisted scanning densitometry was used to analyse the density of the immunoreactive bands.

A sheep antihuman stromelysin-1 polyclonal antibody (1:500 dilution, The Binding Site Ltd, Birmingham, UK) and a monoclonal mouse antihuman TIMP-1 antibody (1μg/ml, CN Biosciences, Nottingham, UK) were used as primary antibodies. Rabbit anti-sheep (1 in 2500 dilution, Dako Ltd, Ely, Cambridgeshire) or rabbit anti-mouse (1 in 1000 dilution) antibodies were conjugated to horseradish peroxidase and were used as secondary antibodies. Ponceau S solution was used to reveal the uniformity of protein loading onto gels.

2.7 RNA methods

2.7.1 RNA extraction and quantification of quality and concentration

Determination of messenger RNA using Taqman realtime quantitative PCR

The Taqman technique uses primers and probes that are designed to detect a specific target region in the gene of interest. The Taqman probe is labeled with a quenching molecule and a fluorescent molecule. Cleavage of the annealed probe by Taq polymerase results in a loss of quenching, and an increase in the intensity of the fluorescent signal. Normal PCR products are formed leading to an accumulation of cleaved probe during each cycle. After each thermocycle the fluorescent signal increases and reaches a threshold (ΔRn) that is set to be the same for the gene of interest and the reference gene. The threshold cycle (C_T) is the number of PCR cycles after which there is a detectable fluorescent signal from the reaction tube.

2.7.2 RNA extraction using Stratagene Absolutely RNA® Microprep Kit

Total cellular RNA was extracted using a Stratagene Absolutely RNA kit (Stratagene, Cedar Creek, Texas, USA), according to the manufacturer's instructions. This kit is specifically designed for the isolation of RNA from samples containing a very small number of cells. Briefly, β-mercaptoethanol and lysis buffer containing guanidine thiocyanate was added to each sample immediately after isolation. Samples were homogenized following which an equal volume of 70% ethanol was added, and the RNA collected in a RNA-binding spin cup. An additional DNase treatment was then undertaken using a DNA-free™ kit (Ambion Europe Ltd, Huntingdon, UK). Three high speed washes using supplied washing buffers were undertaken to remove contaminants, after which the RNA was eluted in a microcentifuge tube by adding 20μl of RNase free water.

2.7.3 DNase treatment of RNA samples

RNA samples were treated with DNase I using a DNA-free™ kit to minimize contamination by genomic DNA. Briefly, during the extraction process RNA was

incubated for 30 minutes in a 37°C water bath with 2 units of DNase I in a 20ul volume. A DNase inactivation reagent was then added and then, following centrifugation at 13,000rpm for 1 minute, the supernatant was decanted and the RNA extraction process completed.

2.7.4 Quantification of RNA

A 5μl aliquot of each sample was diluted ten-fold and its absorbance of light at a wave-length of 260nm was measured using a Beckman DU 530 spectrophotometer (Beckman Coulter). The concentration of RNA was calculated on the basis that 1 absorbance unit at 260nm equates to 40μg/ml RNA.

2.7.5 Synthesis of complementary DNA (cDNA)

cDNA was synthesized from extracted RNA using the Improm-II RT system (Promega UK Ltd, Southampton, UK). 1μg or RNA (or maximal volume of 7.7μl) was incubated with 0.5μg of random hexamers at 70°C for 5 minutes to denature the RNA to enable cDNA synthesis. 10μl of Mastermix was added to the solutions, along with reverse transcriptase using the Improm-II RT enzyme, and the samples heated to 42°C for 1hr, followed by 15 minutes at 70°C to inactivate the enzyme. An RT reaction without RT enzyme was performed for each tissue type as a negative control for quantitative PCR. Samples were then diluted with nuclease-free water and stored at -20°C.

2.7.6 PCR reaction

All PCR reactions were performed quantitatively in an iCycler (Bio-Rad) in triplicate. RT reactions were diluted 1 in 10 in dH_2O and 5μl of template was added to 6.5μl of 2x master mix (Eurogentech, Belgium) containing 1.2 μM forward and reverse primers and 0.248 μM of probe in a total volume of 12.5 μl. The samples were heated to 50°C for 2mins, followed by 90°C for 10 minutes to activate the Hot Goldstar DNA polymerase. This was followed by 45-50 cycles of denaturing (90°C for 15s) and annealing (60°C for 1 min). The PCR probes were labelled with a 5'-reporter dye FAM (6-carboxy-

fluorescein) or yakima yellow and a 3'-quencher dye TAMRA (6-carboxy-N,N,N',N'-tetramethyl-rhodamine). Primer sequences for IL-12p40, IL-18, IL-4, IL-5, IL-10, TGF-β and IFN-γ were used. These had previously been tested for amplification efficiency prior to their use. Primers against 18S, ubiquitin, and glyceraldehyde 3-phosphatase dehyrogenase (GAPDH) were used as normalising controls. All primer sequences were designed to span exon-intron junctions and confirmed using NCBI BLAST software. Following completion of the PCR reaction, the thresholds for fluorescence emission baseline were set just above background levels on the FAM and yakima yellow dye layers. Expression levels were calculated using the $2^{-\Delta\Delta C}{}_T$ method and expressed relative to one of the specimens which was assigned the value 1.

2.7.7 Relative DNA quantification

At the end of each series of reactions an average CT value was obtained from each sample for the cytokines of interest and for the housekeeping genes. Assuming the priming efficiency of the PCR reaction approaches 100% the amount of target RNA normalized to the endogenous reference is given by the formula $2^{-\Delta\Delta C}{}_T$.

$^{\Delta\Delta C}{}_{T=}{}^{\Delta C}{}_T$ (Reference gene) - $^{\Delta C}{}_T$ (gene of interest)

Therefore, if the CT of a housekeeping gene is 10 and the CT of the gene of interest is 18

$^{\Delta\Delta C}{}_{T=}$ 10 - 18 = -8

Thus:

$2^{-\Delta\Delta C}{}_T = 2^{-(-8)} = 256$

Accordingly there is 256 fold more housekeeping signal than the gene of interest. The relative amount of normalized RNA in different samples of different targets could therefore be compared by normalizing for the presence of housekeeping genes and then choosing one sample as the denominator of the other samples.

2.8 ELISA for TNFα, TGFβ, and IgA

TNFα, TGFβ, and IgA were measured by commercial ELISA using, respectively, the Human TNFα Quantikine ELISA kit (R&D Systems Inc., Abingdon, UK), the TGFβ1 Human Biotrak ELISA system (Amersham Biosciences, Buckinghamshire, UK), and the Human IgA ELISA Quantitation kit (Bethyl Laboratories, Montgomery, Texas, USA). All were performed according to the manufacturer's instructions and read using a spectrophotometric microplate reader capable of measuring absorbance at 450nm.

2.9 ELISpot

The ELISpot assay is a simple and highly sensitive assay for analysis of cell-antibody responses at the single-cell level. Briefly, 96-well polyvinylidene difluoride (PVDF) backed plates (Millipore, Watford, England) were pre-wetted with 70% ethanol for 2 minutes then rinsed thoroughly with PBS. Following this they were coated with 15 μg/ml of anti-IgA (BD Pharmigen) or IgG antibody (Abcam, Cambridge, UK) overnight in the dark at 4°C. Plates were then washed 5 times with PBS and blocked with RPMI supplemented with 10% FCS for 1 hour. Cells of interest were added to each pre-coated well in a total volume of 100μl /well at various concentrations ranging from 200 to 50,000 cells/well. Cells were then cultured for 18hrs overnight at 37°C with 5% CO_2. Next, the plates were washed 5 times with PBS, and coated with a secondary goat anti-human IgA or IgG –ALP labeled antibody (Mabtech, Nacka Strand, Sweden) at a concentration of 0.1μg/ml in 100μl/well and incubated for a further two hours. The plates were then washed a further five times with PBS and 100μl of BCIP/NBT (5-bromo,4-chloro,3-indolylphosphate(BCIP)/nitroblue tetrazolium (NBT)) added as a substrate chromogen. The wells were then left for 15-30 minutes until the brownish/purple spots had appeared at sufficient intensity for enumeration. The reaction was stopped by washing in cold running water. All assays were performed in duplicate with appropriate negative controls. Enumeration of spot-forming units (SFUs) was undertaken following magnifying and digitally capturing each well using a dissecting microscope and a Zeiss Axiocam high resolution digital camera.

2.10 Immunofluorescent confocal laser microscopy

Cryostat sections were cut at 5μm thick from colonic tissue samples taken from subjects with CD, UC and normal controls that had previously been snap frozen at -70^{o}C within 1 hr of resection. The sections were then fixed in acetone for 20 minutes, air dried for 10 minutes and then rehydrated in TBS following which they were blocked for twenty minutes in TBS containing 1:5 horse serum. Primary antibodies for either rabbit anti-human IgG (1:400 Dako UK Ltd, Ely, Cambridgeshire) or rabbit anti-human IgA (1:400 Dako UK Ltd, Ely, Cambridgeshire) and mouse anti-human MMP3 (1:100; SL-1 IID4 Chemicon) were applied in PBS. Primary antibodies were then incubated at room temperature for 1-2 hours. The slides were then washed three times with TBS and the secondary antibody applied for a further 1-2 hours. For IgA slides this was goat anti-mouse Alexaflour 546 (1:1000; Molecular Probes, Invitrogen, Paisley, UK) 1 in1000 plus swine anti-rabbit FITC (1:30; Dako UK Ltd, Ely, Cambridgeshire) in 4% horse serum plus TBS. For IgG slides this was goat anti-mouse Alxaflour 546 (1:1000; Molecular Probes, Invitrogen, Paisley, UK) plus rabbit anti-human IgG F(ab')^2 FITC (1:100; Dako UK Ltd, Ely, Cambridgeshire) or with MMP3 alone. The slides were then washed a further three times in TBS. To stain the nuclei TOPRO-3 (1:1000; Molecular Probes, Invitrogen, Paisley, UK) nuclear stain was added in TBS for 5 minutes. The slides were then washed a further three times and mounted using Pro-Long antifade GOLD (Molecular Probes, Invitrogen, Paisley, UK). Sections were then scanned sequentially using a confocal microscope (Leica Microsystems, Wetzlar, Germany) and a series of 1μm thick images were compiled to produce 3D composites.

Chapter 3

3 The effect of thalidomide, and thalidomide analogues, on lamina propria mononuclear cell TNFα and MMP-3 production in patients with IBD

3.1 Introduction

Thalidomide belongs to a unique class of drugs with multiple immunological properties. The best known is its ability to inhibit TNFα production by blood monocytes, initially demonstrated by Sampiao et al in 1991.[251] TNFα is now widely recognized to play a central role in gut inflammation, a fact emphasized by the dramatic improvement seen in patients with CD treated with the anti-TNFα antibody, infliximab. Thalidomide has been demonstrated in several small clinical trials to be effective in the treatment of steroid-resistant CD,[254, 255] and it has been hypothesized that its effectiveness is related to its ability to inhibit TNFα production. However its mechanism of action in IBD is poorly understood. Bauditz et al demonstrated a reduction in TNFα and IL-12 in short term culture of gut LPMCs from patients with CD.[253] Conversely, however, in a larger study Prehn et al were unable to demonstrate any inhibitory effect of thalidomide on TNFα production by normal gut LPMCs.[338]

Recently, several thalidomide analogues have been developed which are reported to be up to 10,000 fold more potent that thalidomide at inhibiting monocyte TNFα production. These can be broadly split into two different classes of compounds according to their properties. One group, the IMiDs (Immunomodulatory Imide Drugs), are potent inhibitors of LPS-induced monocyte IL-1β and IL-12 production, and increase IL-10 production.[325] They are also powerful costimulators of T-cells and increase cell proliferation and TNFα production from T-cells activated with anti-CD3 antibody.[406] The second group, the SelCIDs (Selective cytokine inhibitory drugs), have little effect on IL-12, and cause a smaller rise in IL-10 production. They also have little effect on T-cell activation, and minimally inhibit T-cell proliferation.[406] They appear to be potent inhibitors of PDE4 (phosphodiesterase D4) which plays an important role in degrading cAMP. Inhibition of PDE4 has been shown to inhibit TNFα production.[407] During the

course of this study Prehn et al demonstrated that in contrast to thalidomide several SelCIDs were effective at inhibiting normal gut LPMC TNFα production.[338]

TNFα has been reported to kill epithelial cells, impair barrier function, and cause bowel necrosis.[408-410] However it appears the predominant method by which it damages the gut is by upregulating matrix metalloproteinase production by gut myofibroblasts, resulting in ECM breakdown and mucosal destruction.[289] Of these, MMP-3 is one of the most highly expressed MMPs in IBD being found in abundance in inflamed tissue in both CD and UC,[171-173,284,286] and in fistulous tracts in CD.[174]

In this chapter I have therefore investigated the effect of thalidomide and its derivatives on gut LPMC TNFα and MMP-3 production from patients with IBD.

3.2 LPMCs from patients with IBD produce large quantities of MMP-3

LPMCs from patients with CD and UC secrete significantly more MMP-3 than LPMCs from normal controls. MMP-3 by patients with IBD is predominantly in the activated forms, while in normal subjects it is predominantly in the latent forms (Figure 3.1). Time course experiments demonstrate that after 48hrs culture, MMP-3 production from cultures containing 200,000 LPMCs give optimal results (Figure 3.2). MMP-3 production can be inhibited by the addition of cycloheximide (Figure 3.3).

3.3 DMSO at 0.1% does not affect TNFα or MMP-3 production by LPMCs

Thalidomide and its analogues are very poorly water soluble. Accordingly, for in vitro experiments DMSO is commonly used a solvent vehicle. DMSO itself can mimic the properties of thalidomide and cause a reduction in activity in culture systems due to biological toxicity.[411] Consequently DMSO dose-response experiments were carried out to assess its activity on MMP-3 and TNFα production by LPMCs (Figures 3.4A and 3.4B). DMSO caused a dose dependent decrease in both MMP-3 and TNFα production by LPMCs. DMSO at a concentration of 0.1% did not significantly affect MMP-3 or TNFα production. Accordingly, in all future culture experiments serial dilutions from stock solutions of thalidomide and analogues were made so that all conditions, including controls, contained 0.1% DMSO final solution.

3.4 Thalidomide and analogues inhibit TNFα production by LPS stimulated PBMCs

Assessment of the effect of thalidomide and three analogues (IMiDs CC-5013 and CC-4047, and SelCID CC-10004) on TNFα production by peripheral blood mononuclear cells (PBMCs) from normal volunteers was undertaken. Each drug was used at a concentration of 10μg/ml, which is equivalent to the highest physiological plasma concentrations found clinically using thalidomide. Thalidomide and all three thalidomide analogues significantly reduced TNFα levels. Thalidomide resulted in a mean reduction of 17% (CI -26.6 to -7.4; p=0.02) over vehicle control (DMSO 0.1%). CC-4047 caused a

mean reduction of -71.7% (CI -93.6 to -49.8; p=0.04), CC-10004 a reduction of -81.5% (CI -87.4 to -75.6; p<0.001), compound C -64.5 (-83.7 to -45.3; p=0.004) (Figure 3.5, Table 3.1).

3.5 CC-10004 significantly reduced TNFα production by gut LPMCs

The effect of thalidomide and analogues on TNFα production by LPMCs was investigated next. LPMCs from normal patients were stimulated with PWM to induce TNFα production. PWM 1μg/ml caused an approximately 10 fold rise in TNFα production ($p < 0.01$). SelCID (CC-10004) caused a mean reduction of 63.2% (CI -51.1 to -75.2; $p < 0.001$) in TNFα production. There was no significant difference in TNFα levels between LPMCs treated with thalidomide and the two IMiDs (Compound A and C) and the vehicle control (Figure 3.6, Table 3.2).

3.6 CC-10004 inhibits the production of MMP-3 by LPMCs from patients with CD and UC.

The effect of thalidomide and analogues on the spontaneous production of MMP-3 from LPMCs of IBD patients was investigated next. The SelCID CC-10004 significantly inhibited MMP-3 production by LPMCs from patients with CD and UC. Thalidomide and the two IMiDs did not inhibit MMP-3 production by LPMCs (Figures 3.7A and B). Further studies revealed that CC-10004 significantly inhibited both LPMC MMP-3 RNA and protein production in a dose dependent manner at a concentration range of 0.1-50μg/ml (Figures 3.8 A and B). No effect on LPMC production of TIMP-1 RNA was seen (Figures 3.9 A and B).

3.7 MMP-3 production by cultured myofibroblasts is not inhibited by thalidomide or CC-10004, CC-5013, and CC-4047

Myofibroblasts are the major source of MMP-3 in dispersed mucosal LPMC suspensions. Pro-inflammatory cytokines such as TNFα can upregulate myofibroblast MMP-3 production, and this can be inhibited by a p55 TNFR fusion protein. Accordingly the

inhibition of LPMC MMP-3 production by SelCID CC-10004 may partially result from downregulation of LPMC TNFα production, or from a direct effect of CC-10004 on myofibroblasts themselves. To determine this, the effect of thalidomide and analogues on myofibroblast MMP-3 production was investigated. Myofibroblasts were cultured for 48 hrs in serum free media in the presence or absence of thalidomide or analogues at a concentration of 10μg/ml. All cultures including controls contained a final concentration of 0.1% DMSO. Myofibroblast production of MMP-3 was not inhibited by thalidomide or analogues CC-10004, CC-5013, and CC-4047 (Figure 3.10A and B).

3.8 Discussion

Two major conclusions can be drawn from the studies presented in this chapter. Firstly, thalidomide is a relatively weak inhibitor of TNFα. Though it partially inhibited TNFα production by LPS stimulated blood monocytes, it had no effect on TNFα production by gut LPMCs. Gut LPMCs are in a considerably higher state of activation than PBMCs and is likely this property contributed to the relative refractoriness of the LPMCs to TNFα inhibition by thalidomide. Furthermore thalidomide had no direct or indirect effect on LPMC MMP-3 production. These two facts taken in conjunction suggest that any beneficial effect of thalidomide in the treatment of Crohn's disease does not relate to TNFα inhibition or MMP downregulation. Two other papers investigating the effect of thalidomide on LPMCs were published while this study was ongoing. In the study by Prehn et al, thalidomide was found to have no significant effect on LPMC TNFα production in keeping with the findings reported here.[338] In contrast, Bauditz et al reported that thalidomide resulted in an approximately 60% reduction in TNFα production by PWM stimulated LPMCs.[253] However, this result may be an artifact due to the use of DMSO as a vehicle to dissolve thalidomide. As shown at the start of this chapter DMSO has a profound toxic effect, and concentrations greater than 0.1% caused a dose-related reduction in TNFα and MMP-3 production. Thus in this study, and the study by Prehn *et al*, DMSO concentration was carefully controlled, with all experiments containing no more than 0.1% by volume. However, very unusually for papers using thalidomide in in-vitro studies, no mention of DMSO is made by Bauditz *et al* and they

simply describe using different doses of thalidomide. As thalidomide is always maintained in 100% DMSO stock solution, simple sequential dilution would also result in a sequential dilution of DMSO. This could therefore account for the reported dose dependant reduction in TNFα production reported by Bauditz *et al.* Despite this, it is still feasible that thalidomide downregulates mucosal inflammation in CD via an alternative pathway related to its other reported properties, such as inhibition of angiogenesis, or downregulation of NFκB. However, it is equally possible that its effectiveness in the treatment of chronic active CD has been overstated, as no randomized trial has been undertaken, and it is well known there is a considerable placebo response in such patients. Furthermore a major component of the CDAI used to assess CD activity is stool frequency, and one of the major side-effects of thalidomide is constipation. Overall therefore, this study did not find evidence to support of the use of thalidomide in CD.

The second major finding in this study is that the SelCID CC-10004 is an effective inhibitor of both PBMC and LPMC TNFα, and LPMC MMP-3 production. This was a robust finding with a clear dose response. Though not fully proven, the results from this study suggest that, as CC-10004 had no direct effect on myofibroblast MMP-3 production, the downregulation of MMP-3 inhibition demonstrated in ex-vivo cultures is mediated through inhibition of TNFα production. This, therefore, adds to the evidence cited earlier supporting the central role of TNFα in CD pathogenesis.

The ability of CC-10004 to inhibit LPMC TNFα and MMP-3 production is very interesting as this result was in sharp contrast to the other two thalidomide analogues tested, raising questions about its mechanism of action. CC-10004 is known to be a potent phosphodiesterase-4 (PDE4) inhibitor in contrast with the two IMiDs (CC-5013 and CC-4047) which lack PDE4 activity. Thus PDE4 inhibition may play an important role in suppressing LPMC TNFα production. In support of this, the selective PDE4 inhibitor rolipram has been shown to inhibit TNFα production and abrogate inflammation in both DSS and TNBS rodent models of experimental colitis.[412, 413] However, in a small open label clinical study pentoxifylline, another PDE4 inhibitor, was not effective in the treatment of CD.[414] One potential pitfall in the therapeutic application of PDE4 inhibitors to clinical practice is their narrow therapeutic index. The use of theophylline and rolipram in human subjects has been limited by dose-related nausea and emesis. It is hypothesized

that this results from non-selective PDE inhibition leading to modified adenosine receptor signaling.[415] CC-10004 is potentially a more selective PDE4 inhibitor and is currently undergoing phase I clinical studies. If these are successful then, on the results shown here, a phase II study in the treatment of active CD would be justified.

FIGURE 3.1 MMP-3 (STROMELYSIN-1) PRODUCTION BY LPMCS FROM PATIENTS WITH CD, UC, AND NORMAL CONTROLS

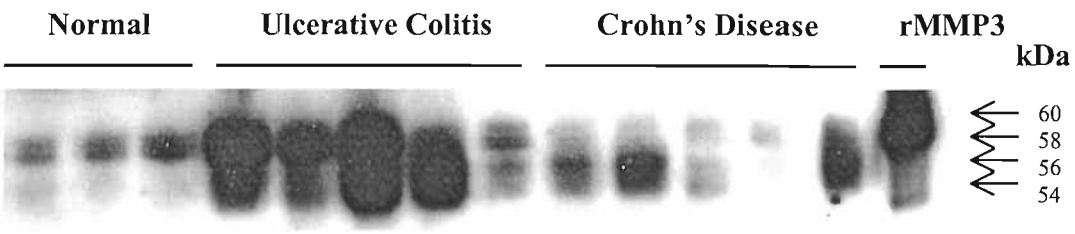


Figure 3.1 Representative western blot of conditioned media from LPMCs of patients with CD, UC, and normal controls following 48hr culture in serum free media. Total MMP-3 protein was increased in samples from patients with CD and UC compared with normal controls. Furthermore, MMP-3 in controls was primarily in the higher molecular weight latent forms (58 and 60kDa bands), while the majority of the MMP-3 in subjects with UC and CD was in the lower molecular weight activated forms (54 and 56 kDa bands). rMMP-3 is a recombinant positive control

FIGURE 3.2 EFFECT OF LENGTH OF TIME IN CULTURE ON LPMC MMP-3 EXPRESSION

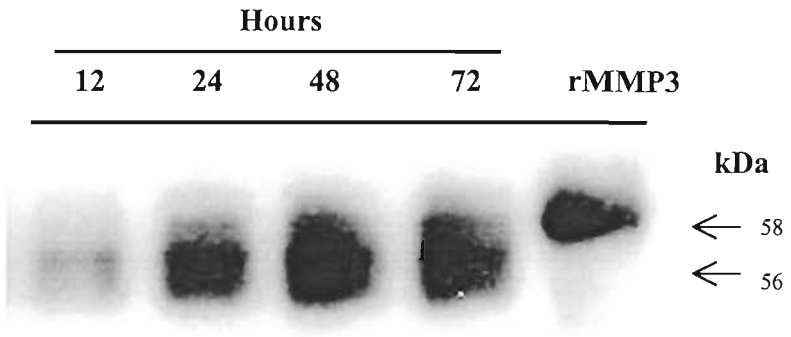


Figure 3.2 Representative western blot of conditioned media from LPMCs from a patients with UC whose LPMCs were cultured for 12, 24, 48, and 72hrs. rMMP-3 is a recombinant positive control.

FIGURE 3.3 EFFECT OF CYCLOHEXIMIDE ON LPMC MMP-3 PRODUCTION

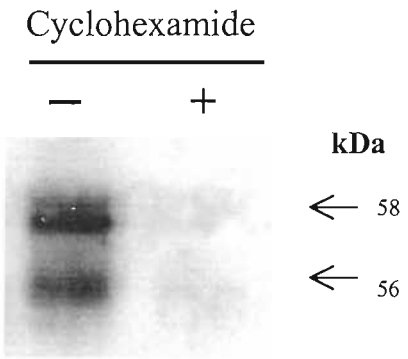


Figure 3.3 Representative western blot of conditioned media from LPMCs of a patient with UC cultured for 48hrs. The addition of cyclohexamide 10μg/ml inhibited synthesis of MMP-3.

FIGURE 3.4A EFFECT OF DMSO ON LPMC MMP-3 PRODUCTION

A

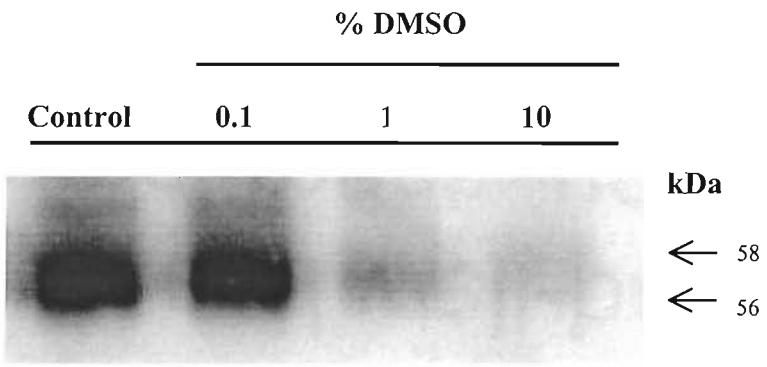


Figure 3.4A Representative western blot of conditioned media of LPMCs from a patient with UC. DMSO (0.1 to 10% concentration) caused a dose dependant reduction in MMP-3 production by IBD LPMCs. MMP-3 production by LPMCs cultured with 0.1% DMSO was not different from controls.

FIGURE 3.4B EFFECT OF DMSO ON LPMC TNFα PRODUCTION

B

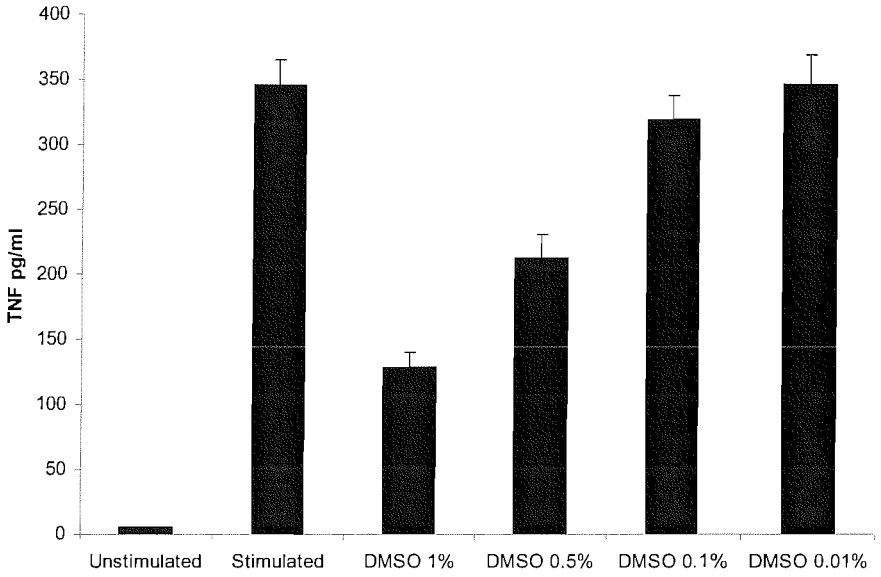


Figure 3.4B LPMCs from normal subjects were stimulated with 5μg/ml PWM and then cultured with an increasing concentration of DMSO. After 48hrs TNFα levels in conditioned media were determined by ELISA.DMSO caused a dose-dependent reduction in TNFα production by LPMCs (Data shown are the mean +/- SEM of 5 experiments).

FIGURE 3.5 EFFECT OF THALIDOMIDE AND ANALOGUES ON PBMC TNFα PRODUCTION

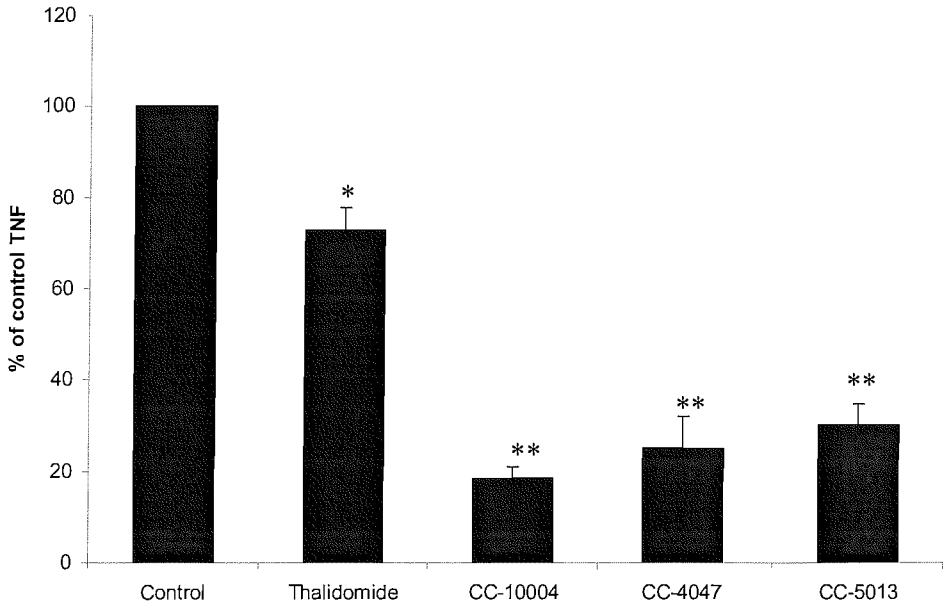


TABLE 3.1 EFFECT OF THALIDOMIDE AND ANALOGUES ON PBMC TNFα PRODUCTION

Compound	Absolute difference %	95% CI for difference	Significance
Thalidomide	-27.3	-11.0 to 43.5	P=0.013; t=5.34
CC-4047	-74.75	-53.7 to 95.7	P=0.001; t=11.34
CC-10004	-81.5	-73.8 to 89.1	P<0.001; t=33.98
CC-5013	-69.7	-54.5 to 84.9	P= 0.001; t=14.57

Figure 3.5 and Table 3.1 TNFα production by LPS stimulated BPMCs was weakly inhibited by thalidomide and strongly inhibited by SelCID CC-10004, and the IMiDs CC-4047 and CC-5013. The graph represents the percentage reduction in TNFα production compared with controls (Data shown are the mean +/- SEM, n=4; *p=0.013, **p<0.001).

FIGURE 3.6 CC-10004 SIGNIFICANTLY INHIBITS TNFA PRODUCTION BY STIMULATED LPMCS

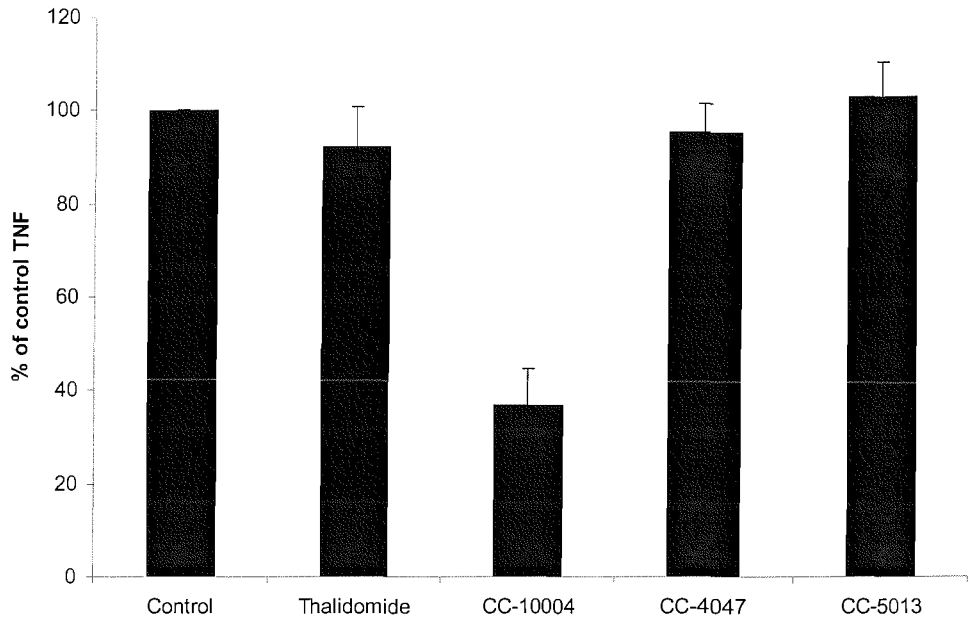


TABLE 3.2 CC-10004 SIGNIFICANTLY INHIBITS TNFα PRODUCTION BY STIMULATED LPMCs

Compound	Absolute difference %	95% CI for difference	Significance
Thalidomide	-7.5	-10.2 to 25.2	P=0.337; t=0.91
CC-4047	-4.3	-12.1 to 20.6	P=0.586; t 0.56
CC-10004	-63.2	-51.1 to -75.2	P < 0.001; t=11.2
CC-5013	3.4	-11.9 to 18.7	P= 0.644; t =-0.47

Figure 3.6 and Table 3.2 PWM activated LPMCS were cultured in the presence of 10μg/ml thalidomide and analogues for 48hrs. Analogue CC-10004 caused a significant reduction in TNFα compared with vehicle controls. TNFα levels from cultures containing thalidomide and the two IMiDs (CC-4047 and CC-5013) were not significantly different from controls (n=8).

FIGURE 3.7A INHIBITION OF LPMC MMP-3 PRODUCTION BY SELCID 10004 BUT NOT BY THALIDOMIDE OR OTHER ANALOGUES

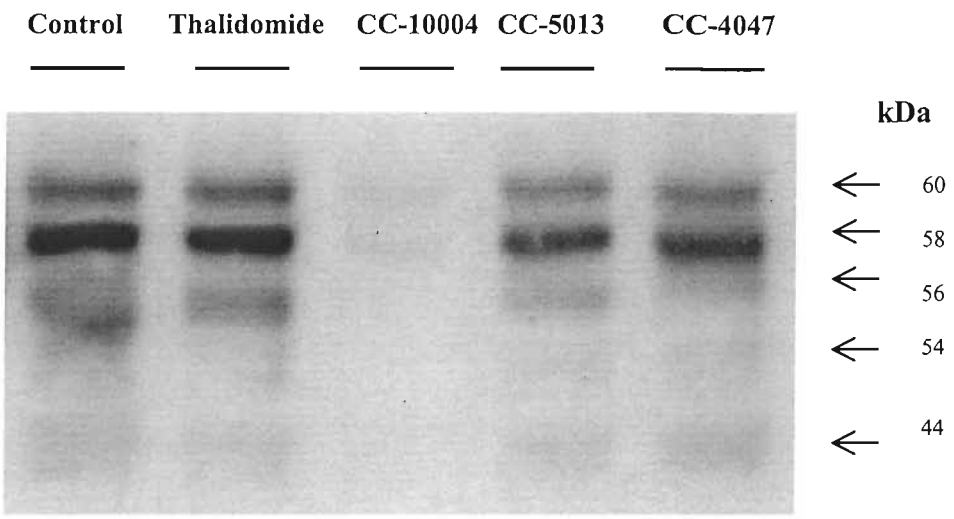


Figure 3.7A Representative western blot for MMP-3 in conditioned media from LPMCs of a patient with UC. Total MMP-3 protein was decreased in conditioned media from LPMCs cultured with CC-10004 10μg/ml compared with controls. MMP-3 production from LPMCs treated with thalidomide or analogues CC-5015 and CC-4047 (all 10μg/ml) was not different from controls.

FIGURE 3.7B QUANTITATIVE ANALYSIS OF MMP-3 PRODUCTION BY LPMCS TREATED WITH THALIDOMIDE AND ANALOGUES

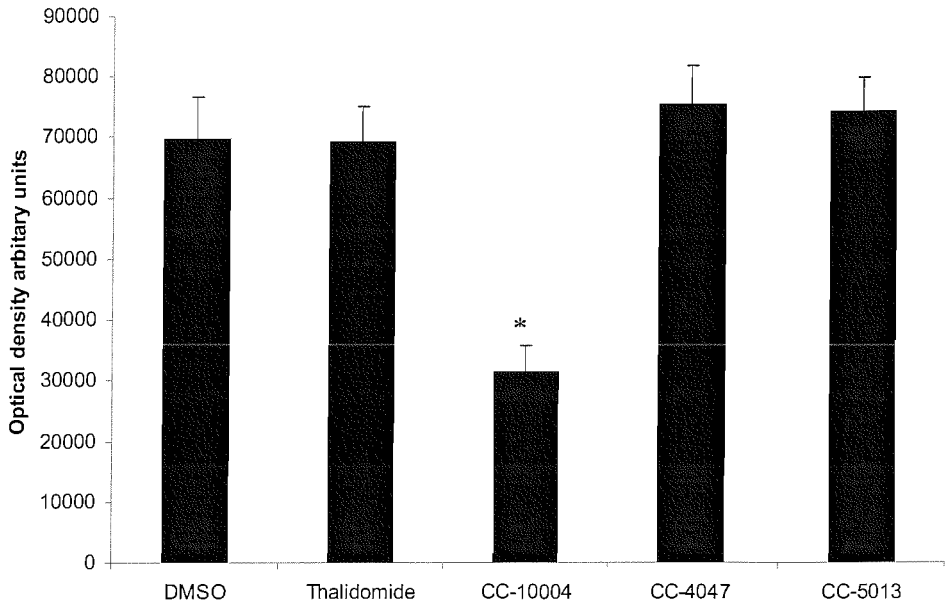
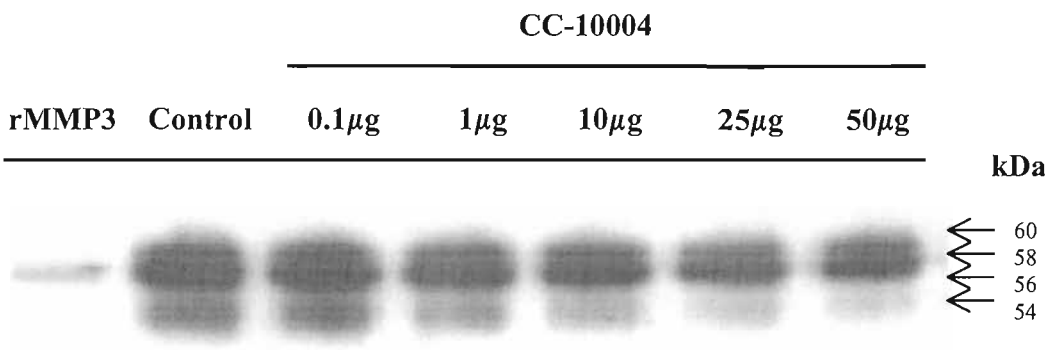


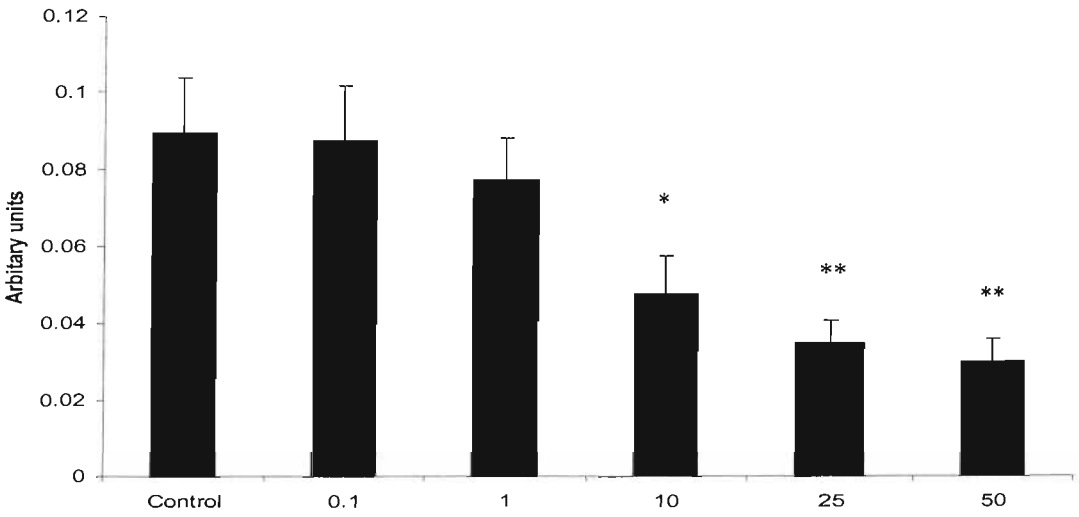
Figure 3.7B Quantitative analysis of MMP-3 protein production from LPMCs. MMP-3 production is significantly inhibited by CC-10004, but not by thalidomide or other analogues (Data shown are the mean +/- SEM; n=4; *p=0.017).

FIGURE 3.8A AND B DOSE RESPONSE OF THE EFFECT OF SELCID CC-10004 ON LPMC MMP-3 PROTEIN PRODUCTION

A



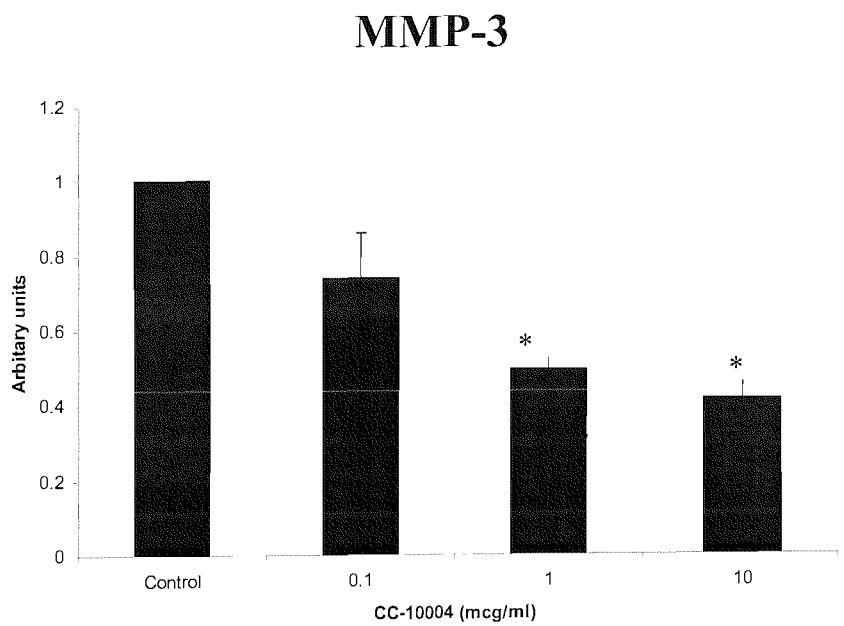
B



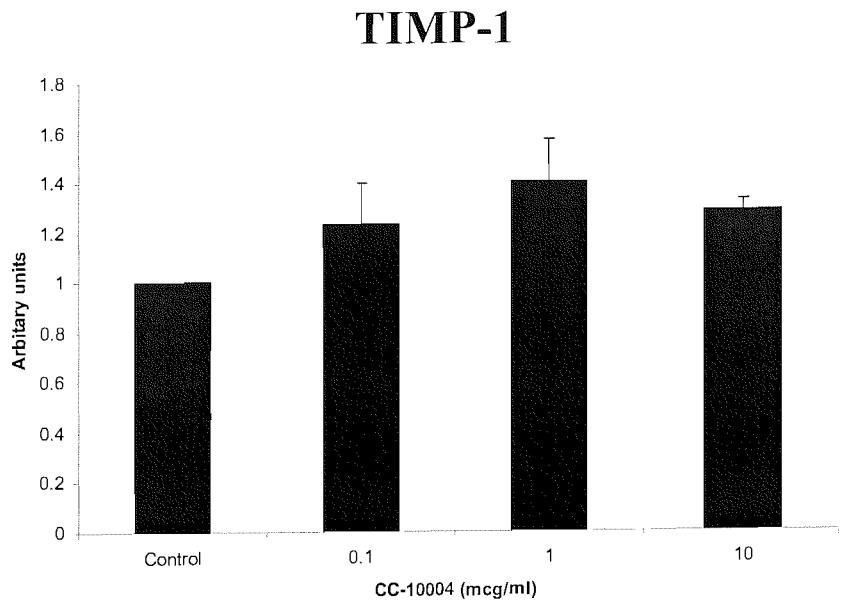
Figures 3.8A and B Representative western blot and quantitative analysis of LPMC MMP3- protein production showing dose dependent inhibition of MMP-3 production by CC-10004 (0.1-50μg/ml) (Data represented are mean +/- SEM; * p=0.02, ** P<0.01 compared with control).

FIGURE 3.9A AND B EFFECT OF SELCID CC-10004 ON LPMC MMP-3 AND TIMP-1 TRANSCRIPTS

A



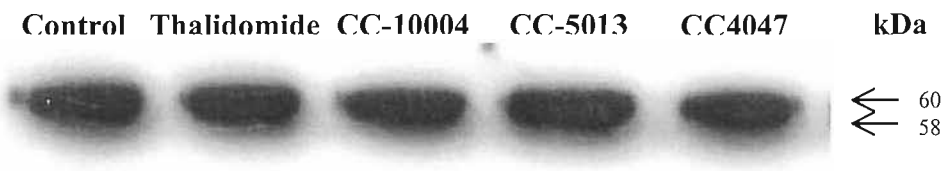
B



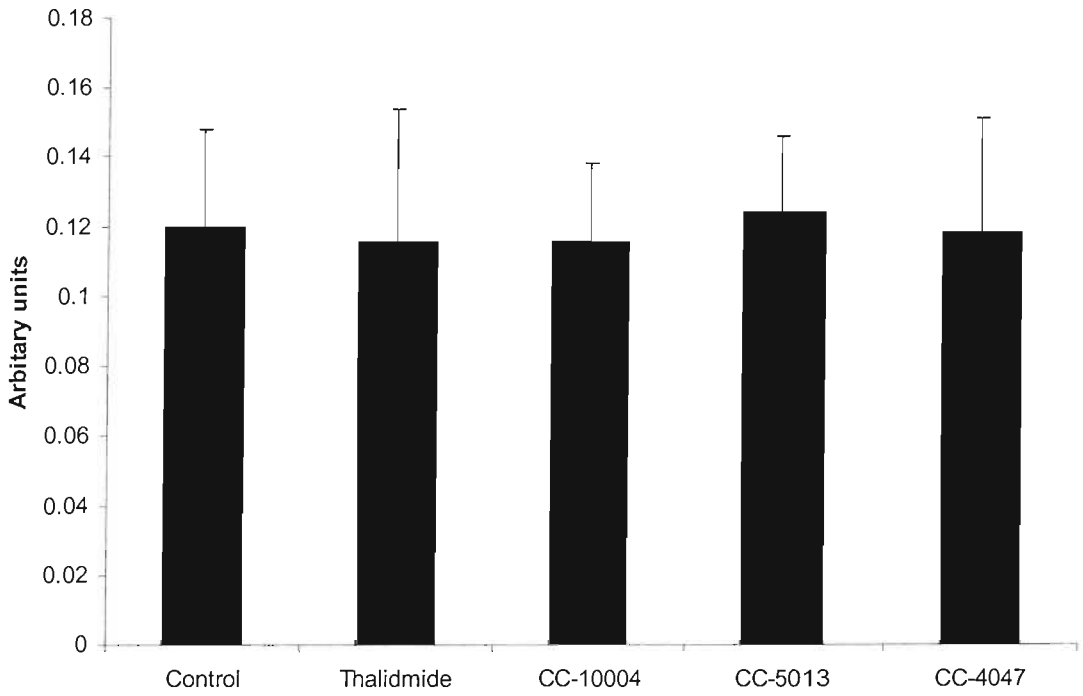
Figures 3.9A and B The addition of CC-10004 (0.1 to 10μg/ml) to short term LPMC cultures resulted in a dose-dependent reduction in MMP-3 RNA. CC-10004 had no effect on TIMP-1 RNA production (Data expressed as mean +/- SEM; n=4. *$p<0.01$).

FIGURE 3.10A AND B EFFECT OF THALIDOMIDE AND ANALOGUES CC-10004, CC-5013, AND CC-4047 ON MYOFIBROBLAST PROTEIN PRODUCTION.

A



B



Figures 3.10A and B Representative western blot and densitometry of conditioned media from myofibroblast cultured in the presence or absence of thalidomide and analogues. Thalidomide and analogues had no effect on MMP-3 protein production (Data shown mean +/- SEM; n=2).

Chapter 4

4 Isolation of functionally active plasma cells from gut lamina propria

4.1 Introduction

Terminally differentiated plasma cells represent the effector arm of the humoral immune system. Following antigenic stimulation germinal centres rapidly appear in inductive lymphoid organs such as the spleen, tonsil, lymph nodes, and Peyer's patches in the gut. In these, antigen-primed B-cells undergo differentiation to become either memory B-cells or plasma cells. Plasma blasts then migrate to specific effector sites, predominantly the bone marrow and spleen for the systemic humoral immune response, and the intestinal lamina propria for the mucosal humoral immune response. Migration is controlled by chemokines and integrins. Peyer's patch derived B-cells express the $\alpha 4\beta 7$ integrin, which by binding to its ligand, MAdCAM1 on gut vascular endothelium, directs extravasation into the gut lamina propria. IgA+ plasma blasts are also attracted by epithelial derived chemokines such as CCL25/TECK and CCL28/MEC. However despite the fact that plasma cells of the mucosal immune system are considerably more numerous and produce more antibody than their systemic counterparts, relatively little is know about their functional capability.

Research into gut mucosal plasma cells has been hampered by the absence of a reliable method for their separation and purification. Classically, investigation of specific gut cell populations is performed by isolating the cell of interest from dispersed lamina propria mononuclear cell suspensions. Gut mucosa obtained from biopsy or resection specimens is first separated from submucosal tissues and the epithelium removed, and then digested for 12-18 hrs in a solution containing collagenase or a related enzyme. The resulting cell suspension contains predominantly lymphocytes, macrophages, occasional neutrophils, plasma cells, and fibroblasts. Pure populations of specific cell types can then be separated by methods such as immunomagnetic selection or FACS sorting using specific cell surface markers. However, there is only one published paper in the literature detailing a method of purifying plasma cells from gut tissue. In this paper CD54 (ICAM-1) was used as a cell surface marker to isolate functionally active plasma cells by immunomagnetic

selection.[416] Despite this being somewhat counter-intuitive, given that ICAM-1 has previously been reported to be expressed on a wide variety of cell types, we used this as a starting point to try and separate out a pure and functionally active population of plasma cells.

4.2 Plasma cell markers

During plasma cell differentiation some B-cell surface markers are progressively lost while other markers are up regulated. The B-cell marker CD20 is rapidly lost, followed more slowly by CD19. During this period there is a correspondingly rapid increase in expression of CD38 so that all human plasma cells express high levels of CD38 ($CD38^{high}$).[417] Thus the gut plasma cell population containing both maturing and fully differentiated plasma cells can be identified by $CD38^{high}/CD19^{+/-}$ surface expression. Human CD38 is a 45 kDa type II transmembrane glycoprotein that has emerged as a multifunctional protein in recent years.[418] It has a discontinuous pattern of expression on leucocytes, being strongly expressed on plasma cells and weakly expressed on other cell types such as activated B-cells, T-cells and monocytes. It is involved in the regulation of cell life-span and migration, through control of lymphocyte adhesion to endothelial cells via interaction with the ligand CD31, a member of the Ig superfamily.[419] In addition, it catalyzes the synthesis and hydrolysis of cyclic ADP, a Ca2+ mobilizing agent that acts independently of inositol triphosphate. Other reported functions include growth stimulation, induction and prevention of apoptosis, induction of cytokines, activation of kinases, and phosphorylation of certain proteins.[420]

All mature plasma cells also express CD138 (Syndecan-1), which is not expressed by any other peripheral blood or bone marrow leucocyte. Anti-CD138 can therefore also be used for as a plasma cell marker.[421] CD138 is a member of a family of transmembrane heparan sulfate proteoglycans. Heparin suphate proteoglycans can bind cell-surface and matrix proteins, and can thus modulate various biological processes such as inflammatory cell maturation and activation, and leucocyte rolling, adhesion, and extravasation.[422] CD138 binds extracellular matrix components and fibroblast growth factors (FGFs) and can modify their function.[423] It is strongly expressed by all plasma cells though not by any

other haematopoietic cell in bone marrow or peripheral blood. In tissue it is constitutively expressed at low levels by epithelial cells and, during growth and regeneration, can be inducibly expressed in fibroblasts by growth factors such as FGF-2, and in keratinocytes by epidermal growth factor (EGF) and keratinocyte growth factor (KGF).

CD54 (ICAM-1) is a cell surface glycoprotein expressed on multiple cell types including leucocytes, epithelial cells, endothelial cells, and fibroblasts.[424] It is a member of the immunoglobulin superfamily of adhesion molecules and is centrally involved in leukocyte trafficking and in T-cell/APC interactions. In the gut it is abundantly expressed on endothelial cells where it plays a crucial role in migration and extravasation of leucocytes through interaction with the ligands LFA-1 and Mac-1, primarily expressed on T-cells and neutrophils respectively.[425] Its expression is upregulated by pro-inflammatory cytokines such as TNFα, and bacterial products. In view of this, anti-sense oligonucleotides to ICAM-1 have been used therapeutically in inflammatory disorders such as ulcerative colitis where excess leucocyte trafficking occurs.[272] In contrast, gut epithelial cells do not constitutively express ICAM-1 though its expression can also be induced by pro-inflammatory cytokines.[424] Interestingly, following its induction in epithelial cells it is only expressed on the apical surface,[426] suggesting that here it may play a role in communicating with luminal antigens, rather than leucocyte trafficking. Additionally, there is also evidence from in-vitro studies that suggest ICAM-1 may be involved in epithelial cell antigen presentation to T-cells. Antibodies to ICAM-1 can block superantigen induced T cell activation in an MHC class-II deficient epithelial cell system, and in another study mouse M cells that only express low levels of HLA-DR develop antigen presenting capacity following the introduction of endogenous ICAM-1.[427, 428] Thus, upregulation of ICAM-1 can result in both leucocyte recruitment, and increased antigen presentation in areas of inflammation. ICAM-1 is also known to be expressed on other lamina propria cell types. In normal colon, 7% of lamina propria cells express ICAM-1, rising to 46% and 69% in active CD and UC respectively.[429] In a more recent study using immunoelectron microscopy gut plasma cells and macrophages from patients with UC expressed high levels of ICAM-1 whereas no expression was detected on lamina propria lymphocytes or granulocytes.[430]

4.3 Immunomagnetic isolation of plasma cells

The first aim of this study was to isolate a purified population of functional plasma cells from the gut mucosa. Medina et al reported that immunomagnetic positive selection of CD54+ cells from dispersed LPMCs resulted in a cell fraction that consisted almost entirely of $CD38^{high}$ cells (94.4 (1)%; mean (SEM); n=4), and could be identified on the basis of morphological appearance and intracellular IgA as plasma cells.[416] Accordingly, this method was replicated exactly using fresh specimens of normal colonic tissue in initial attempts to isolate PCs. The presence of plasma cells in the dispersed cell fractions was analysed by means of staining by flow cytometric analysis. The $CD38^{high}CD19^{+/-}$ fraction was taken to represent the total plasma cell fraction.

4.4 Immunomagnetic selection of CD54+ cells resulted in variable enrichment of the plasma cell population.

$CD38^{high}CD19^{+/-}$ cells accounted for 27.4 ± 9.9% of initial dispersed lamina propria mononuclear cell suspensions (n=8) (Figure 4.1, Table 4.1). Immunomagnetic selection with a monoclonal anti-CD54 antibody enriched the proportion of plasma cells as identified by the $CD38^{high}CD19^{+/-}$ fraction using flow cytometry. The average yield following enrichment was 3.4 x 10^6 (95% CI 1.8-5.1), with a purity of 64% (95% CI 40.5 – 87.4), (n=5) (Figure 4.2, Table 4.2). However the average purity of 64% was not as high as expected and not sufficient for detailed analysis of plasma cells without possible contamination by other cell types. The relatively low average purity resulted from the very wide variation in degree of plasma cell enrichment between subjects.

4.5 CD54 expression on LPMCs is variable and is not confined to the plasma cell compartment

To investigate the wide variability in plasma cell purity following CD54+ immunomagnetic selection, CD54 and CD38 co-expression was examined. On average 73% (range: 61% – 89%) of $CD38^+$ plasma cells expressed CD54, whilst 32% (range: 14% - 59%) of $CD54^+$ cells did not express CD38. Thus in normal LPMCs, contrary to Medina *et al's* data, CD54 was not exclusively expressed on the plasma cell fraction, but

was expressed on average on 13% of CD38-ve LPMCs (Table 4.3). Furthermore 28% of plasma cells did not express CD54. Considerable inter-sample variation in CD54 expression was seen, probably reflecting that the fact that adhesion molecule expression in the gut is a dynamic process.

The percentage of $CD54^{+}/CD38^{-}$ cells in individual LPMC suspensions was then examined in relation to subsequent plasma cell purity achieved by CD54 immunomagnetic selection. As expected the percentage of $CD54^{+}/CD38^{-}$ cells was found to inversely correlate with subsequent plasma cell purity (Figure 4.3). Taken together these results indicated that CD54 was not specific for gut plasma cells, and that expression on other cell types resulted in the low purity of plasma cell achieved when using CD54 as an immunomagnetic marker.

4.6 Alternative markers for immunomagnetic selection of plasma cells

In view of the poor results obtained using an anti-CD54 antibody to purify plasma cells, alternative markers plasma cell markers were investigated. CD138 has been successfully used for the immunomagnetic selection of a highly purified plasma cell population from both blood and bone marrow.[431, 432] However, there is no published data relating to its use in purifying gut plasma cells. One probable reason for this is that CD138 is extremely sensitivity to proteolysis. Accordingly the method of LPMC digestion was modified to see if CD138 could be used to isolate gut plasma cells.

An alternative marker that could be used for immunomagnetic selection is the plasma cell marker CD38 as it is highly expressed on all plasma cells. Again there is no published data on the isolation of gut plasma cells using this marker. As it is also present at low levels on activated T-cells, a two stage process of plasma cell isolation was devised. LPMCs were initially depleted of CD3 +ve cells, after which plasma cells were isolated from the negative effluent using an anti-CD38 antibody.

4.7 CD138 can be detected on LPMCs following short enzymatic digestion, but is rapidly lost with more prolonged collagenase digestion

Following conventional 18hr overnight collagenase digestion, CD138 was not detectable in LPMC suspensions. In view of this the LPMC isolation protocol was modified to use more intensive mechanical tissue disruption, with a considerably shorter period of collagenase digestion. Time course experiments revealed that following a 10-15 minute period of mechanical agitation and digestion, liberated LPMCs still expressed CD138 (Figures 4.4A and B). However, this was rapidly lost with more prolonged periods of digestion. Significant inter-sample variation was also present with CD138 occasionally being undetectable even after 10 minutes of digestion.

4.8 Immunomagnetic selection using CD138 (Syndecan-1) results in a highly purified population of plasma cells

LPMCs were obtained using a modified protocol. This involved a shorter total period of washing in HBSS and EDTA with more frequent (10minute) changes of solution. This was followed by vigorous mechanical disruption with a syringe and 14G blunt needle, and then a 10-15 minute period of enzymatic digestion with further intermittent mechanical disruption. The resulting LPMC suspension was incubated with 20ul of anti-CD138 coated microbeads per 10^7 LPMCs. The cell suspension was then separated using two consecutive cell runs on LS columns. The positively isolated cell fraction represented the plasma cell population.

Immunomagnetic selection using CD138 microbeads resulted in a mean purity of 94.5% (95% C.I. 92.1 – 96.9%; SEM 0.96; n=8) $CD39^{high}/CD19^{+/-}$ cells (Table 4.4, Figures 4.7 and 4.8).

4.9 Immunomagnetic selection using CD3 negative depletion followed by CD38 postitive enrichment

In an attempt to improve the cell yield of plasma cells, an alternative immunomagnetic selection protocol was devised using a two-stage process. The dispersed LPMC population was initially incubated with CD3 microbeads to remove the T-cell fraction.

The negative effluent was then collected and incubated with anti-CD38 mAb (10μg/ml) for 15 minutes at 4°C in the dark. After washing the cells were then incubated with goat anti-mouse magnetic microbeads. The cell suspension was then separated using two consecutive cell runs on LS columns. The positively isolated cell fraction represented the plasma cell population.

Immunomagnetic selection using this two stage selection process resulted in a mean purity of 80.7% (95% C.I. 68.1 – 93.4%; SEM 3.96; n=4) $CD38^{high}/CD19^{+/-}$ cells (Table 4.5, Figure 4.9).

4.10 Confirmation of plasma cell purity and correspondence with $CD38^{high}/CD19^{+/-}$ cell fraction

To confirm the $CD38^{high}/CD19^{+/-}$ cell fraction did represent the plasma cell fraction the enriched cell fractions were cyto-centrifuged onto slides and stained for intracellular IgA. Plasma cells were identified by both typical morphological appearance and the presence of intracellular IgA. CD138 microbead selection resulted in the purest plasma cell population while the other two methods of selection yielded significantly inferior results, confirming the data obtained from flow cytometric analysis (Figures 4.10A and B).

4.11 Confirmation of functional activity of enriched plasma cell fraction

To confirm that cells in the enriched plasma cell fraction were viable and functionally active, purified plasma cells were cultured for two, five, and seven days in vitro. Cell free supernatants were then collected following culture and IgA secretion was tested by enzyme linked immunoabsorbent assay. The enriched plasma cell population secreted significantly more IgA at all time points than the initial non-enriched LPMC population from the same subjects. IgA was actively secreted for at least 7 days and was inhibited by the addition of cycloheximide (Figure 4.11).

4.12 Discussion

Research into gut plasma cell biology has been hampered by the lack of a quick and effective method of isolation a pure and functionally active population of these cells. To date there is only one report in the literature of an effective method of purifying gut plasma cells.[416] In this paper immunomagnetic selection using an antibody directed against the adhesion molecule CD54 (ICAM-1) was reported to result in a highly purified (94.4 ± 1.1%; mean (SEM); n=4) plasma cell population. However our experience gave conflicting results with only moderate and very variable plasma cell enrichment. Further investigation revealed this to be due to variable expression of CD54 on other lamina propria leucocytes effectively negating this as a useful method of plasma cell purification.

Other methods of purifying gut plasma cells were subsequently investigated. CD138 is a specific plasma cell marker that has previously been used to purify plasma cells from blood and bone-marrow. However, the sensitive nature of the antigen and it rapid shedding during collagenase digestion has previously been a barrier to its use in isolating gut plasma cells. To surmount this, a modified protocol was developed to extract LPMCs from tissue following which cell surface CD138 expression could still be identified by flow cytometery. Immunomagnetic selection of gut plasma cells was then undertaken using CD138 microbeads. This resulted in a very highly purified plasma cell population, (94.5%; 95% C.I. 92.1 – 96.9%) and was easily reproducible. However a limitation of this process was the low yield of plasma cells (0.76×10^6 PC from ~ 50×10^6 LPMC). To address this, a second method of plasma cell isolation was devised. This involved a two-step process. An initial CD3 depletion step was undertaken as in addition to being highly expressed on plasma cells, low level CD38 expression can be found on activated T-cells. Following this positive immunomagnetic selection through CD38 was undertaken. This again resulted in a highly purified plasma cell population (80.7%; 95% C.I. 68.1 – 93.4%). Though the purity was not as high as that seen with selection through CD138, the yield was considerably greater with a mean of 2.28×10^6 cells. Confirmation that the $CD38^{high}/CD19^{+/-}$ cell fraction did represent plasma cells was obtained by assessment of the typical morphological appearance of the cells in conjunction with strong intracellular IgA staining which is not seen in other cell types.

Functional capability of the cells was also confirmed by in-vitro IgA secretion. These two methods therefore both represent effect methods of isolating gut plasma cells. As the purity was significantly better following CD138 microbead selection this method was used for all future experiments. Though CD3 depletion followed by CD38 positive selection did result in an enriched cell population the purity was not sufficient for use in future experiments.

An interesting separate finding from this set of experiments was that isolated plasma cells continued to secrete IgA for at least a week in ex-vivo culture experiments. This suggested that some gut plasma cells might be longer lived than previously realised. Though in recent years some evidence has accumulated that suggests gut plasma cells may be long lived, the classical viewpoint is that there is a high turnover of plasma cells in the lamina propria with individual cells only surviving for a few days. The fact that in these experiments plasma cells were functional for at least one week raised the question whether some gut plasma cells were capable of persisting for longer periods of time. This question is addressed in the next chapter.

FIGURE 4.1 CD19 AND CD38 EXPRESSION ON DISPERSED LPMCS FROM NORMAL COLON

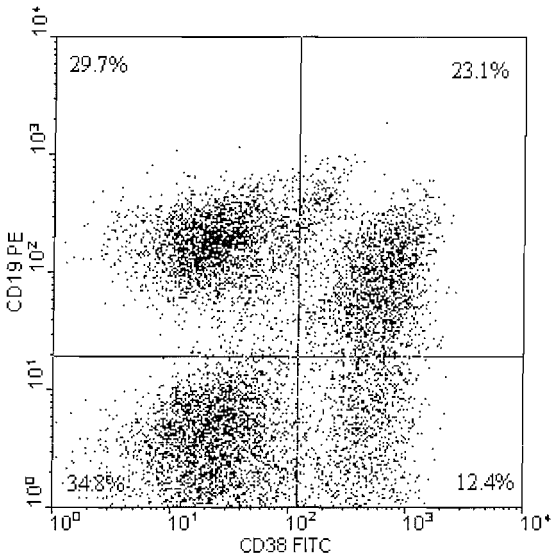


TABLE 4.1 CD19 AND CD38 POSITIVE CELL FRACTIONS AS PERCENTAGE OF TOTAL LPMCS

Cell fraction	Mean ± SEM (%)
$CD38^{-}/CD19^{-}$	52.5 ± 4.7
$CD19^{+}/CD38^{+}$	21.8 ± 4.3
$CD38^{high}/CD19^{+}$	15.2 ± 2.0
$CD38^{high}/CD19^{-}$	10.3 ± 1.4
Total PC ($CD38^{high}/CD19^{+/-}$)	25.6 ± 3.1

Figure 4.1 shows a representative dot-blot of CD19 and CD38 expression on dispersed LPMCs. CD38 staining is along the x-axis and CD19 staining along the y-axis. Table 4.1 shows the proportion of $CD38^{high}CD19^{+/-}$ plasma cells as a percentage of the total LPMC population (data shown are the mean and SEM; n=8).

FIGURE 4.2 PLASMA CELL ENRICHMENT FOLLOWING IMMUNOMAGNETIC SELECTION USING AN ANTI-CD54 ANTIBODY

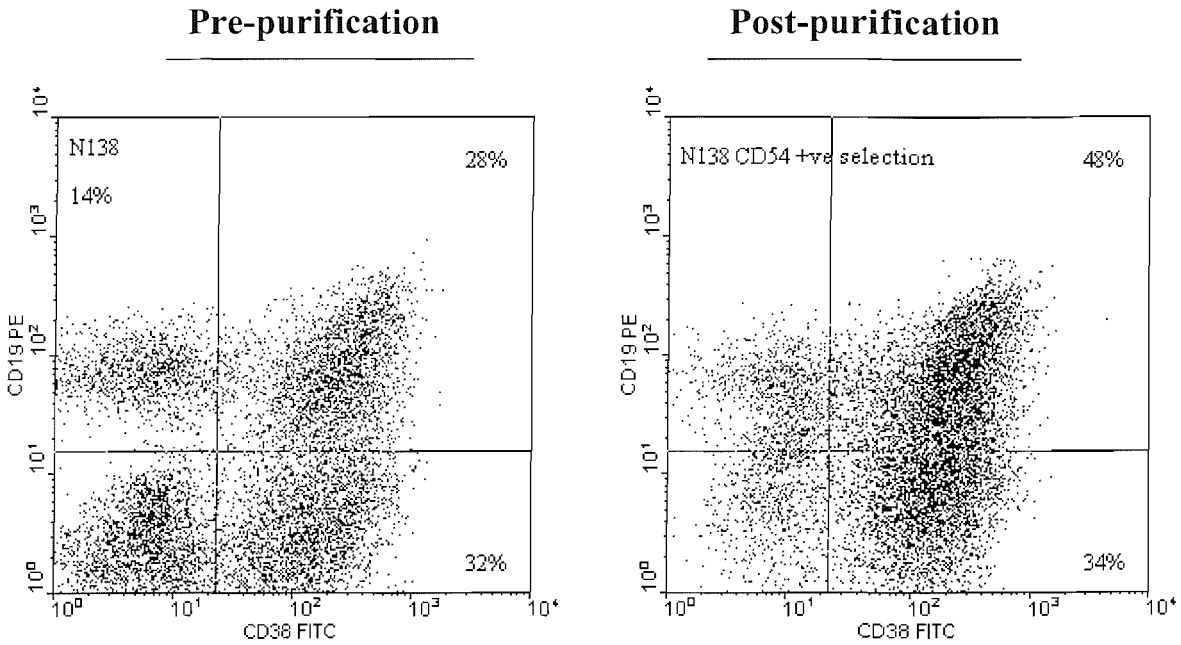


TABLE 4.2 AVERAGE PLASMA CELL PURITY FOLLOWING IMMUNOMAGNETIC SELECTION USING AN ANTI-CD54 ANTIBODY

Cell fraction	Mean % (95% CI)	Average Yield (95% CI)
$CD38^{high}/CD19^{+/-}$	64.0% (40.5 – 87.4)	3.4×10^6 (1.8 – 5.1)

Figure 4.2 shows a representative flow cytometric analysis of CD19 and CD38 expression on dispersed LPMCs before (left graph) and after (right graph) immunomagnetic selection using anti-CD54 antibody. The plasma cell population is enriched from 60% to 82%. Table 4.2 shows the average plasma cell purity and yield following anti-CD54 immunomagnetic selection(Data shown are the mean and 95% confidence intervals, and yield and 95% confidence intervals; n=4).

TABLE 4.3 CD54 AND CD38 POSITIVE CELL FRACTIONS AS PERCENTAGE OF TOTAL LPMCS

Cell fraction	Mean ± SEM (%)	StD
$CD38^{-}/CD54^{+}$	13.0 ± 6.1	12.1
$CD38^{high}/CD54^{+}$	22.7 ± 4.7	9.4
$CD38^{high}/CD54^{-}$	8.9 ± 4.4	8.9

Table 4.3 Proportion of $CD38^{+}$ and $CD54^{+}$ plasma cells as a percentage of the total LPMC population. This demonstrates that on average 36% $CD54^{+}$ cells are not plasma cells, and that 28% of plasma cells do not express CD54 (Data shown are the mean and SEM; n=4).

FIGURE 4.3 CD54 AND CD38 POSITIVE CELL FRACTIONS AS PERCENTAGE OF TOTAL LPMCS

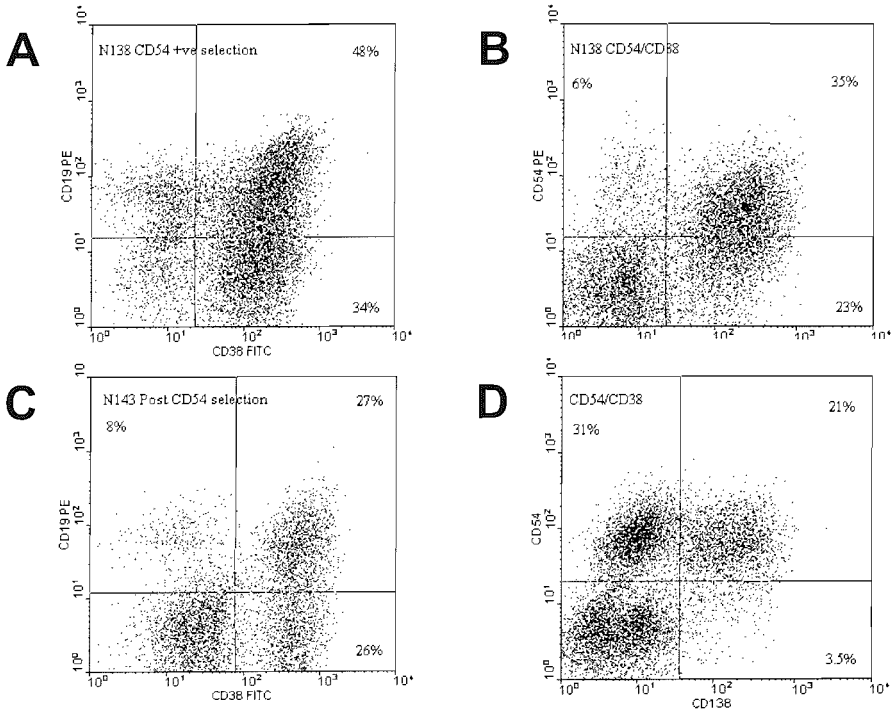


Figure 4.3 Representative dot-blots correlating plasma cell purity following immunomagnetic selection with the proportion of $CD54^{+}/CD38^{-}$ cells in the initial LPMC suspension. In graph A plasma cell purity was 82%, whilst the corresponding percentage of $CD54^{+}CD38^{-}$ cells was 6% (graph B). In graph C the plasma cell purity was 53% whilst the corresponding percentage of $CD54^{+}CD38^{-}$ cells was 31% (graph D).

FIGURE 4.4A AND B TIME COURSE OF THE EFFECT OF TIME ON ENZYMATIC DIGESTION ON CD138 EXPRESSION ON LPMCS

Figure A

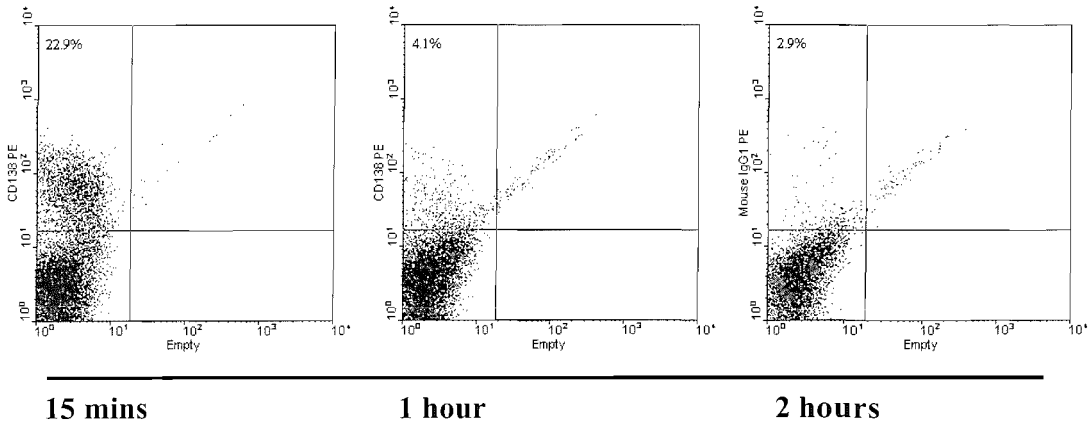


Figure B

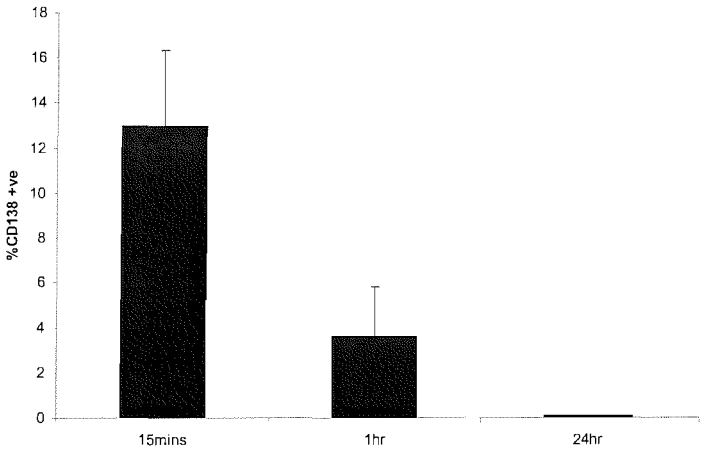


Figure 4.4A and B. Figure A shows a representative series of dot-blots showing CD138 staining clearly visible at 15minutes, but virtually absent at 1hr. Figure B shows the percentage of LPMCs +ve for CD138 rapidly decreases with length of enzymatic digestion (Data shown are mean and SEM; n=5).

TABLE 4.4 AVERAGE PLASMA CELL PURITY FOLLOWING IMMUNOMAGNETIC SELECTION USING CD138 MICROBEADS

Cell fraction	Mean % (95% CI)	Average Yield (95% CI)
$CD38^{+}/CD19^{+/-}$	94.5% (92.1 – 96.9)	0.76×10^{6} (0.23 – 1.3)

Table 4.4 Average plasma cell purity and yield following plasma cell enrichment with CD138 microbeads (Data shown are the mean and 95% confidence intervals, and yield and 95% confidence intervals; n=8).

FIGURE 4.7 PLASMA CELL ENRICHMENT FOLLOWING IMMUNOMAGNETIC SELECTION USING CD138 MICROBEADS

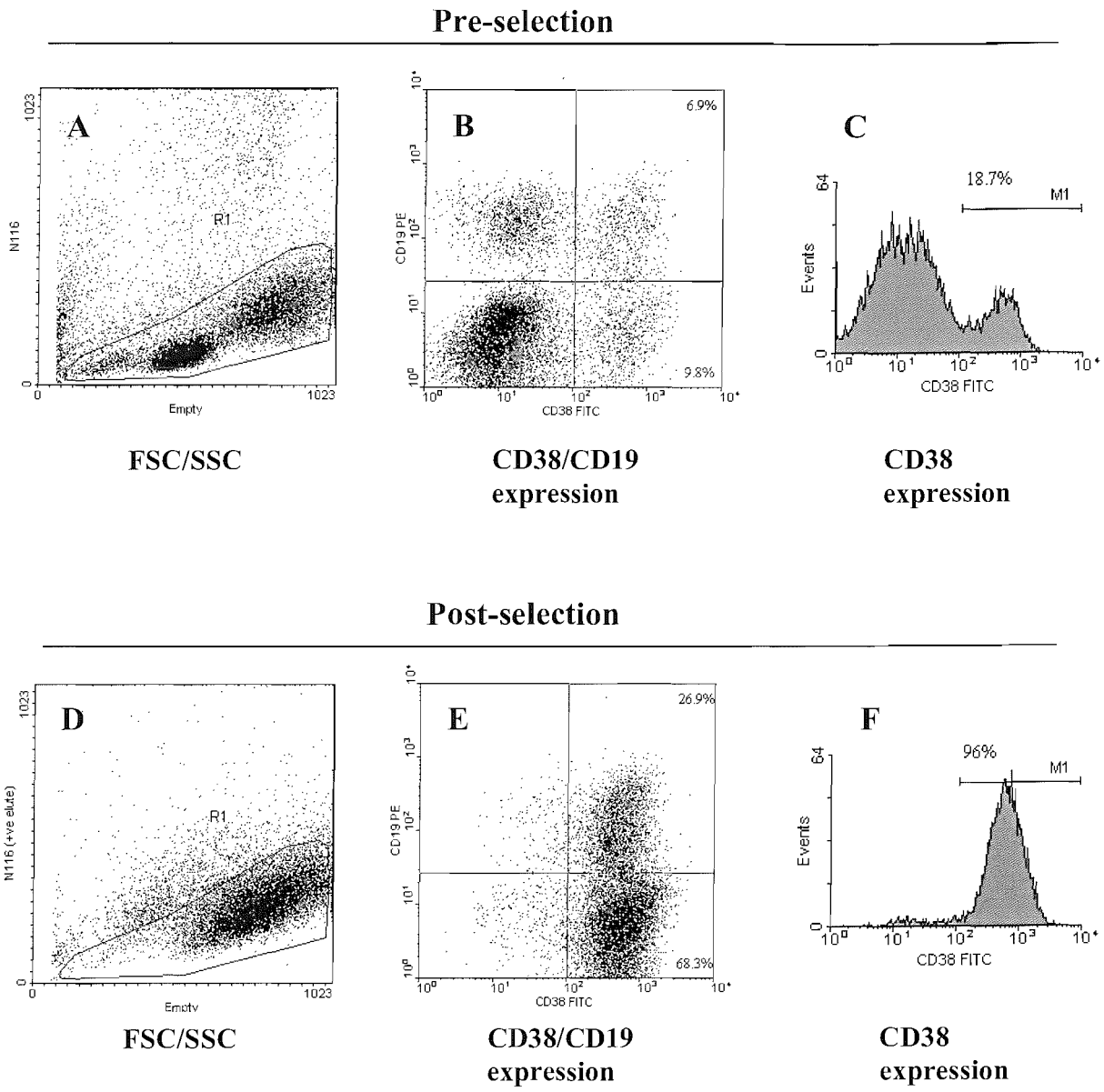
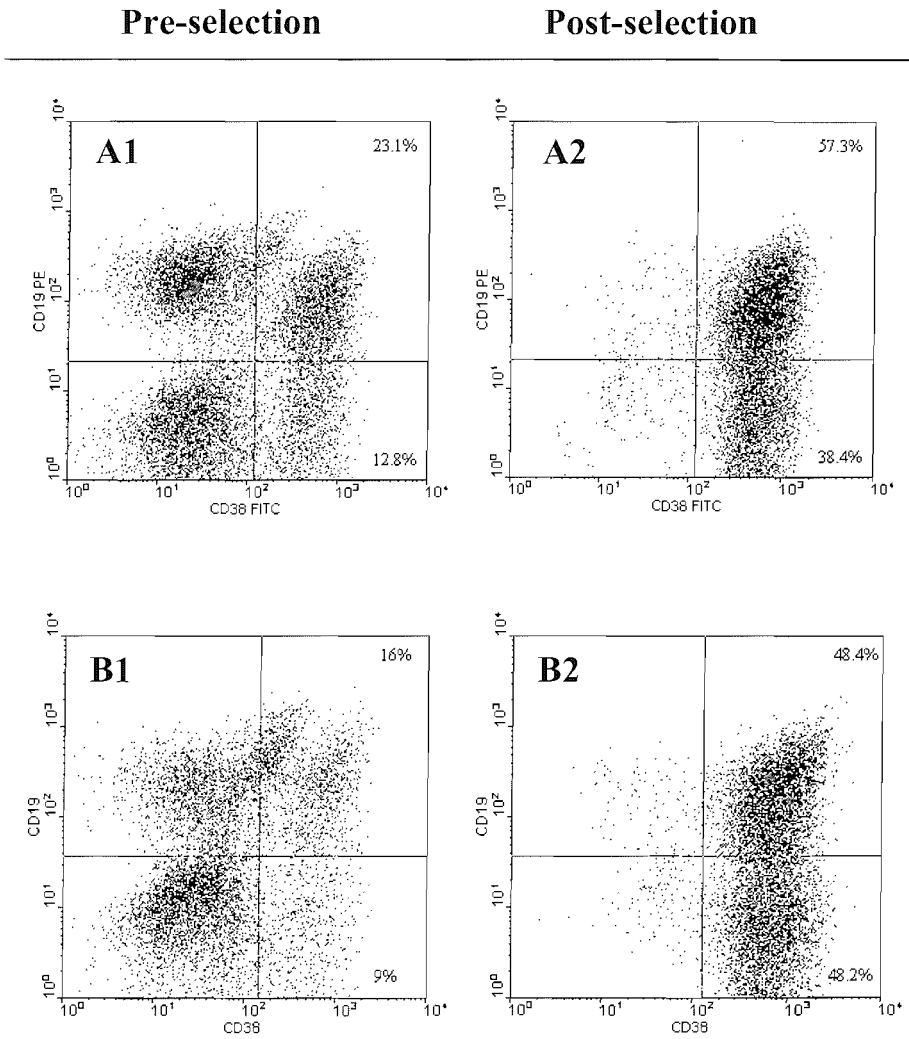


Figure 4.7 shows a representative series of dot-blots from one complete experiment. Graphs A and D show the total cell population defined by forward and sideways scatter before and after plasma cell enrichment using CD138 microbeads. Graphs B and E show the same cell population stained with anti-CD19 and CD38 antibodies to define the plasma cell population. Histograms C and F indicate the total proportion of $CD38^{+}$ve cells before and after enrichment for plasma cells

FIGURE 4.8 PLASMA CELL ENRICHMENT FOLLOWING IMMUNMAGNETIC SELECTION USING CD138 MICROBEADS



Figures 4.8 shows representative FACS analysis of CD38/CD19 staining in two further individual experiments. Panels A1 and B1 shown the pre-selection cell populations and panels A2 and B2 show the post selection cell populations.

TABLE 4.5 PLASMA CELL PURITY AFTER IMMUNOMAGNETIC SELECTION USING CD3 DEPLETION FOLLOWED BY CD38 ENRICHMENT

Cell fraction	Mean % (95% CI)	Average Yield (95% CI)
$CD38^{+}/CD19^{+/-}$	80.7% (68.1 – 93.4)	2.28×10^{6} (0.57 – 3.9)

Table 4.5 shows the average plasma cell purity and yield following plasma cell enrichment using CD3 depletion followed by CD38 enrichment (Data shown are the mean and 95% confidence intervals, and yield and 95% confidence intervals; n=4).

FIGURE 4.9 PLASMA CELL ENRICHMENT AFTER IMMUNOMAGNETIC SELECTION USING CD3 DEPLETION FOLLOWED BY CD38 ENRICHMENT

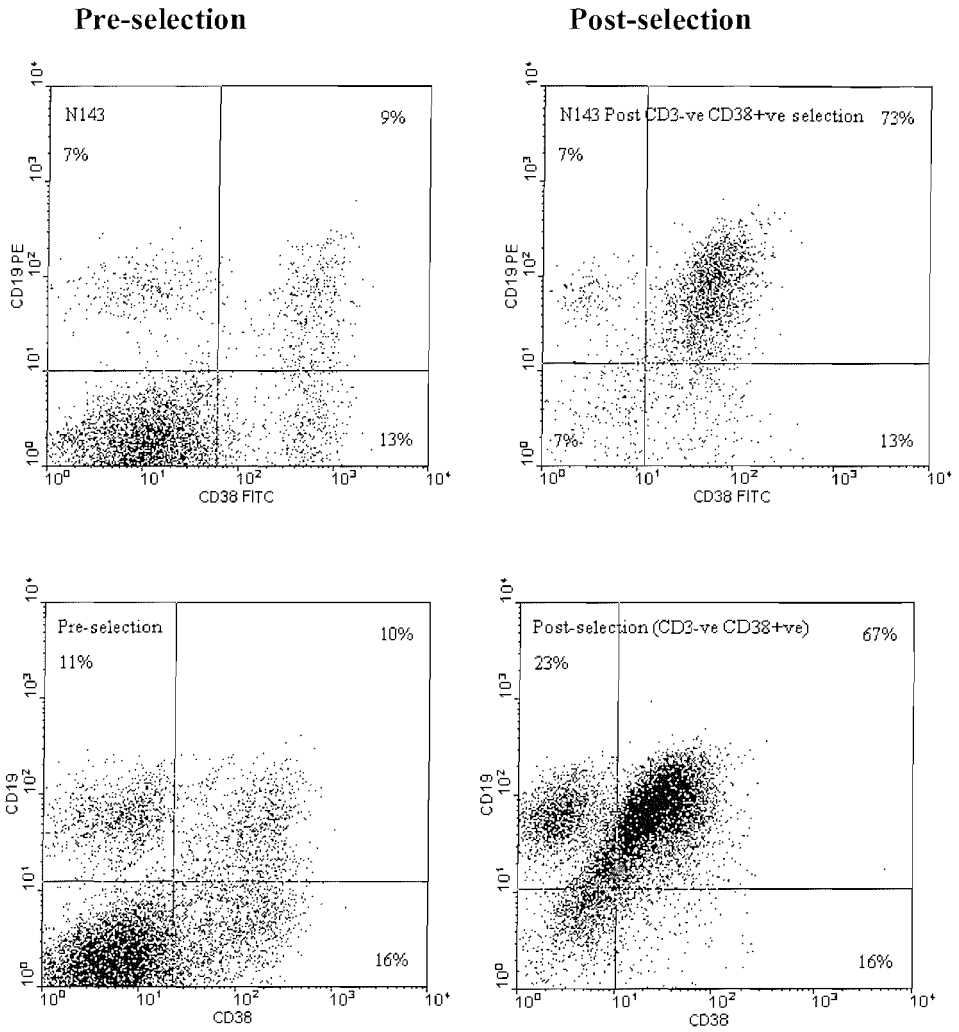


Figure 4.9 Representative flow cytometry analysis from two separate experiments showing plasma cell enrichment from after CD3 depletion step followed by CD38 enrichment.

FIGURE 4.10A INTRACELLULAR IGA^{+} STAINING OF PLASMA CELLS IN NON-ENRICHED DISPERSED LPMC FRACTION

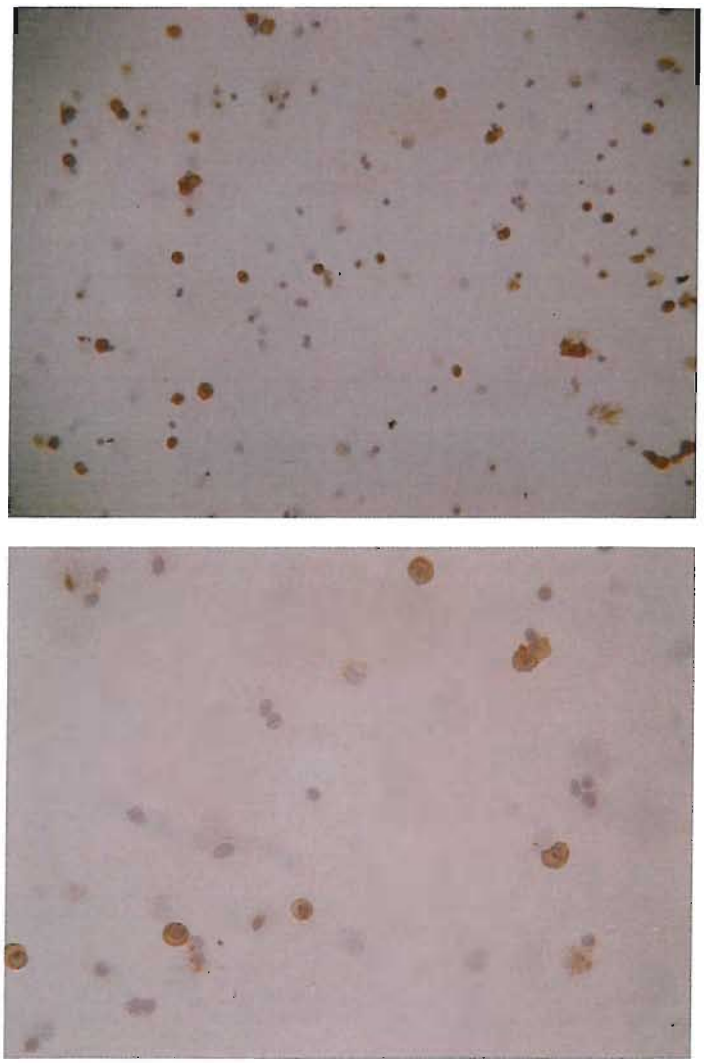


Figure 4.10A Representative cytospin of dispersed gut LPMCs. Plasma cells are be identified by their typical large appearance with eccentric clock face nuclei and brown intracellular IgA staining. They represent 27.2% ± 6.7% of the pre selection lamina propria mononuclear cell population.

FIGURE 4.10B INTRACELLULAR IGA^{+} STAINING OF ENRICHED PLASMA CELL FRACTION FOLLOWING IMMUNOMAGNETIC SELECTION USING CD138 MICROBEADS

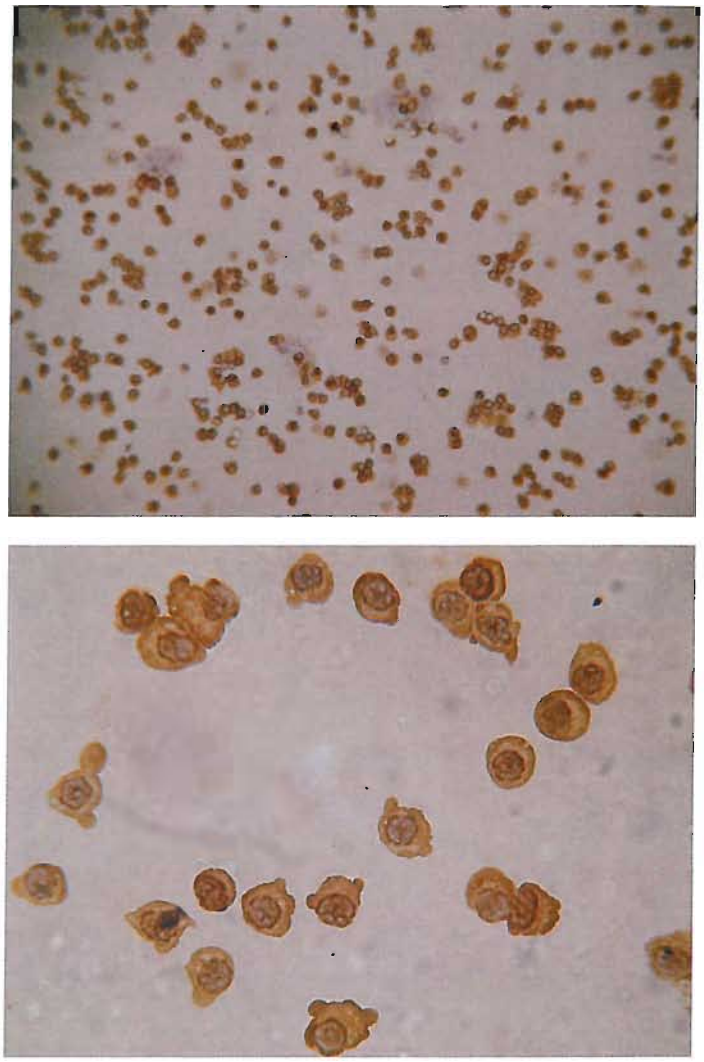


Figure 4.10B Representative cytospin of dispersed gut LPMCs. Plasma cells are be identified by their typical large appearance with eccentric clock face nuclei and brown intracellular IgA staining. They represent 88.2% ± 4.7% of the pre selection lamina propria mononuclear cell population.

FIGURE 4.11 IN VITRO IGA SECRETION BY CD138 ENRICHED PLASMA CELL AND NON-ENRICHED LPMC FRACTIONS

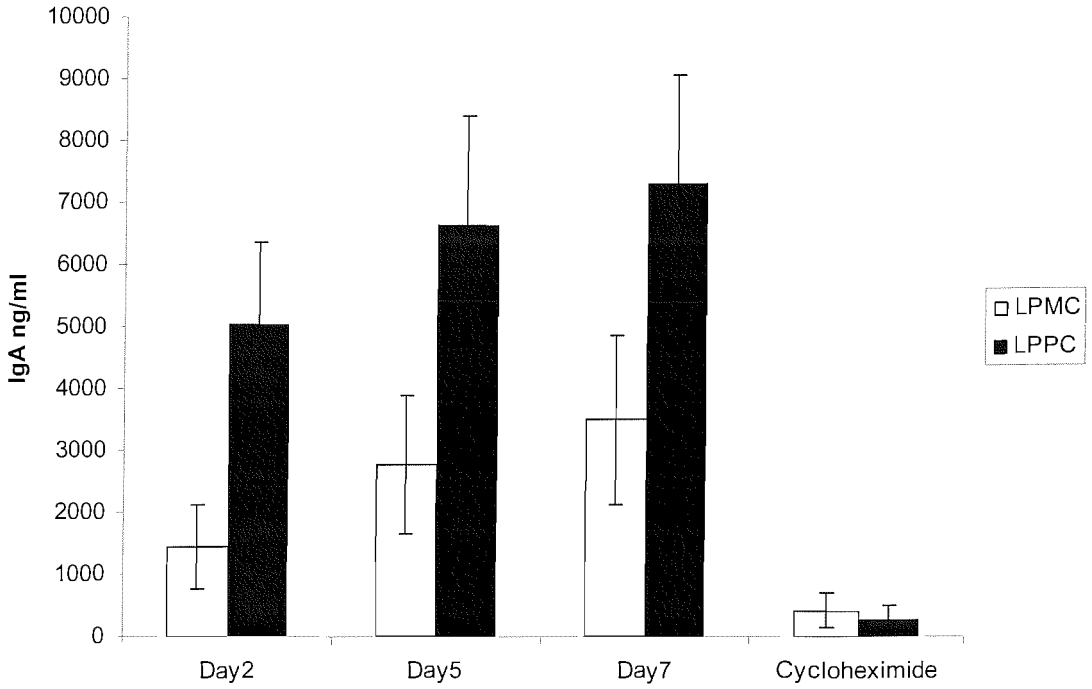


Figure 4.11 Spontaneous IgA production from enriched lamina propria plasma cell (LPPC) fraction compared with that from the initial non-enriched LPMC fraction. Supernatant IgA was measured by ELISA and results expressed in ng/ml (Data shown are the mean and SEM; n=5).

Chapter 5

5 Gut plasma cell lifespan ex-vivo

5.1 Introduction

Long-lasting protective antibody titres against antigens, such as polio and tetanus, can be found in human sera for decades following infection or immunization. These long-term antibody responses have classically been thought to be maintained by the continuous differentiation of memory B-cells into antibody secreting cells.[433] This is based on the premise that plasma cells are short-lived and need to be continually replenished. However, in recent years it has become apparent that long-lived plasma cells reside in specific niche areas such as the bone-marrow and spleen, and that these provide a source of long-lasting high-affinity antibody.[369, 434] These plasma cells, which no longer react to antigen or antigen-antibody complexes have been found to survive for months to years and are not sensitive to irradiation or to cell-division inhibitors.[435] Their survival appears dependant on a complex molecular microenvironment termed the plasma cell survival niche.

There is increasing evidence that it is the cytokine milieu and direct cell-cell interaction in these survival niches that is responsible for plasma cell longevity, rather than an intrinsic property of the plasma cell. Ex vivo, bone marrow plasma cells that are long-lived *in vivo* die within 3 days *in vitro* if cultured in standard medium. Their survival can be prolonged by the addition of various cytokines such as IL-5, IL-6, and TNFα.[370] However, survival cannot be sustained for much over 5 days by the addition of cytokines, which is in stark contrast to the estimated PC lifespan of over six months in vivo. This suggests that other survival factors including direct cell-cell interaction may also be necessary for optimal survival. In support of this concept it has been demonstrated that isolated tonsillar plasma cells undergo apoptosis *in vitro* if not rescued by stromal cells.[371] Plasma cells are also found in large numbers at sites of inflammation, which may provide the correct microenvironment to act as survival niches. In the lupus prone NZW/B mouse, the inflamed kidneys support plasma cells in numbers comparable to the bone marrow.[436]

The gut lamina propria with its large plasma cell population is likely to represent another plasma cell survival niche. However, in contrast to systemic plasma cells, very little is known with regard to gut plasma cell lifespan. In mice treated with tritiated thymidine at birth, IgA gut plasma cells appear to have a half-life of 4.7 days.[437] The life-span of gut IgG plasma cells is unknown. In humans IgA gut plasma cells have been shown to actively secrete IgA for up to two weeks in ex-vivo cultures.[416] This is in keeping with bone marrow derived IgG plasma cells which can secrete IgG for up to two weeks, whereas blood and tonsillar IgG plasma cells can only secrete IgG for 2-3 days.[438]

In the following experiments I have investigated the lifespan of gut plasma cells. This was performed using antibody specific ELISpots. LPMCs isolated from subjects with inflammatory bowel disease and normal controls were cultured overnight on ELISpot membranes to investigate proportion of IgA and IgG antibody secreting cells Captured cells were visualized and enumerated using BCIP/NBT. Each spot-forming unit (SFU) represents one antibody secreting cell.

5.2 Frequency of IgG antibody secreting cells is increased in inflammatory bowel disease

IgA antibody secreting cells from normal and IBD subjects comprised 14.2% and 19.6% of the total LPMC population respectively. IgG antibody secreting cells from normal and IBD subjects comprised 1.3% and 7.3% of the total LPMC population respectively. These figures correlated with cell frequency determined by FACS and immuno-histochemistry (Figures 5.1-5.3).

5.3 IgG secreting plasma cells persist in inflammatory bowel disease

LPMCs from patients with IBD and normal controls were grown in short term-cultures for up to 3 weeks. LPMCs were sampled on a weekly basis and then cultured overnight in isotype specific ELISpots to determine the relative proportion of IgA and IgG plasma cells surviving in culture. IgA secreting plasma cells disappeared rapidly from both IBD and normal cell culture, with very few surviving by 2-3 weeks. However IgG secreting plasma cells from subjects with IBD persisted in culture for at least 3 week (Figures 5.4-5.6).

5.4 Discussion

The experiments in this chapter clearly demonstrate that IgG secreting plasma cells in patients with IBD survive for considerably longer in ex-vivo cell cultures than IgA secreting plasma cells. Furthermore, the fact that a considerable number of plasma cells are still actively secreting IgG after 3 week in culture suggests that these may represent a population of long-lived plasma cells. Thus, it appears that the gut lamina propria can act as a survival niche for gut plasma cells, in a similar manner to the bone marrow. The discrepancy between IgA and IgG antibody secreting cells lifespan suggest that there is likely to also be intrinsic differences between plasma cell isotypes that contribute to the determination of lifespan. These are novel findings that have not previously been reported.

The only published work on plasma cell lifespan relates to bone marrow and spleen IgG antibody producing cells. It is now recognised that the bone marrow microenvironment supports plasma cell survival, and that a long-lived (>6 months) population of plasma cells exists that contributes to immunological memory. Isolated bone marrow plasma cells die rapidly in culture with none recovered at 1 week. In contrast, when non-processed total bone-marrow is cultured approximately 70% of plasma cells are still viable at 1 week.[374] Our results suggest that this may also the case in the gut in inflammatory bowel disease. It appears likely that the inflammatory cytokine and cell milieu in the lamina propria of subjects with active IBD provides signals that prevent IgG

antibody secreting cells from undergoing apoptosis. In support of this, a failure of lamina propria T-cell apoptosis is thought to be one of the mechanisms that result in persistent gut inflammation in Crohn's disease.[439] Further experiments will be required to determine if either individual or combinations of cytokines can prolong gut plasma cell lifespan. It is also possible that the continuing translocation of organisms from the gut flora across the disrupted epithelial surface in IBD may contribute to plasma cells survival by preventing them from undergoing apoptosis. CpG DNA motifs have been shown to activate autoreactive B-cells through BCR cross-linking and stimulation of TLR-9 dependent signalling pathways.[440, 441] Accordingly, it would be pertinent to investigate cell surface expression of toll-like receptors by plasma cells, which has not previously been reported, as their expression would enable plasma cells to respond directly to bacterial components.

Interestingly, there was a suggestion from the ELISpot data that IgG secreting plasma cells from normal subjects could also survive ex-vivo for at least 2 weeks. The data obtained from these experiments was limited however as the frequency of IgG secreting plasma cells was 10-100 fold less in normal gut lamina propria compared with IBD lamina propria. This resulted in very low cell counts with a high degree of variability. In addition, it was not possible to culture normal unselected gut LPMCs beyond two weeks meaning that data on more prolonged normal plasma cell survival could not be obtained. That LPMCs in general did not survive well may have been due to a combination of lower total cell numbers, lower state of cell activation, and a lack of cytokine signalling to prevent apoptosis in normal cell cultures. Therefore additional experiments would need to be done to confirm the observation whether IgG plasma cells also persist in the normal gut.

The differential in lifespan between IgA and IgG plasma cells suggest that though the gut may be acting as a survival niche, intrinsic difference between plasma cells must also exist that contribute to longevity. This has not previously been reported and what these factors are remains to be determined. The expression of pro- and anti-apoptotic proteins such as BAX and Bcl-2 in gut plasma cells has not been well established. Gut plasma

cells express relatively low levels of CD95 and are not sensitive to Fas-mediated apoptosis,[416] in keeping with bone marrow derived plasma cells, and in contrast to more immature tonsillar plasma cells, which express CD95 and undergo Fas-mediated apoptosis.[442] More recently, in an immunohistochemical study of synovium from subjects with rheumatoid arthritis, IgG+ve plasma cells have been shown to stain strongly for the anti-apoptotic protein Bcl-x(L).[443]

There is also an increasing body of evidence that suggest plasma cell apoptosis may be controlled by unique molecular pathways rather than by death receptors or mitochondria. Activation of Bax in the endoplasmic reticulum membrane and subsequent activation of endoplasmic reticulum-associated caspase-4 has been shown to be a key component of programmed plasma cell death.[444] This means that immunoglobulin secretion may be the cardinal mechanism by which plasma cell death is controlled. When antibody production becomes maximal, proteasomal activity decreases leading to an accumulation of polyubiquitinated proteins and the stabilization of endogenous proteasomal substrates such as IκBα and Bax leading to the induction of apoptosis.[445] It is therefore feasible that differences in immunoglobulin secretion kinetics could also account for differences in isotype survival.

Thus, it remains to be elucidated what determines differential gut plasma cell survival. In particular it is unclear whether it is the microenvironment, differences in levels of pro- and anti-apoptotic molecules, or novel apoptotic pathways that result in the differential plasma cell survival seen both between isotypes and between normal subjects and those with IBD.

FIGURE 5.1 PLASMA CELL FREQUENCY BY ISOTYPE IN DISPERSED LPMC POPULATIONS FROM NORMAL AND IBD SUBJECTS

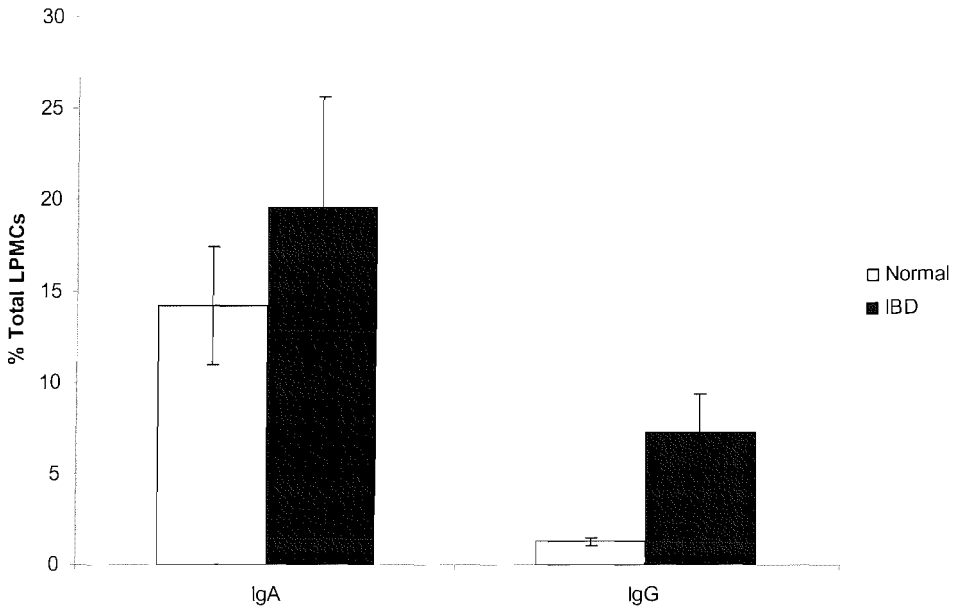
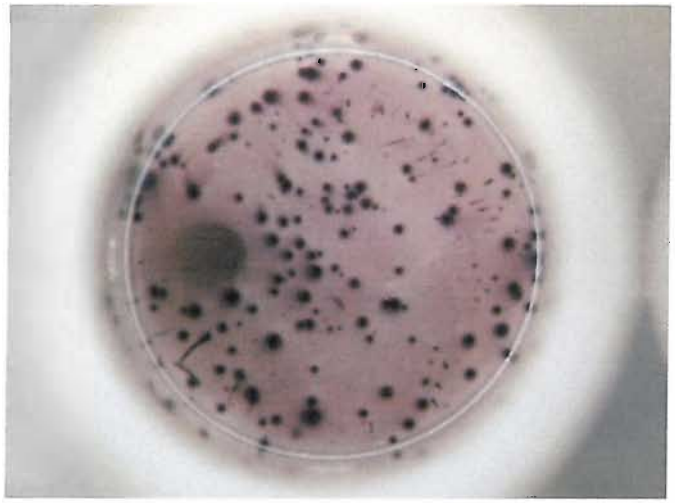


Figure 5.1 IgA and IgG plasma cell frequency expressed as percentage of total LPMC number. The percentage of IgA+ve PCs in IBD subjects was not significantly different from normal controls (19.6% v 14.2%; p=0.23). The percentage of IgG+ve PCs in IBD subjects was significantly greater than in normal controls (7.3% v 1.3%; p=0.034). (Data expressed mean ± SEM; n=3)

FIGURE 5.2 IGA PLASMA CELLS IN DISPERSED LPMC POPULATIONS FROM NORMAL AND IBD SUBJECTS

A



B

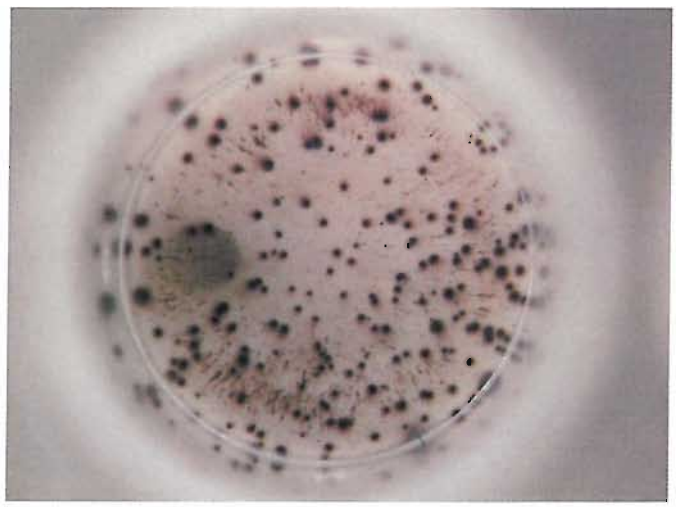
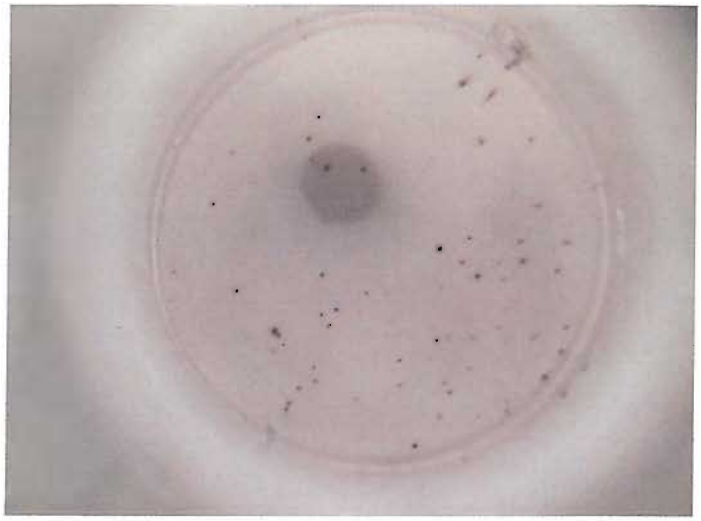


Figure 5.2 Representative ELISpot membranes demonstrating IgA plasma cell SFUs/1000 LPMCs. Figure A is from a normal subject, while Figure B is from a subject with ulcerative colitis (1000LPMCs/well; x10 magnification).

FIGURE 5.3 IGG PLASMA CELLS IN DISPERSED LPMC POPULATIONS FROM NORMAL AND IBD SUBJECTS

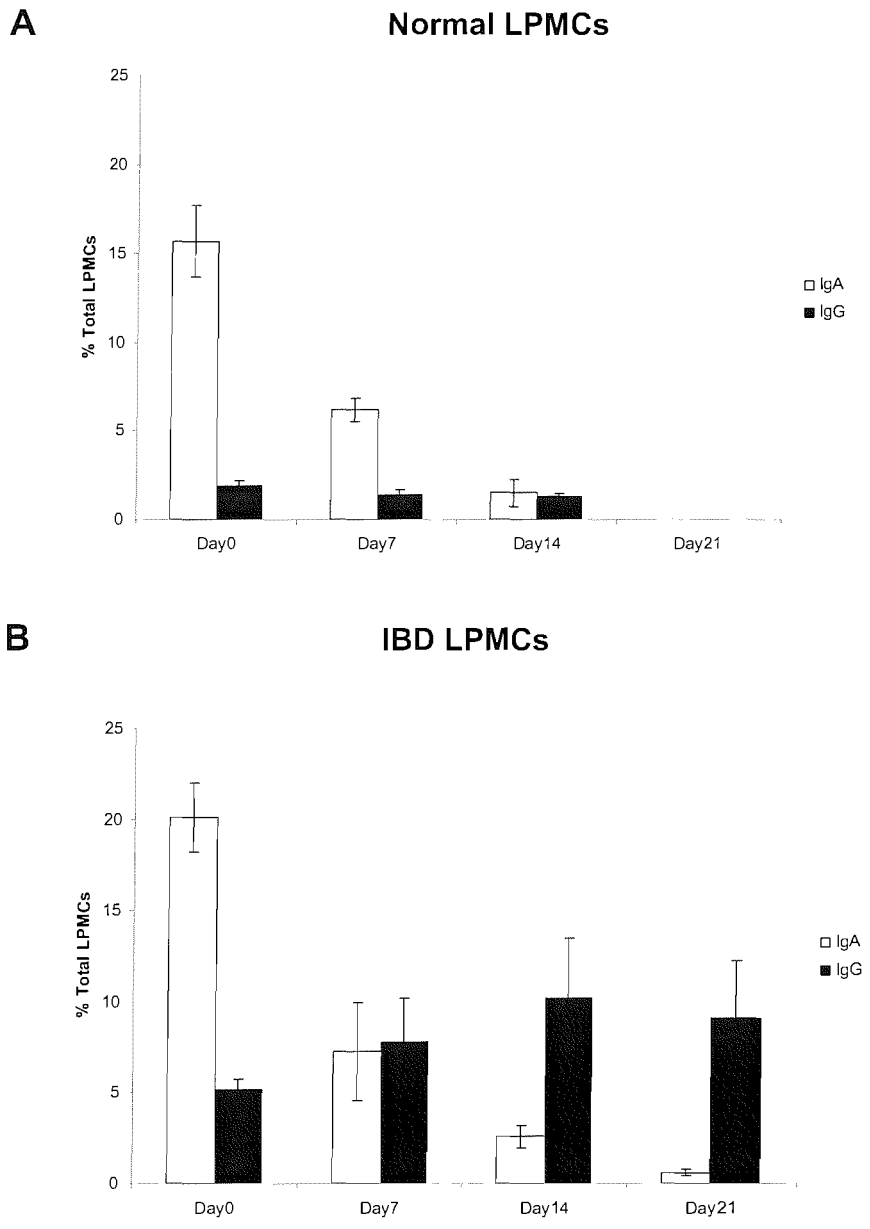
A



B

Figure 5.3 Representative ELISpot membranes demonstrating IgG plasma cell SFUs/1000 LPMCs. Figure A is from a normal subject, while Figure B is from a subject with ulcerative colitis (1000LPMCs/well x10 magnification).

FIGURE 5.4 PLASMA CELL FREQUENCY BY ISOTYPE IN DISPERSED IBD AND NORMAL LPMC POPULATIONS OVER 3 WEEKS EX-VIVO SHORT-TERM CULTURE



Figures 5.4 Panels A and B demonstrate the relative frequency of IgA and IgG secreting plasma cell in LPMCs from normal and IBD subjects respectively over a three week time period (Data shown are the mean and SEM; n=4 for each value).

FIGURE 5.5 IGA PLASMA CELL FREQUENCY IN CULTURED IBD LPMCS OVER THREE WEEKS SHORT-TERM CULTURE

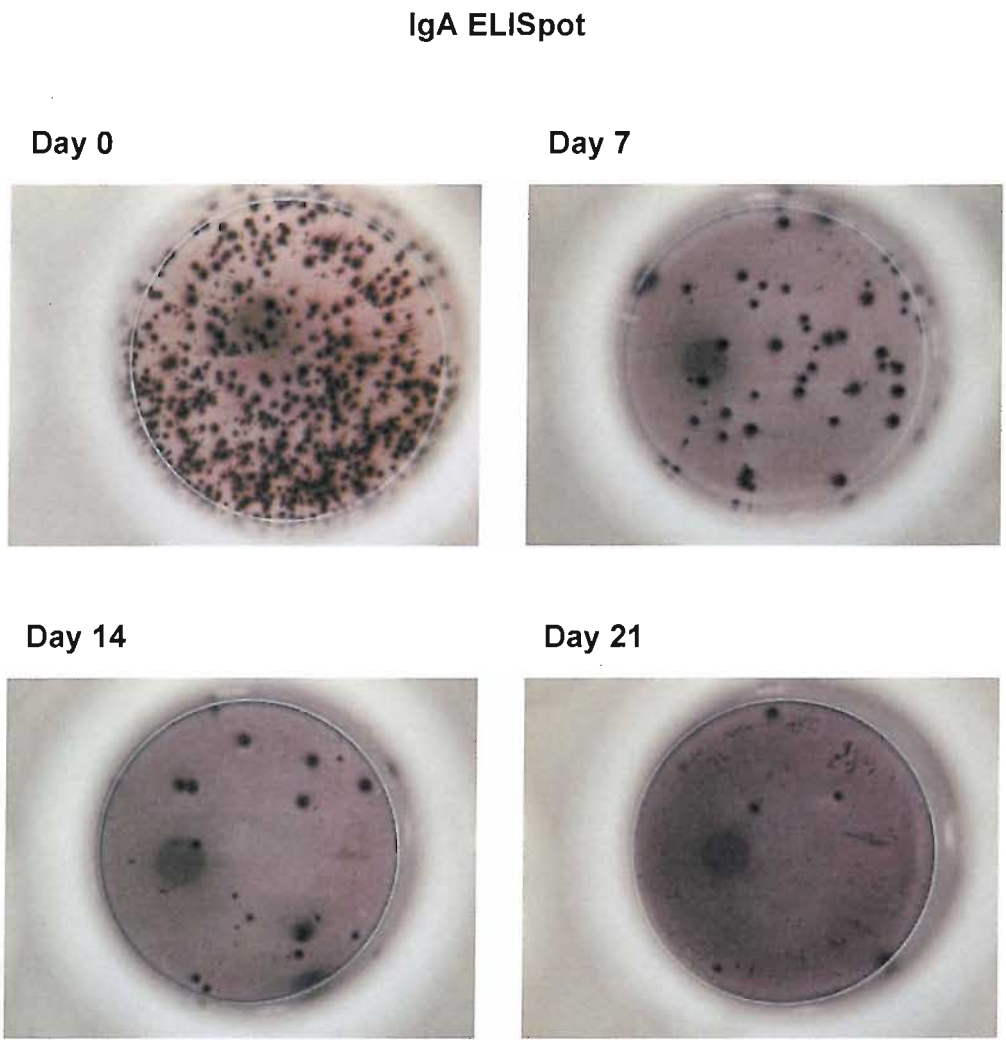


Figure 5.5 Representative ELISpots from a single patient with ulcerative colitis. LPMCs were cultured over a 3 week time period. The ELISpots clearly show a rapid decline in IgA SFUs over the time course of the experiments (1000LPMCs/well x10 magnification).

FIGURE 5.6 IGG PLASMA CELL FREQUENCY IN CULTURED IBD LPMCS OVER THREE WEEKS SHORT-TERM CULTURE

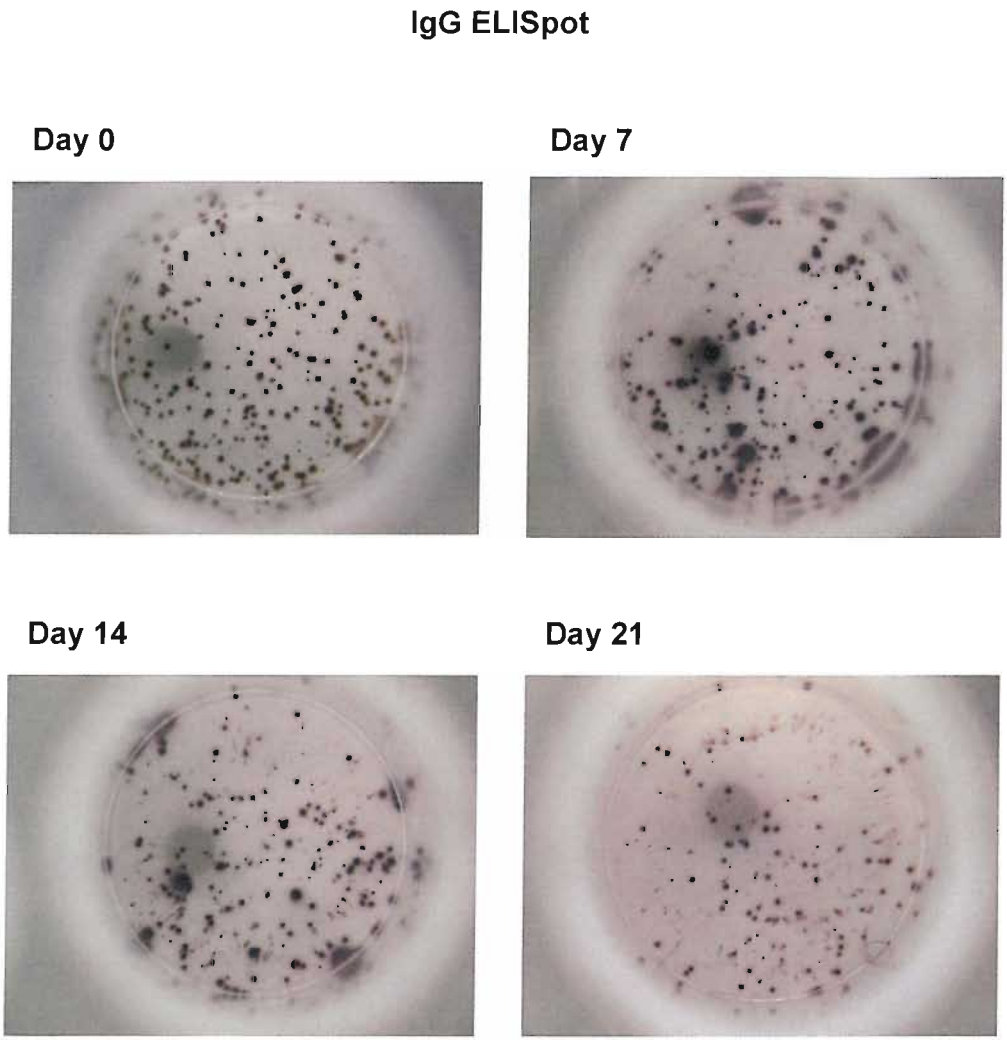


Figure 5.6 Representative ELISpots from a single patient with ulcerative colitis. LPMCs were cultured over a 3 week time period. The ELISpots clearly show a relative persistence in IgG SFUs over the time course of the experiments (1000LPMCs/well x10 magnification).

Chapter 6

6 Gut plasma cell cytokine and matrix metalloproteinase production

6.1 Introduction

There is now clear evidence that, in addition to the production of immunoglobulin, in certain circumstances plasma cells are capable of the production of other immune mediators. As discussed in detail in the introduction, plasma cells from various tissues have been reported to make a variety of cytokines including TNFα, MIF, osteopontin, and TGFβ. Plasma cells from periodontal tissue have also been reported to express MMP-8 and MMP-13, and transformed plasma cell lines and myeloma cells can secrete MMP-3, MMP-2 and MMP-9 in vitro. Accordingly, it appears highly probable that the marked plasma cell infiltrates typically seen in areas of inflammation play a more complex immunological role than previously envisaged.

As plasma cells are abundant in the inflamed mucosa in Crohn's disease and ulcerative colitis they may be capable of producing immune mediators involved in the inflammatory response. Accordingly, in the following experiments I have initially addressed the hypothesis that gut plasma cells in IBD produce inflammatory mediators such as such as cytokines and MMP-3. I have then investigated the role of specific plasma cell isotypes in the production of MMP-3.

6.2 Gut plasma cells from IBD and normal subjects contain abundant transcripts for TGFβ

Previous studies using immuno-histochemistry have indicated that plasma cells can express a variety of cytokines. In addition plasma cell lines can spontaneously secrete cytokines. Therefore, analysis of cytokine gene expression was undertaken on gut plasma cells isolated from subjects with IBD and normal controls. Plasma cells were isolated using CD138 microbead immunomagnetic selection as described in chapter 4, with resultant cell purity confirmed by flow cytometric analysis. IL-10, TGFβ, IFNγ, IL-12p40, IL-4 and IL-5 mRNA expression was determined using real-time RT-PCR.

Both normal and IBD plasma cells very found to contain large numbers of transcripts for TGFβ. No other transcripts were detected in significant quantities though IL-10 and IFNγ were present at around the limit of detection in several samples from both normal and IBD PCs. Relative gene transcription levels for TGFβ were approximately 1000 fold greater than mRNA transcripts for any other cytokines (Figure 6.1).

6.3 Cultured gut plasma cells actively secrete TGFβ

To confirm gut lamina propria plasma cells actively secreted TGFβ, protein expression into culture media was investigated using a commercial ELISA assay. Plasma cells from normal subjects actively secreted TGFβ over the course of 5 days (Figure 6.2).

6.4 Plasma cells isolated from subjects with IBD contained abundant transcripts for MMP-3

Analysis of plasma cell MMP-3 gene expression was also undertaken using Taqman RT-PCR. Plasma cells from patients with Crohn's disease and ulcerative colitis contained abundant transcripts for MMP-3, whereas in normal controls MMP-3 transcripts were barely detectable with a C_T of approximately 40. Relative gene expression was approximately 10,000 fold higher in IBD plasma cells than in normal controls (Figure 6.3) mRNA transcripts for TIMP-1, the endogenous inhibitor of MMP-3, were not significantly different between plasma cells from IBD patients and normal controls (Figure 6.4).

6.5 MMP-3 protein expression is increased in patients with CD and UC compared with normal controls.

Plasma cells from patients with ulcerative colitis, Crohn's disease, and normal controls were placed in short-term cultures for 48hrs. MMP-3 protein production by plasma cells was investigated by western blotting of conditioned medium. MMP-3 production by plasma cells from patients with ulcerative and Crohn's disease was significantly greater

than that from normal plasma cells (Fig 6.5A and B). No difference was seen in TIMP-1 levels between subjects with IBD and normal controls (Fig 6.6).

6.6 IgG+ve gut plasma cells are the predominant source of MMP-3 in both UC and CD

To more precisely identify the cellular source and plasma cell isotype responsible for MMP-3 production, confocal laser microscopy was undertaken on frozen sections of gut lamina propria from subjects with CD, UC, and normal controls. Sections of lamina propria from patients with UC and CD contained numerous MMP-3^+ cells. In contrast, virtually no MMP-3^+ cells were found in normal control lamina propria. Likewise many IgG+ve cells were seen in IBD tissue, whereas they again were virtually absent from normal controls. Both normal and IBD lamina propria contained numerous IgA+ve cells (Figure 6.7).

To characterize the type of plasma cell producing MMP-3, double immunofluorescent staining was undertaken. In IBD tissue the majority of IgG+ve plasma cells stained strongly for MMP-3. A minority of IgA+ve plasma cells in IBD tissue also stained for IgA. In normal controls there were virtually no IgG or MMP-3+ve cells in the lamina propria. The IgA+ve cells in the lamina propria of normal controls did not stain for MMP-3 (Figure 6.8-6.10). Overall, approximately 70% of IgG PCs, and 25% of IgA PCs in IBD tissue expressed MMP-3 (Figure 6.11). IgG^+ and MMP-3^+ cells were so scarce in normal lamina propria that meaningful comparison could not be made.

6.7 Discussion

These studies provide strong evidence that gut plasma cells play a much more complex role in the inflammatory response in IBD that previously appreciated.

Firstly, investigation of plasma cell cytokine expression demonstrated that gut lamina propria cells in both health and disease actively secrete TGFβ. This indicates that plasma cells must play an active physiological role in regulating the immune responses in the gut. TGFβ is a pleiotropic cytokine that regulates growth, differentiation, and function of

immune and non-immune cells. In the gut it plays a crucial role in maintaining immune homeostasis in normal individuals through counteracting the effects of the Th1 pro-inflammatory cytokine response to food and bacterial antigens.[446] Thus, plasma cells may contribute to controlling T-cell immune responses in the gut lamina propria. In addition TGFβ is crucial to plasma cell isotype switching and may therefore contribute in an autocrine manner to the generation of the mucosal humoral immune system. Plasma cells generated in vitro from human B-cells have previously been shown to persistently express TGFβ, but not to limit their own immunoglobulin secretion via this cytokine.[378] In IBD tissue, though increased levels of TGFβ are found in diseased mucosa there appears to be a defect in TGFβ signalling that prevents it from downregulating the inflammatory response. Levels of Smad7, the negative regulator of intracellular TGFβ signaling are increased, rendering cells unresponsive to it.[165] Accordingly the fact that UC and CD plasma cells were also shown to express TGFβ in this study is in keeping with the suggestion that it is a block in TGFβ signaling that results contributes to the excessive inflammatory response seen in IBD.

Secondly, the experiments in this chapter clearly demonstrated that gut plasma cells from subjects with IBD express large quantities of MMP-3. MMP-3 has previously been shown to be one of the major enzymes responsible for the matrix destruction and cell death seen in the mucosa of patients with IBD.[289] The classical pathway by which this occurs is through the upregulation of subepithelial myofibroblast MMP-3 production by pro-inflammatory cytokines, such as TNFα, IL-1β, and IFNγ, resulting in tissue destruction. The findings in this chapter provides evidence for the existence of an additional immunological pathway through which plasma cells can also secrete MMP-3 and thus contribute to the mucosal damage seen in seen in IBD. This is a novel and potentially significant finding.

Finally, I demonstrated that in subjects with IBD the vast majority of IgG gut plasma cells expressed MMP-3 in contrast to around 25% of IgA expressing plasma cells. This suggests that the characteristic IgG plasma cell infiltrate seen in IBD may have a considerably greater pathogenic role than previously recognised. Furthermore, it also

raises the question of whether MMP production is a fundamental property of the IgG plasma cell, with this pathway representing a widespread and important component of the inflammatory response. In support of this concept IgG plasma cell infiltrates are commonly found in areas of inflammation and tissue destruction in a wide-variety of conditions where MMPs may be necessary to facilitate the movement of plasma cells through tissue compartments to sites of inflammation. Furthermore, IgG expressing plasma cells from inflamed synovial and scleral tissue have previously been shown by immunohistochemistry to contain MMP-3 immunoreactivity,[389] and plasma cells from subjects with multiple myeloma secrete a range of MMPs as previously described.

In summary, the work in this chapter demonstrates very clearly that gut plasma cells produce important immunological mediators, and thus almost certainly play a larger role in the immunological cascade than simply that of antibody production. Furthermore MMP production by IgG plasma cells in inflammatory infiltrates represents a hitherto unrecognised pathway of mucosal damage. As such, blockade of this pathway may be an effective target for the treatment of inflammatory disorders such IBD.

FIGURE 6.1 RELATIVE PLASMA CELL CYTOKINE GENE EXPRESSION IN PATIENTS WITH IBD AND NORMAL CONTROLS

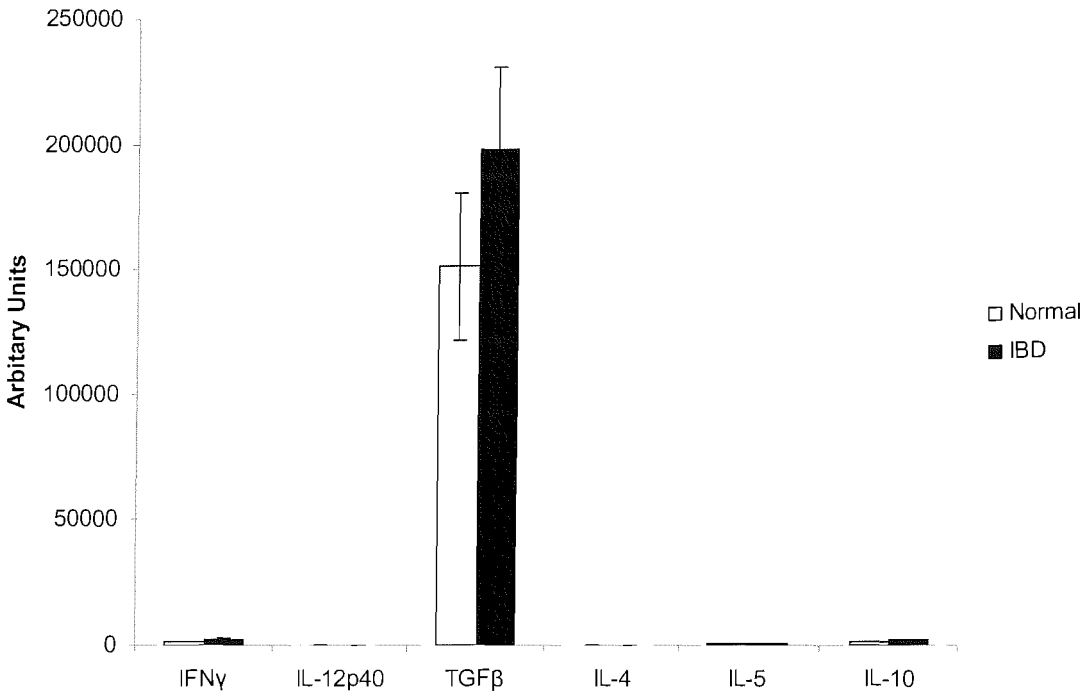


Figure 6.1 Relative cytokine gene expression in plasma cells isolated from subjects with IBD and normal controls measured by Taqman RT-PCR. TGFβ transcripts were markedly elevated in both groups compared to other cytokines which were only just detectable. (Data shown are mean and SEM; Normal n=7; IBD n=6).

FIGURE 6.2 SPONTANEOUS TGFB SECRETION BY CULTURED NORMAL PLASMA CELLS OVER 5 DAYS

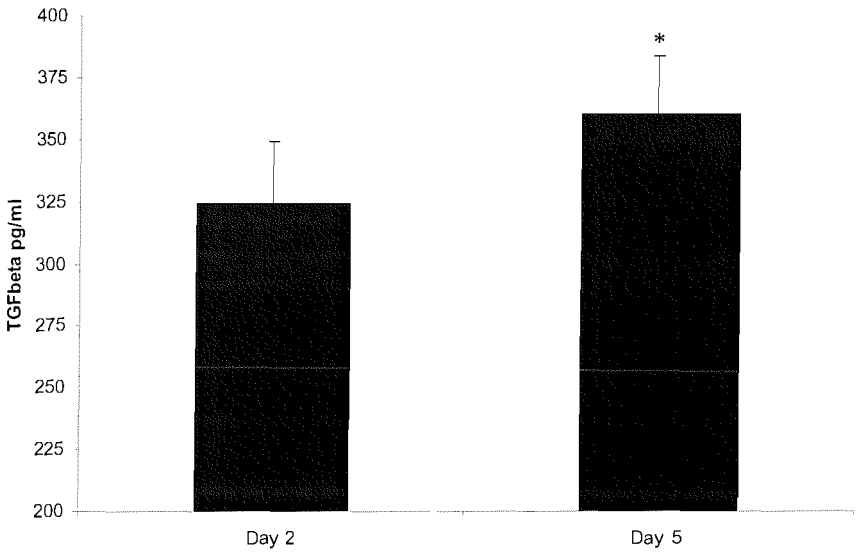


Figure 6.2 Spontaneous TGFβ secretion by isolated normal plasma cells over the course of 5 days. TGFβ levels in conditioned media were significantly higher at day 5 than day 2 ($p=0.031^{*}$; n=5).

FIGURE 6.3 PLASMA CELL MMP-3 GENE EXPRESSION IN PATIENTS WITH ULCERATIVE COLITIS, CROHN'S DISEASE AND NORMAL CONTROLS

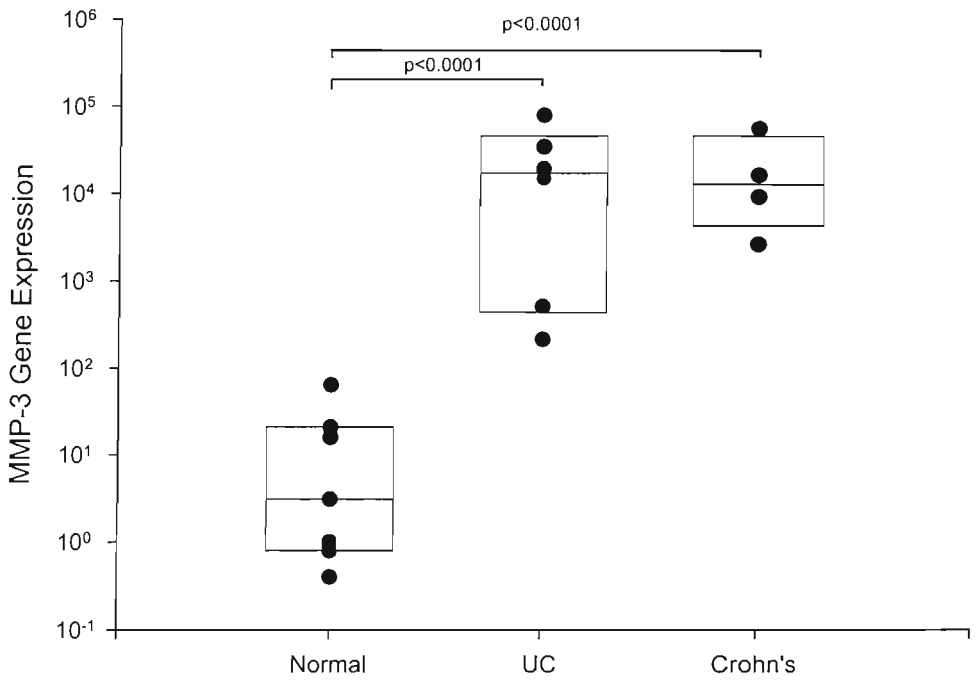


Figure 6.3 Relative MMP-3 gene expression in gut plasma cells isolated from patients with ulcerative colitis, Crohn's disease and normal controls (n=6, 4, and 7 respectively). MMP-3 transcripts were approximately 1000-10,000 fold higher in plasma cells isolated from subjects with UC and CD compared with normal controls (P<0.0001 for both) (Data shown actual values with the boxes indicating the 25th and 75th percentiles, and the line in the bow the median).

FIGURE 6.4 PLASMA CELL TIMP-1 GENE EXPRESSION IN PATIENTS WITH ULCERATIVE COLITIS, CROHN'S DISEASE AND NORMAL CONTROLS

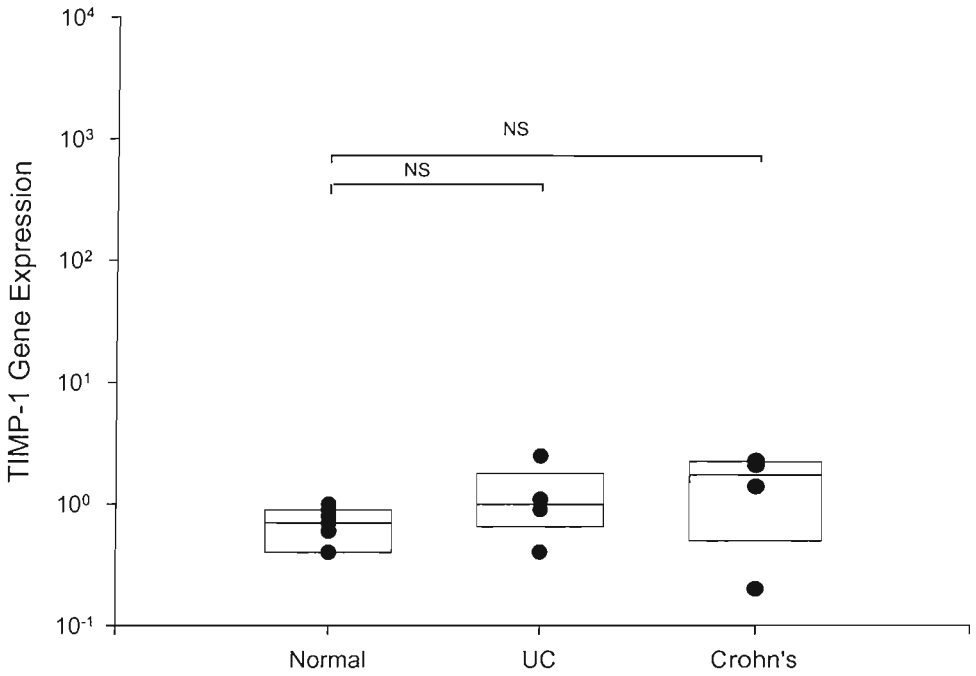


Figure 6.4 Graph demonstrating relative TIMP-1 gene expression in gut plasma cells isolated from subjects with ulcerative colitis, Crohn's disease and normal controls (n=5, 4, and 7 respectively). TIMP-1 transcripts were not significantly different in plasma cells from subjects with Crohn's disease or ulcerative colitis compared with normal controls (Data shown actual values with the boxes indicating the 25th and 75th percentiles, and the line in the bow the median).

FIGURE 6.5A MMP-3 PRODUCTION BY PLASMA CELLS FROM SUBJECTS WITH CROHN'S DISEASE, ULCERATIVE COLITIS, AND NORMAL CONTROLS

A

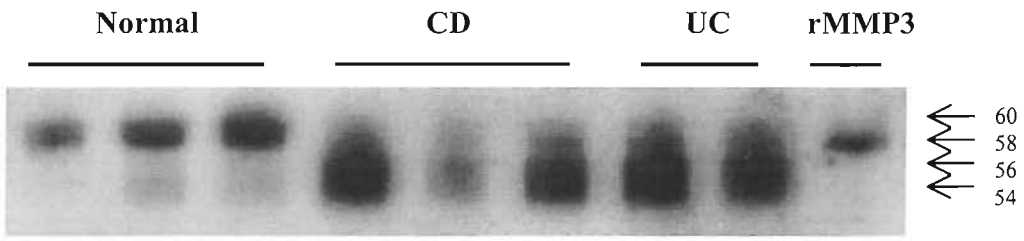


Figure 6.5A Representative western blot for MMP-3 in cultured media from PCs isolated from patients with CD, UC, and normal subjects following 48hr culture in serum free media. Plasma cells from patients with both UC and CD produced significantly more MMP-3 protein than plasma cells from normal subjects. In addition more of the MMP-3 was in the lower molecular weight activated forms (54 and 56 kDa bands) than the normal controls. rMMP-3 is a recombinant positive control.

FIGURE 6.5B QUANTATIVE ANALYSIS OF MMP-3 PROTEIN PRODUCTION BY PLASMA CELLS FROM SUBJECTS WITH ULCERATIVE COLITIS, CROHN'S DISEASE, AND NORMAL CONTROLS

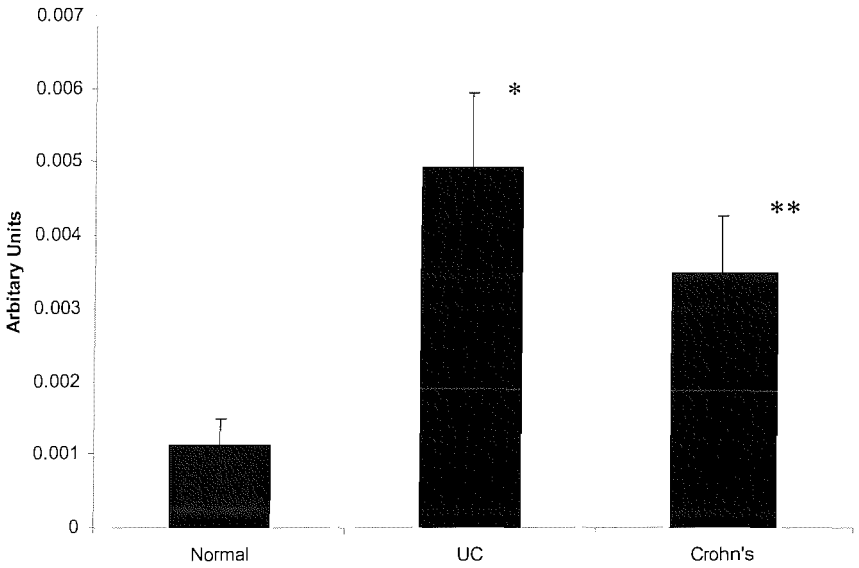


Figure 6.5B Quantitative analysis of MMP-3 protein production by isolated plasma cells from subjects with CD, UC and normal controls. Total MMP-3 protein was significantly increased in cultured media containing plasma cells from subjects with CD and UC compared with normal controls (*p=0.02; **p=0.04) (Data shown are the mean +/- SEM; n=3).

FIGURE 6.6 TIMP-1 PRODUCTION BY PLASMA CELLS FROM SUBJECTS WITH CROHN'S DISEASE, ULCERATIVE COLITIS, AND NORMAL CONTROLS

A

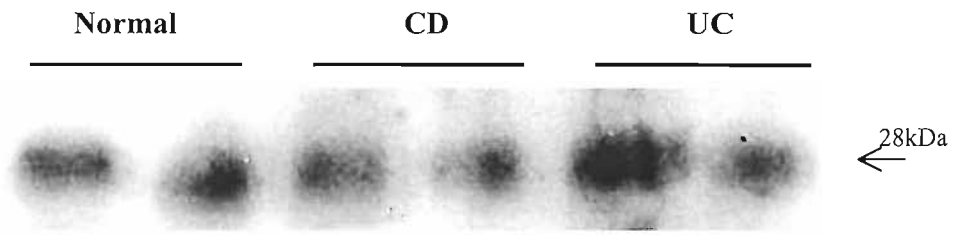


Figure 6.6 Representative western blot for TIMP-1 in cultured media from PCs isolated from patients with CD, UC, and normal subjects. No significant difference was seen in TIMP-1 levels between subjects with IBD and normal controls.

FIGURE 6.7 CONFOCAL IMAGING OF COLONIC MUCOSA IN NORMAL CONTROLS AND SUBJECTS WITH ULCERATIVE COLITIS AND CROHN'S DISEASE

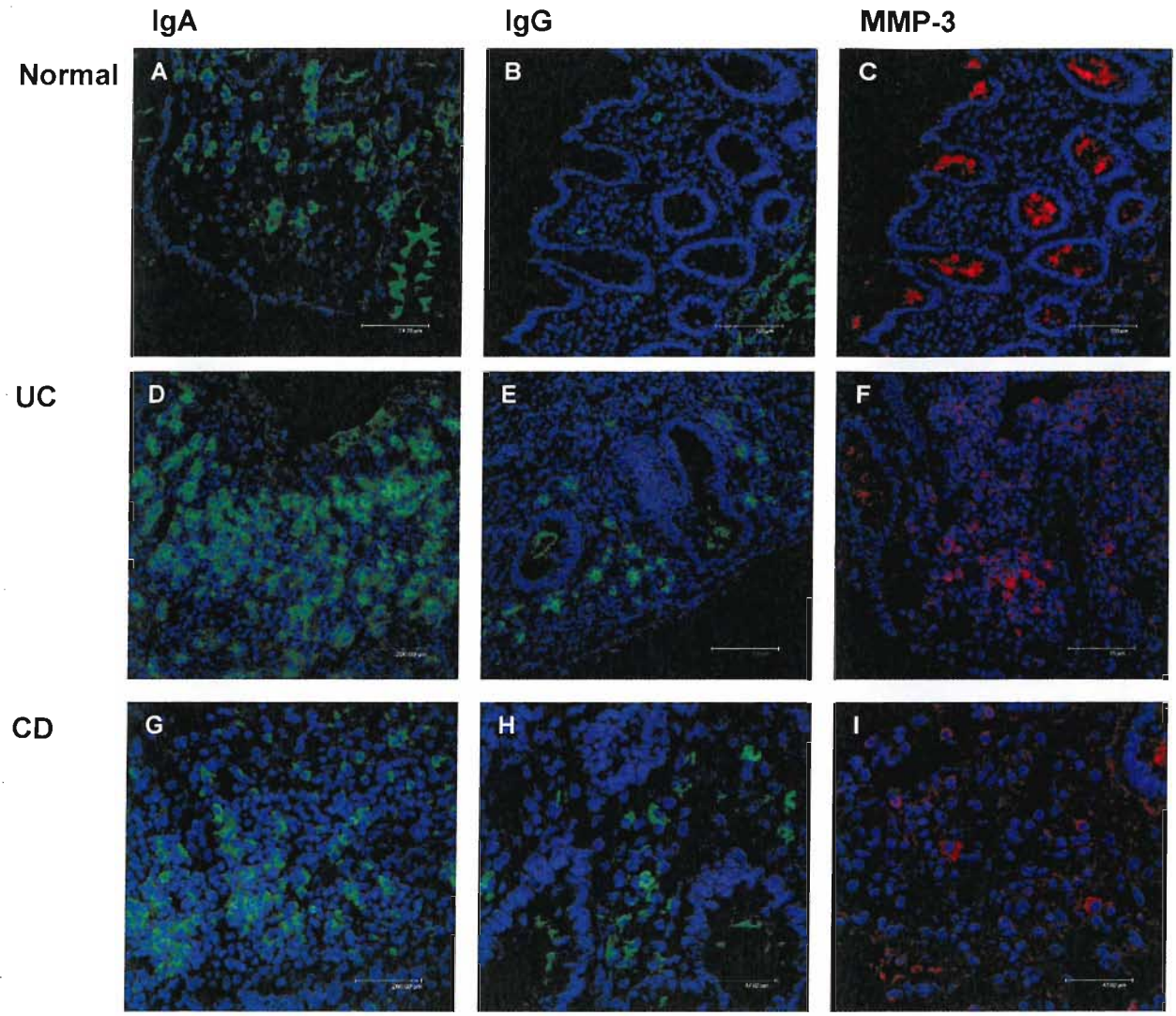


Figure 6.7 Representative colonic mucosa sections from patients with IBD and normal controls. Abundant IgA expressing cells (green) are seen throughout the lamina propria of all subjects (Panels A, D and G), in contrast to IgG expressing cells (green) and MMP3 expressing cells (red) which are seen in subjects with UC and CD, but are extremely rare in normal subjects (IgG: Panels B, E and H, MMP3: Panels C, F, and I).

FIGURE 6.8 CONFOCAL IMAGING OF IGA AND IGG COLOCALISATION WITH MMP-3 IN COLONIC MUCOSA IN NORMAL CONTROLS

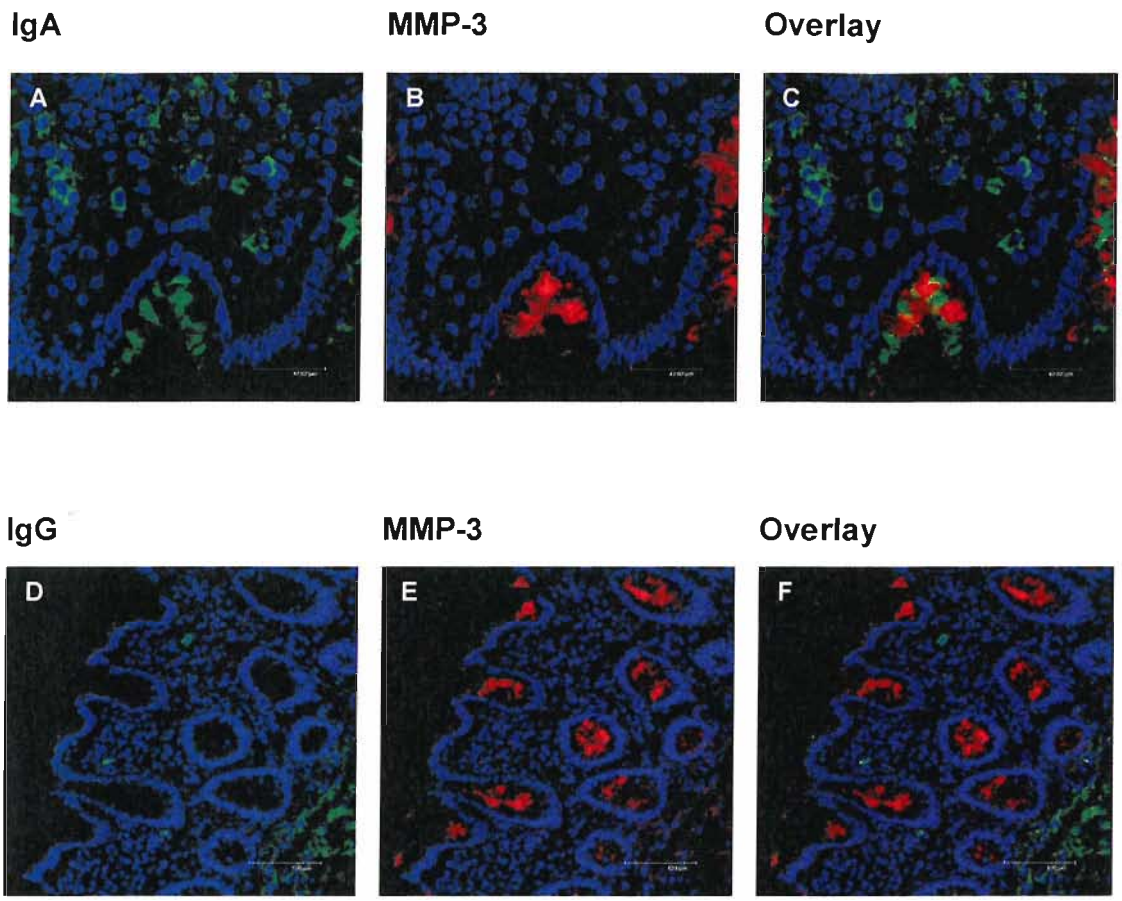


Figure 6.8 Representative colonic mucosa sections from normal controls with Ig expressing cells staining red and MMP-3 expressing cells staining green.. Abundant IgA staining is seen throughout the LP, though no MMP-3 and no co-localisation (Panels A-C). In contrast to IgA very few IgG expressing cells are seen in the LP (Panel D). Again, there is no MMP-3 expressing cells and no co-localisation (Panels E and F) (Images are representative of 5 different experiments, magnification x 40).

FIGURE 6.9 CONFOCAL IMAGING OF IGA COLOCALISATION WITH MMP-3 IN COLONIC MUCOSA IN IBD

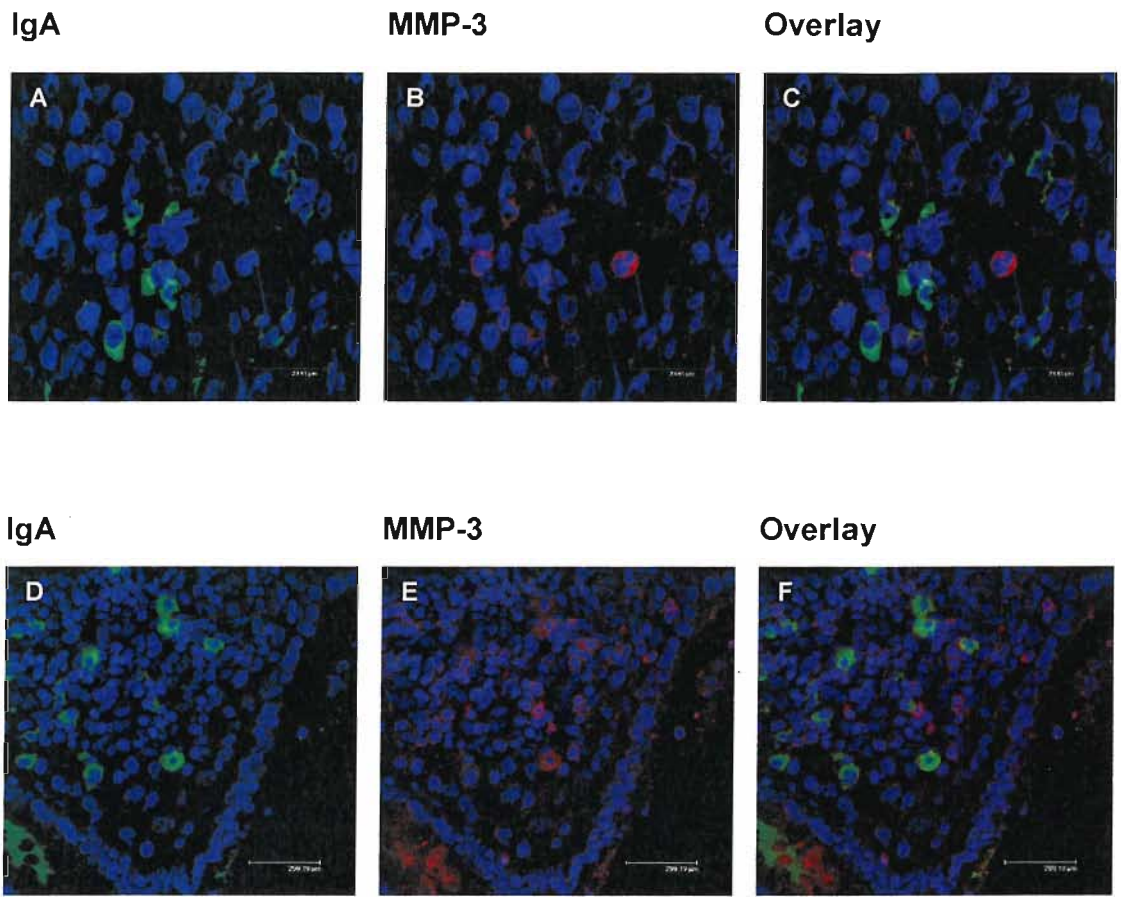


Figure 6.9 Representative colonic mucosa sections from normal controls with Ig expressing cells staining red and MMP-3 expressing cells staining green. Abundant IgA (Panels A and D) and MMP-3 (Panels B and E) staining is seen throughout the LP. The majority of IgA expressing cells do not co-localise with MMP-3 (Panel C) though occasional IgA expressing cells also express MMP-3 (Panel F) (Images are representative of 8 different experiments, magnification x 126 (Panels A-C) and x 80 (Panels D-F).

FIGURE 6.10 CONFOCAL IMAGING OF IGG COLOCALISATION WITH MMP-3 IN COLONIC MUCOSA IN IBD

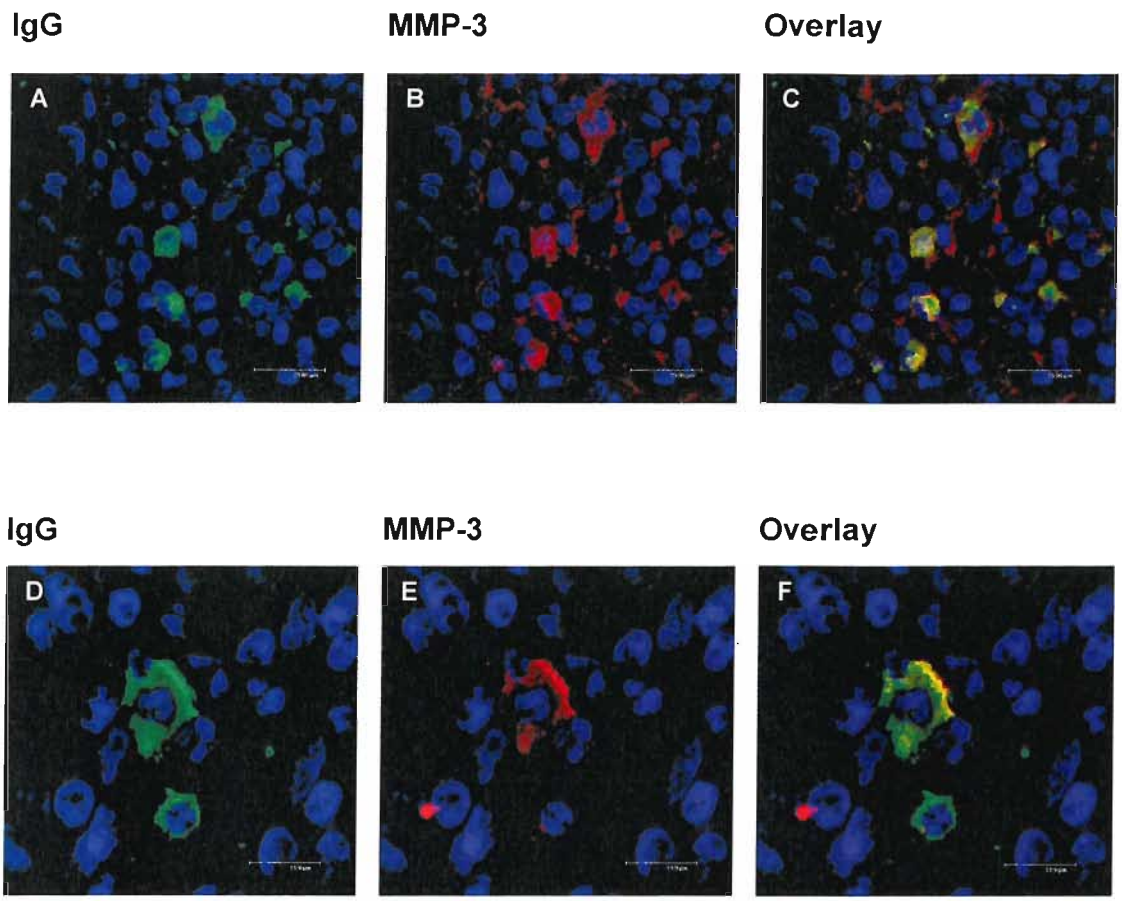


Figure 6.10 Representative colonic mucosa sections from normal controls with Ig expressing cells staining red and MMP-3 expressing cells staining green. Abundant IgG (Panels A and D) and MMP-3 (Panels B and E) staining is seen throughout the LP. The vast majority of IgG expressing cells also express MMP-3 (Panels C and F) (Images are representative of 8 different experiments, magnification x 126 (Panels A-C) and x 80 (Panels D-F).

FIGURE 6.11 THE PERCENTAGE MMP-3 EXPRESSION BY PLASMA CELL ISOTYPE IN COLONIC MUCOSA FROM SUBJECTS WITH UC, CD AND NORMAL CONTROLS.

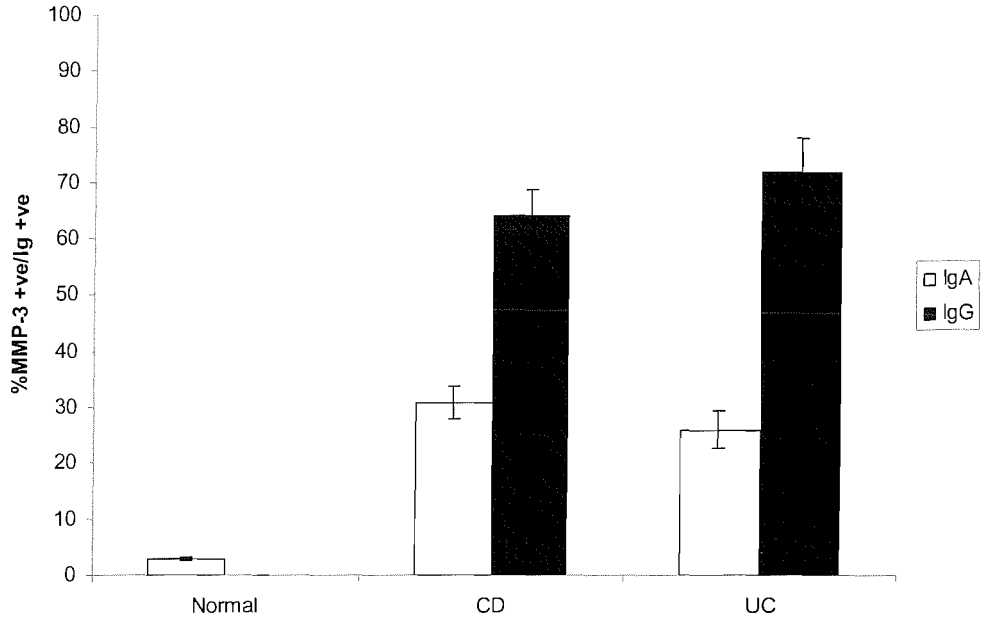


Figure 6.11 Graph demonstrating percentage mmp-3 expression by IgA and IgG expressing plasma cells from subjects with UC, CD, and normal controls. In subjects with UC and CD, the majority of IgG plasma cells expressed MMP-3 compared with around 30% of IgA expressing plasma cells. In contrast, in normal subjects very few IgA expressing plasma cells from normal subjects co-expressed MMP-3 and IgG plasma cells were undetectable (Data expressed as mean +/- SEM; n=3 (N), n=6 (UC), n-4 (CD).

Chapter 7

7 Final comments and future work

7.1 Introduction

In this work I have looked at two separate but related areas of IBD research. In the first I investigated the effect of thalidomide and related analogues on specific biological pathways known to be important in the pathogenesis of IBD. In the second I have investigated the role of plasma cells in IBD with the view to identifying new immunological pathways, and thus potential new therapeutic targets.

The development of new therapies for the treatment of IBD is crucially dependent on basic and translational science research. Both studies presented in this thesis approach this problem, though in two different ways. In the first study, assessing the effect of specific therapies on immunological pathways central to IBD pathogenesis is a pre-requisite to identifying new treatments that can then be taken forward to clinical studies in IBD. In contrast, in the second study, the identification of a new immunological pathway in IBD opens up a potential new therapeutic target for further research. Thus, both these studies may in due course contribute to the ultimate goal of IBD research, namely the introduction of more effective therapies for the treatment of IBD. This will be reviewed in the rest of this chapter

7.2 The study of thalidomide and analogues in IBD

In this study I showed that thalidomide is capable of inhibiting TNFα production by blood mononuclear cells, but not by gut lamina propria mononuclear cells. This demonstrated that though thalidomide can inhibit TNFα production in certain cells it is not able to inhibit gastrointestinal TNFα production and thus is unlikely to be beneficial in the treatment of IBD. The additional finding that thalidomide did not affect gut lamina propria MMP-3 production provided further strong support for this conclusion.

In contrast, the finding that SelCID CC-10004 did inhibit both gut lamina propria TNFα and MMP-3 production strongly suggests that it may be an effective treatment for IBD. It

is also interesting that only CC-10004 effected gut TNFα production while the other two thalidomide analogues (CC-4047 and CC-5013) did not, even though all were equally effective at inhibiting blood mononuclear cell TNFα production. This presumably related to the different modes of action of the three compounds, and the fact that it is harder to inhibit TNFα from activated gut white cells than from relatively naïve blood white cells. The reasons for this would be worthy of further study.

An additional important corroboratory finding from this study is that inhibition of TNFα production in the gut does in turn inhibits MMP-3 production. This provides further support for the hypothesis that of the main reasons anti-TNF therapy is effective in IBD is that it is able to turn off downstream MMP production, promoting mucosal healing.

7.3 Future work with CC-10004

The results of the in-vitro thalidomide studies do not provide support for the rationale of its use in the treatment of IBD. The absence of effect on MMP-3 inhibition found in this study raises considerable doubt over its ability to promote mucosal healing, which is now widely accepted as a hard endpoint in the treatment of IBD. However, this does not exclude the possibility that thalidomide exerts a beneficial effect in IBD through an alternative pathway such as the induction of apoptosis, or inhibition of angiogenesis, both properties that are biologically plausible to be beneficial in the treatment of IBD. However, thalidomide has a very significant side-effect profile over and above its well-publicised teratogenic effects. When these are taken in conjunction with its lack of effect on MMP-3 production demonstrated in this study other therapies begin to appear worthier of further study in clinical trials. Thus though there remains further scope to investigate the role of thalidomide on alternative pathways, such as angiogenesis, in vitro studies, at present there is little evidence to justify its use in clinical trials in IBD.
In contrast, the results with CC-10004 are very encouraging and provide good biological plausibility to support testing it in patients with IBD. Though no data has yet been published on its use in humans, phase I trials have been completed and phase II trials are

underway in psoriasis and asthma.[447] Thus it would be feasible to undertake a phase II study in patients with IBD.

However, a current problem in IBD research is that a logjam is developing between the identification of potential new therapies and their subsequent translation to clinical studies. At a time when ever more therapies are currently being discovered due to huge advances in biological therapies the pool of potential patients to trial them on is diminishing. New therapies are currently trialed on patients with severe IBD unresponsive to conventional therapies. As treatment for IBD has improved specifically following the clinical introduction of monoclonal antibody therapy this group of patients with severe unresponsive disease is diminishing. In addition, this also means that patients that are eligible for clinical trials now have extremely resistant disease, and are not representative of IBD patients as a whole. Thus, as the current pool of eligible trial patients is finite, only the most promising new therapies will be able to reach the stage of clinical trials. CC-10004 needs to be considered in the context of the other new predominantly monoclonal therapies that are currently awaiting trial. In this regard, CC-10004 does have a major advantage over monoclonal technology in that is a "small molecule". As such, it does not suffer from the main disadvantages of monoclonal technology, namely high cost, immunogenicity, and restriction to non-oral routes of administration. Overall, this leads to the conclusion that CC-10004 is a promising novel therapy for IBD and a phase II clinical trial should be undertaken.

7.4 The pathogenic role of plasma cells in IBD

In this second study I investigated the role of the plasma cell in the pathogenesis of IBD. Prior to this study very little work has been published on the role of plasma cells in IBD. In order to investigate their potential role I first had to develop a method of isolating plasma cells from gut tissue. Following this I demonstrated that both normal and IBD plasma cells produce TGFβ. I then showed that, in contrast with plasma cells from normal subjects, plasma cells from patients with IBD produce bioactive MMP-3 that is likely to contribute to the tissue destruction seen in active colitis. Taken together, these two findings are important. They demonstrate that plasma cells produce immunologically

active mediators in both health and disease and play a far more active role in the immunological cascade than previously appreciated. In addition, I found that though both IgA and IgG plasma cells could produce MMP-3, the predominant source was the IgG plasma cell. Furthermore, I also demonstrated that in patients with IBD that the IgG plasma cell was long-lived. This provides strong evidence for a novel pathogenic role for the IgG plasma cell infiltrate in IBD.

7.5 Future experiments with gut plasma cells.

The results from the work on plasma cells in IBD have opened a new area of IBD research and have accordingly raised further questions. The most obvious question is what other immune mediators do plasma cells make? Though I looked at a variety of different cytokines using quantitative PCR the list was by no means exhaustive. It is probable plasma cells make other cytokines as witnessed by the recently published work showing that the also make the Th1 cytokine osteopontin.[402] Furthermore, I only looked at one MMP, namely stromelysin (MMP-3). Though this is the most important with regard to tissue destruction in IBD other MMPs such as MMP-9 and MMP-12 also play an important role. Thus, in addition to looking at the production of other cytokines by plasma cells it would be crucial to also investigate the production of other MMPs as well. Instead of using QPCR, which only allows one gene to be investigated at any time, gene expression profiling using microarray technology would allow multiple genes to be looked at in parallel. This would have the advantage of being able to screen to identify the major products of plasma cells and allow a comparison of gene expression between plasma cells from IBD patients and normal subjects.

A second important question is do all IgG plasma cells make MMP-3 or is it only gut plasma cells? Due to the rarity of IgG plasma cells in IBD it was not possible to determine if they also made MMP-3. Intuitively, one would expect IgG plasma cells from other inflamed areas of tissue to do so. Are there any other specific differences dependent on plasma cell location? To answer these questions IgG plasma cells would have to be isolated from various sources. IgG plasma cells could be isolated from peripheral blood

from patients with inflammatory disorders and normal controls. Plasma cells could also be isolated from inductive sites such as the tonsils in patients undergoing tonsillectomy on account of chronic tonsillitis. It would also be possible to isolate plasma cells from patients with liver disease and patients undergoing splenectomy. Finally, it would be informative to isolate plasma cells from draining mesenteric lymph nodes in resected gut specimens from subjects undergoing colonic resection for non-inflammatory disorders.

Another important finding in this work was that gut IgG plasma cells from subjects with IBD appear to be long-lived. It is not clear whether this is an intrinsic or extrinsic property of these cells. It would therefore be important to look at this area in more detail, in addition using plasma cells isolated from the various sites already discussed above. This has been discussed in detail at the end of chapter five.

7.6 The plasma cell in IBD as a potential novel therapeutic target

The discovery that plasma cells in patients with IBD make MMP-3 raises the question as to whether killing these cells *in vivo* may be of therapeutic benefit? Plasma cells could be targeted by using a cytotoxic monoclonal antibody that binds to one of the specific plasma cell markers, CD38 or CD138. CD138 appears to be the most attractive target as it is the most specific plasma cell marker. However, it is also highly expressed on the basolateral surface of epithelial cells which could give rise to unwanted epithelial cytotoxicity. Furthermore, there is marked heterogeneity of CD138 in humans with most current antibodies only recognizing certain forms of the molecule,[448] and to date there is no report of an anti-CD138 antibody being used in either animal or human studies. In contrast, cytotoxic anti-CD38 antibodies have already been developed, and have been used safely in phase II clinical studies in patients with myeloma and lymphoma.[449] Thus, it would be logical to initially investigate the use of anti-CD38 antibody to deplete plasma cells in subjects with IBD.

Prior to undertaking human studies it would be necessary to demonstrate that a humanized monoclonal anti-human CD38 antibody can kill plasma cells in an ex-vivo

plasma cell system. This could be performed using a similar culture setup to that used in this study. Plasma cell killing can easily be assessed using a combination of FACS, immunohistochemistry, and apoptosis measurement. In addition, it would be useful to determine if treatment with an anti-plasma cell antibody lead to a reduction in active secretion of cytokines and MMPs. It would also be feasible to undertake animal studies to further verify if plasma cell reduction improved IBD. This could be undertaken in rodents that express either a CD or a UC phenotype such as TNBS colitis in SJL/J mice (CD) or $TCR\alpha^{-/-}$ mice (UC).

An alternative approach would be to use a B-cell depleting antibody to such as an anti-CD19 or anti-CD20 antibody. Though CD19 and CD20 are primarily found on pre-B and mature B lymphocytes, they are also present on the cell surface of plasmablasts and some activated plasma cells.[450] In this study I demonstrated that approximately half the gut plasma cell population expressed CD19 though CD20 was not stained for. It is not clear what proportion of IgG^+ plasma cells specifically express CD19 and CD20, though this could easily be determined using 2 colour flow cytometry with intracellular Ig staining. If a sizeable proportion of IgG^+ cells express CD20 then would be possible to test its ability to kill plasma cells in an ex-vivo gut system with a view to a subsequent clinical trial in IBD. The benefit of using an anti-CD20 antibody is that there is currently one commercially available. Rituximab is a cytotoxic humanized anti-CD20 monoclonal antibody that has been extensively tested and is approved for the treatment of B-cell lymphoma in adults.[450] In addition it is currently undergoing evaluation in the treatment of a variety of other autoimmune disorders including rheumatoid arthritis, myasthenia gravis, and SLE.

7.7 Conclusions

The two areas of research covered in this thesis have produced significant new findings that further both our understanding of the immunopathogenesis of IBD, and open avenues to investigate potential new therapies. Thus, this thesis contains translational research that crosses boundaries between basic science and clinical application. Though further

questions remain to be answered in each of the areas explored in this thesis, it is hoped that the work presented here may ultimately contribute to the development of more effective therapies for the treatment of Crohn's disease and ulcerative colitis.

Appendix

8 Appendix 1

Stock solutions

RPMI 1640 + 10%FCS

RPMI 1640	1 litre bottle
$NaHCO_3$	2g
Gentamicin (40mg/ml)	1.2ml
Penicillin/Streptomycin	10ml
FCS	100ml

Hanks Medium

HBSS	1 litre bottle
$NaHCO_3$	2g
Gentamicin (40mg/ml)	1.2ml
Penicillin/Streptomycin	10ml

Percoll gradient

Percoll 100%	Percoll (neat)	45mls
	10 x PBS	4.1mls
	CMF-HBSS	0.9mls
Percoll 60%	Percoll 100%	12mls
	HBSS	8mls
Percoll 40%	Percoll 100%	8mls
	1 x PBS	12mls
Percoll 30%	Percoll 100%	6mls
	HBSS	14mls

30mM EDTA stock solution

EDTA	2.23g
HBSS	200mls

Adjust pH of HBSS to 8.0 prior to addition of EDTA

Dissolve overnight, adjust pH to 7.4, sterile filter

Phosphate-buffered saline (PBS)

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
dH_2O	1 litre

FACS wash

PBS	500mls
BSA	2.5g
Na Azide 0.1%	0.5g

FACS blocking solution

FACS wash	9mls
HUSE (heat inactivated)	1ml

Sterile filter, pH 7.5

9 Appendix 2

While undertaking the research presented in this thesis, I had the opportunity to participate in a related collaborative study with Dr J Spencer from the Department of Immunobiology, Kings College London, London. The study was performed to investigate whether in situ class switching of plasma cells occurred in the lamina propria in humans, and was published in Gastroenterology.[451] My particular contribution to the study was to provide highly purified plasma cell preparations from normal gut where then examined for the presence of AID mRNA. The study is summarised below.

It has been reported in mice, contrary to traditional views, that IgA class switching can occur in the lamina propria as well as in the organised GALT.[361] However, this finding has been questioned by a more recent study where class switch recombination (CSR) molecules essential for isotype switching could only be detected in the GALT and not in the lamina propria.[362] To date, the existence of this pathway in human gut has not been investigated though there is some evidence to support its existence. Clonally related isotype switched plasma cell variants have been identified in small mucosal samples from both gut and nasal mucosa.[452, 453] Activation-induced cytidine deaminase (AID) mRNA has also been identified in nasal mucosa though its exact localisation was not investigated. The presence of AID is essential for class switch recombination to take place.

Accordingly, we investigated whether in situ class switching occurred in the human lamina propria by analysis of the tissue microenvironment for molecular evidence of class switching, and by tracking clonally related B and plasma cells. To look for the presence of AID in plasma cells it was necessary to be able to isolate a pure population of plasma cells from the gut LP. This was achieved using CD138 microbead immunomagnetic selection as described in chapter 4.

We found no evidence of AID expression outside the organised GALT, and proliferating B-cells were not found in the lamina propria. In addition clonally related IgA and IgM isotype switched cells were identified in multiple samples found to be free of AID. These results support the dissemination of cells from a common set of precursors and were inconsistent with the hypothesis that IgA class switching occurs in the lamina propria.

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