The Role of *Mycobacterium avium* ss *paratuberculosis* (MAP) in patients with Crohn's Disease.

Jacqueline Turton

BSc (Hons)

Doctor of Philosophy (PhD)

December 2011

Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

Acknowledgements

I would like to firstly thank my supervisor, Emeritus Prof. Robert Clancy, for his guidance and patience throughout my PhD project. His understanding of my personal circumstances and belief in me enabled me to complete this thesis. Thank you Robert.

I wish to thank to Dr Zhigang Ren, for his help in the continued collection and processing of samples whilst I was on maternity leave. Also, thank you Ren for your technical support in the lab and also with the statistical analysis of my raw data.

Thank you to Dr Thomas Borody of the Centre for Digestive Diseases, Five Dock NSW, for his invaluable contribution. Without his passion for helping people with Crohn's disease and other bowel disease, this thesis would not have happened. He was able to supply me biological samples from his patients and their clinical data. Thank you also to the staff of the Centre for Digestive Diseases which help prepare the ethics applications and provide all the samples for this project. A special mention to Mrs. Evelyn Vigano for her skillful technical assistance in the CDD laboratory.

Thank you to Professor John Herman-Taylor for supplying our positive PCR control, IS900 + pIDL60.

A big thanks to my friend Kath Pink for her help in proof reading this thesis, you were a life saver Kath!

Thanks you to Mr Patrick McElduff, HMRI Biostatistician, for his expertise and advice concerning my statistical analysis.

Thank you to Dr Ira Shafran at Department of Molecular Biology and Microbiology and Center for Discovery of Drugs and Diagnostics, University of Central Florida, Orlando, Florida USA who

analysed serum samples for me using techniques he has developed to identify MAP infected patients using *M. paratuberculosis* antigens as serological markers (p35 and p36).

Thank you to the Mark Turner and Leslie Reddacliff from the Elizabeth Macarthur Agriculture Institute (Camden, NSW, Australia) for providing us with soluble MAP antigens.

Lastly, I must thank my family…my husband Richard, my beautiful children Flynn and Claire, and my parents Sandra and Ian Turton. They have put up with me over the course of this thesis and without them I wouldn't have been able to continue.....Love you all xx

It hasn't been smooth sailing for me during this thesis…I got married, gave birth to two beautiful, amazing kids who I love more then anything. In January 2006 we almost lost our son from undiagnosed Type 1 Diabetes at 14 months, which completely turned our world upside down. But I am proud to say I've finally finished!!

Dedication

I dedicate this thesis to my two beautiful children Flynn and Claire….I love them with all my heart.

List of publications

Clancy R, Ren Z, Turton J, Pang G, Wettstein A. Molecular evidence for Mycobacterium avium subspecies paratuberculosis (MAP) in Crohn's disease correlates with enhanced TNF-alpha secretion. . Dig Liver Dis. 2007 May;39(5):445-51.

Ren Z, Turton J, Borody T, Pang G, Clancy R. Selective Th2 pattern of cytokine secretion in Mycobacterium avium subsp. paratuberculosis infected Crohn's disease. J Gastroenterol Hepatol. 2008 Feb;23(2):310-4.

Foell D, Wittkowski H, Ren Z, Turton J, Pang G, Daebritz J, Ehrchen J, Heidemann J, Borody T, Roth J, Clancy R. Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. J Pathol. 2008 Oct;216(2):183-92.

ABSTRACT

The cause of chronic inflammation in the gut of subjects with Crohn's Disease (CD) is unclear; however most would agree that 3 interacting factors are critical to mucosal inflammation: genetic susceptibility, enteric microflora and the host immune system. The most controversial theory is whether or not a particular microbe(s) infects and maintains in intestinal tissues, resulting in chronic inflammation. The most discussed microbe is *Mycobacterium avium* ss *paratuberculosis* (MAP). The aim of this thesis was to better document the natural history of MAP infection in subjects with Crohn's disease, IBS, "non-Crohn's colitis" and normal subjects, correlating clinical status with parameters relating to MAP, to the mucosal T cell response and to the genetic susceptibility gene CARD15/NOD2.

MAP is an obligate intracellular pathogen, which causes chronic inflammation in the intestine of many species, including primates. MAP was first identified as the causative agent in Johnes Disease, a gastrointestinal disease in ruminants and primates. The infection of domestic livestock with MAP is now widespread, which has increased the risk of transmission of MAP to humans via milk products. The involvement of MAP in human patients with Crohn's disease (CD) has been difficult to prove, due to the difficulties in isolating and detecting this organism.

By using nested PCR (Polymerase Chain Reaction) on DNA extracted from fresh human intestinal mucosal biopsy samples the presence of MAP can be verified. Bacteria contain unique insertion elements, which play a role in virulence, pathogenicity and antibiotic resistance. Within MAP a unique 1.4 kb insertion element was identified, IS900. By using DNA primers for PCR within this unique insertion element we were able to identify MAP in tissue. There was found to be no statistical significance among the disease groups tested.

The next objective was to determine whether the presence of MAP in gut biopsies was associated with a different cytokine secretion profile as observed in organ and whole blood culture, using an ELISA assay. Significantly higher levels of TNF-α

were found in culture supernatants from organ culture for Crohn's Disease when compared to ulcerative colitis ($p<0.05$), irritable bowel ($p<0.01$) and controls ($p<0.0001$). When TNF-α levels were correlated with the presence of MAP, significantly greater concentrations were only found in MAP positive Crohn's Disease patients ($p<0.05$). In whole blood culture significantly higher levels of IL-4 ($p<0.05$) and IL-2 ($p<0.05$) were found in MAP positive Crohn's Disease patients compared to MAP negative CD, which is consistent with a skewed Th2 immune response. This data provided the first evidence of an abnormal macrophage handling of MAP and an altered T cell function linked to MAP infection.

Finally, the co-existence of MAP infection and NOD2/CARD15 mutation status was investigated. SNP analysis of the three most common NOD2/CARD15 variants: missense mutations R702W (2104C→T, SNP8) and G908R (2722G→C, SNP12) and the frameshift mutation 1007fs (3020insC, SNP13); was performed on trial participants and compared to MAP status to determine if genetic susceptibility to CD predisposes them to MAP infection. Analysis of CD patients NOD2 status and cytokine profile found no correlation. Given that there is no clear link between NOD2 gene mutation, presence of MAP and cytokine secretion in our CD patients the suggestion that defective handling of MAP is due to NOD2/CARD15 gene mutations is not relevant to our group.

We can conclude that

1. MAP is not essential for CD,
2. That MAP is present in IBD and non-IBD patients,
3. There is a defect in the cellular handling of MAP in CD and
4. MAP in CD has the capacity to enhance drive a Th2 response, which in turn down regulates the protective Th1 response and enhances mucosal permeability which leads to increased inflammation.

Table of Contents

1.1 Crohn's Disease

Inflammatory Bowel Disease (IBD) is a term which is used to describe 2 diseases, Crohn's Disease (CD) and Ulcerative Colitis (UC). Whilst these 2 diseases are both chronic inflammatory diseases, they have many differences. Ulcerative colitis is a disease which involves diffuse mucosal inflammation of the rectum and extends to all or part of the colon (Chacon, et al, 2004; Bouma and Strober, 2003).

In Crohn's disease however, the disease can affect any part of the gastrointestinal tract, from the mouth to the anus. It is characterised by transmural inflammation, meaning all layers of the bowel wall are affected. This inflammation can often extend to the serosa resulting in sinus tract or fistula formations. It is also characterised by the presence of "skip lesions", which are diseased segments separated by normal bowel, this may produce the characteristic "cobblestone" appearance (Chacon, et al, 2004; Bouma and Strober, 2003). Another characteristic which is used in the diagnosis of CD is the presence of granulomas (Chacon, et al, 2004).

Farmer et al (1975) found a pattern to the sites involved with CD when he reviewed 615 patients with CD; the ileocolic site is the most commonly involved (41%), followed by the small bowel alone (27%) and colon alone (27%). People with Crohn's can develop abdominal pain, diarrhoea and weight loss. Symptoms and their severity vary from person to person and will flare up or improve with time.

Treatment is multifaceted, including anti-diarrhoeals and anti-inflammatory agents to treat symptoms, immunosuppressive drugs, and surgery to remove diseased bowel or alleviate complications such as fistulas, stricture or obstructions (Podolsky, 2002). Patients with Crohn's disease are a heterogeneous group and the aetiology may not be the same for all patients (Grant, 2005).

In the past decades the incidence of CD has slowly increased. In Australia it is estimated that there are between 1 and 5 new cases/100,000 population/year, with a prevalence of 10 – 50/100,000 people. It is most common in Western countries and more common in Caucasians than other races (Selby, 2000). CD usually manifests in young people, the mean annual incidence is highest in people aged 15 to 30 years and this incidence does not differ between the sexes (Duchmann and Zeitz, 1999).

1.2 Pathogenesis of Crohn's Disease

The pathogenesis of CD is still unclear; however, most would agree that 3 interacting factors are critical to mucosal inflammation: genetic susceptibility, enteric microflora and the host immune system.

There are several theories about the pathogenesis of CD. Is it an autoimmune response to self-antigens? Or is there a dysfunctional immune response to commensal bacterium (Hendrickson, 2002; Chamberlin and Naser, 2006)? One of the most controversial theories is whether a pathogen infects and maintains in the intestinal tissues, which results in a chronic inflammatory response. The most

discussed microbe is *Mycobacterium avium* ss *paratuberculosis* (MAP). A variation on this theory is that inflammation persists due to an abnormality in immune regulation following infection with a pathogen (Hendrickson, 2002).

1.3 Mycobacterium avium ss *paratuberculosis - MAP*

The data which support a specific role for MAP infection include an analogy with Johnes Disease (JD) in ruminants. MAP was first recognised as the causative agent of chronic enteritis in cattle in 1895 by Johne and Frothingham, now known as Johnes disease (JD). JD has been documented in other ruminants, such as sheep and goats, and also in primates. JD is prevalent in domestic animals worldwide and is considered to be the most serious disease affecting dairy cattle (Harris and Barletta, 2001). Neonatal and juvenile animals are at the highest risk of MAP infection and they are most commonly infected through the faecal-oral route. This occurs when the organism is ingested through contaminated milk or food products or by accidental ingestion from contaminated surfaces (Harris and Barletta, 2001).

MAP is similar to other pathogenic mycobacteria, in that it targets the mucosa-associated lymphoid tissues of the upper G.I. It is endocytosed by the M cells of the ileal Peyers patches and is subsequently phagocytosed by subepithelial and intraepithelial macrophages. MAP bacilli then remain and multiply within the phagosome. The appearance of an intestinal granuloma appears after cytokine production and the initiation of a cellular immune response. Within the lymph nodes a cellular immune response occurs in an attempt to clear the infection.

These inflammatory responses lead to the classic clinical manifestations associated with JD; corrugated intestinal epithelium and malnutrition syndrome (Harris and Barletta, 2001).

In 1913 Thomas Dalziel (Dalziel, 1913) had observed that the histological characteristics of human granulomatous were similar to those of Johnes disease and he proposed “the diseases may be the same”.

Mycobacteria include a group of high GC gram positive micro-organisms (Chacon, et al, 2004). They are characterised by their acid-fastness and lipid rich cell wall. MAP is a slow-growing, hydrophobic rod shaped bacterium (Gould, 2004 report). It is an obligate intracellular pathogen that can only survive in a host that provides iron, as MAP lacks the iron-chelating compound mycobactin.

The problem that Dalziel faced was that he could not see acid-fast mycobacteria in the diseased intestines of humans. Ziehl-Neelsen staining is a technique described in 1886 by Ziehl and Neelsen which identifies the cell-wall of intact mycobacteria by acid-alcohol-fast-staining. If MAP is in the cell-wall deficient form then the Ziehl-Neelsen staining will be negative. In Johnes disease numerous acid-fast mycobacteria are generally visible in diseased tissue. However, Johnes disease in animals can show a range of histopathological types from a multibacillary form with large numbers of acid fast bacilli, to a paucimicrobial form characterised by chronic granulomatous inflammation in which MAP cannot be seen and cannot be cultured (Clarke, CJ and Little, D, 1996).

In 1932 the first description of Crohn's Disease was published by Crohn, Oppenheimer and Ginzburg, where CD was distinguished from intestinal tuberculosis through a lack of detectable tubercle bacilli (Crohn, BB et al, 1932). The inability to identify and culture acid-fast bacilli from infected tissues from patients with CD led to Dalziel's theory being discounted.

Interest in MAP having an aetiological role in CD was revived in 1984 when Chiodini successfully isolated aerobic acid fast rods from 3 patients with Crohn's disease. These isolates had identical biochemical profiles, they were mycobactin dependent and they belonged to the Mycobacterium genus. These organisms were initially isolated as cell wall defective forms. They were similar to MAP in both cultural and biochemical characteristics. The fastidiousness of the organism, the mycobactin dependency, the long incubation periods for culture, and the special isolation methods required were postulated to account for the earlier failure to identify the organism in Crohn's patients (Chiodini RJ et al, 1984). MAP was again on the map as a candidate pathogen.

Chiodini subsequently identified a spheroplastic phase of the Mycobacterium isolated from CD patients (Chiodini RJ et al, 1986). The spheroplasts were isolated from tissue samples from patients with CD after several months of incubation. They were then subcultured and later developed a cell-wall that was acid-fast positive. This spheroplast form persists for longer periods and replicates slowly leading to chronic infection. These characteristics were considered to account for the long culture incubation period and difficulty found in identifying

acid-fast bacilli in tissues. Spheroplasts may transform into the bacillary form over time. He found that the 5S rDNA gene restriction fragments in isolates from CD patients were identical to that of MAP DNA, with both the spheroplast and bacillary forms having identical restriction fragments. Spheroplasts were only isolated from CD tissue and not from normal or UC tissue.

The inability to successfully culture MAP has resulted in inconsistencies noted in published results. Schwartz et al (2000) investigated the short-term culture of MAP from CD tissues and considered that MAP might reside in the sub-mucosa of ulcerated tissues in CD patients, rather then in the superficial mucosa sampled at colonoscopy (Schwartz D, et al, 2000). MAP was cultured within several weeks from resected tissues, as compared to the several months taken for biopsies, leading to a conclusion that MAP in deep tissue is virulent and metabolically active, while MAP on the mucosal surface may be avirulent and metabolically inactive.

In-situ hybridisation has enabled the cell wall-defective mycobacteria to be detected in tissue specimens where Ziehl-Neelsen staining has been negative (Hulten K, et al, 2001). A specific in-situ hybridisation method was developed which was tailored to detect cell wall-defective forms of MAP. The results were that 40% of the CD patients with granulomas tested were positive, 9.5% of 21 ulcerative colitis patients were positive and none of the control patients were positive. These results are consistent with the hypothesis that CD is caused by cell wall-defective MAP.

The ability to verify the identity of the cell wall deficient organisms as MAP was made possible by the application of PCR. MAP contains 17 IS900 insertion elements which are unique to MAP. Insertion sequence (IS) elements are small mobile genetic elements which contain genes only related to insertion functions (Green et al, 1989). Green et al (1989) first identified the DNA sequence IS900, and reported that it consists of 1451 bp of which 66% is G + C. They also found that it lacks terminal inverted and direct repeats, and as a result cannot form stem loop structures which means that they cannot transpose by any of the classical IS element mechanisms (Green et al, 1989). Insertion elements, in bacteria, play a role in the bacteria's virulence, pathogenicity and antibiotic resistance. The IS900 insertion element has enabled researchers to identify MAP in both CD tissue and in MAP culture.

The use of paraffin embedded tissues is of limited value as DNA fragmentation may occur. The IS900 positivity rate has varied. Review of the literature showed an overall positivity rate of MAP identification by IS900 PCR to be 38% for CD and 7% for non-inflammatory bowel disease (including UC). El-Zaatari et al (2001) noted that the majority of IS900 positives from CD were from resected tissues compared to biopsies, again suggesting that this might be due to a bacterial infection deep in the tissue and not a contaminate passing through the gut, or a question of tissue volume. Ryan et al (2002) developed a method using laser capture microdissection (LCM) and IS900 PCR to detect MAP in CD granulomas. MAP DNA was detected in microdissected granulomas in 40% of cases and in

whole tissue sections in 20% of cases, indicating that isolation of granulomas by LCM allowed detection of MAP, not seen with whole tissue examinations.

Others however, would explain these differences in detection, in terms of sensitivity of assay and quality of tissue extraction. For example, Bull et al (2003) have reported a detection rate in mucosal biopsies of IS900 PCR positives of 92%, claiming they had increased sensitivity by greatly increasing the number of IS900 copies extracted. What has not been demonstrated however, is specificity of infection for MAP. These results were obtained by optimising steps in the DNA extraction process which involved using only fresh tissue, using ribolysation, resuspending DNA pellets overnight at 4^{o}C and with nested PCR using Roche Expand HF Taq Polymerase. The commercially available MGIT culture system (BD) was also shown to be a useful system for culturing MAP from mucosal biopsies. Bull et al (2003) suggested that PCR of MGIT culture is a useful addition to visual MAP detection. This paper reported an optimised, standard MAP detection system.

This high rate of detection is being reported by other groups in different geographic areas (Romero C, et al, 2005; Lisby G, et al, 1994). However others have failed to find MAP in Crohn's tissues (Baksh FK et al, 2004; Chiba M, et al, 1998; Rowbotham DS et al, 1995) or report detection in a minority of subjects (Dumonceau JM et al, 1997; Clarkston WK et al, 1998). Culture of MAP from breast milk of women with Crohn's disease and recently from blood buffy coat cells in Crohn's but not in controls supports the argument that MAP is not simply an

environmental contaminant appearing in diseased mucosa due to disruption of the epithelial barrier (Naser SA et al, 2000; Naser SA et al, 2004). Regardless however of the 'cause or effect' argument, the two issues that need resolution remain first the variable reported incidence of MAP, and second the high level of detection in normal and diseased controls.

The humoral immune response to MAP in CD patients was analysed by Naser who used recombinant clones of MAP (Naser et al, 1999). It was found that antibodies to p35 and p36 recombinant antigens were significantly more frequent and of higher titre in CD patient's (Naser SA et al, 2000). In 77% of CD patient's seroreactivity to p35 and p36 was found, compared to 8% of UC patients and 0% of normal controls. Nucleotide and amino acid analysis of p35 antigen showed 97% homology to a MAP specific protein previously identified and isolated by Cocito (Shafran I et al, 2002). Other groups, however, reported different results. Bernstein's study did not show any difference in serum antibody levels against MAP antigen among CD patients (37.8%), UC patients (34.7%) and healthy controls (33.6%) (Bernstein CN et al, 2004), while Collins et al (2000) reported that in an American population only small differences in CD patients (20.7%) compared to UC patients (6.1%) and healthy controls (3.8%), in Danish patients no difference in antibody detection was found among the study groups (Collins MT et al, 2000), results similar to those found in our Australian population (unpublished observation). The variable use of the bacillus Calmette-Guérin (BCG) vaccine in different countries may account for high antibody detection in some normal populations due to cross reactivity between MAP and *M. bovis* protein.

It has been suggested that MAP enters the human host via contaminated water, meat or dairy supplies. Studies in the US and UK have shown that normal pasteurisation (i.e. 72^{o}C for 15 sec) does not destroy MAP. In a study into retail milk supplies in England and Wales it was found that 7% were MAP positive by IS900 PCR (Millar D et al, 1996). Viability of the organisms was confirmed by positive culture 50% of IS900 positive specimens (Millar D et al, 1996). It was noted however, that 16% of the PCR negatives were also found to be culture positive. Subsequent studies by Grant et al (1999) showed that milk samples spiked with MAP and then pasteurised, contained viable MAP. They confirmed the presence and viability of MAP in approximately 10% of retail milk samples in the UK. Millar et al (1996) reported that in clinically "healthy" cattle, approximately 3.5% were IS900 positive in the absence of any clinical evidence of JD. These cattle also shed MAP in their faeces and milk. The issue of milk contamination and its significance with respect to human infection remains controversial. For instance, while Crohn's disease occurs throughout Australia, Johnes disease is common only in south-eastern Australia (Selby W, 2003).

Infection of cattle with MAP does not always result in JD. In cattle JD can take up to 15 years to develop a model that may reflect the situation in man (Thompson DE, 1994). It has been proposed that most individuals have repeated exposure to MAP without disease, while a small minority develops clinical disease due to additional genetic factors that modulate the handling of the organism and the subsequent inflammatory response (Greenstein RJ, 2003). This pattern is similar to that of tuberculosis in man where only a minority of those infected get clinical

disease, and where the load of environmental exposure influences the prevalence of disease. Thus the issue of environmental 'load' of MAP may be an important one.

The UK Food Standards Agency has taken a precautionary step, based upon growing evidence, to eradicate MAP from the food chain. It believes this action should be taken now irrespective of whether the link between MAP and Crohn's disease has been proved or disproved (Grant, 2005).

The concept of infection, particularly by MAP, in CD has encouraged treatment by antibiotics. Traditional therapy for CD is based on suppression of the inflammatory response by immune suppression and judicious surgical resection, with antibiotics having only a limited role (El-Zaatari FAK et al, 2001). MAP is generally resistant to "typical" antituberculosis drugs, and clinical trials using these drugs have been unsuccessful (Bonen DK and Cho JH, 2003; Prantera C and Scribano ML, 1999). Driven by antibiotic developments for MAC infections in HIV immunocompromised patients, rifabutin (a rifamycin derivative) and clarithromycin (a novel macrolide) in combination were found to be effective against MAP (Chiodini RJ, 1991; Rastogi N et al, 1992; Ghebremichael S et al, 1996). In 1997 Gui et al conducted a 2-year trial of 46 patients with CD with a combination of rifabutin and a macrolide antibiotic (clarithromycin or azithromycin). Treatment with this multidrug regimen resulted in a substantial clinical improvement. Shafran et al in 2002 carried out an open clinical trial using low dose rifabutin and clarithromycin in 36 patients with CD. They reported that more than 50% of the patients achieved a sustained remission

with a drop of more than 70 points in the CDAI (Crohn's Disease Activity Index) using antibiotics alone. Borody et al (2002) used 3 drugs (rifabutin, clarithromycin, and clofazimine) in patients with refractory CD that had failed maximal immunosuppressant therapy. Patient progress was monitored for up to 54 months using colonoscopy, histology, clinical response and the Harvey-Bradshaw Activity Index. They found that 6 of the 12 patients went into complete remission assessed clinically, colonoscopically and histologically when treated with antibiotics without immunosuppression. Three of the 6 patients remained CD-free, off all therapy for between 2-4 years. Recent colonoscopic review of those in complete remission, however, showed new mucosal lesions, raising the issue of whether cure is a realistic outcome in CD.

Not all groups claim clinical benefit from the use of combination 'anti-MAP' strategies. A randomised case-controlled study using clarithromycin and ethambutol for 3 months and a 12 month follow-up did not show any benefit in patients with Crohn's disease (Goodgame RW et al, 2001). Swift et al (1994) reported a double blind, placebo controlled study, using ethambutol, isoniazid and rifampicin for 2 years in 130 patients; no difference was observed between drug and placebo groups. Thomas et al (1998) reported a combination therapy with antibiotics and 5-ASA, but failed to reveal any benefit compared to patients with Crohn's who were receiving placebo during a five-year follow-up study. Although one can criticise these studies as not including optimal antibiotic combinations or an appropriate dose, small group size and chosen parameters of assessment, it remains that no clear conclusion can be drawn from the available literature. A large

(over 200 recruits) multisite study using a three drug regimen of clarithromycin, rifabutin and clofazimine, has been concluded recently in Australia (Selby W, 2005). A significant improvement was reported within an initial 16 week treatment period. The remission rate was 66% in treatment group vs. 51% in placebo group, though no additional long-term benefit was noted at a 2 year follow-up period, in terms of numbers of relapses, time to relapse, and endoscopic score.

Many of the anti-mycobacterial drugs first administered to CD patients were developed for treatment of *Mycobacterium tuberculosis* infections. Although effective in managing tuberculosis and leprosy, they are not effective in CD. Infection caused by Mycobacterium avium complex (MAC) organisms (to which group MAP belongs) is resistant to these standard antituberculosis drugs. MAC organisms can rapidly develop mutations that confer drug resistance (Chamberlin W et al, 2001). Mycobacteria are intracellular parasites residing within macrophages and other host cells, which these antibiotics are unable to penetrate. For effective antimicrobial therapy, agents that have intracellular activity must be used (Hermon-Taylor J et al, 2000). Recent data suggesting defective macrophage function in CD further supports the necessity of having highly intracellular concentrations of antibiotic to suppress MAP infection.

The treatment of MAC infections in both HIV and non-HIV infected patients was advanced by the development of a new series of therapeutic agents with intracellular activity. MAP is a MAC organism, thus lessons learnt from the treatment of MAC are relevant to the treatment of MAP (Borody TJ et al, 2002).

The two main agents are the chemical derivative of rifamycin, rifabutin, a RNA synthesis inhibitor, and the macrolide clarithromycin which inhibits protein synthesis. Both these antibiotics have good activity against MAC and MAP alone and in combination with other agents (Borody TJ et al, 2002). These drugs are also concentrated within macrophages and other cells (Chamberlin W et al, 2001). About one-quarter of cases of active Crohn's disease appear to be clinically resistant to rifabutin and clarithromycin (Chamberlin W et al, 2001). A logical approach based on many years experience in the management of human Mycobacterial infection, would be the use of more than two antibiotics in combination, chosen following *in vitro* sensitivity assay to maximise the chance of remission, while minimising the risk of acquired drug resistance. This strategy has not been used in CD.

1.4 Immune response in Crohn's disease

Based upon the pattern of cytokine production CD4+ T cells can be characterised into 2 different phenotypes: Th1 responses and Th2 responses. Th1 responses are characterised by the secretion of IL-2, TNF-α and IFN-γ, which are cytokines important for cellular immunity. Th2 responses typically secrete IL-4, IL-5 and IL-10 (Camoglio, L, et al, 1998; Romagnani, S, 1999). Th1 cells mainly develop following infections by intracellular bacteria and some viruses (Romagnani, S, 1999).

IL-12 has been found to play an important role in the generation of a Th1 immune response. IL-12 is produced by antigen-presenting cells (APC) following the interaction of the CD40 molecule on the APC with its ligand CD40L on T cells. IL-12 induces IFN-γ synthesis which drives Th1 differentiation (Pallone, F and Monteleone, G, 1998 and Van Den Brande, JMH et al, 2002).

IL-12 is a heterodimer consisting of 2 covalently linked polypeptide subunits (p35 and p40). Transcripts for both these subunits have been identified in CD lamina propria mononuclear cells (LPMC) (Monteleone, G, 1997). Immunohistochemical analysis has also shown that IL-12 production in the human intestine is restricted to macrophages infiltrating lamina propria (Parronchi, P et al, 1997). Parronchi (1997) also found that culturing T cells from gut biopsies from CD patients in the presence of neutralising anti-IL-12 antibody, down-regulated the development of IFN-γ producing CD4+ T cells. The up regulation of IL-12 production appears to account for the Th1 response in CD.

The Th2 response is responsible for strong antibody production, eosinophil activation and inhibition of macrophage functions, which provides phagocyte-independent protective responses (Romagnani, S, 1999). The cytokine IL-4 acts to promote Th2 differentiation. The T-cell response in UC appears to be Th2 (Van Den Brande, JMH, et al, 2002).

Upon activation lymphocytes rapidly proliferate and subsequently decline to preimmunization levels. This regulation is the termination of the immune response by apoptosis (Shanahan F, 2002 and Van Den Brande, JMH, 2002). Evidence has emerged that in CD there may be defective T-cell apoptosis (Ina, K, et al, 1999 and Boirviant, M, 1999). In CD, activated T-cells are resistant to apoptosis and this leads to their accumulation and the perpetuation of inflammation (Ina, K, et al, 1999; Boirviant, M, 1999 and Shanahan, F, 2002).

1.5 CARD15/NOD2 Gene Mutations

In 2001 2 independent groups identified 3 mutations within the NOD2 gene (renamed CARD15 - Caspase activating recruitment domain 15, by the International Nomenclature Committee), which were found to be associated with CD (Hugot, et al, and Ogura, et al, 2001). The identified mutations included 2 missense mutations R702W (2104C→T; SNP8), and G908R (2722G→C; SNP12) and one frameshift mutation 1007fs (3020insC; SNP13) which introduces a stop codon resulting in a truncated protein. This gene has been localised to chromosome 16q12. These 3 main mutations of CARD15 have been found in patients with CD but not in patients with UC.

From data found by Hugot et al (2001) it has been found that the relative risk for developing CD in simple heterozygotes is 3, whereas in homozygotes it is 38. Compound heterozygotes, individuals that have one mutation on one chromosome and one on the other, function as homozygotes and have a relative risk of 44 (Bouma, G and Strober, W, 2003).

The CARD15 gene is comprised of 12 exons which encode for a protein composed of 2 NH2-terminal caspase recruitment domains (CARDs), a nucleotide-binding domain (NBD) and 10 COOH-terminal leucine-rich repeats (LRR) (Ogura, et al, 2001b).

The CARD15/NOD2 cytoplasmic proteins are expressed in phagocytes, including monocytes, macrophages, dendritic cells and polymorphonuclear cells, and in intestinal epithelial cells. Recently, it has been found that Paneth cells express CARD15/NOD2 in mouse and human intestine (Lala, S et al, 2003). Paneth cells specialise in host defence by producing antibacterial peptides and lysozyme at the bottom of the crypts. CARD15/NOD2 appears to be involved in defensin production; as a result it is thought to limit the invasion of bacteria present in the gut lumen (Hugot, 2006). Thus it appears to have a pivotal role in innate immune defence.

The exact function of CARD15/NOD2 is still unknown, however what is known is that through its C-terminal LRR it allows cells to recognise and react to bacterial component peptidoglycan, muramyl dipeptide (MDP). The 3 variants of the CARD15 gene occur around the LRR which suggest that they alter the capacity of the host to respond to bacterial components. The CARD domain is also known to be involved in signal transduction, via the caspases which lead to apoptosis.

Under MDP activation CARD15 activates the NF-κB pathway. Activation of NF-κB induces the expression of the pro-inflammatory cytokines TNF-α and IFN–γ (Caprilli, R and Guagnozzi, D, 2003). Expression of TNF-α and IFN–γ in turn regulates the expression of CARD15. Activated NF-κB has also been shown to play an important role in eliminating invasive intracellular bacteria (Caprilli, R and Guagnozzi, D, 2003).

The peptidoglycan component of bacterial cell walls also activate the Toll-like receptor (TLR) pathway. TLRs are expressed on the cell surface of human intestinal mucosal and epithelial cells. CARD15 receptor protein is the functionally intracellular counterpart of the TLR. Once TLRs are activated by specific antigen it too leads to the activation of NF-κB.

The 3 variants of the CARD15 gene occur around the LRR which suggests that they alter the capacity of the host to respond to bacterial components. Since CARD15 function is impaired, the innate immune system is no longer able to fulfil its role and hyperactivity of the adaptive immune systems occurs (Caprilli R, Guagnozzi D, 2003). Lack of intolerance may ensue with amplification of the inflammatory process (Caprilli R, Guagnozzi D, 2003). Disruption of the delicate balance with the gastrointestinal mucosa could lead to chronic intestinal inflammation.

1.6 Hypothesis

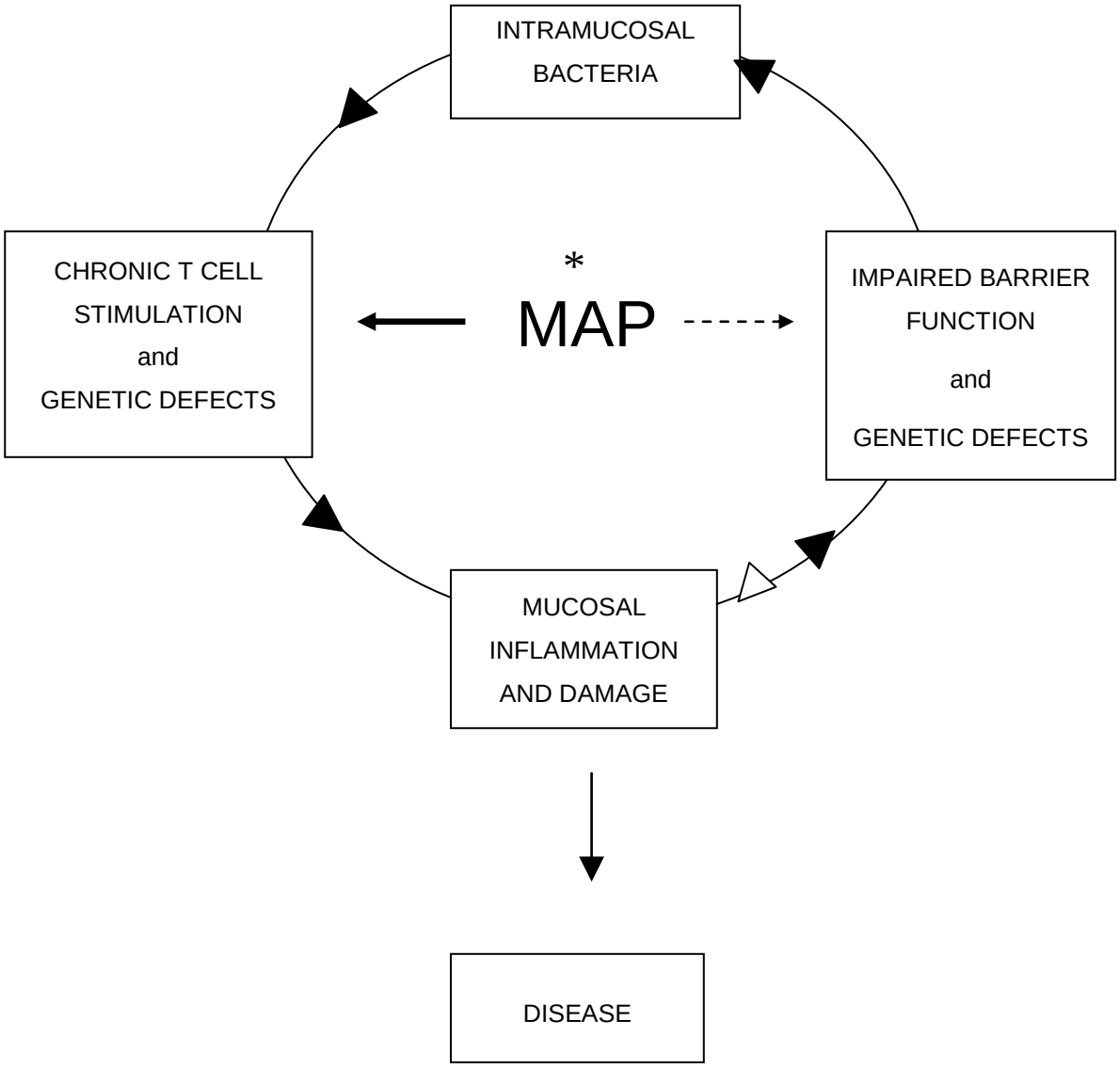
Transmural inflammation of CD is promoted by the presence of MAP within the gut wall (Figure 1). This thesis tests the hypothesis by:

1. Detecting the incidence of MAP in the gut wall of CD patients, compared with normal and inflammatory controls.

2. MAP correlation with proinflammatory cytokines in CD.

3. Correlation of MAP presence and an inflammatory response with NOD2 mutations.

Figure 1: Possible role of *Mycobacterium avium* ss *paratuberculosis* (MAP) in Crohn's Disease.



* initiating aetiology

CHAPTER TWO: *MAP detection and culture from mucosal biopsies.*

2.1 Introduction

In this chapter the prevalence of MAP infection in CD, compared with IBS, UC and normal subjects is determined. A nested polymerase chain reaction (PCR) is used on DNA which has been extracted from fresh human intestinal mucosal biopsy samples to detect the unique 1.4 kb insertion element IS900. By designing DNA primers for PCR within the IS900 insertion element it has enabled researchers to identify MAP in CD tissue.

A review of literature showed an average isolation rate of MAP to be 38% from CD and 7% from non-inflammatory bowel disease including UC patients (El-Zaatari, et al 2001). In 2004 Professor John Hermon-Taylor's group in the UK published results which reported a 92% detection rate of MAP in CD patients and 26% in non-CD (Bull et al, 2003). They claim to have increased sensitivity of detection by optimising steps in the DNA extraction process which greatly increased the number of IS900 copies extracted. The commercially available MGIT Culture System from BD Biosciences was also shown to be a useful system for culturing MAP from mucosal biopsies. Their results were obtained by using only fresh tissue, using ribolysation, resuspending pellets overnight at 4^{o}C and with nested PCR using Roche Expand High Fidelity Taq Polymerase.

By using the methods described by Bull et al (2003) we hope to optimise the technique of detection of MAP from colonic mucosal biopsies and thus determine the prevalence of MAP infection in CD, UC, IBS and normal subjects.

2.2 Materials and Methods

Following the published methodology of Bull, et al (2003) the MAP DNA is extracted from biopsy samples by manual disruption, using a Fastprep Ribolyser (Qbiogene, CA, USA), followed by phenol:chloroform extraction. DNA was then used for nested PCR. PCR products were then viewed by Ethidium Bromide staining following agarose gel electrophoresis. Biopsy samples were also set up for culture using the BD Biosciences MGIT Culture System.

2.2.1 Mucosal biopsy specimen collection

The study was approved by the Ethics Committee of the Centre for Digestive Diseases, Sydney, and Human Research Ethics Committee, University of Newcastle, Australia. Informed consent was obtained from all patients. A total of 259 patients were recruited into the study. Subjects were assessed in four groups: ulcerative colitis (UC) (n=57), Crohn's disease (CD) (n=70), irritable bowel syndrome (IBS) (n=49) and normal subjects (n=83). The diagnosis was based on classical clinical, endoscopic and histological data. Disease activity was assessed as active by the presence of inflamed gut at endoscopic examination of 70 patients with CD; 64 subjects had active inflammation and 6 had no inflammation. Of 57 patients with UC, 37 had active inflammation and 20 had no inflammation observed at colonoscopy. The mucosa was normal in both normal controls and subjects with IBS. During colonoscopic examination, biopsy specimens of inflamed mucosa were obtained from the colon or terminal ileum for histopathology and the detection of *Mycobacterium avium* subsp *paratuberculosis* (MAP) by nested PCR. Subjects with no macroscopic inflammation had random colonic biopsy specimens collected.

Patients presenting both for review and at initial diagnosis were included. For each specimen collected, 2 negative controls were also processed, these are 2 ribolyser tubes containing Mycobacteria Lysis Buffer (MLB) and no biopsy.

2.2.2 DNA Extraction from mucosal biopsies

1. Biopsies are collected into 500μl 0.85% (biological) saline.
2. Biopsy transferred to the laboratory where it is to be processed on the same day.
3. Centrifuge biopsy in saline at 10,000 rpm for 2-3mins.
4. Remove supernatant.
5. Resuspend biopsy in 600μl Mycobacteria Lysis Buffer (MLB) (Appendix) with 33 μg/ml proteinase K (Promega) and 0.6% SDS (Sigma-Aldrich).
6. Transfer all directly to a Lysing Matrix B (blue capped) ribolyser tube (Qbiogen).
7. Incubate (on an angle to increase surface area for better tissue digestion) at 37°C for at least 2 hours with gentle shaking.
8. At this point the tube can be frozen at –20^{o}C or processed immediately.
9. Ice the ribolyser tubes for 5mins (or defrost the samples if they have been stored at –20^{o}C).
10. Ribolise at 6.5m/s^{-2} for 45secs using FastPrep Instrument (Qbiogene, CA, USA).
11. Ice immediately for 15mins to allow the sample to settle.
12. Add 600μl Phenol @ pH 6.7 (saturated in 1X TE pH 8.0) (Sigma-Aldrich).

13. Vortex for 20secs.
14. Centrifuge 13,000g for 1 min.
15. Pipette aqueous layer (about 550μl) to a new tube.
16. Add 550μl Phenol/Chloroform/Isoamylalcohol (25:24:1) saturated in 1X TE pH 8.0 (Sigma-Aldrich).
17. Vortex 20secs.
18. Centrifuge 13,000g for 1 min.
19. If there is a layer of fat on the top (from large tissue samples) this should be removed at this stage.
20. Pipette aqueous layer (about 500μl) to a new tube.
21. Add 500μl Chloroform/Isoamylalcohol (24:1) (Sigma-Aldrich).
22. Vortex for 20 secs.
23. Centrifuge 13,000g for 1 min.
24. Pipette aqueous layer (about 450μl) to a new tube.
25. Add 90μl (1/5 vol) of RT 10M NH_4Ac (Sigma-Aldrich) and mix well.
26. Add 1ml (2x vol) Ice cold 100% ethanol.
27. Precipitate at RT for at least 1 hr.
28. Centrifuge 13,000g for 20mins.
29. Pour off supernatant and wash in 750μl ice cold 70% ethanol.
30. Centrifuge 13,000g for 5mins.
31. Pour off and air-dry pellets for up to 30mins.
32. Resuspend in 50μl 1 x TE (Appendix) and store at 4°C overnight.
33. Use 5μl of sample for each PCR reaction.

2.2.3 IS900 + pIDL60 Plasmid preparation

Positive DNA Control, consisting of a plasmid (pIDL60) containing a single copy of IS900, was kindly donated by Professor J. Hermon-Taylor's research group (Bull et al 2003). This plasmid was cloned into *E.coli* DH5α and selected with ampicillin. Plasmid purified using Wizard® *Plus* Minipreps DNA Purification System (Promega).

Wizard® Plus Minipreps DNA Purification System Method

1. Prepared LB media with ampicillin 100 μg/ml.
2. 5mls of LB media inoculated with *E.coli*/plasmid.
3. Left overnight at 37^{o}C, with shaking.
4. Pellet cells for 10min at 2500 to 3000 rpm in 50ml Falcon tube.
5. To cell pellet 300μl of cell resuspension solution added, cell suspension mix then transferred to 1.5ml Eppendorf tube.
6. 300μl cell lysis solution added to Eppendorf tube and mixed by inverting 4 times.
7. 300μl neutralisation solution then added to Eppendorf tube and mixed by inverting 4 times.
8. Centrifuge for 5 min in microcentrifuge at 12000 rpm.
9. Connect syringe and minicolumn.
10. Add 1ml resin to minicolumn/syringe assembly.
11. Transfer the clear lysate from Step 8 to the resin.
12. Push lysate through column using syringe.

13. Wash column with 2 ml of column wash solution.
14. Transfer the minicolumn to a 1.5ml Eppendorf tube. Centrifuge for 2min.
15. Transfer minicolumn to new 1.5ml Eppendorf tube and add 50µl of dH_2O to the minicolumn. Wait 1 min and then centrifuge for 20 sec to collect Plasmid DNA.
16. Discard minicolumn. Store plasmid DNA at -20^{o}C.

2.2.4 IS900 Nested PCR

Nested PCR to detect the IS900 insertion element was performed using primers designed by Bull et al (2003). Mycobacterial lysis buffer (MLB) processed in parallel with each specimen batch and PCR reagent only were used as negative controls. Positive and negative controls for both first round PCR and nested PCR were run on each PCR reaction. Extensive precautions were taken as described by Bull et al (2003).

First round PCR

For the first round of PCR the following primers were used, Liz1: 5'- CTT TCT TGA AGG GTG TTC GG - 3'; Liz2: 5'- ACG TGA CCT CGC CTC CAT -3'. (Invitrogen, Custom Primers).

Reaction Mix

Final concentrations (50 ul reaction mix) 3.5U Expand High Fidelity Taq polymerase enzyme mix (Roche, Roche Expand High Fidelity PCR System).

1x Expand HF reaction buffer containing 1.5mM $MgCl_2$

2μM of each primer

100μM dNTP (A, C, G, and T for primary PCR, *BUT* in nested reaction mix dTTP is replaced with **200μM** of dUTP) (Promega) (Appendix)

10% DMSO (Sigma-Aldrich) is added to each of the samples to stabilise the G and C rich template

PCR master mix: First Round PCR

REAGENT	**STOCK CONC.**	**VOL (ul)**
10 x Reaction buffer	10 x	5
Forward Primer	20 uM	5
Reverse Primer	20 uM	5
DMSO	100%	5
dNTP	2.5 mM	2
dH_20		22
Taq (Roche High Fidelity)	100 U	1
DNA		5
FINAL VOLUME		50 ul

Cycling conditions were 1 cycle at 94^{o}C for 5 mins, then 30 cycles of 94^{o}C for 1 min, 58^{o}C for 1 min and 72^{o}C for 3 mins, finished with 1 cycle of 72^{o}C for 7 mins.

Following amplification first-round PCR products were treated with 1 U of uracil-DNA glycosylase (Roche) at room temperature for 10 min, followed by 10 min at 95^{o}C.

Nested PCR

Second round PCR primers: AV 1: 5'- ATG TGG TTG CTG TGT TGG ATG G -3'; AV 2: 5'- CCG CCG CAA TCA ACT CCA G -3' (Invitrogen, Custom Primers).

Reaction Mix

5 μl of PCR product from first round of PCR was added to nested PCR mix. In the nested reaction mix dTTP is replaced with **200μM** of dUTP (appendix).

PCR master mix: Nested PCR

REAGENT	**STOCK CONC.**	**VOL (μl)**
10 x Reaction buffer	10 x	5
Forward Primer	20 μM	5
Reverse Primer	20 μM	5
DMSO	100%	5
dNTP (*dUTP)	2.5 mM	2
dH_20		22
Taq (Roche High Fidelity)	100 U	1
DNA		5
FINAL VOLUME		50 ul

Cycling conditions were 1 cycle at 94^{o}C for 5 mins, then 40 cycles of 94^{o}C for 1 min, 58^{o}C for 1 min and 72^{o}C for 3 mins, finished with 1 cycle of 72^{o}C for 7 mins.

Electrophoresis

Following nested PCR the product is visualised with Ethidium Bromide staining of agarose gels.

1. Dissolve 1.5 g per 100 mls of DNA Grade agarose (Quantum Scientific) in 1 x TBE (appendix) by microwaving. Allow to cool.
2. Add 2 μl Ethidium Bromide to gel.
3. Pour gel into cast and allow to set.
4. Run out 10-20μl nested product alongside 100 bp DNA ladder (Promega) on the agarose gel at 80 V for 45 minutes in 1 x TBE.
5. View under UV light and take photo.

The IS900 nested product is 298bp (Figure 1) in length. Results are given as a negative (in the absence of 298 bp band) and positive (in the presence of 298 bp band).

2.2.5 DNA Sequencing

Positive samples were confirmed by DNA sequencing analysis. DNA Sequencing was performed by the Biomolecular Research Facility, Newcastle DNA, of the University of Newcastle. Sequence data was then analysed performing a BLAST search on the NCBI, National Centre for Biotechnology Information website. Basic

Local Alignment Search Tool (BLAST) compares gene and protein sequences against others in public databases.

Purification of PCR product from agarose gel

The QIAquick Gel Extraction Kit (Qiagen) was used to purify the nested PCR product from the agarose gel.

1. Excise the DNA from the agarose gel using a clean, sharp scalpel.
2. Weigh the gel slice in a colourless tube. Add 3 volumes of Buffer QG to 1 volume of gel.
3. Incubate at 50^{o}C for 10 minutes (or until gel has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2-3 minutes during incubation.
4. After the gel slice has completely dissolved, check that the colour of the mixture is yellow (similar to Buffer QG without dissolved agarose).
5. Add 1 gel volume of isopropanol to the sample and mix.
6. Place a QIAquick spin column in a provided 2 ml collection tube.
7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute.
8. Discard flow-through and place QIAquick column back in the same collection tube.
9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.
10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 minute.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at 13,000 rpm.

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 μl of Buffer EB (10 mM Tris-Cl, pH 8.5) or H_2O to the centre of the QIAquick membrane and centrifuge the column for 1 min.

To calculate the concentration of PCR product, the purified product and a DNA ladder of known DNA amounts was electrophoresed and concentration was estimated in the presence of Ethidium Bromide. The optimal amount for DNA sequencing is 10ng/100 bases in a volume of less than 10 μl.

The second round PCR primers: AV 1: 5'- ATG TGG TTG CTG TGT TGG ATG G -3'; AV 2: 5'- CCG CCG CAA TCA ACT CCA G -3' were supplied at a concentration of 10 pmol/μl. Concentration was calculated by using a spectrophotometer and determining the absorbance at 260 nm. These primers were used for the sequencing reaction.

The chemistry used in Automated DNA sequencing relies on the use of 4 fluorescent dyes as labels for the reactions. The dyes are incorporated into the dideoxynucleotides using PCR. The labelled DNA is electrophoresed through a narrow bore capillary filled with a separation matrix. Fluorescence from the dye labelled reaction products is excited by light from the laser, and the resulting data is recorded as four colour raw data plots and an analysed data file.

The DNA sequence was then analysed by using the NCBI website BLAST tool. Basic Local Alignment Search Tool (BLAST) compares gene and protein sequences against others in public databases.

2.2.6 Culture of mucosal biopsy specimens

MAP cultures will be performed according to the method of Bull et al, 2003. Biopsies will be cultured using the commercially available MGIT (Mycobacteria Growth Indicator Tubes) system (BD Biosciences, Becton Dickinson).

1. Transfer biopsy to sterile 1.5 ml screw cap tube.
2. Add 500μl BBL Mycoprep (BD Bioscience) and incubate at RT for 20mins with occasional mixing.
3. Centrifuge at 10000 rpm for 5-10mins.
4. Remove supernatant.
5. Resuspend in 500μl MLB (appendix) minus SDS and Proteinase K or PBS (appendix).
6. Add to prepared BBL MGIT (BD Bioscience) tube for culture (See appendix).
7. Incubate at 37^{o}C for 14 – 88 weeks.
8. Once a culture has established, 0.5ml of the culture is used for DNA extraction and PCR (as per method previously described in 2.2.2).

Mycobacterial growth is detected by visually locating tubes that have fluorescence when viewed under UV fluorescence. Growth can also be detected by the

presence of a non-homogeneous turbidity, small grains or flakes in the culture medium. Tubes which were thought to be positive were then sub cultured and also tested for MAP by DNA extraction and PCR detection.

2.2.7 Analysis of sera by recombinant p35 and p36.

Serum samples from all the disease groups were sent to Dr Ira Shafran at Department of Molecular Biology and Microbiology and Center for Discovery of Drugs and Diagnostics, University of Central Florida, Orlando, Florida USA, using *M. paratuberculosis* antigens as serological markers (p35 and p36) to identify MAP infected patients. Recombinant clones of MAP, designated p35 and p36 and expressing 35- and 36-kDa proteins, were used to screen 197 patients (44 colitis, 41 Crohn's, 40 IBS and 72 normal) by Western blotting. Both p35 and p36 must be positive to be regarded positive.

2.3 Results

2.3.1 IS900 PCR Results

The IS900 nested PCR product is approximately 298 bp in size (Figure 2). DNA sequencing of the positive nested PCR products confirmed their identity as being from the IS900 insertion element unique to MAP (Figure 3).

Figure 2: IS900 nested PCR gel photo. Lane A contains the MAP positive PCR control. Lane B contains PCR negative control. In lanes P and S, a 298 bp product is evident. These subjects are positive for MAP.

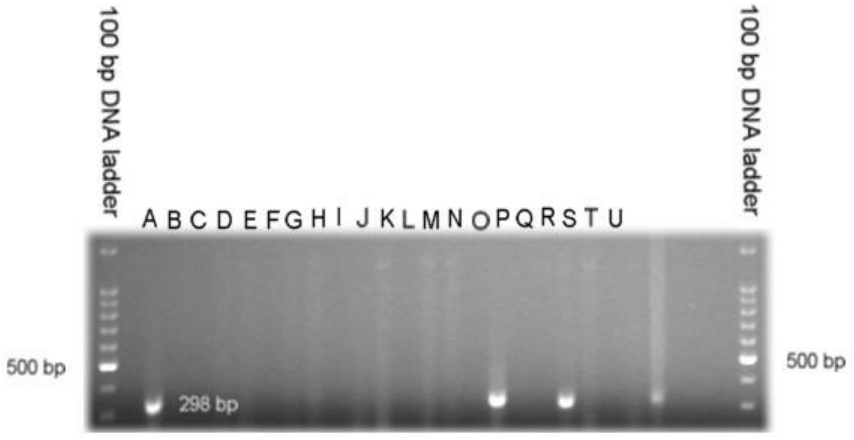


Figure 3: BLAST search results of IS900 PCR product sequencing. DNA sequence data was analysed by performing a BLAST search using the NCBI, National Centre for Biotechnology Information, website. Basic Local Alignment Search Tool (BLAST) compares gene and protein sequences against others in public database. 95% identity with *Mycobacterium avium* ss *paratuberculosis* IS900 transposase (p43) gene found, confirming that the PCR product is IS900.

```
gb|AF416985.1|  Mycobacterium avium subsp. paratuberculosis insertion
sequence
IS900 putative transposase (p43) gene, complete cds
Length=1453

 Score =  425 bits (470),  Expect = 6e-116
 Identities = 269/281 (95%), Gaps = 9/281 (3%)
 Strand=Plus/Plus

Query  4    AGGAAATNGGCCGNCCGGCGCTCCCGCGGACGGACTCGTACCGCTAATTGGAGAGATGCG
63
            |||| || ||||| |||||| ||||||| ||| ||||| |||||||||| |||||||||
Sbjct  105  AGGAGATTGGCCGCCCGGCG-TCCCGCG-ACG-ACTCG-ACCGCTAATTG-AGAGATGCG
159

Query  64   ATTGGCATCGCTGTGGTAAGGNACACGTCGGCGTGGTCGTCTGCTGGGTTGATCTGGACA
123
            ||||| ||||||||| ||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  160  ATTGG-ATCGCTGTG-TAAGG-ACACGTCGGCGTGGTCGTCTGCTGGGTTGATCTGGACA
216

Query  124  ATGGACGGTTACGGAGGTGGTTGTGGCACAACCTGTCTGGGCGGGCGTGGACGCCGGTAA
183
            ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  217  ATG-ACGGTTACGGAGGTGGTTGTGGCACAACCTGTCTGGGCGGGCGTGGACGCCGGTAA
275

Query  184  GGCCGACCATTACTGCATGGTTATTAACGACGACGCGCAGCGATTGCTCTCGCAGCGGGT
243
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  GGCCGACCATTACTGCATGGTTATTAACGACGACGCGCAGCGATTGCTCTCGCAGCGGGT
335

Query  244  GGCCAACGACGAGGCCGCGCTGCTGGAGTTGATTGCGGCGG  284
            |||||||||||||||||||||||||||||||||||||||||
Sbjct  336  GGCCAACGACGAGGCCGCGCTGCTGGAGTTGATTGCGGCGG  376
```

2.3.2 IS900 Detection in biopsy specimens

The detection rate of the MAP specific DNA IS900 by nested PCR was 12.3% (7/57) in ulcerative colitis subjects, 20% (14/70) in CD subjects, 18.4% (9/49) in IBS and 14.4% (12/83) in normal subjects. There was no statistically significant difference among the disease groups (X^2= 1.743, P = 0.6274).

At the time of study, treatment of those with CD was: 13 subjects were taking combination antibiotics (rifabutin, clarithromycin, and clofazimine: 4 for MAP^+ and 9 for MAP^-), 37 subjects were taking azathioprine, and/or mesalazine formulations, and/or prednisone (6 for MAP^+ and 31 for MAP^-) and 20 subjects were on no treatment (4 for MAP^+ and 16 for MAP^-).

2.3.3 Mucosal biopsy culture

Within the colitis group only 2 (n=57) were thought to be positive, CD 5 possible positives (n=70), IBS all were negative (n=49) and the normal subjects 2 possible positives (n=83).

However, upon subculture and DNA extraction/PCR analysis only 2 MAP positive cultures were found: 1 in the colitis group and 1 in the CD group. There was no statistical significance.

2.3.4 Analysis of sera by p35 and p36 MAP antigens

As seen in Table 1 there was no statistical significance among the disease groups when analysing sera by p35 and p36 antigens. There was a trend for Crohn's sera to be higher in reactivity against both antigens than the other groups, positivity rate for Crohn's 39.02%, Colitis 25%, IBS 35% and normal 30.96%, however it was not statistically significant.

When comparing MAP PCR results to the p35 and p36 results in the Crohn's group only 6 of the 40 (14.6%) were found to be positive for both, this was not statistically significant. The other groups were also not statistically significant; Colitis 1/44 (2.3%), IBS 3/41 (7.3%/) and normal 1/72 (1.4%).

Table 1: Results for reactivity of patient sera to p35 and p36 antigens. Both p35 and p36 must be positive to be regarded positive.

		Negative	**Positive**		**Positive rate (%)**
Colitis		**33**	**11**		**25.00**
(n=44)	MAP PCR+	5	1	X^2=0.257	
	MAP PCR-	28	10	p=0.612	
Crohn's		**25**	**16**		**39.02**
(n=41)	MAP PCR+	4	6	X^2=2.445	
	MAP PCR-	21	10	p=0.118	
IBS		**26**	**14**		**35.00**
(n=40)	MAP PCR+	5	3	X^2=0.027	
	MAP PCR-	21	11	p=0.868	
Normal		**52**	**20**		**27.78**
(n=72)	MAP PCR+	9	1	X^2=1.829	
	MAP PCR-	43	19	p=0.176	
Total		**136**	**61**		**30.96**
					X^2=2.625 p=0.453

2.4 Discussion

This chapter was designed to test the previously published methods of Bull (2003) who described an optimised technique for extracting and detecting MAP by PCR and culture from mucosal biopsies. By ensuring that the biopsy was processed immediately after collection during colonoscopy and that the biopsy sample was mechanically disrupted by ribolysation, and by using the optimal PCR conditions published we hoped to increase the sensitivity of the PCR reaction by increasing the number of copies of IS900 available for detection.

In our study MAP was found in only 20% of our CD population and 14% in normal subjects which was not statistically significant. This is in contrast to the results published by Bull (2003) who detected IS900 in 92% and 26% in CD and non-IBD patients, respectively. We had hoped that by using the optimised methods of Bull (2003) to reproduce their results.

This variability in results between laboratories is well documented, several studies have found IS900 in gut tissues (Romero C, et al, 2005; Bull, et al 2003; Lisby G, et al, 1994) and many groups have failed to find MAP (Baksh FK et al, 2004; Chiba M, et al, 1998; Rowbotham DS et al, 1995). In 2005, 2 independent groups, Sechi et al and Autschbash et al, reported results for the detection of IS900 using the optimised detection system. Sechi et al found MAP in 83% of CD patients by PCR and in 63% by culture. Autschbash et al detected MAP in 52% of CD patients by PCR. Autschbash also reported that even with using the optimised protocol they did find negative test results in known MAP positive patients, which they

suggested to be a result of heterogeneous distribution of MAP DNA within the tissue.

Our detection rate may be lower than the true infection rate for a number of reasons. Firstly, we used biopsy specimens obtained at colonoscopy rather than resected gut. In 2000 Schwartz published a paper on the use of short term culture for identifying MAP and compared the use of mucosal biopsies versus resected tissue in establishing cultures. They found that MAP was present in 86% of surgically resected tissue compared to 20% of biopsies. They suggested that MAP may reside in the submucosal layer of ulcerated tissue in CD patients rather than in the surface of the mucosal layer (Schwartz, et al 2000). It was also suggested that due to the fact that MAP in resected tissue was cultured in several weeks compared to several months in biopsies that MAP in deep tissue is virulent and metabolically active, while MAP on the surface of the mucosal layer, may be avirulent and inactive metabolically (Schwartz, et al 2000). Surgically resected tissues are a larger quantity of tissue and in theory may result in a higher number of MAP organisms available for PCR detection and culture. A number of our CD patients were also on or had taken anti-MAP therapy, a combination of rifabutin and macrolides. This of course would result in a negative nested PCR result.

MAP was found in 14.4% of our control group. This group however is not essentially a “healthy” group as they were undergoing colonoscopy for diagnostic and diverse reasons. They didn’t have an established diagnosis IBD, etc. As a

result positive MAP detection within this group could possibly be from a person with undiagnosed CD.

The IS900 nested PCR has been designed to detect as little as 2 copies of the MAP genome. It could also be possible that positive normal controls are a result of contamination. However, this was eliminated by running negative controls at all stages of the PCR assay, including DNA extraction. These negative controls were found to always give a negative result.

Our PCR and culture results highlight the difficulties in detecting this organism, due to its fastidious and slow growing nature and the complexity of its cell wall.

3.1 Introduction

The cause of chronic inflammation in the gut of patients with CD has not yet been identified, though two hypotheses are widely held. Firstly a heightened inflammatory response to enteric bacteria that have accessed the gut mucosal compartment has occurred as a result of genetic defects in the mucosal immune regulation and/or mucosal barrier protection (Podolsky, 2002). The second hypothesis is that a specific infection drives mucosal inflammation, the most popular candidate being Mycobacterium avium ss paratuberculosis (MAP) (Green, et al, 1989; Collins, et al, 2000; Naser, et al, 2004).

Through the study of patient and mouse models it has been found that CD is a Th-1 mediated inflammation (Chacon, Bermudez and Barletta, 2004). Th1 responses are characterised by the secretion of IL-2, TNF-α and IFN-γ, cytokines important for cellular immunity. Th1 responses are marked by transmural cellular infiltration that in some cases are associated with granulomas; this describes the histopathologic picture of CD. Th1 cells mainly develop following infection with intracellular bacteria and some viruses (Romagnani S, 1999).

In this chapter the pattern of cytokine secretion in both gut organ culture and blood are described. In a subsequent chapter, a link between pattern of cytokine secretion in the gut and diagnosis of MAP presence or absence is described.

3.2 Materials and Methods

Whole blood cultures and organ cultures were set up within 2 hours of sample collection. Cytokine levels were measured by ELISA; IL-4, IL-12, IFN-γ, IL-10, IL-2 and TNF-α from culture supernatants were quantitated using paired antibodies (BD PharMingen, San Diego, USA) according to manufacturer's protocols. Recombinant IL-4, IL-12, IFN-γ, IL-10, IL-2 and TNF-α (BD PharMingen) were used as standards. The limit of sensitivity was 7.8 pg/ml for IL-4, IL-12, IFN-γ, and TNF-α, and 15 pg/ml for IL-2 and IL-10. The absorbance was read at 450 nm in an ELISA plate reader (Bio-Rad 680, Japan). The amount of cytokine in samples was determined using Microplate Manager (5.2) software (Hercules, CA, USA).

Soluble antigens were supplied from Elizabeth Macarthur Agriculture Institute (Camden, NSW, Australia). They were from MAP strain 316 V prepared by mechanical disruption and purified by ammonium sulfate precipitation. Soluble antigens were used for whole blood culture. They were supplied at a concentration of 2.16mg/mL.

Total number of subjects was 116.

3.2.1 Organ Culture

The cytokines IL-12, IL-2, IL-10 and IFN-g were quantitated by ELISA following organ culture.

Day One

1. For each patient 2 tubes are set up (5ml round bottom tubes).
2. 1 ml serum-free AIM-V medium (Life Technology, Melbourne, VIC, Australia) added to each tube.
3. Transfer 2 biopsy samples into each tube. Each biopsy weighed about 10 mg (with little variation)
4. Incubate at 37^{o}C with 5% CO_2 overnight.

Day Two

1. Remove supernatant into sterile Eppendorf tube.
2. Freeze supernatant at -20^{o}C. Supernatants were used for cytokine quantitation by ELISA.

3.2.2 Prepare MAP antigen for culture stimulation

1. Make first antigen concentration at 10μg/mL with AIM-V.
2. Then prepare serial dilutions as follows:

1.5 mL Tube	Concentration μg/mL	Volume to be diluted (μL)	Antigen to be diluted	Volume AIM-V (μL)
Tube 1	10	-	-	-
Tube 2	1	100	Tube 1	900
Tube 3	0.1	100	Tube 2	900
Tube 4	0	-	-	1000

3.2.3 Whole Blood Culture

1. Pipette 150 μl of whole blood into well of tissue culture plate. Each specimen has 3 rows.

ROW 1: CONTROL

150 μl of whole blood

150 μl of AIM-V

ROW 3: MAP ANTIGEN (CONCENTRATION =)

150 μl of whole blood

150 μl of AIM-V + Antigen

ROW 4: MAP ANTIGEN (CONCENTRATION =) + ConA (Sigma-Aldrich).

150 μl of whole blood

150 μl of AIM-V + Antigen + ConA.

2. Incubate at 37^{o}C with CO_2 for 24 hours.
3. Collect all supernatants into labelled tubes (3 tubes per patient).
4. At this point the tube can be frozen at –20^{o}C or processed immediately.

The cytokines IL-2, TNF-α and IFN-γ were quantitated by ELISA following whole blood culture. Method found in section 3.2.5.

3.2.4 Whole blood culture and IL-4 Cytokine ELISA

Reagents IL-4 ELISA

IL-4 Capture Ab	0.5 mg	554515
IL-4 Detector Ab (Biotinylated)	0.5 mg	554483
Recombinant IL-4 Human Std	5 μg	554605

Dry coating plates with IL-4 antibody

1. Place plate(s) in a biosafety hood and turn on UV light to sterilise plate(s) while hood lid is on for 20 minutes.
2. Dilute the IL-4 antibody (0.5mg/mL) to 1μg/mL in carbonate buffer as follows: Capture antibody is diluted 1:500 (stock concentration = 0.5 mg/mL). Load 100μL per well onto plate and incubate overnight at 4-8^{o}C. Coat few plates with coating buffer only as assay control.

No of Plates	**1**	**5**	**10**
Volume coating buffer (mL)	12	55	105
Volume stock capture antibody (μL)	24	110	210
Volume of 1%BSA-PBS-T (μL)	12	55	105

3. Flick out coating antibody or carbonate buffer (without washing) and blot to dry on paper towel.
4. Place plate(s) in a 37˚C incubator, upside down and without plate sealer on, for a maximum of 20 minutes.
5. Wrap dry-coated plate(s) in foil and seal it properly in plastic bag with silica gel. Attach a paper card with labelling date and type of capture antibody in each bag.
6. Store dry-coated plates in 4-8˚C in refrigerator (up to 1 year).

Day One

Set up whole blood culture on IL-4 coated plate

1. Record the number of samples being run on the Workbook.
2. Remove dry-coated strips from a sealed bag according to the number of samples to be run. For 1-2 samples, three IL-4-coated strips and 1 uncoated strip are needed; for 3-4 samples, six IL-4-coated strips and 2 uncoated strips are needed. Place IL-4-coated strips in to column 1-3 and 4-7; and place uncoated strips in to column 4 and 8 on a frame.
3. Add 150 µL of AIM-V to each well at row A and row E from column 1-8; add 120 µl of AIM-V to each well at row B, C, D and row F, G, H from column 1-8.
4. Add 30 µL of antigen (0.1µg/mL) to wells at row B and F from column 1-8 (gives final concentration of 0.01µg/mL when blood has been added); and add 30 µL of antigen (1 µg/mL) to wells at row C and G from column 1-8, and 30 µL of antigen (10µg/mL) to wells at row D and H from column 1-8 (gives final

concentration of 0.1µg/mL and 1µg/mL respectively when blood has been added). Load 150 µL of whole blood to each well in quadruplicate as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A1	A1	A1	C1	C1	C1	C1				
B	A2	A2	A2	A2	C2	C2	C2	C2				
C	A3	A3	A3	A3	C3	C3	C3	C3				
D	A4	A4	A4	A4	C4	C4	C4	C4				
E	B1	B1	B1	B1	D1	D1	D1	D1				
F	B2	B2	B2	B2	D2	D2	D2	D2				
G	B3	B3	B3	B3	D3	D3	D3	D3				
H	B4	B4	B4	B4	D4	D4	D4	D4				

Note: Each shading is for one subject. Leave column 9-12 empty for standards

5. Incubate plate at 37˚C for 18-20 hours in an incubator with 5% CO2 and air.

Day Two

Prepare IL-4 standards

For working solution, dilute 1ng/mL IL-4 stock 2 fold as follows:

Tube Number	Tube for diluting	Volume of Standard (μl)	Volume of Diluent (μl)	Final Conc. (pg/ml)
S1		1500	0	1000
S2	S1	750	750	500
S3	S2	750	750	250
S4	S3	750	750	125
S5	S4	750	750	62.5
S6	S5	750	750	31.25
S7	S6	750	750	15.6
S8	S7	750	750	7.8

1. Place two IL-4-coated strips in to the frame at column 9-10. Load 300 µL/well of the standards as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A									S1	S1		
B									S2	S2		
C									S3	S3		
D									S4	S4		
E									S5	S5		
F									S6	S6		
G									S7	S7		
H									S8	S8		

2. Incubate plate for 90 minutes at room temperature.
3. Label 1.5 ml Eppendorf tubes for each subject. In a biosafety hood, set pipette to 205 µL and take supernatant out of well as much as you can (but avoid red blood cells). Pool supernatants from the same stimulation concentration into an eppendorf tube. Store sample at -70˚C. Samples collected for cytokine assays, IL-2, IFN-γ and TNF-α.
4. In a biosafety hood, add 150 µL of PBS-T to each sample well by using a multichannel pipette, pipette up and down for 3-4 times and remove blood sample to a container containing 30% bleach. Then wash plate 5 times by loading 300 µL/well with a plate washer containing PBS-T. Blot dry plate on paper towel.
5. Prepare monoclonal rat anti-human IL-4 biotinylated antibody at 0.5 µg/mL by diluting 1:1000 with 1%BSA-PBS-T (ie.10µL IL-4 Biotin in 10mL diluent). Load 100µL/well with biotinylated antibody and incubate for 90 minutes at room temperature.
6. Wash plate 5x with PBS-T, then blot dry plate on paper towel.
7. Prepare streptavidin-HRP conjugate by diluting 1:1000 with diluent.
8. Add 100µL/well. Incubate for 1 hour at RT. **NB: take out substrate buffer from fridge now, and allow to come to room temperature.**
9. Wash plate 5 times with PBS-T.
10. Prepare developer (TMB + Substrate buffer) 100 µL TMB + 100 mL substrate buffer. Add 100 µl/well. Incubate for 10 min RT. **Note**: this step does NOT have to be done in the dark, but does need to be accurately timed.

11. Stop development by adding 50µL 1 M H_2SO_4 to each well.

12. Read absorbance at 450 nm within 30 minutes of stopping the reaction.

3.2.5 Cytokine ELISA – IL-12, IFN-γ, IL-10, IL-2 and TNF-α

Reagent Preparation

Paired antibodies (BD PharMingen, San Diego, USA) used according to manufacturer's protocols. Recombinant IL-12, IFN-γ, IL-10, IL-2 and TNF-α (BD PharMingen) were used as standards.

Antibodies	**Size**	**Catalogue Number (BD)**
IL-12 (p70) Capture Ab	0.5 mg	555065
IL-12 (p40/70) Detector Ab (Biotinylated)	0.5 mg	554660
Recombinant IL-12 Std	5 µg	554613
IL-10 Human Capture Ab	0.5 mg	554705
IL-10 Human Detector Ab (Biotinylated)	0.5 mg	554499
Recombinant IL-10 Human Std	5 µg	554611
IFN-γ Capture Ab	1.0 mg	551221
IFN-γ Detector Ab (Biotinylated)	0.5 mg	554550
Recombinant IFN-γ Human Std	52 µg	554616
IL-2 Capture Ab	0.5 mg	555051
IL-2 Detector Ab (Biotinylated)	0.5 mg	555040
Recombinant IL-2 Human Std	10 µg	554603
TNF Capture Ab	1.0 mg	551220
TNF Detector Ab (Biotinylated)	0.5 mg	554511
Recombinant TNF Human Std	10 µg	554618

Day One

1. Prepare human capture antibody. Antibody diluted in carbonate buffer to the following concentrations.

Antibody	Stock Conc.	Dilution	Final Conc.
IL-2	0.5 mg/ml	1:500	1 μg/ml
IL-12	1 mg/ml	1:500	2 μg/ml
IL-10	0.5 mg/ml	1:250	2 μg/ml
TNF	1 mg/ml	1:500	2 μg/ml
IFN-γ	1 mg/ml	1:500	2 μg/ml

2. Load 100 μl per well onto plate and incubate overnight at 4^{o}C.

Day Two

1. Wash plate 3 times with washing buffer.
2. Block with diluent for 1 hour at room temperature.
3. Wash plate 3 times with wash buffer.
4. Dilute all standards with diluent (PBS/1% BSA/Tween 20) to a final concentration of 2 ng/ml.

Standard	Stock Concentration (μg/ml)
IL-2	200
IL-12	200
IL-10	100
IFN-γ	250
TNF	200

For the working solution of standard, the 2 ng/ml stock is diluted as follows.

Tube Number	Tube for diluting	Volume of Standard (μl)	Volume of Diluent (μl)	Final Concentration (pg/ml)
S1		1000	0	2000
S2	S1	500	500	1000
S3	S2	500	500	500
S4	S3	500	500	250
S5	S4	500	500	125
S6	S5	500	500	62.5
S7	S6	500	500	31.25
S8		0	1000	0

5. Dilute samples with diluent, if needed. Record dilution factor.
6. Load 100 μl of standard and samples to appropriate wells. Record plate set up in lab book/worksheet.
7. Incubate for 2 hours at room temp.
8. Wash with washing buffer 3 times.
9. Prepare biotinylated antibody by diluting with diluent as follows.

Antibody	Stock Concentration (mg/ml)	Dilution
IL-2	0.5	1:1000
IL-12	0.5	1:500
IL-10	0.5	1:500
IFN-γ	0.5	1:500
TNF	0.5	1:1000

10. Add 100μl/well. Incubate 90 min at RT.
11. Wash plate 3 times with wash buffer.
12. Prepare streptavidin-HRP conjugate by diluting with diluent as follows.

Cytokine	Streptavidin Dilution
IL-2	1:2000
IL-12	1:1000
IL-10	1:1000
IFN-γ	1:1000
TNF	1:2000

13. Add 100μl/well. Incubate for 1 hour at RT.
14. Wash plate 3 times with wash buffer.
15. Prepare developer (TMB+Substrate buffer) 100 μl TMB + 100 ml buffer.
16. Add 100 μl/well. Incubate for 10 min RT.
17. Stop reaction by adding stop buffer, 50 μl 1M H_2SO_4.
18. Read absorbance at 450 nm in an ELISA plate reader (Bio-Rad 680, Japan).

3.2.5 Statistical Analysis

The non-parametric Kruskal-Wallis was used to compare levels of TNF-α, IL-12, IFN-γ, IL-10, IL-4 and IL-2 across the four patient groups followed by Mann Whitney when the Kruskal-Wallis test was significant. All statistical analyses were performed using Prism® (Version 4) software (GraphPad Software Inc., San Diego CA, USA) and a 5% level of significance was used.

3.2 Results

3.3.1 Organ Culture

Subjects were assessed in four groups: ulcerative colitis (UC) (n = 53), Crohn's disease (CD) (n = 63), irritable bowel syndrome (IBS) (n = 45) and normal (n = 74). The diagnosis was based on classical clinical, endoscopic and histological criteria.

Of the 63 CD patients, 52 had active inflammation observed at colonoscopy and 11 had no inflammation. Within the 53 UC subjects 32 had active inflammation and 21 had no inflammation observed at colonoscopy. The mucosa in both normal controls and IBS subjects was normal.

Within the CD group 31 subjects had lesions in the terminal ileum (5 involving caecum), 24 had lesions in the colon, 5 had lesions in small bowel/or near the anastomosis after right hemi colectomy and 3 had a pouch following resection.

At the time of study, treatment of those with CD was: ten subjects were taking combination antibiotics (rifabutin, clarithromycin and clofazimine), 35 subjects were taking azathioprine, and/or mesalazine formulations and/or prednisone, and eighteen subjects were no longer on treatment.

3.3.2 Cytokine secretion from organ culture supernatant

There was no significant difference in secretion of IL-2, IL-12, IL-10 and IFN-γ (Table 2) among the disease groups. There was a trend for an increase in IL-12 in CD subjects.

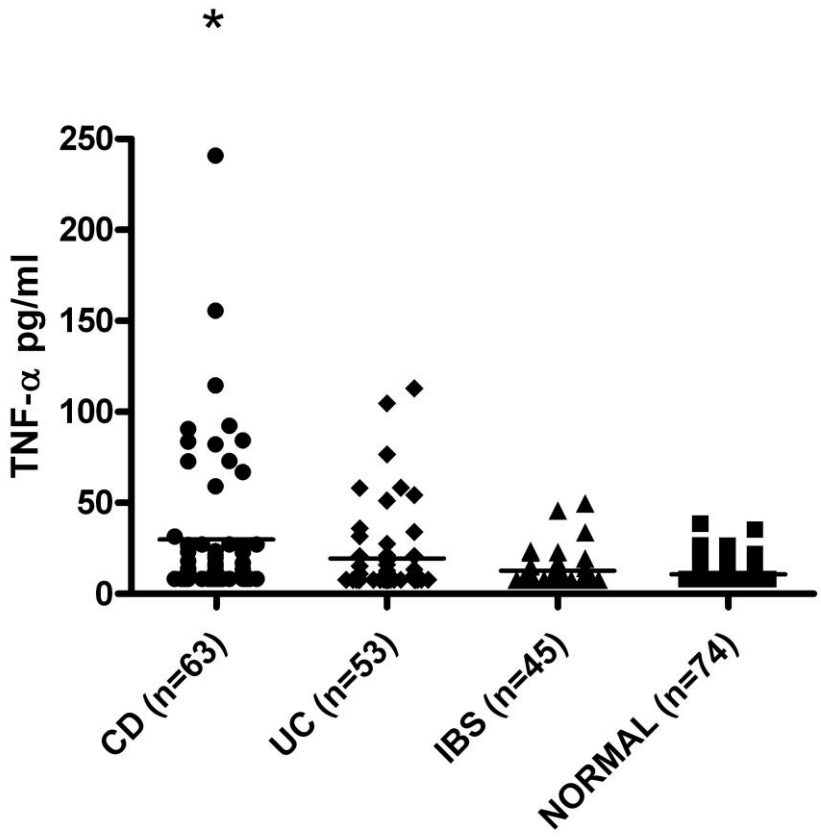
Significantly greater levels of TNF-α were found for CD, compared to UC, IBS and normal mucosa (Figure 4).

With respect to treatment in CD, those treated with combination antibiotics and corticosteroids had lower levels of TNF-α secretion compared to untreated subjects. However only a trend was noted as numbers were small and levels variable. The levels of TNF-α were 21.0 $\pm$ 8.2, 29.9 $\pm$ 6.1 and 34.8 $\pm$ 13.3 pg/ml in subjects with combination antibiotic therapy, azathioprine and/or mesalazine formulations and/or prednisone and no treatment, respectively. There was no statistical significance noted in active versus inactive CD as assessed at colonoscopy (30.98 $\pm$ 6.0 pg/ml versus 24.5 $\pm$ 9.2 pg/ml). The levels of TNF-α were not statistically significant between those with diseased ileal, diseased colon and those studied post-surgery (29.8 $\pm$ 8.9, 31.6 $\pm$ 7.3 and 24.8 $\pm$ 7.3, respectively).

Table 2: IL-12, IFN–γ, IL-10 and IL-2 cytokine secretion in organ culture from all disease groups. The non-parametric Kruskal-Wallis test was used to compare levels of IL-12, IFN-γ, IL-10 and IL-12 across the disease group. Results are expressed as Mean ± S.E. NS: not significant.

	IL-12 (pg/ml)	***IFN-γ (pg/ml)***	***IL-10 (pg/ml)***	***IL-2 (pg/ml)***
CD (n = 63)	82.2 ± 23.3	56.3 ± 9.4	25.8 ± 3.5	29.8 ± 3.4
UC (n = 53)	56.6 ± 10.2	57.3 ± 12.4	22.9± 2.9	24.1 ± 2.0
IBS (n=45)	51.5 ± 8.8	45.0 ± 7.0	20.9 ± 1.4	25.3 ± 2.0
Normal (n = 74)	51.3 ± 8.3	64.9 ± 19.3	22.6 ± 1.2	26.4 ± 1.7
p value	NS	NS	NS	NS

Figure 4: TNF-α levels in gut organ culture supernatant. The culture supernatant was determined for TNF-α by ELISA. Horizontal bars denote the mean. (CD n=63, UC n=55, IBS n=44 and normal n=74). (*) $p < 0.05$, $p < 0.01$, $p < 0.0001$ compared with the value of UC, IBS and normal controls subjects, respectively.



3.3.3 Whole Blood Culture

Subjects were assessed in 4 groups: UC (n=30), CD (n=46), IBS (n=22) and normal subjects (n=18). Of 46 patients with CD, 37 subjects had active disease and 9 were inactive. Of the 30 UC patients, 25 had active disease and 5 were inactive. Treatment of those with CD at the time of study was as follows: 25 subjects were taking azathioprine, and/or mesalazine formulations and/or prednisone, 7 subjects were taking anti-MAP antibiotics (rifabutin, clarithromycin and clofazimine) and 14 subjects were on no treatment.

3.3.4 Cytokine secretion in Whole Blood Culture

There was no statistical significance in IL-4, IFN-γ, TNF-α and IL-2 levels across the disease groups (Table 3). Analysis of cytokine secretion patterns in relation to presence of active disease (mucosal inflammation with or without ulceration) showed no significant differences for IFN–γ, IL-2 and IL-4 secretion. No difference in secretion pattern of any cytokine was noted in any group in relation to therapy.

Table 3: Cytokine secretion in blood cultures of CD, UC, IBS and normal subjects. Results are expressed as median (first quartile, third quartile) . The non-parametric Kruskal-Wallis test was used to compare levels of IL-4, IFN-γ, TNF-α and IL-2 across the disease group. NS: Not significant.

	IL-4 (pg/ml) ***Median (95% CI)***	***IL-2 pg/ml)*** ***Median (95% CI)***	***IFN-γ((pg/ml)*** ***Median (95% CI)***	***TNF-α (pg/ml)*** ***Median (95% CI)***
CD ***(n = 46)***	11.0 (7.8, 44.7)	15.2 (15.0, 27.9)	15.4 (7.8, 27.2)	7.8 (7.8, 30.1)
UC ***(n = 30)***	7.8 (7.8, 42.2)	21.1 (15.0, 34.5)	18.1 (11.4, 56.4)	7.8 (7.8, 22.4)
IBS ***(n=22)***	8.4 (7.8, 29.2)	15.0 (15.0, 16.9)	8.3 (7.8, 29.5)	7.8 (7.8, 24.2)
Normal ***(n = 18)***	7.8 (7.8, 39.0)	15.1 (15.0, 34.5)	14.8 (7.8, 51.8)	7.8 (7.8, 22.5)
P-value	NS	NS	NS	NS

3.3.5 Effect of MAP Antigen on Cytokine Secretion

The effect of the MAP antigen on Th1 and Th2 cytokine secretion was observed by measuring the levels of IFN-γ and IL-4 after whole blood culture with soluble MAP antigen. There was no significant increase in IL-4 and IFN-γ levels in antigen-stimulated cultures (Table 4 and 5, respectively).

Table 4: The effect of MAP antigen on IL-4 secretion in normal, UC, CD and IBS subjects. Results expressed as mean ± SE. ns: not significant.

	Control	**0.1 (μg/ml)**	**1.0 (μg/ml)**	**10.0 (μg/ml)**
Normal	85.7 ± 77.9	61.7 ± 53.9	91.8 ± 84.0	79.4 ± 63.63
UC	92.8 ± 33.3	176.0 ± 40.6	198.6 ± 56.2	54.02 ± 19.7
CD	352.7 ± 87.5	282.3 ± 85.92	377.7 ± 107.4	78.63 ± 29.04
IBS	31.07 ± 15.4	42.7 ± 23.12	66.01 ± 35.4	65.78 ± 14.29
p value	ns	ns	ns	ns

Table 5: The effect of MAP antigen on IFN-γ secretion in normal, UC, CD and IBS subjects. Results expressed as mean ± SE. ns: not significant.

	Control	**0.1 (μg/ml)**	**1.0 (μg/ml)**	**10.0 (μg/ml)**
Normal	49.89 ± 16.34	96.36 ± 29.59	62.62 ± 16.6	78.37 ± 16.5
UC	50.34 ± 12.5	312.52 ± 91.6	130.7 ± 34.97	212.4 ± 59.7
CD	181.59 ± 134.36	1946.9 ± 263.1	207.3 ± 140.0	263.4 ± 131.5
IBS	55.71 ± 25.15	2217.8 ± 379.5	459.13 ± 246.2	635.8 ± 277.0
p value	ns	ns	ns	ns

3.4 Discussion

The aim of this chapter was to determine the pattern of cytokine secretion across the disease groups. It was found that in CD patients there are significantly higher levels of TNF-α in culture supernatants. This is not surprising as TNF-α has been found as a marker of inflammation in stool (Braegger, CP et al 1992), in intestinal mucosal cells (Breese EJ et al 1994), in the stool of children with Crohn's disease increased TNF-α have been found (Nicholls, S, et al, 1993) and has also been identified as a secretion product from lamina propria cells from IBD patients (Reinecker HC et al 1993).

TNF-α has been found to be a crucial proinflammatory cytokine in various inflammatory diseases and is a well characterised effector molecule in CD. Therefore it has been approached for therapeutic use. By neutralising TNF it has been shown to decrease the recruitment of inflammatory cells and granuloma formation in animal models (van Dullemen HM et al 1995). Van Dullemen et al (1995) investigated the potential efficacy of anti-TNF in the treatment of CD and found that TNF is of major importance in the pathogenesis of CD and treatment with anti-TNF was safe and useful in patients with CD that are unresponsive to steroid treatment and is now a central management tool. The chimeric monoclonal antibody to TNF-α, Infliximab, was introduced into clinical practice for the treatment of CD in 1997. Infliximab causes rapid suppression of mucosal inflammation, as indicated by a reduction in the number of lamina propria cells producing TNF-α (Radema SA, et al, 1996). It is a well documented treatment option in patients

with active CD who have not responded to traditional therapy (Mortimore M et al 2001; Keating GM and Perry CM 2002; Lin J et al 2007). Infliximab and anti-TNF biologics are the first drugs shown to induce endoscopic and histologic healing in patients with CD (Lin J et al 2007). In 2002, ten Hove reported that treatment with Infliximab causes a rapid and specific increase in apoptosis of T lymphocytes in the gut mucosa. Resistance to T-cells against apoptosis is a potential key factor in the pathogenesis of CD.

In the mucosa immune responses are characterised by major expansions of antigen-specific T-cells that have potent effector function. Whilst this is important for host defence, it can lead to effector cells populations with substantial autoreactivity and the capacity to cause mucosal inflammation (Neurath, et al, 2001). To deal with this, the mucosal immune system has evolved strategies to control mucosal immune responses (Neurath, et al, 2001). Among these strategies is apoptosis, programmed cell death, which can occur via active mechanisms following T-cell receptor (TCR) stimulation. In CD it appears that bacterial antigens in the gut induce T-cell activation and Th1 cell differentiation via IL-12. The mediator substances from Th1 cells such as TNF and IFN-γ activates macrophages to release TNF, IL-6 and IL-12, which in turn mediate T-cell resistance against apoptosis in the gut (Neurath, et al, 2001). This results in prolonged production of cytokines, leading to resistance to apoptosis and finally disease perpetuation (Neurath, et al, 2001). In the 2002 paper by ten Hove et al, they reported that in CD patients Infliximab rapidly increased the number of

apoptotic T cells in the inflamed mucosa. Their data suggested that Infliximab functions as an “immunotoxin” that specifically targets mucosal T lymphocytes involved in CD pathogenesis.

In CD patients the secretion of TNF-α is significantly higher than in UC, IBS and normal subjects. In the following chapter we determine whether the presence of MAP contributes to the pathogenesis of Crohn's disease by promoting cytokine secretion within the gut mucosa.

4.1 Introduction

The aim of this chapter is to determine whether MAP detected in gut biopsies is associated with a different cytokine secretion profile in whole blood and organ culture. The method of assay used a whole blood culture technique that detects secretion from T cells committed to a pattern of cytokine secretion which is amplified by ligation of CD40L on the surface of the T cell by CD40 expressed on platelets (Hein, V, et al, 1998; Leiva, LE, et al, 2001; Danese S, et al, 2004).

Recent evidence has shown a spheroplastic form of MAP within the cytoplasm of macrophages from CD patients by Ziehl-Neelsen staining and confirmed by PCR (Gearry, RB, et al, 2005). Macrophages are phagocytic cells that function by containing the infecting micro-organism, once contained they then form granulomata to wall off foreign material from the remainder of the body and secrete cytokines that prime and amplify the immune system (Van Den Brande, 2002). A chronic inflammation characteristic of CD is granulomas (Van Den Brande, 2002; Marks, et al, 2006).

By showing a link of an altered T cell function to MAP infection in CD it would provide a link between detection of MAP and disease.

1.2 *Materials and methods*

IL-4 from culture supernatants were quantitated using paired antibodies (BD PharMingen, San Diego, USA) according to manufacturer's protocols. Recombinant IL-4 (BD PharMingen) was used as a standard, the limit of sensitivity was 7.8 pg/ml. Whole blood cultures were set up within 2 hours of sample collection and cytokine levels were measured by ELISA. The amount of IL-4 in samples was determined using a Microplate Management program (Bio-Rad, Japan).

Organ culture, whole blood culture and cytokine ELISA for IFN-γ, TNF-α, IL-10, IL-12 and IL-2 was performed using methods described in Chapter 3.

Soluble antigens were supplied from Elizabeth Macarthur Agriculture Institute (Camden, NSW, Australia). They were from MAP strain 316 V prepared by mechanical disruption and purified by ammonium sulfate precipitation. Soluble antigens were used for whole blood culture.

Total number of subjects was 116.

Reagents

IL-4 Capture Ab	0.5 mg	554515
IL-4 Detector Ab (Biotinylated)	0.5 mg	554483
Recombinant IL-4 Human Std	5 μg	554605

Antibodies (BD Biosciences)

4.2.1 Whole Blood Culture And IL-4 ELISA

Dry coating plates with IL-4 antibody

1. Place plate(s) in a biosafety hood and turn on UV light to sterilise plate(s) while hood lid is on for 20 minutes.
2. Dilute the IL-4 antibody (0.5mg/mL) to 1µg/mL in carbonate buffer as follows: Capture antibody is diluted 1:500 (stock concentration = 0.5 mg/mL). Load 100µL per well onto plate and incubate overnight at 4-8^{o}C. Coat few plates with coating buffer only as assay control.

No of Plates	**1**	**5**	**10**
Volume coating buffer (mL)	12	55	105
Volume stock capture antibody (µL)	24	110	210
Volume of 1%BSA-PBS-T (µL)	12	55	105

3. Flick out coating antibody or carbonate buffer (without washing) and blot to dry on paper towel.
4. Place plate(s) in a 37˚C incubator, upside down and without plate sealer on, for a maximum of 20 minutes.

5. Wrap dry-coated plate(s) in foil and seal it properly in plastic bag with silica gel. Attach a paper card with labelling date and type of capture antibody in each bag.
6. Store dry-coated plates in 4-8˚C in refrigerator (up to 1 year).

Prepare JD antigen for culture stimulation

1. Make first antigen concentration at 10μg/mL with AIM-V.
2. Then prepare serial dilutions as follows:

1.5 mL Tube	Concentration μg/mL	Volume to be diluted (μL)	Antigen to be diluted	Volume AIM-V (μL)
Tube 1	10	-	-	-
Tube 2	1	100	Tube 1	900
Tube 3	0.1	100	Tube 2	900
Tube 4	0	-	-	1000

Day One

Set up whole blood culture on IL-4 coated plate

1. Record the number of samples being run on the Workbook.
2. Remove dry-coated strips from a sealed bag according to the number of samples to be run. For 1-2 samples, three IL-4-coated strips and 1 uncoated strips are needed; for 3-4 samples, six IL-4-coated strips and 2 uncoated strips are needed. Place IL-4-coated strips in to column 1-3 and 4-7; and place uncoated strips in to column 4 and 8 on a frame.

3. Add 150 µL of AIM-V to each well at row A and row E from column 1-8; add 120 µl of AIM-V to each well at row B, C, D and row F, G, H from column 1-8.
4. Add 30 µL of antigen (0.1µg/mL) to wells at row B and F from column 1-8 (gives final concentration of 0.01µg/mL when blood has been added); and add 30 µL of antigen (1 µg/mL) to wells at row C and G from column 1-8, and 30 µL of antigen (10µg/mL) to wells at row D and H from column 1-8 (gives final concentration of 0.1µg/mL and 1µg/mL respectively when blood has been added). Load 150 µL of whole blood to each well in quadruplicate as follows:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	A1	A1	A1	A1	C1	C1	C1	C1				
B	A2	A2	A2	A2	C2	C2	C2	C2				
C	A3	A3	A3	A3	C3	C3	C3	C3				
D	A4	A4	A4	A4	C4	C4	C4	C4				
E	B1	B1	B1	B1	D1	D1	D1	D1				
F	B2	B2	B2	B2	D2	D2	D2	D2				
G	B3	B3	B3	B3	D3	D3	D3	D3				
H	B4	B4	B4	B4	D4	D4	D4	D4				

Note: Each shading is for one subject. Leave column 9-12 empty for standards

5. Incubate plate at 37˚C for 18-20 hours in an incubator with 5% CO2 and air.

Day Two

Prepare IL-4 standards

For working solution, dilute 1ng/mL IL-4 stock 2 fold as follows:

Tube Number	Tube for diluting	Volume of Standard (μl)	Volume of Diluent (μl)	Final Conc. (pg/ml)
S1		1500	0	1000
S2	S1	750	750	500
S3	S2	750	750	250
S4	S3	750	750	125
S5	S4	750	750	62.5
S6	S5	750	750	31.25
S7	S6	750	750	15.6
S8	S7	750	750	7.8

1. Place two IL-4-coated strips in to the frame at column 9-10. Load 300 µL/well of the standards as follows:

	1	2	3	4	5	6	7	8	9	10	11	12
A									S1	S1		
B									S2	S2		
C									S3	S3		
D									S4	S4		
E									S5	S5		
F									S6	S6		
G									S7	S7		
H									S8	S8		

2. Incubate plate for 90 minutes at room temperature.
3. Label 1.5 ml eppendorf tubes for each subject. In a biosafety hood, set pipette to 205 µL and take supernatant out of well as much as you can (but avoid red blood cells). Pool supernatants from the same stimulation concentration into an eppendorf tube. Store sample at -70˚C. Samples collected for cytokine assays, IL-2, IFN-γ and TNF-α, using methods described in Chapter 3.
4. In a biosafety hood, add 150 µL of PBS-T to each sample well by using a multichannel pipette, pipette up and down for 3-4 times and remove blood sample to a container containing 30% bleach. Then wash plate 5 times by loading 300 µL/well with a plate washer containing PBS-T. Blot dry plate on paper towel.
5. Prepare monoclonal rat anti-human IL-4 biotinylated antibody at 0.5 µg/mL by diluting 1:1000 with 1%BSA-PBS-T (ie.10µL IL-4 Biotin in 10mL diluent). Load 100µL/well with biotinylated antibody and incubate for 90 minutes at room temperature.

6. Wash plate 5 times with PBS-T, then blot dry plate on paper towel.
7. Prepare streptavidin-HRP conjugate by diluting 1:1000 with diluent. Add 100μL/well. Incubate for 1 hour at RT. **NB: take out substrate buffer from fridge now, and allow to come to room temperature.**
8. Wash plate 5 times with PBS-T.
9. Prepare developer (TMB + Substrate buffer) 100 μL TMB + 100 mL substrate buffer. Add 100 μl/well. Incubate for 10 min RT. Note: this step does NOT have to be done in the dark, but does need to be accurately timed.
10. Stop development by adding 50μL 1 M H_2SO_4 to each well.
11. Read absorbance at 450 nm within 30 minutes of stopping the reaction.

4.2.2 Statistical Analysis

The non-parametric Kruskal-Wallis test for unpaired samples was used among disease groups. The Mann-Whitney test was used to compare cytokine data between MAP+ and MAP- subjects, and between disease categories. Spearman's rank correlation was used to assess correlation between IL-4 and IL-2 variables. Statistical analyses were performed using Prism software (Version 4; GraphPad Software, San Diego, CA, USA).

4.3 Results

Subjects were assessed in 4 groups: UC (n=30), CD (n=46), IBS (n=22) and normal subjects (n=18). Of 46 patients were CD, 37 subjects had active disease and 9 were inactive. Of the 30 UC patients, 25 had active disease and 5 were inactive. Treatment of those with CD at the time of study was as follows: 25 subjects were taking azathioprine, and/or mesalazine formulations and/or prednisone (5 were MAP+ and 20 were MAP-); 7 subjects were taking anti-MAP antibiotics (rifabutin, clarithromycin and clofazimine); 3 were MAP+ and 4 were MAP-) and 14 subjects were on no treatment (4 MAP+ and 10 MAP-).

Detection rates of MAP by IS900 PCR for these subject groups were as follows: 10% (3/30) in UC, 26.1% (12/46) in CD, 13.6% in IBS (3/22) and 16.7% (3/18) in normal subjects. There was no statistical significance among the disease groups (χ^2=3.63, P=0.305).

4.3.1 IL-4 Secretion in Whole Blood Culture

There was no statistical significance in IL-4 levels amongst the disease groups (Table 6). However, when comparing MAP+ CD to MAP- CD subjects the levels of IL-4 were significantly different, where significantly higher levels of IL-4 were detected in MAP+ CD subjects (Table 7). The levels of IL-4 in UC, IBS and normal subjects who were MAP+ were not significantly higher.

Table 6: Cytokine secretion in blood cultures in subjects with gastroenterological disease and normal controls. Values shown as median (first quartile;third quartile) NS not significant.

Disease	n	IL-4 (pg/ml)	IL-2 (pg/ml)	IFN-γ (pg/ml)	TNF-α (pg/ml)
Ulcerative Colitis	30	7.8 (7.8; 42.2)	21.1 (15.0;34.5)	18.1 (11.4;56.4)	7.8 (7.8;22.4)
Crohn's disease	46	11.0 (7.8;29.2)	15.2 (15.0;27.9)	15.4 (7.8;27.2)	7.8 (7.8;30.1)
Irritable Bowel syndrome	22	8.4 (7.8;29.2)	15.0 (15.0;16.9)	8.3 (7.8;29.5)	7.8 (7.8;24.2)
Normal	18	7.8 (7.8;39.0)	15.1 (15.0;34.5)	14.8 (7.8;51.8)	7.8 (7.8;22.5)
***P*-value**	-	NS	NS	NS	NS

4.3.2 IL-2 Secretion from Whole Blood Culture Supernatants

Within all the disease groups and the normal controls similar levels of IL-2 were found (Table 6). When MAP+ CD patients were compared to MAP- CD patients significantly higher levels were found in MAP+ CD (Table 7). No statistical significance was found in IL-2 secretion between MAP+ and MAP- subjects in the UC, IBS and normal groups (Table 7).

4.3.3 IFN-γ and TNF-α Secretion in Whole Blood Culture

There was no statistical significance of IFN-γ or TNF-α secretion within the disease groups and normal controls (Table 6). In regard to MAP status there was no difference in secretion of IFN-γ or TNF-α in each group (Table 7).

4.3.4 Effect of MAP Antigen on Cytokine Secretion

The effect of the MAP antigen on Th1 and Th2 cytokine secretion was observed by measuring the levels of IFN-γ and IL-4 after whole blood culture with soluble MAP antigen. There was no significant increase in IFN-γ levels of stimulated cultures from MAP+ subjects. No significant increase in IL-4 levels was also observed in antigen-stimulated cultures from MAP+ subjects.

Table 7: Cytokine secretion in whole blood culture from MAP+ and MAP- Crohn's disease and Ulcerative Colitis subjects. Results are expressed as medians and 95% confidence intervals. NS: Not significant.

	IL-4 (pg/ml) ***Median (95% CI)***	***IL-2 pg/ml)*** ***Median (95% CI)***	***IFN-γ((pg/ml)*** ***Median (95% CI)***	***TNF-α (pg/ml)*** ***Median (95% CI)***
CD ***MAP-*** ***MAP+***	7.8 (7.8,25.2) 30.1 (13.0,82.5)	15.0 (15.0,22.0) 24.4 (16.3,48.4)	11.3 (7.8,24.8) 16.4 (7.8, 37.5)	7.8 (7.8,30.1) 12.1 (7.8, 56.8)
P-value	0.02	0.03	NS	NS
UC ***MAP-*** ***MAP+***	7.8 (7.8, 57.7) 7.8 (7.8, 12.4)	21.0 (15, 31.4) 21.3 (19.1, 100)	19.1 (10.4,60.5) 18.1 (17.3, 18.2)	7.8 (7.8, 22.8) 7.8 (7.8, 7.8)
P-value	NS	NS	NS	NS

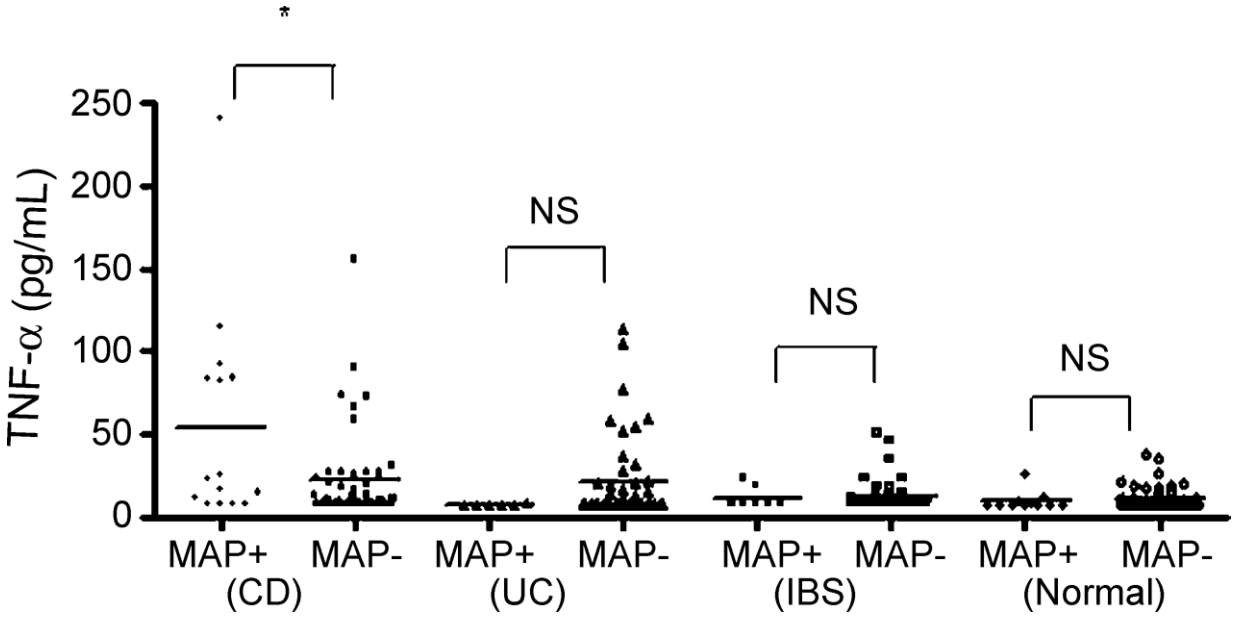
4.3.5 Cytokine secretion in organ cultures in relation to MAP status

There were no significant differences in IL-2, IL-12, IL-10, IFN-γ and TNF-α secretions in MAP-positive versus MAP-negative subjects with IBS, UC or normal mucosa. In CD, there were no significant differences in the secretion of IL-2, IL-12, IL-10 or IFN-γ in MAP-positive versus MAP-negative subjects, though a trend existed for an increase in IL-12 for MAP-positive subjects (Table 8). However, TNF-α secretion was more than two times higher in MAP-positive subjects ($p<0.05$, Figure 5). Furthermore, levels of TNF-α were significantly higher in MAP-positive CD than MAP-positive UC ($p<0.01$), MAP-positive IBS ($p<0.05$) and MAP-positive normal subjects ($p<0.01$), respectively. Subjects with CD who were MAP-positive but not treated with antibiotics (n=4) had higher levels of TNF-α than did MAP-negative subjects with CD (n=14) ($p<0.05$). CD subjects who were MAP-positive and who were treated with antibiotics for at least 3 months (n=3) secreted TNF-α at 40% of that of untreated MAP-positive CD but numbers were small. Similar treatment of MAP-negative CD was not associated with a difference in TNF-α secretion.

Table 8: Cytokine secretion organ culture from MAP+ and MAP- subjects. Results are expressed as medians and 95% confidence intervals. NS: Not significant.

	IL-12 (pg/ml) ***Median (95% CI)***	***INF-γ pg/ml)*** ***Median (95% CI)***	***IL-10 ((pg/ml)*** ***Median (95% CI)***	***Il-2 (pg/ml)*** ***Median (95% CI)***
CD				
MAP-	75.8±23.2	57.9+10.9	26.4±4.4	30.5±3.9
MAP+	102.7±65.5	51.3±19.3	23.8±3.5	27.6±6.5
P-value	NS	NS	NS	NS
UC				
MAP-	52.7±10.6	56.0±13.3	23.7±3.2	23.0±1.8
MAP+	60.8±36.5	67.2±37.3	16.8±1.8	32.8±10.2
P-value	NS	NS	NS	NS
IBS				
MAP-	49.8±10.0	47.1±7.9	20.5±1.4	26.1±2.2
MAP+	60.3±17.3	33.5±13.5	22.9±4.0	20.8±3.3
P-value	NS	NS	NS	NS
Normal				
MAP-	51.3±9.3	68.3±22.6	22.9±1.4	25.1±1.6
MAP+	51.5±17.6	45.7±11.6	20.6±1.8	33.4±6.8
P-value	NS	NS	NS	NS

Figure 5: TNF-a levels in gut organ supernatants. Culture supernatants were collected after 24 h culture of intestinal biopsy tissues and measured for TNF-a by ELISA. Horizontal bars denote the mean (*) $p<0.05$ compared with values of MAP-negative subjects. NS: Not significant.



4.4 Discussion

This chapter aimed to determine whether the pattern of cytokine secretion from CD4+T cells in CD was modified in those subjects positive for MAP in gut biopsies. To determine this a whole blood culture technique was used that detects secretion from T cells committed to a pattern of cytokine secretion which is amplified by ligation of CD40L on the surface of the T cell by CD40 expressed on platelets (Henn V et al, 1998; Leiva LE et al, 2001; Danese S et al, 2004). Supernatants from organ culture were also collected and measured for cytokine by ELISA.

The demonstration of an enhanced TNF-α secretion in the gut of subjects with CD who are positive for MAP is the first potential evidence of a mechanism that could link the presence of MAP with gut disease. The selective effect of MAP infection of TNF-α secretion in CD compared to an absence of such an effect in normal and disease controls suggests a specific defect in the cellular handling of MAP in CD. The relatively frequent occurrence of MAP detection in both normal and disease control groups is consistent with the idea that MAP is a common environmental contaminant that does not trigger inflammation in gut mucosa that has a normal capacity to handle the microbe. Thus infection of those capable of 'normal handling' of MAP, does not lead to an increase in TNF-α secretion.

Analysis of IL-2 and IL-4 results according to the presence or absence of MAP in the gut mucosa, found that in MAP positive CD subjects a significant and selective increase in secretion for both cytokines occurred. In MAP + subjects in the other

disease and normal control groups this was not found. This could suggest that these high cytokine levels in MAP+ CD are not a direct consequence of MAP infection, but rather an additional and constitutive difference in subjects with CD. What is unclear from the data is whether patients with CD have a potential 'Th2' secretion profile or is this found only in those who are MAP+. Also, is the difference in IL-2 and IL-4 secretion between MAP+ and MAP- patients with CD only due to stimulation *in vivo* of circulating MAP antigen of primed CD4+T cells.

IL-4 is an important cytokine in mucosal immunity. There is genetic evidence that subjects with CD have IL-4 and IL-4 receptor gene (-34T and Q576R, respectively) polymorphisms (Aithant GP, et al, 2001). The variant IL-4 and IL-4 receptor alleles have been suggested to be associated with increased transcriptional and signal activity. In humans, administration of IL-4 in cancer therapy is often associated with diarrhoeal illness and mucosal ulceration (Margolin K, et al, 1994) Also in CD patients the early ileal lesions after resection are associated with increased IL-4 mRNA secretion in mucosal biopsies (Desreumaux P, Brandt E, Gambiez L, et al, 1997). It is thought that high IL-4 concentrations in the gut mucosa promote inflammation as IL-4 disrupts the intestinal epithelial barrier to increase permeability (Di Leo V, Yang PC, Berin MC, Perdue MH, 2002) thus enabling the transmigration of enteric bacteria into the gut mucosa (Hollander D, 1999).

An increased IL-4 secretion has been claimed to promote *M. tubercuolis* in man and mouse infection (Seah et al, 2001; van Crevel et al 2001) which suggests that IL-4 dominance promotes mycobacterial disease (Lienhardt C, et al 2002). In

tuberculosis the eradiction of *Mycobacteria* is facilitated by cell mediated immunity, the infected macrophages are activated by Th1 cytokines, in particular IFN-γ. However, this may be antagonised by Th2 cytokines, in particular IL-4. The balance between Th1 and Th2 responses may influence mycobacterial growth.

It cannot be ascertained from these results whether the association of increased IL-4 secretion and the presence of MAP in CD patients is due to stimulation of T cells by circulating MAP or whether MAP persists due to impaired immunity which has resulted from high IL-4 levels. Another option is that in CD MAP infection increases IL-4 due to defective handling of antigen presenting cells.

From these observations it can be concluded that MAP in CD mucosa may promote disease by enhanced IL-4 secretion which affects mucosal permeability and by withdrawing the IFN-γ drive needed for effective control of mucosa infection by defective macrophage.

CHAPTER FIVE: *Analysis of CARD15/NOD2 polymorphisms by Real Time PCR .*

5.1 Introduction

In 2001 two groups, Hugot et al and Ogura et al, both reported the first susceptibility gene in CD, NOD2 now commonly known as CARD15. The gene is located on chromosome 16q12. Since this discovery numerous studies have shown that coding region polymorphisms in the NOD2/CARD15 are associated with CD. More than 100 mutations have been described in the CARD15 gene, but only 3 are associated with CD. They are the missense mutations R702W (2104C→T, SNP8), G908R (2722G→C, SNP12) and the frameshift mutation 1007fs (3020insC, SNP13) which introduces a stop codon resulting in a truncated protein (Hugot, et al, 2003). Hugot (2001) found that the relative risk of developing CD in simple heterozygotes is 3, in homozygotes it is 38. Compound heterozygotes, individuals that have one mutation on one chromosome and one on another, have a relative risk of 44 (Bouma G and Strober W, 2003). Genotype analysis of patients has shown that NOD2 variants are more significantly associated with ileal disease (Ahmad T et al, 2002).

The protein product of NOD2 is composed of 2 NH2 terminal caspase recruitment domains (CARD), a centrally located nucleotide binding domain and multiple COOH terminal leucine rich repeats (Ogura, et al 2001). The NOD family are a group of cytosolic proteins implicated in intracellular recognition of bacteria. NOD2/CARD15 is expressed by antigen presenting cells (APC) (eg macrophages,

dendritic cells) and epithelial cells (Ogura, et al, 2003) in Paneth cells, which are specialised intestinal cells located at the base of crypts of Liberkühn in the small intestine. The Paneth cells secrete defensins and other antimicrobial peptides into the lumen of the crypt to protect epithelial stem cells against microbes (Ogura et al 2003).

The bacterial peptidoglycan (PGN) derivates muramyldipeptide (MDP) have been shown to be ligands for NOD2/CARD15. The MDP binds to the LRR (leucine-rich repeats) of NOD2/CARD15 and is transported across the IEC (intestinal epithelial cell) membrane. Ligand binding then facilitates recruitment activation of RICK (receptor-interacting serine/threonine-protein kinase) through a CARD-CARD interaction which subsequently activates NF-κB transcription factor via the IKK complex, IFF-γ/NEMO. This then leads to phosphorylation, ubiquitination and degradation of IκB, which leads to production of cytokines, cryptdins and defensins (van Limbergen et al 2007a, van Limbergen et al 2007b, and Ogura et al 2001).

The exact mechanism that NOD2/CARD15 mutations contribute to the development of CD is still not completely known. The variants of NOD2/CARD15 have been shown to impair activation of NFκB *in vitro*. The variant 1007fs has been shown *in vitro* to be associated with a reduced activation of NFκB in mononuclear cells.

It has led to the hypothesis that in many people it is related to a dysfunction in the innate immune response. The frameshift mutation 1007fs which produces a truncated protein has been shown to be associated with ileal involvement of CD, young age onset, granuloma formation and fistulating phenotypes (Berrebi D et al 2003; Cuthbert AP et al 2002; Hampe JP et al 2001).

One hypothesis is that the truncated protein results in a defective activation of NFKB. However, it has been shown (Maeda S et al 2005) that in mice whose NOD2/CARD15 locus harboured the 1007fs allele NFKB activity was elevated. They argue that enhanced responsiveness to bacterial PGN predisposes a person to CD with NOD2/CARD15 mutations.

It has also been found that the NOD2 variants are found to be associated with a diminished α-defensin expression (Wehkamp J et al 2004; Kobayashi KJ et al 2005). The α-defensins are effector molecules that are predominantly expressed in the Paneth cells of the ileum, which also express NOD2/CARD15. They play an important role in maintaining the integrity of the gut mucosa. In patients with CD this integrity is impaired; it is thought that defensins deficiency may lead to impaired mucosal barrier function and susceptibility to bacterial invasion. This would trigger inflammation and loss of homeostasis. As a result a local inflammatory response, ie high TNF-α or NFKB production may cause barrier break (Buhner S et al 200%).

In this chapter the co-existence of MAP infection and NOD2/CARD15 mutation status was investigated. SNP analysis of the three most common NOD2/CARD15 variants: missense mutations R702W (2104C→T, SNP8), G908R (2722G→C, SNP12) and the frameshift mutation 1007fs (3020insC, SNP13); was performed on trial participants and compared to MAP status to determine if genetic susceptibility to CD predisposes them to MAP infection.

5.2 Materials and methods

5.2.1 Preparation of DNA

DNA was extracted from blood samples using the MagAttract^{TM} 96 Miniprep Core Kit (QIAGEN Pty Ltd, Australia) and BioRobot M48 workstation (QIAGEN Pty Ltd, Australia).

Purification of total DNA MagAttract DNA Blood Mini M48 kit.

Kit Contents

- MagAttract Suspension B
- Buffer ML
- Buffer MW1
- Buffer MW2
- RNase-free water

1 Thaw whole blood samples and equilibrate at room temperature.

2 Ensure Buffer ML does not contain a white precipitate by shaking bottle. If necessary incubate for 30 min at 37^{o}C with occasional shaking.

3 Add 26ml of ethanol to bottle of Buffer MW1 (contains approximately 77ml of buffer). Mix bottle by shaking five times. Tick the check box on bottle to indicated ethanol has been added.

4 Shake bottle of MagAttract Suspension B and vortex for 3 minutes to fully resuspend silica particles.

5 Place 200 μl of blood into 1.5 ml sample tube.

6 Switch on BioRobot M48.

7 Switch on computer and monitor.

8 Launch the QIAsoft M Operating system.

9 Select the protocol group “Genotyping” from drop-down menu, by clicking on the dark green arrow, then select “gDNA”.

10 Select the protocol “200 μl Blood” and click “Select” button to choose the elution tube type. Enter the number of samples, and sample and elution volumes into the software.

The QIAsoft M software guides you through the remaining steps required to set up the BioRobot M48 for the MagAttract DNA Blood Mini M48 protocol, these steps include the option of entering names of samples.

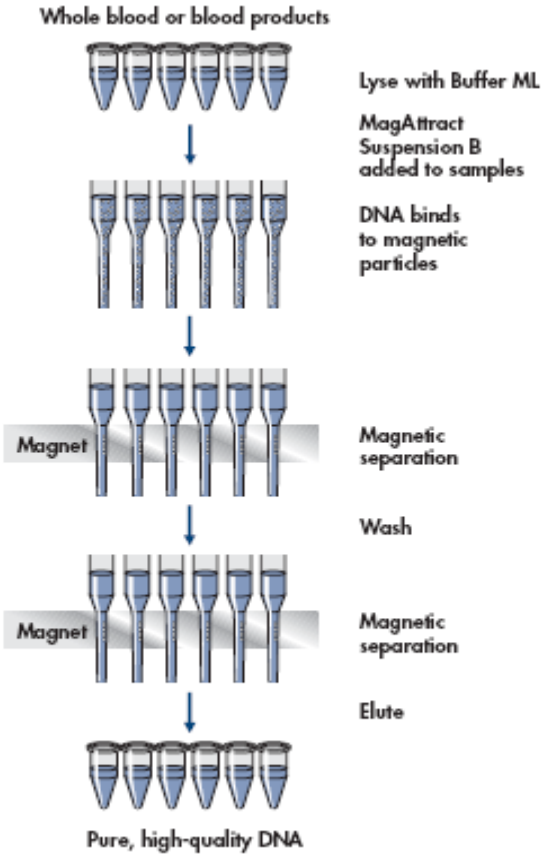
11 Place sample tubes on the worktable, plus reagent containers and plasticware, according to the software.

12 Close the workstation door and start purification protocol. All steps fully automated, and software message on screen will indicate when protocol is finished.

13 Retrieve elution tubes containing purified DNA from cooling block. DNA now ready to be used or stored.

14 A 20μl DNA aliquot was transferred to a 96 well plate. Sample number and position on plate was recorded. The plate was sealed and stored at 4^{o}C.

Figure 6: Flowchart of MagAttract DNA Blood M48 procedure. (MagAttract DNA Blood M48 Handbook, July 2005, QIAGEN Pty Ltd, Australia).



5.2.2 CARD15/NOD2 SNP analysis

Real time PCR allelic discrimination assays were developed to test for the presence of single nucleotide polymorphisms (SNPs) in the CARD15/NOD2 gene. SNPs for the gene CARD15/NOD2 were found using the SNP500Cancer Database (http://snp500cancer.nci.nih.gov/home). The three SNP sequences and the corresponding SNP identifier (dbSNP ID) are as follows: 1007fs (rs2066847), R702W (rs2066844) and G908R (rs2066845). The primers and probes are documented in Table 9. The assay reagents for SNP geonotyping were designed using Applied Biosystems Assays-by-Design Service^{SM} (Applied Biosystems, CA, USA). The assay reagents for SNP genotyping consisted of a 40X mix of unlabelled PCR primers and TaqMan® MGB probes (FAM^{TM} and VIC® dye-labelled). Each assay enables scoring of both alleles in a single well and are optimised to work with TaqMan® Universal PCR Master Mix.

Each assay used two specific, fluorescent-labelled probes to identify each allele. The probes for the wildtype alleles were labelled with 6-carboxyfluorescein (6-FAM). The probes for the variant alleles were labelled with VIC^{TM}. Wildtype and variant probes for each assay are also documented in Table 9.

Table 9: Sequences of probes and primers used to detect CARD15/NOD2 SNPs 1007fs, R702W and G908R.

Polymorphism		**Sequence**
1007fs	Primer Forward (F)	GTCCAATAACTGCATCACCTACCT
	Primer Reverse (R)	ACTTCCAGGATGGTGTCATTCC
	Probe Wildtype (WT)	CTGCAGGCCCTTG
	Probe Mutant (M)	CTGCAGGCCCCTTGA
R702W	Primer F	GCTGAGTGCCAGACATCTGAGA
	Primer R	AGACACCAGCGGGCACAG
	Probe WT	CCTGCTCCGGCGC
	Probe M	CCCTGCTCTGGCGC
G908R	Primer F	CTGTTGACTCTTTTGGCCTTTTCAG
	Primer R	CCACCTCAAGCTCTGGTGATC
	Probe WT	CTGTTGCGCCAGAAT
	Probe M	CTCTGTTGCCCCAGAAT

PCR Master Mix

1 Set up PCR master mix in PCR set up room as follows:

Reagent	**X 1**	**X 100**
40 x Assay Mix	0.125 μl	12.5 μl
2 x TaqMan® PCR Universal Master Mix (Applied Biosystems)	2.5 μl	250 μl
Sterilised MilliQ water (Millipore, Sydney, Australia)	1.375 μl	137.5 μl
DNA	1 μl	
TOTAL	5 μl	400 μl

2 To each well of a 96 well plate 4 μl of master mix is added.

3 1 μl of DNA added to corresponding well of 96 well plate.

4 Plate sealed with clear film.

5 Plate pulse spun.

5.2.3 Allelic Discrimination Analysis Procedure using ABI Prism 7900HT Sequence Detection System.

PCR reactions were conducted on the ABI PRISM® 7900HT sequence detection system (Applied Biosystems). Allelic discrimination assays are multiplexed end-point assays that detect variants of a single nucleic acid sequence. The presence of 2 primer/probe pairs in each reaction allows genotyping of the two possible variants at the SNP site in a target template sequence. Fluorescence emitted from the sequence specific probes is proportional to the level of sequence specific PCR product and is detected spectrophotometrically at intervals during the cycle.

1 Launch SDS2.2 Software and open a new document from file menu.

2 Select Absolute Quantification, Blank template from drop down list.

3 Highlight any wells on the plate grid that are not being analysed and deselect In Use box.

4 Select 96 well plate.

5 Open instrument by clicking on Instrument tab, select Open/Close and load plate onto tray once tray holder is presented, making sure to position the compression pad over the sealed tray. Press Open/Close to close.

6 Set up detectors (probe descriptions). From Tools menu select Detector manager.

7 Click File and then select New. The Add Detector dialogue box appears.

8 In the Name text field type a name for the detector, ie FAM™ Wildtype R702W and VIC® Mutant R702W.

9 Click the Reporter Dye and Quencher Dye drop-down list and select appropriate dyes for the assay.

10 Click the “Color” box to select a colour to represent the detector using the “Color Picker” dialogue box and Click OK.

11 Click OK to save the detector and return to Detector Manager dialogue box.

12 Repeat steps 7 to 11 to create detectors for all remaining assays on the plate.

13 Highlight wells on the plate grid and label with the appropriate detector(s) by ticking the Use box next to desired detector.

14 Select the detector Task relevant for each well from the drop down list, eg: Set the detector Task to NTC for no DNA controls, set to unknown for all other controls and samples.

15 From the SDS software, click the Instrument tab of the plate document.

16 Change the default thermal profile to 40 cycles, sample volume to 5 μl and untick 9600 emulsion button. The following thermal cycling conditions were used for 40 cycles: 50^{o}C for 2 min, 95^{o}C for 10 min, 92^{o}C for 15 sec, and 60^{o}C for 1 min.

17 Go to Setup and enter sample details into corresponding wells and save.

18 Save the document as a template.

19 Initiate run by selecting Start. The tray is taken into the instrument and the door closes. Do not use mouse or keyboard while instrument is operating.

Allelic Discrimination (End point read)

1 When run has completed click on OK and save the run.

2 Click on the large green analysis arrow on the tool bar,  . Highlight the patient samples on the plate grid. The amplification plot (Figure 6) (log graph) of the real time data can be viewed by licking on the Results tab.

View plots for each dye group by selecting the reporter dye from the dye drop down menu.

In the AD plot, using the lasso tool, circle the groups and select the appropriate detector name from the Call drop down menu. Repeat for any remaining assays performed on the same plate.

3 Select the sample cluster on the AD plot and from the Call drop down list select the Allele X call. Repeat to apply Calls to the rest of the samples within the plot, ie Allele X, Allele Y, Both, NTC and Undetermined.

4 Verify the calls by going to the plate grid and selecting wells containing NTC samples, the software highlights the datapoints with the allele plot. Check that the datapoints cluster is in the expected position on the plot. Also repeat for the wells containing positive controls for Allele X and Y.

See Figure 1 for an example of an Allelic Discrimination Plot.

5 Save the real time document. From the instrument tab select Open/Close, wait for plate to be presented and remove from holder. Select Open/Close and wait for door to close.

6 Open a new SDS document.

7 Select existing template or enter in laboratory numbers.

8 Highlight wells and add markers. Press save.

9 Press  again for results.

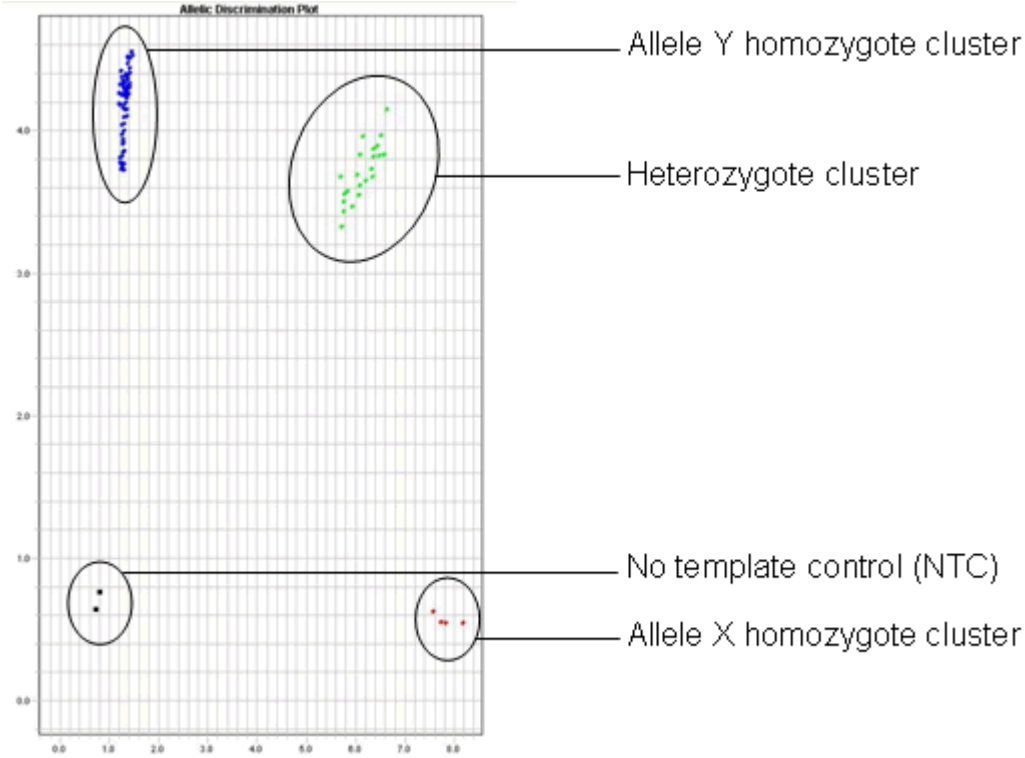
10 From the File menu save results as a *.txt file and export file. Go to Excel and open exported file from selected directory.

5.2.4 Statistical analysis

All statistical analyses were performed using Prism® (Version 4) software (GraphPad Software Inc., San Diego CA, USA) and a 5% level of significance was used. www.graphpad.com.

Figure 7: **A Normal Allelic Discrimination Plot (for SNP Genotyping).**

An allelic discrimination plot, also known as a "cluster plot" or an "AD plot" is shown below. Ideally, these plots show three clusters, and near the origin, the No Template Control (NTC). These clusters are described in the table below. The points in each cluster are grouped closely together, and each cluster is well separated from the other clusters.



A Typical three-cluster allelic discrimination plot

Assignment of clusters in an allelic discrimination plot

Samples Containing...	Are grouped in...
Allele X (homozygote), labeled with VIC® dye	Lower right corner of the plot
Allele Y (homozygote), labeled with FAM™ dye	Upper left corner of the plot
Both (allele X and allele Y—heterozygote)	Approximately midway between the allele X and allele Y clusters
No template control (NTC)	Bottom left corner of the plot
Undetermined	Anywhere on the plot

Source: Applied Biosystems website, http://www3.appliedbiosystems.com/WebTroubleshooting/SNPGoodExample/index.htm

5.3 Results.

5.3.1 CARD15/NOD2 mutation analysis

A total of 99 individuals were analysed for the NOD2/CARD15 SNP's; 1007fs, R70W and G908R. Of these 8 individuals were found to be heterozygous for 1007fs, 9 heterozygous for R70W and 5 heterozygous for G908R (Table 10). No individuals were homozygous for any of the SNP's. Two people were found to be compound heterozygotes, one was found to have both the 1007fs and G908R SNPs and the other had 1007fs and R70W. Both individuals have CD and clinically displayed inflammation with the terminal ileum.

The detection rate of the 3 variants by SNP analysis was 37.5% (12/32) in CD; 15.8% (3/19) in UC; 4.3% (1/23) in IBS and 16% (4/25) in normal individuals (Table 11). It was found that there was a statistical significance across the disease groups (X^2=10.03, P value = 0.0183) (Figure 8).

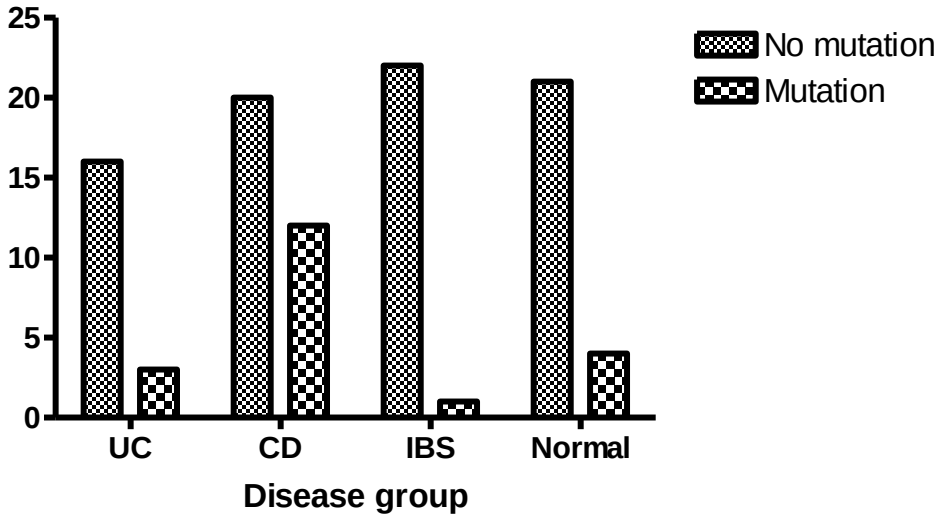
Table 10: Summary of SNP analysis of 99 individuals.

SNP	HETEROZYGOTES	HOMOZYGOTES
1007fs	8	0
R70W	9	0
G908R	5	0

Table 11: Mutation detection in Crohn's disease, Ulcerative Colitis, IBS and normal groups.

	NOD -	NOD +
Crohn's disease	20	12
Ulcerative Colitis	16	3
IBS	22	1
Normal	21	4

Figure 8: Chi square distribution of mutation vs no mutation across the disease groups.



When CD was compared against UC, IBS and the normal group (P value 0.1233, P value 0.0043 and P value 0.085, respectively) a statistical significance was only found when compared to the IBS group. When UC was compared to the IBS and normal groups no statistical significance was found, P value 0.3129 and 1.000 respectively. The IBS and normal group was compared and no statistical significance was found, P value 0.3622.

5.3.2 CARD15/NOD2 SNPs and MAP status

The correlation between NOD2/CARD15 and MAP in the disease groups was examined, Table 12. There was found to be no correlation between NOD2/CARD15 variants and MAP. Having a mutation in the NOD2/CARD15 gene did not increase the risk of MAP infection.

Table 12: Correlation between NOD2 and MAP. Fishers exact test performed to test statistical significance.

		MAP +	MAP -	P value
Colitis	NOD2+	0	3	1
	NOD2-	2	14	NS
Crohn's	NOD2+	2	10	1
	NOD2-	4	16	NS
IBS	NOD2+	0	1	1
	NOD2-	2	20	NS
Normal	NOD2+	0	4	1
	NOD2-	1	20	NS
ALL	NOD2+	2	18	1
	NOD20-	9	70	NS

5.3.3 CARD15/NOD2 and cytokine analysis

The effect of NOD2/CARD15 variants on cytokine secretion in the organ cultures and whole blood culture supernatants was then analysed. The Mann-Whitney test was used to compare these groups, Table 13 and 14.

There was no statistical significance found in any of the cytokine groups when compared to NOD2/CARD15 status.

Table 13: Whole Blood Culture Cytokine scretion compared to NOD2/CARD15 status.

NS – not significant

	NOD2/CARD15 – **(n=56)**	**NOD2/CARD15 +** **(n=13)**	***P* value**
IL-4 **q1, q3 interquartile range**	8.32 (7.8; 40.56)	12.59 (7.8;30.52)	0.3804 NS
IL-2 **q1, q3 interquartile range**	16.9 (15; 53.56)	15 (15; 27.66)	0.771 NS
IFN-γ **q1, q3 interquartile range**	17.74 (7.8; 53.09)	12.25 (7.8; 37.01)	0.0942 NS
TNF-α **q1, q3 interquartile range**	7.8 (7.8; 19.99)	7.8 (7.8; 21.69)	0.7422 NS

Table 14 : Organ Culture Cytokine secretion compared to NOD2/CARD15 status.

NS – not significant

	NOD2/CARD15 – **(n=79)**	**NOD2/CARD15 +** **(n=19)**	**P value**
IL-10 **Q1, q3 interquartile range**	17.65 (15; 26.75)	17.45 (15; 27.29)	0.6276 NS
IL-2 **Q1, q3 interquartile range**	16.98 (15; 30.76)	23.54 (15; 35.79)	0.1688 NS
IFN-γ **Q1, q3 interquartile range**	29.99 (16.86; 64.54)	37.02 (19.37; 142.2)	0.2017 NS
TNF-α **Q1, q3 interquartile range**	11.69 (7.8; 22.82)	10.03 (7.8; 26.68)	0.9906 NS
IL-12 **Q1, q3 interquartile range**	30.61 (15.73; 82.7)	30.28 (17.18; 92.79)	0.5004 NS

The effect of NOD2/CARD15 variants in Crohn's disease patient cytokine secretion was analysed in organ culture and whole blood culture supernatants (Table 15 and 16). No statistical significance was found when comparing NOD2/CARD15 positives to NOD2/CARD15 negatives in the Crohn's disease group.

Table 15: Comparison of organ culture cytokine secretion and NOD2/CARD15 status in Crohn's disease patients. Wilcoxon Rank Sum test performed.

	IL-12	**IFN-γ**	**IL-10**	**IL-2**	**TNF-α**
NOD – **q1, q3** **interquartile range**	20.54 (10.31; 61.78)	34.68 (14.74; 72.08)	18.30 (15; 30.18)	15.0 (15; 25.68)	14.74 (7.8; 57.03)
NOD + **q1, q3** **interquartile range**	42.17 (17.92; 94.80)	52.34 (16.73; 160.8)	24.17 (16.31; 51.14)	24.0 (15.14;45.7)	22.65 (9.34;72.43)
***P* value**	0.5879 NS	0.0681 NS	0.1763 NS	0.2783 NS	0.6845 NS

Table 16: Comparison of whole blood culture supernatant cytokine secretion and NOD2/CARD15 status in Crohn's disease patients. Wilcoxon Rank Sum Test performed.

	IL-4	**IL-2**	**IFN-γ**	**TNF-α**
NOD + **q1, q3** **interquartile range**	11.49 (7.8; 28.38)	15.0 (15.0; 27.78)	14.89 (7.8; 39.49)	13.03 (7.8; 31.68)
NOD - **q1, q3** **interquartile range**	12.62 (7.8; 61.06)	17.58 (15; 40.87)	22.63 (10.73; 31.37)	7.80 (7.8;25.10)
***P* value**	0.1309 NS	0.3125 NS	0.2754 NS	0.5703 NS

5.4 Discussion

This chapter aimed to determine whether genetic susceptibility to CD predisposes people to MAP infection and what effect the NOD2/CARD15 mutation has on cytokine expression. To do this 3 commonly analysed SNPs (single nucleotide polymorphisms) in the NOD2/CARD15 gene were analysed by real time PCR allelic discrimination. These 3 SNPs were 1007fs, R702W and G908R. No homozygotes were identified but 2 individuals with CD, who display inflammation in the terminal ileum, were found to be compound heterozygotes. Hugot, et al (2001) determined that the relative risk for developing CD in simple heterozygotes is 3, whereas in homozygotes it is 38. Compound heterozygotes have an even higher risk of 44. There is a view that having a simple recessive mutation in NOD2/CARD15 might be sufficient for the development of disease. However, it is possible for normal individuals to have mutations in the absence of disease (Bouma and Strober, 2003). It has been reported that the polymorphisms show very limited penetrance and occur in only 40% of CD patients, as well as in 15% of healthy individuals (Cuthbert, AP et al, 2002; Marks DJB, et al 2006), which correlates to this study in which, in the CD patient group 37.5% was found to have NOD2/CARD15 mutations and 16% of our control group had NOD2/CARD15 mutations.

It has also been found that in Japanese, Chinese, Korean and Indian populations that individuals with CD do not have any of the NOD2/CARD15 mutations (Yamazaki, et al (2002), Croucher, et al (2003), Leung et al (2003) and Pugazhendhi et al (2008)). A study was conducted in 2004 by Karban et al, which

compared Ashkenazi Jews to Sephardic Jewish populations in Israel. Ashkenazi Jews are of Middle Eastern descent while Sephardic Jews are of Asian descent. It was found that the Ashkenazi Jews had an increased carrier rate, 47.4% compared to Sephardic Jews, 27.45% (P = 0.034). This could contribute to the higher incidence of CD in Ashkenazi Jews. These results highlight the fact that NOD2/CARD15 mutations are not necessary in the development of CD and the importance of ethnic comparisions in identifying susceptibility genes.

In 2001 Cavanaugh and the IBD International Genetic Consortium demonstrated that in Australia there is strong evidence of a linkage between CD and IBD1 locus, which has been mapped to the 16q12 chromosome. They demonstrated that the 3 risk alleles were more frequent in CD than in controls.

The NOD2/CARD15 protein is implicated in recognition of muramyl dipeptides (MDP), a component of peptidoglycan that is present in cell walls of bacteria. It is a cytoplasmic protein expressed in mononuclear phagocytes. Binding of NOD2 to MDP activates the transcription factor nuclear factor, NF-KB, which induces proinflammatory genes (Marks DJB, et al, 2006). Polymorphisms in the NOD2 gene should therefore abolish the response to MDP and inflammation. This however contrasts the pathology of CD, which is inflamed and contains pro-inflammatory cytokines. A previous study (Schreiber, et al 1998) also reported that in colonic tissue from CD patients that increased rather then decreased NF-KB activity compared to controls was found.

In a genetic susceptible host the ability to down regulate inflammation and to clear bacterial infection would be lacking. It is known that the immunoadjuvant effect of Mycobacteria is dependent on MDP (Bahr and Chedid, 1986). It could be assumed then in a subject with a NOD2/CARD15 mutation the innate response to Mycobacteria would be inadequate, which would lead to establishment of a chronic Mycobacterial infection. This then would activate the inflammatory response characteristic of CD (Behr, et al 2004). The results found in this study however, do not support MAP as a bacterial trigger for development of CD in individuals with NOD2 mutations as no statistical significance was found when comparing MAP status to NOD mutation status.

The cytokine profiles were analysed according to presence or absence of the NOD2/CARD15 mutation. In the whole blood culture cytokines no statistical significance was found in IL-4, IL-2, IFN-γ or TNF-α. The same result was found in the organ culture cytokines; IL-10, IL-2, IL-12, IFN-γ or TNF-α. This suggests that NOD2 mutations are unrelated to cytokine balance. Analysis of CD patients' NOD2 status and cytokine profile also found no correlation. Given that there is no clear link between NOD2 gene mutation, presence of MAP and cytokine secretion in our CD patients the suggestion that defective handling of MAP is due to NOD2/CARD15 gene mutations is not relevant to our group.

CHAPTER SIX: *Final Discussion*

The work in this thesis tested the hypothesis that transmural inflammation of CD is promoted by the presence of MAP within the gut wall. The 3 studies were constructed to:

1) Correlate the presence of MAP to CD.
2) Correlate MAP in CD to enhanced proinflammatory cytokine secretion.
3) Demonstrate a correlation between MAP and CARD15/NOD2 mutations.

A likely explanation of chronic inflammation and associated gut mucosal damage in CD could be an impaired handling of gut bacteria within the mucosa. MAP has gained much attention because of an observed association of MAP with active CD. However, it is still unclear what role, if any, MAP plays as it is a frequent gut microbe and is commonly associated with the mucosa of CD, other inflammatory bowel diseases and normal tissues.

The first objective of this thesis was to correlate the presence of MAP to CD. The involvement of MAP in CD has been difficult to prove due to difficulties in the isolation and detection of the bacterium, which may be a reflection of the different methodologies being used. By using the optimised methodology published by Bull, et al (2003) which reported to increase the number of MAP IS900 copies extracted from mucosal biopsies, MAP was detected in 20% of CD patients, 12.3% of UC, 18.4% of IBS and 14.4% of normal patients. This is in contrast to the results of

Bull, et al (2003) who detected MAP in 92% of CD patients and 26% of non-IBD patients using the same method. The fact that MAP was found in all disease groups, IBD and non-IBD, suggests that all patients are regularly exposed to MAP.

Our result was surprising and raises the question whether our results were due to a technical issue in our method. However, our results were confirmed by serum analysis for MAP p35 and p36. The p35 and p36 recombinant antigen analysis demonstrates a humoral immune response of CD to MAP. Analysis performed by Nasers group on our patient groups serum found no statistical significance when comparing the different disease groups. Thus, confirming our initial PCR results.

The second objective of the study was to determine what effect MAP had on the pattern of cytokine response within the disease groups. In CD patients those positive for MAP had enhanced TNF-α secretion in gut mucosa, and a significant increase in the secretion of both IL-2 and IL-4 in blood. The high TNF-α concentration in MAP positive CD patients suggests a defect in MAP handling. In normal patients they have the capacity to handle the microbe and therefore no inflammatory response is triggered. These findings are in agreement with those of Sibartie, S et al (2010) who found that in CD patients there were significantly higher levels of TNF-α in response to MAP. They concluded that there is an increased frequency of MAP-reactive T-cells due to prior exposure to MAP in CD patients (Sibartie, et al, 2010).

It has previously been described that there is a dominant Th2 cytokine profile in the gut muscularis in CD (Akiho H et al 2005) which is consistent with the selective activation of Th2 CD4+T cells in our CD patient group. CD patients also have been shown to have polymorphisms in 2 gene's which are linked to an increase in IL-4 expression. They have been found to have -34T (IL-4 gene) and Q576R (IL-4 receptor gene) polymorphisms (Aithal GP et al 2001). A high concentration of IL-4 in the gut mucosa promotes inflammation as it disrupts the intestinal epithelial barrier causing an increased permeability which could enable enteric bacteria to migrate into the gut mucosa (Di Leo V, et al 2002; Hollander D, 1999). Therefore, the increase in IL-4 secretion found in our CD group would enhance permeability of the mucosa which would allow MAP to enter the gut mucosa, thus increasing inflammation.

The final objective of this thesis was to determine whether there is a correlation between MAP infection and CARD15/NOD2 mutations. Gene associations with IBD are nearing 100, although many have modest odds ratio's (Flanagan P, et al, 2011). The CARD15/NOD2 polymorphisms remain the only mutation with an odds ratio greater than 3 (in simple heterozygotes) in its link to CD (Hugot, et al, 2001).

CARD15/NOD2 polymorphisms incapacitate the NOD2 pathway that can compensate for impairment of innate inflammation. The NOD2 protein acts as a intracellular receptor of MDP (muramyl dipeptide),which is a component of both Gram positive and Gram negative bacterial cell wall peptidoglycans (Flanagan, P, et al, 2011). MDP-engaged NOD2 plays an important role in autophagosome

generation, recruiting ATG16L1 to the cell surface membrane where bacterial engulfment occurs. Mutations in NOD2 related to CD have complex effects; impaired autophagy and bacterial killing by macrophages, reduced defensin production and reduced mononuclear cell IL-8 response to bacteria (Flanagan, P, et al, 2011). It was found that MAP is not disease specific, but rather a consequence of a primary defect in macrophages. In this study it was shown that there was a skew away from IFN-γ in MAP positive CD patients which could allow MAP to persist in defective macrophages, which are not being stimulated by the levels of IFN-γ needed to compensate for their impaired function.

The results do not support MAP as a bacterial trigger for development of CD in individuals with NOD2 mutations as no statistical significance was found when comparing MAP status to NOD mutation status.

Analysis of the cytokine profiles according to presence or absence of the NOD2/CARD15 mutation was performed to determine whether the exaggerated inflammatory response was a consequence of genetic factors. In the whole blood culture cytokines no statistical significance was found in IL-4, IL-2, IFN-γ or TNF-α. No statistical significance was found in the organ culture cytokines; IL-10, IL-2, IL-12, IFN-γ or TNF-α. This suggests that NOD2 mutations are unrelated to cytokine balance. Analysis of CD patients NOD2 status and cytokine profile also found no correlation. Given that there is no clear link between NOD2 gene mutation,

presence of MAP and cytokine secretion within the CD patients, mutations in CARD15/NOD2 does not result in defective handling of MAP.

It is then concluded that;

1. MAP is not essential for CD.
2. MAP is present in IBD and non-IBD patients.
3. Defect in the cellular handling of MAP in CD.
4. MAP in CD has the capacity to enhance drive a Th2 response, which in turn

    i. Down regulates the protective Th1 response

    ii. Enhances mucosal permeability which leads to increased inflammation.

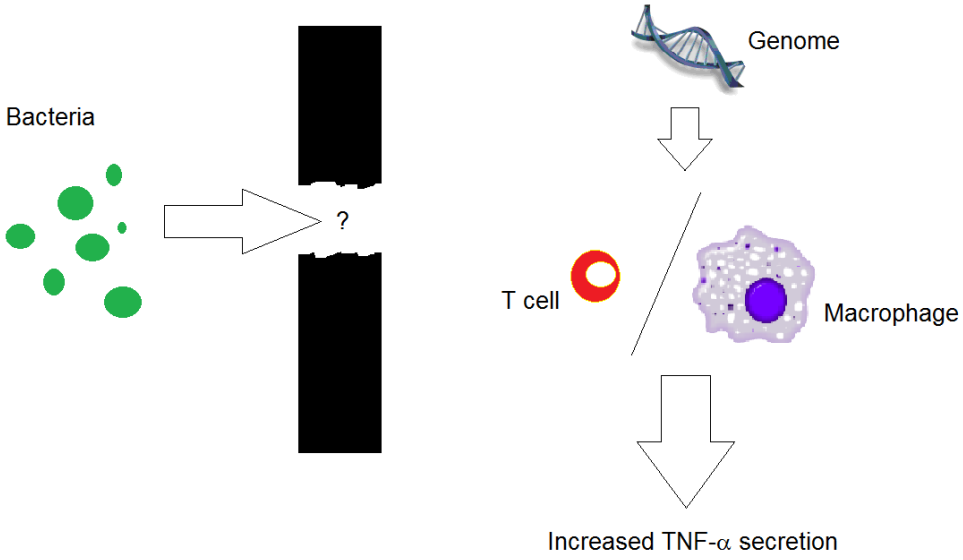
Therefore, MAP does not have a causal role but rather influences an abnormal inflammatory response (Figure 9). The MAP microbe passes through the mucosal barrier where it is taken up by the macrophages, which in turn secretes TNF-α - promoting an inflammatory response. The Th2 response is also triggered, increasing the secretion of IL-2 and IL-4 into the blood. The Th2 response acts by down regulating the Th1 response which leads to chronic inflammation of the mucosal barrier.

Figure 9. Models of a Crohn's Disease patient (1) and a patient with Crohn's Disease infected with *Mycobacterium avium* ss *paratuberculosis* (MAP) (2).

1) Crohn's Disease Patient.

At the onset of thesis it was stated that 3 factors were required to produce CD.

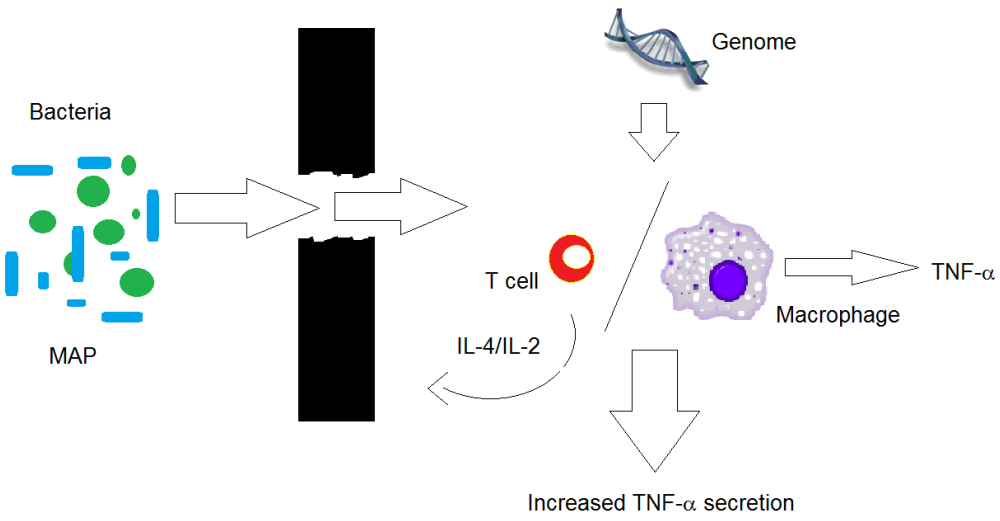
1. Genetics
2. Bacteria
3. Abnormal immune response



2) Crohn's Disease infected with MAP.

The main outcomes of current study were:

1. That in CD (not normal or disease controls) mucosal cultures secreted an increase in TNF-α. Whereas, other T-cell cytokines were not increased.
2. CD (MAP Positive)
    - Significantly greater amounts of TNF-α than MAP negative.
    - Significantly greater IL-4/IL-2 in circulative blood cytokines.
    - Macrophage defect results in abnormal handling of MAP which results in an inappropriate excessive secretion of TNF-α.
    - Switch in circulative Th1/Th2 balance with high IL-4 secretion, resulting in increasing mucosal permeability which then enables more bacteria to activate T cell/macrophage secretion of TNF-α.



Questions raised by this thesis:

1. Is MAP one of many other bacteria that can promote CD? Do other bacteria, i.e. *Yersinia* species, explain regional variations in incidence of MAP associations?
2. The essential defect and influence of genetic factors remains unclear. The possibility that the 'sum' of genetic variations influence the net balance of pro-inflammatory cytokine secretions – that a number of pathogenic factors will ultimately be shown to contribute, and that the particular contributions may vary in individuals.
3. Does TNF–α increased secretion come from defective macrophages? What is the T-cell/macrophage relationship in CD mucosa?
4. The clear evidence of promotion of Th2 activity in blood is not immediately understood. Nor is it clear how this unbalance may contribute to gut pathology.

REFERENCES:

Ahmad, T., J. Satsangi, et al. (2001). "Review article: the genetics of inflammatory bowel disease." Aliment Pharmacol Ther **15**(6): 731-748.

Aithal, G. P., C. P. Day, et al. (2001). "Association of single nucleotide polymorphisms in the interleukin-4 gene and interleukin-4 receptor gene with Crohn's disease in a British population." Genes and immunity **2**(1): 44-47.

Akiho, H., Y. Deng, et al. (2005). "Mechanisms underlying the maintenance of muscle hypercontractility in a model of postinfective gut dysfunction." Gastroenterology **129**(1): 131-141.

Amre, D. K. and E. G. Seidman (2003). "DNA variants in cytokine and NOD2 genes, exposures to infections and risk for Crohn's disease." Paediatr Perinat Epidemiol **17**(3): 302-312.

Autschbach, F., S. Eisold, U. Hinz, S. Zinser, M. Linnebacher, T. Giese, T. Loffler, MW. Buchler and J. Schmidt (2005) "High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease." Gut **54** (7): 944-949.

Bahr, G. M. and L. Chedid (1986). "Immunological activities of muramyl peptides." Federation proceedings **45**(11): 2541-2544.

Baksh, F. K., S. D. Finkelstein, et al. (2004). "Absence of Mycobacterium avium subsp. paratuberculosis in the microdissected granulomas of Crohn's disease." Mod Pathol **17**(10): 1289-1294.

Behr, M. A., M. Semret, et al. (2004). "Crohn's disease, mycobacteria, and NOD2." The Lancet infectious diseases **4**(3): 136-137.

Bernstein, C. N., J. F. Blanchard, et al. (2004). "Population-based case control study of seroprevalence of Mycobacterium paratuberculosis in patients with Crohn's disease and ulcerative colitis." J Clin Microbiol **42**(3): 1129-1135.

Berrebi, D., R. Maudinas, et al. (2003). "Card15 gene overexpression in mononuclear and epithelial cells of the inflamed Crohn's disease colon." Gut **52**(6): 840-846.

Bonen, D. K. and J. H. Cho (2003). "The genetics of inflammatory bowel disease." Gastroenterology **124**(2): 521-536.

Borody, T. J., S. Leis, et al. (2002). "Treatment of severe Crohn's disease using antimycobacterial triple therapy--approaching a cure?" Dig Liver Dis **34**(1): 29-38.

Bouma, G. and W. Strober (2003). "The immunological and genetic basis of inflammatory bowel disease." Nature reviews. Immunology **3**(7): 521-533.

Braegger, C. P., S. Nicholls, et al. (1992). "Tumour necrosis factor alpha in stool as a marker of intestinal inflammation." Lancet **339**(8785): 89-91.

Breese, E. J., P. Kumar, et al. (1994). "Interleukin-2 and interferon-gamma producing cells in the lamina propria in celiac disease." Digestive diseases and sciences **39**(10): 2243.

Buhner, S., C. Buning, et al. (2006). "Genetic basis for increased intestinal permeability in families with Crohn's disease: role of CARD15 3020insC mutation?" Gut **55**(3): 342-347.

Bull, T. J., J. Hermon-Taylor, et al. (2000). "Characterization of IS900 loci in Mycobacterium avium subsp. paratuberculosis and development of multiplex PCR typing." Microbiology **146 (Pt 9)**: 2185-2197.

Bull, T. J., E. J. McMinn, et al. (2003). "Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease." J Clin Microbiol **41**(7): 2915-2923.

Bull, T. J., K. Sidi-Boumedine, et al. (2003). "Mycobacterial interspersed repetitive units (MIRU) differentiate Mycobacterium avium subspecies paratuberculosis from other species of the Mycobacterium avium complex." Mol Cell Probes **17**(4): 157-164.

Camoglio, L., A. A. Te Velde, et al. (1998). "Altered expression of interferon-gamma and interleukin-4 in inflammatory bowel disease." Inflammatory bowel diseases **4**(4): 285-290.

Cangemi, J. R. (1999). "The role of antibiotics in Crohn's disease." Dig Dis **17**(1): 1-5.

Caprilli, R. and D. Guagnozzi (2003). "CARD 15 and toll-like receptors: the link with Crohn's disease." Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver **35**(11): 753-757.

Cavanaugh, J. A., K. E. Adams, et al. (2003). "CARD15/NOD2 risk alleles in the development of Crohn's disease in the Australian population." Ann Hum Genet **67**(Pt 1): 35-41.

Chacon, O., L. E. Bermudez, et al. (2004). "Johne's disease, inflammatory bowel disease, and Mycobacterium paratuberculosis." Annual review of microbiology **58**: 329-363.

Chamberlin, W., D. Y. Graham, et al. (2001). "Review article: Mycobacterium avium subsp. paratuberculosis as one cause of Crohn's disease." Aliment Pharmacol Ther **15**(3): 337-346.

Chamberlin, W. M. and S. A. Naser (2006). "Integrating theories of the etiology of Crohn's disease. On the etiology of Crohn's disease: questioning the hypotheses." Med Sci Monit **12**(2): RA27-33.

Chang, P. and R. W. Schwartz (2001). "Crohn's disease: current concepts in diagnosis and treatment." Curr Surg **58**(3): 288-292.

Chiba, M., T. Fukushima, et al. (1998). "No Mycobacterium paratuberculosis detected in intestinal tissue, including Peyer's patches and lymph follicles, of Crohn's disease." J Gastroenterol **33**(4): 482-487.

Chiodini, R. J. (1991). "Antimicrobial activity of rifabutin in combination with two and three other antimicrobial agents against strains of Mycobacterium paratuberculosis." J Antimicrob Chemother **27**(2): 171-176.

Chiodini, R. J., H. J. Van Kruiningen, et al. (1984). "Characteristics of an unclassified Mycobacterium species isolated from patients with Crohn's disease." J Clin Microbiol **20**(5): 966-971.

Chiodini, R. J., H. J. Van Kruiningen, et al. (1986). "Spheroplastic phase of mycobacteria isolated from patients with Crohn's disease." J Clin Microbiol **24**(3): 357-363.

Chiodini, R. J., H. J. Van Kruiningen, et al. (1984). "In vitro antimicrobial susceptibility of a Mycobacterium sp. isolated from patients with Crohn's disease." Antimicrob Agents Chemother **26**(6): 930-932.

Chiodini, R. J., H. J. Van Kruiningen, et al. (1984). "Possible role of mycobacteria in inflammatory bowel disease. I. An unclassified Mycobacterium species isolated from patients with Crohn's disease." Dig Dis Sci **29**(12): 1073-1079.

Clarke, C. J. and D. Little (1996). "The pathology of ovine paratuberculosis: gross and histological changes in the intestine and other tissues." J Comp Pathol **114**(4): 419-437.

Clarkston, W. K., M. E. Presti, et al. (1998). "Role of Mycobacterium paratuberculosis in Crohn's disease: a prospective, controlled study using polymerase chain reaction." Dis Colon Rectum **41**(2): 195-199.

Collins, M. T., G. Lisby, et al. (2000). "Results of multiple diagnostic tests for Mycobacterium avium subsp. paratuberculosis in patients with inflammatory bowel disease and in controls." J Clin Microbiol **38**(12): 4373-4381.

Croucher, P. J., S. Mascheretti, et al. (2003). "Lack of association between the C3435T MDR1 gene polymorphism and inflammatory bowel disease in two independent Northern European populations." Gastroenterology **125**(6): 1919-1920; author reply 1920-1911.

Croucher, P. J., S. Mascheretti, et al. (2003). "Haplotype structure and association to Crohn's disease of CARD15 mutations in two ethnically divergent populations." European journal of human genetics : EJHG **11**(1): 6-16.

Cuthbert, A. P., S. A. Fisher, et al. (2002). "The contribution of NOD2 gene mutations to the risk and site of disease in inflammatory bowel disease." Gastroenterology **122**(4): 867-874.

Dale, J. W. (1995). "Mobile genetic elements in mycobacteria." Eur Respir J Suppl **20**: 633s-648s.

Dalziel, T. K. (1989). "Thomas Kennedy Dalziel 1861-1924. Chronic interstitial enteritis." Dis Colon Rectum **32**(12): 1076-1078.

Danese, S., F. Scaldaferri, et al. (2004). "CD40L-positive platelets induce CD40L expression de novo in endothelial cells: adding a loop to microvascular inflammation." Arteriosclerosis, thrombosis, and vascular biology **24**(9): e162.

Desreumaux, P., E. Brandt, et al. (1997). "Distinct cytokine patterns in early and chronic ileal lesions of Crohn's disease." Gastroenterology **113**(1): 118-126.

Di Leo, V., P. C. Yang, et al. (2002). "Factors regulating the effect of IL-4 on intestinal epithelial barrier function." International archives of allergy and immunology **129**(3): 219-227.

Dumonceau, J. M., A. Van Gossum, et al. (1997). "Detection of fastidious mycobacteria in human intestines by the polymerase chain reaction." Eur J Clin Microbiol Infect Dis **16**(5): 358-363.

El-Zaatari, F. A., M. S. Osato, et al. (2001). "Etiology of Crohn's disease: the role of Mycobacterium avium paratuberculosis." Trends Mol Med **7**(6): 247-252.

Englund, S., G. Bolske, et al. (2001). "Detection of Mycobacterium avium subsp. paratuberculosis in tissue samples by single, fluorescent and nested PCR based on the IS900 gene." Vet Microbiol **81**(3): 257-271.

Englund, S., G. Bolske, et al. (2002). "An IS900-like sequence found in a Mycobacterium sp. other than Mycobacterium avium subsp. paratuberculosis." FEMS Microbiol Lett **209**(2): 267-271.

Esters, N., M. Pierik, et al. (2004). "Transmission of CARD15 (NOD2) variants within families of patients with inflammatory bowel disease." Am J Gastroenterol **99**(2): 299-305.

Fang, Y., W. H. Wu, et al. (2002). "Comparison of real-time, quantitative PCR with molecular beacons to nested PCR and culture methods for detection of Mycobacterium avium subsp. paratuberculosis in bovine fecal samples." J Clin Microbiol **40**(1): 287-291.

Farmer, R. G., W. A. Hawk, et al. (1975). "Clinical patterns in Crohn's disease: a statistical study of 615 cases." Gastroenterology **68**(4 Pt 1): 627-635.

Flanagan, P., B. J. Campbell, et al. (2011). "Bacteria in the pathogenesis of inflammatory bowel disease." Biochemical Society transactions **39**(4): 1067-1072.

Gearry, R. B., J. M. Aitken, et al. (2005). "Images of interest. Gastrointestinal: Mycobacterium avium paratuberculosis and Crohn's disease." Journal of gastroenterology and hepatology **20**(12): 1943.

Ghadiali, A. H., M. Strother, et al. (2004). "Mycobacterium avium subsp. paratuberculosis strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns." J Clin Microbiol **42**(11): 5345-5348.

Ghebremichael, S., S. B. Svenson, et al. (1996). "Antimycobacterial synergism of clarithromycin and rifabutin." Scand J Infect Dis **28**(4): 387-390.

Goodgame, R. W., K. Kimball, et al. (2001). "Randomized controlled trial of clarithromycin and ethambutol in the treatment of Crohn's disease." Aliment Pharmacol Ther **15**(12): 1861-1866.

Gordin, F. M., P. M. Sullam, et al. (1999). "A randomized, placebo-controlled study of rifabutin added to a regimen of clarithromycin and ethambutol for treatment of disseminated infection with Mycobacterium avium complex." Clin Infect Dis **28**(5): 1080-1085.

Grant, I. R., H. J. Ball, et al. (1999). "Effect of higher pasteurization temperatures, and longer holding times at 72 degrees C, on the inactivation of Mycobacterium paratuberculosis in milk." Lett Appl Microbiol **28**(6): 461-465.

Grant, I. R., C. M. Pope, et al. (2000). "Improved detection of Mycobacterium avium subsp. paratuberculosis In milk by immunomagnetic PCR." Vet Microbiol **77**(3-4): 369-378.

Grant, I. R., A. G. Williams, et al. (2005). "Efficacy of various pasteurization time-temperature conditions in combination with homogenization on inactivation of Mycobacterium avium subsp. paratuberculosis in milk." Applied and environmental microbiology **71**(6): 2853-2861.

Green, E. P., M. L. Tizard, et al. (1989). "Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease isolate of Mycobacterium paratuberculosis." Nucleic Acids Res **17**(22): 9063-9073.

Greenstein, R. J. (2003). "Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease." Lancet Infect Dis **3**(8): 507-514.

Gui, G. P., P. R. Thomas, et al. (1997). "Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics." J Antimicrob Chemother **39**(3): 393-400.

Hampe, J., A. Cuthbert, et al. (2001). "Association between insertion mutation in NOD2 gene and Crohn's disease in German and British populations." Lancet **357**(9272): 1925-1928.

Hampe, J., H. Frenzel, et al. (2002). "Evidence for a NOD2-independent susceptibility locus for inflammatory bowel disease on chromosome 16p." Proceedings of the National Academy of Sciences of the United States of America **99**(1): 321-326.

Hampe, J., J. Grebe, et al. (2002). "Association of NOD2 (CARD 15) genotype with clinical course of Crohn's disease: a cohort study." Lancet **359**(9318): 1661-1665.

Harris, N. B. and R. G. Barletta (2001). "Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine." Clin Microbiol Rev **14**(3): 489-512.

Hayashi, T., A. Catanzaro, et al. (1997). "Apoptosis of human monocytes and macrophages by Mycobacterium avium sonicate." Infect Immun **65**(12): 5262-5271.

Heifets, L. (2002). "Susceptibility testing of Mycobacterium tuberculosis to pyrazinamide." J Med Microbiol **51**(1): 11-12.

Helio, T., L. Halme, et al. (2003). "CARD15/NOD2 gene variants are associated with familially occurring and complicated forms of Crohn's disease." Gut **52**(4): 558-562.

Hendrickson, B. A., R. Gokhale, et al. (2002). "Clinical aspects and pathophysiology of inflammatory bowel disease." Clin Microbiol Rev **15**(1): 79-94.

Henn, V., J. R. Slupsky, et al. (1998). "CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells." Nature **391**(6667): 591-594.

Heresbach, D., V. Gicquel-Douabin, et al. (2004). "NOD2/CARD15 gene polymorphisms in Crohn's disease: a genotype- phenotype analysis." Eur J Gastroenterol Hepatol **16**(1): 55-62.

Hermon-Taylor, J. (2000). "Mycobacterium avium subspecies paratuberculosis in the causation of Crohn's disease." World J Gastroenterol **6**(5): 630-632.

Hermon-Taylor, J. (2002). "Treatment with drugs active against Mycobacterium avium subspecies paratuberculosis can heal Crohn's disease: more evidence for a neglected public health tragedy." Dig Liver Dis **34**(1): 9-12.

Hermon-Taylor, J., N. Barnes, et al. (1998). "Mycobacterium paratuberculosis cervical lymphadenitis, followed five years later by terminal ileitis similar to Crohn's disease." Bmj **316**(7129): 449-453.

Hermon-Taylor, J. and T. Bull (2002). "Crohn's disease caused by Mycobacterium avium subspecies paratuberculosis: a public health tragedy whose resolution is long overdue." J Med Microbiol **51**(1): 3-6.

Hermon-Taylor, J., T. J. Bull, et al. (2000). "Causation of Crohn's disease by Mycobacterium avium subspecies paratuberculosis." Can J Gastroenterol **14**(6): 521-539.

Hermon-Taylor, J., M. Moss, et al. (1990). "Molecular biology of Crohn's disease mycobacteria." Baillieres Clin Gastroenterol **4**(1): 23-42.

Hollander, D. (1999). "Intestinal permeability, leaky gut, and intestinal disorders." Current gastroenterology reports **1**(5): 410-416.

Huggett, J., K. Dheda, et al. (2004). "Crohn's disease and MAP." Lancet **364**(9452): 2178; author reply 2178-2179.

Hugot, J. P. (2006). "CARD15/NOD2 mutations in Crohn's disease." Annals of the New York Academy of Sciences **1072**: 9-18.

Hugot, J. P., C. Alberti, et al. (2003). "Crohn's disease: the cold chain hypothesis." Lancet **362**(9400): 2012-2015.

Hugot, J. P., M. Chamaillard, et al. (2001). "Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease." Nature **411**(6837): 599-603.

Hulten, K., A. Almashhrawi, et al. (2000). "Antibacterial therapy for Crohn's disease: a review emphasizing therapy directed against mycobacteria." Dig Dis Sci **45**(3): 445-456.

Hulten, K., H. M. El-Zimaity, et al. (2001). "Detection of Mycobacterium avium subspecies paratuberculosis in Crohn's diseased tissues by in situ hybridization." Am J Gastroenterol **96**(5): 1529-1535.

Ina, K., J. Itoh, et al. (1999). "Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance." Journal of immunology **163**(2): 1081-1090.

Inohara, N., Y. Ogura, et al. (2003). "Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease." J Biol Chem **278**(8): 5509-5512.

Janowitz, H. D. (1981). "Crohn's disease--50 years later." N Engl J Med **304**(26): 1600-1602.

Kalis, C. H., H. W. Barkema, et al. (2002). "Evaluation of two absorbed enzyme-linked immunosorbent assays and a complement fixation test as replacements for fecal culture in the detection of cows shedding Mycobacterium avium subspecies paratuberculosis." J Vet Diagn Invest **14**(3): 219-224.

Karban, A., M. Waterman, et al. (2004). "NOD2/CARD15 genotype and phenotype differences between Ashkenazi and Sephardic Jews with Crohn's disease." The American journal of gastroenterology **99**(6): 1134-1140.

Kaufmann, S. H. (1993). "Immunity to intracellular bacteria." Annu Rev Immunol **11**: 129-163.

Keating, G. M. and C. M. Perry (2002). "Infliximab: an updated review of its use in Crohn's disease and rheumatoid arthritis." BioDrugs : clinical immunotherapeutics, biopharmaceuticals and gene therapy **16**(2): 111-148.

Klitgaard Nielsen, K. and P. Ahrens (2002). "Putative in vitro expressed gene fragments unique to Mycobacterium avium subspecies paratuberculosis." FEMS Microbiol Lett **214**(2): 199-203.

Kobayashi, K. S., M. Chamaillard, et al. (2005). "Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract." Science **307**(5710): 731-734.

Kurzawski, G., J. Suchy, et al. (2004). "The NOD2 3020insC mutation and the risk of colorectal cancer." Cancer Res **64**(5): 1604-1606.

Lala, S., Y. Ogura, et al. (2003). "Crohn's disease and the NOD2 gene: a role for paneth cells." Gastroenterology **125**(1): 47-57.

Langelaar, M., A. Koets, et al. (2002). "Mycobacterium paratuberculosis heat shock protein 70 as a tool in control of paratuberculosis." Vet Immunol Immunopathol **87**(3-4): 239-244.

Leiva, L. E., B. Butler, et al. (2001). "Up-regulation of CD40 ligand and induction of a Th2 response in children immunized with pneumococcal polysaccharide vaccines." Clinical and diagnostic laboratory immunology **8**(2): 233-240.

Lesage, S., H. Zouali, et al. (2002). "CARD15/NOD2 mutational analysis and genotype-phenotype correlation in 612 patients with inflammatory bowel disease." Am J Hum Genet **70**(4): 845-857.

Lienhardt, C., A. Azzurri, et al. (2002). "Active tuberculosis in Africa is associated with reduced Th1 and increased Th2 activity in vivo." European journal of immunology **32**(6): 1605-1613.

Lin, J., D. Ziring, et al. (2008). "TNFalpha blockade in human diseases: an overview of efficacy and safety." Clinical immunology **126**(1): 13-30.

Lindsay, J. O. and H. J. Hodgson (2001). "Review article: the immunoregulatory cytokine interleukin-10--a therapy for Crohn's disease?" Aliment Pharmacol Ther **15**(11): 1709-1716.

Lisby, G., J. Andersen, et al. (1994). "Mycobacterium paratuberculosis in intestinal tissue from patients with Crohn's disease demonstrated by a nested primer polymerase chain reaction." Scand J Gastroenterol **29**(10): 923-929.

Lugton, I. (1999). "Mucosa-associated lymphoid tissues as sites for uptake, carriage and excretion of tubercle bacilli and other pathogenic mycobacteria." Immunol Cell Biol **77**(4): 364-372.

MacDonald, T. T., A. Di Sabatino, et al. (2005). "Immunopathogenesis of Crohn's disease." JPEN J Parenter Enteral Nutr **29**(4 Suppl): S118-124; discussion S124-115, S184-118.

Madsen, K. (2002). "Combining T cells and IL-10: a new therapy for Crohn's disease?" Gastroenterology **123**(6): 2140-2144.

Maeda, S., L. C. Hsu, et al. (2005). "Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing." Science **307**(5710): 734-738.

Marks, D. J., M. W. Harbord, et al. (2006). "Defective acute inflammation in Crohn's disease: a clinical investigation." Lancet **367**(9511): 668-678.

McFadden, J. J., P. D. Butcher, et al. (1987). "Crohn's disease-isolated mycobacteria are identical to Mycobacterium paratuberculosis, as determined by DNA probes that distinguish between mycobacterial species." J Clin Microbiol **25**(5): 796-801.

McFadden, J. J., P. D. Butcher, et al. (1987). "Determination of genome size and DNA homology between an unclassified Mycobacterium species isolated from patients with Crohn's disease and other mycobacteria." J Gen Microbiol **133**(1): 211-214.

McGovern, D. P., D. A. Van Heel, et al. (2003). "Further evidence of IBD5/CARD15 (NOD2) epistasis in the susceptibility to ulcerative colitis." Am J Hum Genet **73**(6): 1465-1466.

Meenan, J., M. Mevissen, et al. (1996). "Mechanisms underlying neutrophil adhesion to apical epithelial membranes." Gut **38**(2):

Millar, D., J. Ford, et al. (1996). "IS900 PCR to detect Mycobacterium paratuberculosis in retail supplies of whole pasteurized cows' milk in England and Wales." Appl Environ Microbiol **62**(9): 3446-3452.

Miller, J. M., A. L. Jenny, et al. (2002). "Polymerase chain reaction detection of Mycobacterium tuberculosis complex and Mycobacterium avium organisms in formalin-fixed tissues from culture-negative ruminants." Vet Microbiol **87**(1): 15-23.

Mishina, D., P. Katsel, et al. (1996). "On the etiology of Crohn disease." Proc Natl Acad Sci U S A **93**(18): 9816-9820.

Monajemi, H., J. Meenan, et al. (1996). "Inflammatory bowel disease is associated with increased mucosal levels of bactericidal/permeability-increasing protein." Gastroenterology **110**(3): 733-739.

Monteleone, G., L. Biancone, et al. (1997). "Interleukin 12 is expressed and actively released by Crohn's disease intestinal lamina propria mononuclear cells." Gastroenterology **112**(4): 1169-1178.

Morgan, K. L. (1987). "Johne's and Crohn's. Chronic inflammatory bowel diseases of infectious aetiology?" Lancet **1**(8540): 1017-1019.

Mortimore, M., P. R. Gibson, et al. (2001). "Early Australian experience with infliximab, a chimeric antibody against tumour necrosis factor-alpha, in the treatment of Crohn's disease: is its efficacy augmented by steroid-sparing immunosuppressive therapy? The Infliximab User Group." Internal medicine journal **31**(3): 146-150.

Moss, M. T., J. D. Sanderson, et al. (1992). "Polymerase chain reaction detection of Mycobacterium paratuberculosis and Mycobacterium avium subsp silvaticum in long term cultures from Crohn's disease and control tissues." Gut **33**(9): 1209-1213.

Naser, S., I. Shafran, et al. (1999). "Mycobacterium avium subsp. paratuberculosis in Crohn's disease is serologically positive." Clin Diagn Lab Immunol **6**(2): 282.

Naser, S. A. (2005). "Mycobacterium in Crohn's disease is hard to digest." Gastroenterology **129**(4): 1359-1360; author reply 1360-1351.

Naser, S. A., G. Ghobrial, et al. (2004). "Culture of Mycobacterium avium subspecies paratuberculosis from the blood of patients with Crohn's disease." Lancet **364**(9439): 1039-1044.

Naser, S. A., K. Hulten, et al. (2000). "Specific seroreactivity of Crohn's disease patients against p35 and p36 antigens of M. avium subsp. paratuberculosis." Vet Microbiol **77**(3-4): 497-504.

Naser, S. A., D. Schwartz, et al. (2000). "Isolation of Mycobacterium avium subsp paratuberculosis from breast milk of Crohn's disease patients." Am J Gastroenterol **95**(4): 1094-1095.

Neurath, M. F., S. Finotto, et al. (2001). "Regulation of T-cell apoptosis in inflammatory bowel disease: to die or not to die, that is the mucosal question." Trends in immunology **22**(1): 21-26.

Nicholls, S., S. Stephens, et al. (1993). "Cytokines in stools of children with inflammatory bowel disease or infective diarrhoea." Journal of clinical pathology **46**(8): 757-760.

Ogura, Y., D. K. Bonen, et al. (2001). "A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease." Nature **411**(6837): 603-606.

Ogura, Y., S. Lala, et al. (2003). "Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis." Gut **52**(11): 1591-1597.

Ohkusa, T., I. Okayasu, et al. (2003). "Induction of experimental ulcerative colitis by Fusobacterium varium isolated from colonic mucosa of patients with ulcerative colitis." Gut **52**(1): 79-83.

O'Mahony, J. and C. Hill (2002). "A real time PCR assay for the detection and quantitation of Mycobacterium avium subsp. paratuberculosis using SYBR Green and the Light Cycler." J Microbiol Methods **51**(3): 283-293.

Pallone, F. and G. Monteleone (1998). "Interleukin 12 and Th1 responses in inflammatory bowel disease." Gut **43**(6): 735-736.

Parronchi, P., P. Romagnani, et al. (1997). "Type 1 T-helper cell predominance and interleukin-12 expression in the gut of patients with Crohn's disease." The American journal of pathology **150**(3): 823-832.

Pickup, R. W., G. Rhodes, et al. (2005). "Mycobacterium avium subsp. paratuberculosis in the catchment area and water of the River Taff in South Wales, United Kingdom, and its potential relationship to clustering of Crohn's disease cases in the city of Cardiff." Appl Environ Microbiol **71**(4): 2130-2139.

Plant, D., J. Lear, et al. (2004). "CARD15/NOD2 single nucleotide polymorphisms do not confer susceptibility to type I psoriasis." Br J Dermatol **151**(3): 675-678.

Podolsky, D. K. (2002). "Inflammatory bowel disease." N Engl J Med **347**(6): 417-429.

Prantera, C. and M. L. Scribano (1999). "Current treatment for prevention of relapse and recurrence in Crohn's disease." Ital J Gastroenterol Hepatol **31**(6): 515-518.

Pugazhendhi, S., A. Amte, et al. (2008). "Common NOD2 mutations are absent in patients with Crohn's disease in India." Indian journal of gastroenterology : official journal of the Indian Society of Gastroenterology **27**(5): 201-203.

Rastogi, N., K. S. Goh, et al. (1992). "Activity of clarithromycin compared with those of other drugs against Mycobacterium paratuberculosis and further enhancement of its extracellular and intracellular activities by ethambutol." Antimicrob Agents Chemother **36**(12): 2843-2846.

Reinecker, H. C., M. Steffen, et al. (1993). "Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease." Clinical and experimental immunology **94**(1): 174-181.

Romagnani, S. (1999). "Th1/Th2 cells." Inflammatory bowel diseases **5**(4): 285-294.

Romero, C., A. Hamdi, et al. (2005). "Evaluation of surgical tissue from patients with Crohn's disease for the presence of Mycobacterium avium subspecies paratuberculosis DNA by in situ hybridization and nested polymerase chain reaction." Inflamm Bowel Dis **11**(2): 116-125.

Rowbotham, D. S., N. P. Mapstone, et al. (1995). "Mycobacterium paratuberculosis DNA not detected in Crohn's disease tissue by fluorescent polymerase chain reaction." Gut **37**(5): 660-667.

Ryan, P., M. W. Bennett, et al. (2002). "PCR detection of Mycobacterium paratuberculosis in Crohn's disease granulomas isolated by laser capture microdissection." Gut **51**(5): 665-670.

Ryan, P., R. G. Kelly, et al. (2004). "Bacterial DNA within granulomas of patients with Crohn's disease--detection by laser capture microdissection and PCR." Am J Gastroenterol **99**(8): 1539-1543.

Saito, H., Y. Fukuda, et al. (2003). "Isolation of peptides useful for differential diagnosis of Crohn's disease and ulcerative colitis." Gut **52**(4): 535-540.

Sanderson, J. D., M. T. Moss, et al. (1992). "Mycobacterium paratuberculosis DNA in Crohn's disease tissue." Gut **33**(7): 890-896.

Schreiber, S., S. Nikolaus, et al. (1998). "Activation of nuclear factor kappa B inflammatory bowel disease." Gut **42**(4): 477-484.

Schwartz, D., I. Shafran, et al. (2000). "Use of short-term culture for identification of Mycobacterium avium subsp. paratuberculosis in tissue from Crohn's disease patients." Clin Microbiol Infect **6**(6): 303-307.

Sechi, L. A., M. Mura, et al. (2004). "Mycobacterium avium sub. paratuberculosis in tissue samples of Crohn's disease patients." New Microbiol **27**(1): 75-77.

Sechi, L. A., M. Mura, et al. (2001). "Identification of Mycobacterium avium subsp. paratuberculosis in biopsy specimens from patients with Crohn's disease identified by in situ hybridization." J Clin Microbiol **39**(12): 4514-4517.

Selby, W. (2000). "Pathogenesis and therapeutic aspects of Crohn's disease." Vet Microbiol **77**(3-4): 505-511.

Selby, W. S. (2003). "Current issues in Crohn's disease." Med J Aust **178**(11): 532-533.

Selby, W. S. (2004). "Mycobacterium avium subspecies paratuberculosis bacteraemia in patients with inflammatory bowel disease." Lancet **364**(9439): 1013-1014.

Shafran, I., L. Kugler, et al. (2002). "Open clinical trial of rifabutin and clarithromycin therapy in Crohn's disease." Dig Liver Dis **34**(1): 22-28.

Shafran, I., C. Piromalli, et al. (2002). "Seroreactivities against Saccharomyces cerevisiae and Mycobacterium avium subsp. paratuberculosis p35 and p36 antigens in Crohn's disease patients." Dig Dis Sci **47**(9): 2079-2081.

Shanahan, F. (2002). "Crohn's disease." Lancet **359**(9300): 62-69.

Shanahan, F. (2002). "Gut flora in gastrointestinal disease." The European journal of surgery. Supplement. : = Acta chirurgica. Supplement(587): 47-52.

Shanahan, F. and J. O'Mahony (2005). "The mycobacteria story in Crohn's disease." Am J Gastroenterol **100**(7): 1537-1538.

Sibartie, S., P. Scully, et al. (2010). "Mycobacterium avium subsp. Paratuberculosis (MAP) as a modifying factor in Crohn's disease." Inflammatory bowel diseases **16**(2): 296-304.

Stabel, J. R. (2000). "Transitions in immune responses to Mycobacterium paratuberculosis." Vet Microbiol **77**(3-4): 465-473.

Stainsby, K. J., J. R. Lowes, et al. (1993). "Antibodies to Mycobacterium paratuberculosis and nine species of environmental mycobacteria in Crohn's disease and control subjects." Gut **34**(3): 371-374.

Stephan, R., K. Buhler, et al. (2002). "Incidence of Mycobacterium avium subspecies paratuberculosis in bulk-tank milk samples from different regions in Switzerland." Vet Rec **150**(7): 214-215.

Suenaga, K., Y. Yokoyama, et al. (1999). "Serum antibodies to Mycobacterium paratuberculosis in patients with Crohn's disease." Dig Dis Sci **44**(6): 1202-1207.

Suenaga, K., Y. Yokoyama, et al. (1995). "Mycobacteria in the intestine of Japanese patients with inflammatory bowel disease." Am J Gastroenterol **90**(1): 76-80.

Suh, Y. and C. Cantor (2005). "Single nucleotide polymorphisms (SNPs): detection, interpretation, and application." Mutat Res **573**(1-2): 1-2.

Swift, G. L., E. D. Srivastava, et al. (1994). "Controlled trial of anti-tuberculous chemotherapy for two years in Crohn's disease." Gut **35**(3): 363-368.

ten Hove, T., C. van Montfrans, et al. (2002). "Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease." Gut **50**(2): 206-211.

Thayer, W. R., Jr., J. A. Coutu, et al. (1984). "Possible role of mycobacteria in inflammatory bowel disease. II. Mycobacterial antibodies in Crohn's disease." Dig Dis Sci **29**(12): 1080-1085.

Thomas, G. A., G. L. Swift, et al. (1998). "Controlled trial of antituberculous chemotherapy in Crohn's disease: a five year follow up study." Gut **42**(4): 497-500.

Thompson, D. E. (1994). "The role of mycobacteria in Crohn's disease." J Med Microbiol **41**(2): 74-94.

Tizard, M. L., M. T. Moss, et al. (1992). "p43, the protein product of the atypical insertion sequence IS900, is expressed in Mycobacterium paratuberculosis." J Gen Microbiol **138 Pt 8**: 1729-1736.

Valentine, J. F. (2003). "A map to the diagnosis of Crohn's disease." Inflamm Bowel Dis **9**(2): 133-134.

Van Damme, N., F. De Keyser, et al. (2001). "The proportion of Th1 cells, which prevail in gut mucosa, is decreased in inflammatory bowel syndrome." Clin Exp Immunol **125**(3): 383-390.

Van Den Brande, J. M., M. P. Peppelenbosch, et al. (2002). "Treating Crohn's disease by inducing T lymphocyte apoptosis." Annals of the New York Academy of Sciences **973**: 166-180.

van Dullemen, H. M., S. J. van Deventer, et al. (1995). "Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2)." Gastroenterology **109**(1): 129-135.

van Heel, D. A., I. A. Udalova, et al. (2002). "Inflammatory bowel disease is associated with a TNF polymorphism that affects an interaction between the OCT1 and NF(-kappa)B transcription factors." Hum Mol Genet **11**(11): 1281-1289.

Van Limbergen, J., R. K. Russell, et al. (2007). "Genetics of the innate immune response in inflammatory bowel disease." Inflammatory bowel diseases **13**(3): 338-355.

Van Limbergen, J., R. K. Russell, et al. (2007). "The genetics of inflammatory bowel disease." The American journal of gastroenterology **102**(12): 2820-2831.

van Montfrans, C., M. Peppelenbosch, et al. (2002). "Inflammatory signal transduction in Crohn's disease and novel therapeutic approaches." Biochem Pharmacol **64**(5-6): 789-795.

Van Soolingen, D. (2001). "Molecular epidemiology of tuberculosis and other mycobacterial infections: main methodologies and achievements." J Intern Med **249**(1): 1-26.

Vermeire, S. and P. Rutgeerts (2004). "Antibody responses in Crohn's disease." Gastroenterology **126**(2): 601-604.

Verspaget, H. W., A. M. van der Zon, et al. (2001). "Infliximab for Crohn's disease." Aliment Pharmacol Ther **15**(12): 2041-2042.

Wall, S., Z. M. Kunze, et al. (1993). "Identification of spheroplast-like agents isolated from tissues of patients with Crohn's disease and control tissues by polymerase chain reaction." J Clin Microbiol **31**(5): 1241-1245.

Walmsley, R. S., J. P. Ibbotson, et al. (1996). "Antibodies against Mycobacterium paratuberculosis in Crohn's disease." Qjm **89**(3): 217-221.

Watanabe, T., A. Kitani, et al. (2004). "NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses." Nat Immunol **5**(8): 800-808.

Wehkamp, J., J. Harder, et al. (2004). "NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression." Gut **53**(11): 1658-1664.

Whittington, R. J., I. Marsh, et al. (1998). "Rapid detection of Mycobacterium paratuberculosis in clinical samples from ruminants and in spiked environmental samples by modified BACTEC 12B radiometric culture and direct confirmation by IS900 PCR." J Clin Microbiol **36**(3): 701-707.

Yamazaki, K., M. Takazoe, et al. (2002). "Absence of mutation in the NOD2/CARD15 gene among 483 Japanese patients with Crohn's disease." Journal of human genetics **47**(9): 469-472.

APPENDIX

SOLUTIONS

IS900 DNA EXTRACTION

Mycobacterial Lysis Buffer (MLB)

	Volume	**Final Concentration**
0.5 M EDTA	400 μl	2 mM
5 M NaCl	8 ml	400 mM
1 M Tris HCl	1 ml	10 mM
H_2O	83.27 ml	
10% SDS	6 ml	0.6%
10 mg/ml Proteinase K	330 μl	33 μg/ml

PCR AND GEL ELECTROPHORESIS

2.5 mM dNTP

12.5 μl of each dNTP

450 μl dH_20

Nested PCR dNTP mix

12.5 μl dATP

12.5 μl dCTP

12.5 μl dGTP

25.0 μl dUTP

PRIMERS

nmoles x 5 = 200 μM

10 x TE

FOR 1 Litre

	Volume (ml)	**Final Concentration**
1 M Tris pH 8.0	100 ml	10 mM
0.5 M EDTA pH 8.0	20 ml	1 mM
D. H_2O	Make up to 1000ml	

Autoclave

Dilute to 1 x with sterile water before use.

EDTA 0.5 M pH 8.0

For 1 Litre

Final Concentration	Amount
0.5 M EDTA	186.1 g
Add to 800 ml D. H_2O	

Adjust pH with ~20 g NaOH pellets, make to 1000 ml with D. H_2O.

Autoclave prior to use.

NB WILL DISSOLVE ONLY WHEN AT CORRECT pH.

10 x TBE

	Amount
TRIS base	108g
Boric Acid	55 g
0.5M EDTA (pH 8.0)	40 ml
D. H_2O make up to	1000 ml

1 x TBE

Dilute 10 x TBE with D. H_2O as follows:

	Volume (ml)	Volume (ml)
10 x TBE	100	200
D. H_2O	900	1800
Final volume	1000 ml	2000 ml

DNA LADDER

Defrost ladder

Add 25 ul of loading dye to tube containing stock.

For working DNA ladder solution:

10 ul of ladder (with dye)

10 ul of D. H_2O

LB Medium

1. Add the following to 800ml H_2O
    - 10g Bacto-tryptone.
    - 5g yeast extract.
    - 10g NaCl.
2. Adjust pH to 7.5 with NaOH.
3. Adjust volume to 1L with dH_2O
4. Sterilize by autoclaving

2% AGAROSE GEL

	Amount	Amount	Amount
Agarose	2 g	4 g	6 g
1 x TBE	100 ml	200 ml	300 ml

Microwave on high for 2 – 3 minutes (making sure it doesn't bubble over)

Allow to cool (cool enough to handle without gloves).

Add Et.Br. (approximately 2 ul)

Mix and pour. Allow to set.

GEL–LOADING BUFFER

	Amount	Final Vol.
Bromophenol blue	0.06g	0.25%
Ficoll	6.125 ml	
1M Tris pH 8.0	0.25ml	
0.5M EDTA	5 ml	
D. H_2O	Make up to 25 mls	

MAP Culture

Mycobactin J (Allied Monitor, Inc)

1 ml 95% Ethanol

Final concentration = 2 mg/ml

2mg/ml enough to supplement IL

2 μl of Mycobactin J/ml

Therefore add 8 μl to MGIT

BBL PANTA

Reconstitute in 3 ml of sterile dH_20.

Aliquot and freeze.

BBL MGIT

Add to tube 0.5 ml OADC

0.1 ml PANTA

Asceptically add 8 μl Mycobactin J.

ELISA

CARBONATE BUFFER

BUFFER A – 8.4 g $NaHCO_3$ in 100 ml of dH_20 = 1 M $NaHCO_3$

BUFFER B – 10.6 g Na_2CO_3 in 100 ml of dH_20 = 1 M Na_2CO_3

Solutions can be stored for up to 1 year, store at 4^oC.

4.53ml of 1 M $NaHCO_3$ (Buffer A)

1.8 ml of 1 M Na_2CO_3 (Buffer B)

Make up to 100 ml with dH_20 and then pH to 9.6 with Na_2CO_3 (acid) or $NaHCO_3$ (base).

Can be used for 2 days, store at 4^oC.

TMB + DMSO

0.1 g TMB (Sigma-Aldrich)

10 ml DMSO (Sigma-Aldrich)

Transfer into an opaque container (light sensitive) or cover with foil.

Label as toxic.

WASHING BUFFER

2 L PBS

Add TWEEN 20 – 200 ul/L

DILUENT

PBS/BSA/TWEEN 20

100 ml PBS/TWEEN + 1g BSA

BLOCKING BUFFER

Use diluent

SUBSTRATE BUFFER

1 tablet (citrate) to 100 ml dH_20.

STOP SOLUTION

1 M H_2S0_4

PUBLISHED PAPERS

Available online at www.sciencedirect.com
ScienceDirect
Digestive and Liver Disease 39 (2007) 445–451
Digestive and Liver Disease
www.elsevier.com/locate/dld

Alimentary Tract

Molecular evidence for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Crohn's disease correlates with enhanced TNF-α secretion

R. Clancy [a,b,c,*], Z. Ren [a,b], J. Turton [b], G. Pang [b], A. Wettstein [c]

[a] *Hunter Immunology Unit, Hunter New England Area Health Service, John Hunter Hospital, Newcastle, NSW, Australia*
[b] *Discipline of Immunology and Microbiology, School of Biomedical Sciences, University of Newcastle, Newcastle, NSW, Australia*
[c] *Centre for Digestive Diseases, Five Dock, NSW, Australia*

Received 15 September 2006; accepted 5 December 2006
Available online 21 February 2007

Abstract

Background. Support for a role of *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's disease is largely based on epidemiological evidence, as no data on mechanisms linking the presence of *M. avium* subspecies *paratuberculosis* with gut damage is available.

Aims. To determine whether the presence of *M. avium* subspecies *paratuberculosis* contributes to the pathogenesis of Crohn's disease by promoting cytokine secretion within gut mucosa.

Patients and methods. A total of 235 subjects were recruited: 63 with Crohn's disease, 53 with ulcerative colitis, 45 with irritable bowel syndrome and 74 normal controls. *M. avium* subspecies *paratuberculosis* status was defined by nested PCR using *IS900* sequence. Gut mucosal organ cultures were established to detect cytokine secretion patterns.

Results. Significantly higher tumour necrosis factor-α concentrations were found in culture supernatants for Crohn's disease compared to ulcerative colitis ($p < 0.05$), irritable bowel syndrome ($p < 0.01$) and controls ($p < 0.0001$). When tumour necrosis factor-α levels were correlated with the presence of *M. avium* subspecies *paratuberculosis*, significantly greater concentrations were only found in *M. avium* subspecies *paratuberculosis*-positive Crohn's disease patients ($p < 0.05$). Tumour necrosis factor-α levels in *M. avium* subspecies *paratuberculosis*-positive Crohn's disease were significantly higher than in *M. avium* subspecies *paratuberculosis*-positive ulcerative colitis ($p < 0.01$), *M. avium* subspecies *paratuberculosis*-positive irritable bowel syndrome ($p < 0.05$) and *M. avium* subspecies *paratuberculosis*-positive controls ($p < 0.01$) and all *M. avium* subspecies *paratuberculosis*-negative specimens.

Conclusions. The data link *M. avium* subspecies *paratuberculosis* with a pathogenic mechanism in Crohn's disease and is consistent with abnormal macrophage handling of *M. avium* subspecies *paratuberculosis*.

Keywords: Crohn's disease; Inflammatory bowel disease; *Mycobacterium avium* subspecies *paratuberculosis*; TNF-α; Ulcerative colitis

1. Introduction

The cause of chronic inflammation in the gut of subjects with Crohn's disease (CD) is unclear, though two hypotheses are widely held. The first is that genetically influenced defects in mucosal immune regulation and/or mucosal barrier protection, underpin a heightened inflammatory response to enteric bacteria that have accessed the gut mucosal compartment [19]. The second is that a specific infection drives mucosal inflammation, with the most popular candidate being *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*). The prevalence of *MAP* in CD would appear to vary, although much of this variation may be a reflection of the different methodologies used [13,10,18]. Any relationship between

* Corresponding author at: Discipline of Immunology and Microbiology, University of Newcastle, Level 4, David Maddison Clinical Sciences Building, Newcastle, NSW 2300, Australia. Tel.: +61 2 49236185; fax: +61 2 4923 6998.
E-mail address: robert.clancy@newcastle.edu.au (R. Clancy).

1590-8658/$30 © 2007 Published by Elsevier Ltd on behalf of Editrice Gastroenterologica Italiana S.r.l.
doi:10.1016/j.dld.2006.12.006

MAP infection and disease is complicated by the observations that *MAP* may not be necessary for CD; that *MAP* is found in gut disease other than CD, as well as in normal subjects [10,18]; and that the clinical benefits claimed for rifabutin/macrolide antibiotic combinations developed to eradicate *MAP* have not yet been correlated with the presence of *MAP* infection [4]. The argument for a role of *MAP* in the pathogenesis of CD would be strengthened by identifying a mechanism whereby *MAP* could induce tissue damage. The aim of this study was to determine whether the presence of *MAP* in CD correlated with a particular pattern of cytokine secretion by the gut mucosa. We report data that link the presence of *MAP* in CD with a selective enhancement of tumour necrosis factor-α (TNF-α) secretion from the gut mucosa, a known contributor to the inflammatory response that mediates tissue damage characteristic of this disease.

2. Materials and methods

2.1. Subjects

The study was approved by the Centre for Digestive Diseases Human Research Ethics Committee, Sydney and Human Research Ethics Committee, University of Newcastle, Australia, and was performed in accordance with the principles of the 2000 Declaration of Helsinki. Informed consent was obtained from all subjects. Diagnosis was based on clinical criteria and the results of the pathological examination [15]. Patients presenting both for review and initial diagnosis were included. During colonoscopic examination, multiple biopsy specimens of mucosa (areas of inflammation when present) from the colon or terminal ileum were collected to be used for intestinal organ culture (two biopsies), histopathology (two biopsies) and the detection of *MAP* by nested PCR (two biopsies). Normal controls were asymptomatic and were having colonoscopy to exclude cancer. Random colonic biopsy specimens were collected. Histological examination was normal. Histological examination of gut in CD and UC confirmed clinical assessment of 'active' or 'inactive' disease.

2.2. Intestinal organ culture

The methodology was modified from that previously described for gastric biopsies [21]. Each biopsy weighed about 10 mg (with little variation). Two intestinal biopsy tissues were cultured in 1 mL of serum-free AIM-V medium (Life Technology, Melbourne, VIC, Australia) for 24 h at 37 °C in an incubator with 5% CO_2. The culture supernatants were collected and centrifuged. Aliquots were stored at −20 °C until assay. The total DNA was then extracted from cultured biopsy tissues using a FastDNA kit (Qbiogene, Carlsbad, CA, USA) and quantitated in a spectrophotometer (BioSpec-mini, Japan) at 260 nm/280 nm. Biopsy size varied only slightly with a DNA content of 64 ± 3 μg/mL.

2.3. Cytokine quantitation

IFN-γ, TNF-α, IL-2, IL-10 and IL-12 from culture supernatants were quantitated using paired antibodies (BD PharMingen, San Diego, USA) according to the manufacturer's protocols. Recombinant human IFN-γ, TNF-α, IL-2, IL-10 and IL-12 (BD PharMingen) were used as standards. The limit of sensitivity was 7.8 pg/mL for IFN-γ, TNF-α and IL-12, and 15 pg/mL for IL-2 and IL-10. The absorbance was read at 450 nm in an ELISA plate reader (Bio-Rad 680, Japan). The amount of cytokine in samples was determined using Microplate Manager (5.2) software (Hercules, CA, USA).

2.4. DNA extraction and IS900 PCR

DNA extraction used a method described elsewhere [7]. Briefly, fresh biopsies (within 1 h) in mycobacterial lysis buffer (2 mM sodium EDTA, 400 mM NaCl, 10 mM Tris–HCl, 0.6% sodium dodecyl sulfate, and 33 μg of proteinase K [Sigma–Aldrich, St. Louis, MO] per mL) were incubated for 2 h at 37 °C and then were ribolysed on site in a Lysing Matrix B Ribolyser tube (Qbiogene, NY, USA) in a FastPrep Ribolyser (FP120) (Qbiogene, NY, USA). The DNA was extracted using phenol (pH 6.7), phenol–chloroform–isoamyl alcohol (25:24:1) and chloroform–isoamyl alcohol (24:1). DNA was re-suspended in 50 μl of 1× TE buffer. *IS900* PCR specific for *MAP* was conducted as a nested primer PCR, as previously described by Bull et al. [7]. The primers for the first round PCR were Liz1 (5′-CTT TCT TGA AGG GTG TTC GG-3′) and Liz2 (5′-ACG TGA CCT CGC CTC CAT-3′). The primers for the nested PCR were AV 1 (5′-ATG TGG TTG CTG TGT TGG ATG G-3′) and AV 2 (5′-CCG CCG CAA TCA ACT CCA G-3′). To reduce the risk of amplicon contamination, the first round PCR products were treated with 1 U of uracil-DNA glycosylase (Invitrogen, VIC, Australia) at room temperature for 10 min. A control plasmid (pIDL60) containing a single copy of *IS900* cloned from a genome library *MAP* strain was used for positive control (a gift from Dr. Tim Bull). Mycobacterial lysis buffer processed in parallel with each specimen batch and PCR reagent only were used as negative controls. Positive and negative controls for both first round PCR and nested PCR were run on each PCR reaction. Extensive precautions were taken as described to prevent carryover contamination [7].

2.5. Statistical analysis

The non-parametric Kruskal–Wallis test was used to compare levels of TNF-α, IL-12, IFN-γ, IL-10 and IL-2 across the four patient groups followed by the Mann–Whitney test when the Kruskal–Wallis test was significant. A Chi-square test was used to compare the difference in *MAP*-positive rates among the disease groups. The Mann–Whitney test was used to determine significance of differences in cytokine secretion in

MAP-positive versus *MAP*-negative biopsies for each disease classification group. All statistical analyses were performed by using Prism® (Version 4) software (GraphPad Software Inc., San Diego CA, USA) and a 5% level of significance was used.

3. Results

Subjects were assessed in four groups: Crohn's disease (CD) ($n = 63$), ulcerative colitis (UC) ($n = 53$), irritable bowel syndrome (IBS) ($n = 45$) and normal controls ($n = 74$). The diagnosis was based on classical clinical, endoscopic and histological criteria. Of the 63 subjects with CD, 52 subjects had active inflammation (defined at colonoscopy as inflammation and/or ulceration and confirmed in biopsies) (11 for *MAP*-positive and 41 for *MAP*-negative) observed at colonoscopy and 11 had no inflammation (4 for *MAP*-positive and 7 for *MAP*-negative). Of the 53 subjects with UC, 32 had active inflammation and 21 had no inflammation as observed at colonoscopy. The mucosa was normal in both normal controls and subjects with IBS. Thirty-one subjects with CD had lesions in the terminal ileum (five involving the caecum), 24 had lesions in the colon, 5 had lesions in the small bowel/near the anastomosis after right hemi colectomy and 3 had a pouch following resection.

At the time of study (and for at least 3 months prior to study), treatment of those with CD was as follows: 10 subjects were taking combination antibiotics (rifabutin, clarithromycin and clofazimine: 3 *MAP*-positive and 7 *MAP*-negative), 35 subjects were taking azathioprine, and/or mesalazine formulations and/or prednisone (8 *MAP*-positive and 27 *MAP*-negative), and 18 subjects were not on treatment (4 *MAP*-positive and 14 *MAP*-negative). Of 11 subjects with CD, with no active inflammation at the time of study, 5 were taking combined antibiotics and 6 were taking azathioprine, and/or mesalazine formulations.

3.1. IS900 DNA detection in biopsy specimen

The detection rate of *MAP* specific *IS900* DNA by nested PCR was 11.3% (6/53) in subjects with ulcerative colitis, 23.8% (15/63) in subjects with Crohn's, 15.6% (7/45) in subjects with IBS and 14.9% (11/74) with normal controls.

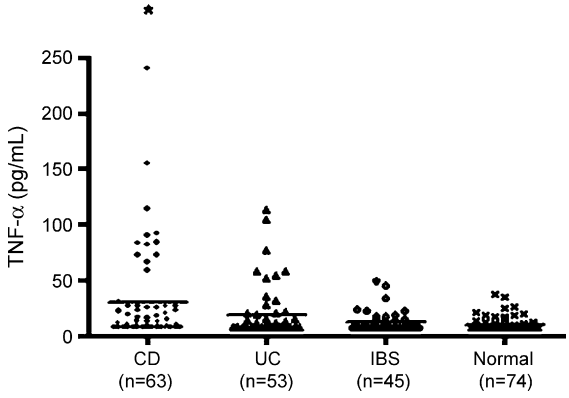


Fig. 1. TNF-α levels in gut organ culture supernatants. The culture supernatants were determined for TNF-α by ELISA. Horizontal bars denote the mean. (*) $p < 0.05$, $p < 0.01$ and $p < 0.0001$ compared with the value of UC, IBS and normal control subjects, respectively.

There was no significant difference among the disease groups.

3.2. Cytokine secretion from organ culture supernatant

There were no significant differences in the secretion of IL-2, IL-12, IL-10 and IFN-γ among the study groups, though a trend for an increase in IL-12 was observed in CD subjects (Table 1).

3.3. TNF-α secretion from organ culture supernatant

Significantly greater levels of TNF-α were found for CD, compared to UC, IBS and normal gut mucosa (Fig. 1). With respect to treatment in CD, those treated with combination antibiotics and corticosteroids had lower levels of TNF-α secretion compared to untreated subjects. However, only a trend was noted as numbers were small and levels variable. The levels of TNF-α were 21.0 ± 8.2, 29.9 ± 6.1 and 34.8 ± 13.3 pg/mL in subjects with combination antibiotic therapy, azathioprine and/or mesalazine formulations and/or prednisone and no treatment, respectively. No significant difference existed between those with diseased ileal, diseased colon and those studied post-surgery. The levels of TNF-α were 29.8 ± 8.9, 31.6 ± 7.3 and 24.8 ± 7.3 pg/mL for ileal disease, colon disease and post surgery, respectively. No significant difference was noted in active versus

Table 1
Cytokine secretion in organ culture[a]

	IL-12 (pg/mL)	IFN-γ (pg/mL)	IL-10 (pg/mL)	IL-2 (pg/mL)
CD ($n = 63$)	82.2 ± 23.3	56.3 ± 9.4	25.8 ± 3.5	29.8 ± 3.4
UC ($n = 53$)	56.6 ± 10.2	57.3 ± 12.4	22.9 ± 2.9	24.1 ± 2.0
IBS ($n = 45$)	51.5 ± 8.8	45.0 ± 7.0	20.9 ± 1.4	25.3 ± 2.0
Normal ($n = 74$)	51.3 ± 8.3	64.9 ± 19.3	22.6 ± 1.2	26.4 ± 1.7
p value[b]	NS	NS	NS	NS

[a] The non-parametric Kruskal–Wallis test was used to compare levels of IL-12, IFN-γ, IL-10 and IL-2 across the disease groups. Results are expressed as mean ± S.E.

[b] NS: not significant.

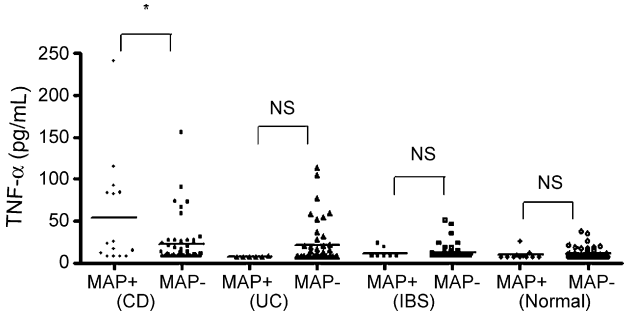


Fig. 2. TNF-α levels in gut organ culture supernatants. Culture supernatants were collected after 24 h culture of intestinal biopsy tissues and measured for TNF-α by ELISA. Horizontal bars denote the mean. (*) $p < 0.05$ compared with values of *MAP*-negative subjects. NS: not significant.

inactive Crohn's disease as assessed at colonoscopy (30.98 ± 6.0 pg/mL versus 24.5 ± 9.2 pg/mL).

3.4. Cytokine secretion in relation to MAP status

There were no significant differences in IL-2, IL-12, IL-10, IFN-γ or TNF-α secretions in *MAP*-positive versus *MAP*-negative subjects with IBS, UC or normal mucosa (Table 2 and Fig. 2). In CD, there were no significant differences in the secretion of IL-2, IL-12, IL-10 or IFN-γ in *MAP*-positive versus *MAP*-negative subjects, though a trend existed for an increase in IL-12 for *MAP*-positive subjects (Table 2). However, TNF-α secretion was more than two times higher in the *MAP*-positive subjects ($p < 0.05$, Fig. 2). Furthermore, levels of TNF-α were significantly higher in *MAP*-positive CD than in *MAP*-positive UC ($p < 0.01$), *MAP*-positive IBS ($p < 0.05$) and *MAP*-positive normal controls ($p < 0.01$), respectively. Subjects with CD who were *MAP*-positive but not treated with antibiotics ($n = 4$) had higher levels of TNF-α than did *MAP*-negative subjects with CD ($n = 14$) ($p < 0.05$, 240 pg/mL versus 10 pg/mL). CD subjects who were *MAP*-positive and who were treated with antibiotics for at least 3 months ($n = 3$) secreted TNF-α at 40% of that of untreated *MAP*-positive CD (92 pg/mL versus 240 pg/mL) but numbers were small. Similar treatment of *MAP*-negative CD was not associated with a difference in TNF-α secretion (10 pg/mL in both sets; 14 with no treatment and 7 with antibiotics).

4. Discussion

The demonstration of enhanced TNF-α secretion in the gut of subjects with CD who are positive for *MAP* is the first potential evidence of a mechanism that could link the presence of *MAP* with gut disease. The selective effect of *MAP* infection on TNF-α secretion in CD compared to an absence of such an effect in normal and disease controls, points to a specific defect in the cellular handling of *MAP* in CD.

The high concentration of TNF-α in the supernatants of gut mucosal cultures in subjects with inflammatory bowel disease (IBD) is not surprising as TNF-α has been found in stool as a marker of inflammation [5], in intestinal mucosal cells [6], and identified as a secretion product from isolated lamina propria cells from patients with IBD [20]. The method of organ culture used in the current study to detect cytokine secretion was developed to retain cell relationships and mimic in vivo conditions in an effort to avoid the discrepancies that have occurred with more indirect methods [30]. TNF-α is an important and well-characterized effector molecule in CD, with significant clinical improvement following treatment with both chimeric monoclonal anti-tumour necrosis factor (anti-TNF-α) [31], and thalidomide [2] which selectively degrades TNF-α mRNA [25].

Table 2
Cytokine secretion in organ culture from *MAP*+ and *MAP*− subjects[a]

	IL-12 (pg/mL)	IFN-γ (pg/mL)	IL-10 (pg/mL)	IL-2 (pg/mL)
CD				
MAP− ($n = 48$)	75.8 ± 23.2	57.9 ± 10.9	26.4 ± 4.4	30.5 ± 3.9
MAP+ ($n = 15$)	102.7 ± 65.5	51.3 ± 19.3	23.8 ± 3.5	27.6 ± 6.5
p value[b]	NS	NS	NS	NS
UC				
MAP− ($n = 47$)	52.7 ± 10.6	56.0 ± 13.3	23.7 ± 3.2	23.0 ± 1.8
MAP+ ($n = 6$)	60.8 ± 36.5	67.2 ± 37.3	16.8 ± 1.8	32.8 ± 10.2
p value[b]	NS	NS	NS	NS
IBS				
MAP− ($n = 38$)	49.8 ± 10.0	47.1 ± 7.9	20.5 ± 1.4	26.1 ± 2.2
MAP+ ($n = 7$)	60.3 ± 17.3	33.5 ± 13.5	22.9 ± 4.0	20.8 ± 3.3
p value[b]	NS	NS	NS	NS
Normal				
MAP− ($n = 63$)	51.3 ± 9.3	68.3 ± 22.6	22.9 ± 1.4	25.1 ± 1.6
MAP+ ($n = 11$)	51.5 ± 17.6	45.7 ± 11.6	20.6 ± 1.8	33.4 ± 6.8
p value[b]	NS	NS	NS	NS

[a] The non-parametric Mann–Whitney test was used to compare cytokine levels between *MAP*+ and *MAP*− subjects. Results are expressed as mean ± S.E.
[b] NS: not significant.

Infection by atypical mycobacteria has been proposed to be a cause of CD since the report by Thomas Dalziel in 1913 [11]. More recently *MAP* has been identified in gut tissue of subjects with CD by both PCR and culture [26,7] resulting in a polarized debate between those supporting *MAP* as being the major cause of CD and those who attach no special significance to its presence because of a high detection rate in normal subjects and in disease controls [10,27], as well as an absence of data relevant to a mechanism whereby *MAP* may contribute to the disease process. The polarity of the argument appears to require that most, if not all subjects with CD have *MAP* infection [9], which is not an observation in accordance with the findings of many experienced scientists [23,1,10]. We detected *MAP* in 23.8% of our CD population, which may be lower than the true infection rate as we used biopsies obtained at colonoscopy rather than resected gut, and 10 subjects had taken rifabutin and macrolides which have anti-*MAP* activity. However, an apparent biomodal distribution of TNF-α levels between *MAP*-positive and *MAP*-negative CD subjects (the current data), together with attention to methodological detail with an 'on site' processing laboratory, and precise, reproducible results, suggest it is unlikely that many detectable *MAP*-positive CD subjects are missed. Preliminary study failed to demonstrate culture of biopsies added significantly to the diagnosis for *MAP*, and detection of antibody to P35 and P36 antigens (courtesy of Dr. S. Naser) failed to differentiate between Crohn' disease and control groups. These latter observations are similar to those found by Bernstein et al. [3] though methodological differences make precise comparison difficult. Thus, if *MAP* is to contribute to the pathogenesis of CD, it is probable that it does so in the context of a complex host–parasite relationship involving defects in mucosal barrier function and/or mucosal immune regulation [19], rather than being the aetiological agent that it is in ruminants with Johne's disease [8].

High TNF-α secretion rates in *MAP*-positive CD could not be explained in terms of regional involvement or therapy. *MAP*-specific high TNF-α secretion was seen in CD, but was not found in normal or disease control groups, suggesting that a specific defect in handling *MAP* exists in CD. The relatively frequent occurrence of *MAP* detection in both normal and diseased control groups is consistent with the idea that *MAP* is a common environmental contaminant that does not trigger inflammation in gut mucosa that has a normal capacity to handle the microbe. Thus infection of those capable of 'normal handling' of *MAP*, does not lead to an increase in TNF-α secretion.

New information points to two heretofore-unappreciated differences with respect to *MAP* infection described in CD. First, *MAP* has been detected in a greater proportion of subjects by many groups [13,10,18]. Second, the current studies show significantly higher amounts of TNF-α are secreted by the gut mucosa in *MAP*-positive subjects. The association of *MAP* with granulomata in CD has been reported with in situ hybridization [14,24], showing that *MAP* is localized to macrophages and myofibroblasts [14], suggesting retention in defective macrophages, which in turn secrete high levels of TNF-α. Whole blood cultures from *MAP*-positive subjects with CD showed an increase in TNF-α secretion which was short of statistical significance, and a significant increase in IL-4 secretion, suggesting that the presence of *MAP* in CD (but not in normal or disease controls) is associated with a complicated shift in cytokine secretion commensurate with the systemic nature of CD, with pathology focused on the gut [22]. The suggestion that defective handling of *MAP* is due to NOD2/CARD15 gene mutations [32,16] would not appear relevant to our CD group, in which haplotype mutations occur in only about 30%. TNF-α levels were similar in both those with and those without NOD2 mutations (unpublished data). However, it appears that defective destruction of ingested *MAP* by phagocytes occurs in many CD subjects [12]. The failure of macrophages to destroy *MAP* may in part be due to evolved microbial survival mechanisms in the context of constitutionally defective phagocytic function [17]. Data demonstrating that *MAP* can drive TNF-α secretion from a human monocyte cell line [29] and that in *MAP*-positive CD subjects, separated peripheral blood mononuclear cells are sensitised to *MAP* [28] are consistent with our findings. The use of PCR to detect *MAP* DNA, however, does not allow us to conclude as to whether *MAP* needs to be alive to have a pathogenic effect.

What are the implications of these findings with respect to *MAP* and the management of CD? Clinical studies using combination antibiotic regimens designed to eradicate *MAP* have shown variable results, but none have correlated outcome with the presence of *MAP*. The link between *MAP* and high levels of TNF-α secretion not only supports a cause and effect role for *MAP* infection in CD, but also provides another parameter that can be used to monitor anti-*MAP* antibiotic therapy. The finding that the level of TNF-α secretion in subjects given combination antibiotic therapy was lower, though short of statistical significance, encourages further study of the effect of this therapy in larger numbers of *MAP*-positive and *MAP*-negative subjects. Indeed, while different therapies between the groups could not explain differences in TNF-α secretion, an effect of therapy on cytokine secretion remains likely.

It is concluded that, for the first time, the epidemiological argument for a role for *MAP* in the pathogenesis of CD may be supported by the demonstration of a mechanism likely to contribute to gut inflammation and damage. It is possible that not all subjects with CD are necessarily infected by *MAP*, and the reason why more CD subjects are *MAP*-positive than is found in normal and disease controls (as described by others, e.g. [13,18] but not found in this study), is probably due to defective clearance in CD. By strengthening the evidence for a role of *MAP* in the pathogenesis of a proportion of subjects with CD, support is given to the continued evaluation of controlled anti-*MAP* therapeutic trials. In these trials it is important that appropriate antibiotic regimens are given and

that results are correlated with the presence and eradication of *MAP*, and an effect on TNF-α levels, to determine not only the degree of clinical benefit, but also its specificity. The low incidence of the presence of *MAP* in our CD population compared to the high levels described in northern hemisphere studies has been a window of opportunity enabling comparison of cytokine secretion between *MAP*-positive and *MAP*-negative subjects.

Practice points

- The pathogenesis of Crohn's disease (CD) is a topic of intense debate. A particular focus has been whether or not infection with *Mycobacterium avium* subspecies *paratuberculosis* (*MAP*) plays a role in the pathogenesis of CD.
- Cytokine secretion from gut cultured biopsies, was studied from CD and from normal and disease controls, and correlated with the presence or absence of *MAP* as detected by nested PCR.
- The demonstration of enhanced TNF-α secretion in the gut of subjects with CD who are positive for *MAP* is the first evidence of a mechanism that links the presence of *MAP* DNA with gut disease.
- The selective effect of MAP infection on TNF-α secretion in CD when compared to detectable MAP DNA in normal and disease controls, is consistent with a defect in the cellular handling of MAP in CD.

Research agenda

- The complicated pathogenesis of CD is beginning to unravel. Prospective studies in different geographical areas with appropriate controls are needed to clarify the relative contribution of *MAP* and other bacteria (e.g. Yersinia species) in relation to the other abnormalities now described (including genetic abnormalities, defects in cell handling of gut derived bacteria, and disturbed cytokine secretion patterns).
- Prospective antibiotic trials ("anti-MAP" therapy) need to include MAP detection and cytokine secretion assays, to increase understanding of the contribution of MAP to the pathogenesis of CD.

Conflict of interest statement

None declared.

Acknowledgements

The study was funded by the Broad Medical Research Program of the Eli and Edythe L. Broad Foundation, USA. Authors are grateful to Evelyn Vigano for her skillful technical assistance. We thank Professor Tom Borody for providing clinical data and comments.

References

[1] Al-Shamali M, Khan I, Al-Nakib B, Al-Hassan F, Mustafa ASA. A multiplex polymerase chain reaction assay for the detection of *Mycobacterium paratuberculosis* DNA in Crohn's disease tissue. Scand J Gastroenterol 1997;32:819–23.
[2] Bariol C, Meagher AP, Vickers CR, Byrnes DJ, Edwards PD, Hing M, et al. Early studies on the safety and efficacy of thalidomide for symptomatic inflammatory bowel disease. J Gastroenterol Hepatol 2002;17:135–9.
[3] Bernstein CN, Blanchard JF, Rawsthorne P, Collins MT. Population-based case control study of seroprevalence of *Mycobacterium paratuberculosis* in patients with Crohn's disease and ulcerative colitis. J Clin Micobiol 2004;42:1129–35.
[4] Borody TJ, Leis S, Warren EF, Surace R. Treatment of severe Crohn's disease using antimycobacterial triple therapy—approaching a cure? Dig Liver Dis 2002;34:29–38.
[5] Braegger CP, Nicholls S, Murch SH, Stephens S, MacDonald TT. Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. Lancet 1992;339:89–91.
[6] Breese EJ, Michie CA, Nicholls SW, Murch SH, Williams CB, Domizio P, et al. Tumor necrosis factor alpha-producing cells in the intestinal mucosa of children with inflammatory bowel disease. Gastroenterology 1994;106:1455–66.
[7] Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, et al. Detection and verification of *Mycobacterium avium* subspecies *paratuberculosis* in fresh ileocolonic mucosal biopsies from people with and without Crohn's disease. J Clin Microbiol 2003;41:2915–23.
[8] Chacon O, Bermudez LE, Barletta RG. Johne's disease, inflammatory bowel disease, and *Mycobacterium paratuberculosis*. Annu Rev Microbiol 2004;58:329–63.
[9] Chamberlin W, Graham DY, Hulten K, El-Zimaity HM, Schwartz MR, Naser S, et al. *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. Aliment Pharmacol Ther 2001;15:337–46.
[10] Collins MT, Lisby G, Moser C, Chicks D, Christensen S, Reichelderfer M, et al. Results of multiple diagnostic tests for *Mycobacterium avium* subsp. *paratuberculosis* in patients with inflammatory bowel disease and in controls. J Clin Microbiol 2000;38:4373–81.
[11] Dalziel DK. Chronic interstitial enteritis. Br Med J 1913;2:1068–70.
[12] Gearry RB, Aitken JM, Roberts RL, Ismail S, Keenan J, Barclay ML. Gastrointestinal: *Mycobacterium avium paratuberculosis* and Crohn's disease. J Gastroenterol Hepatol 2005;20:1943.
[13] Green EP, Tizard MLV, Moss MT, Thompson J, Winterbourne DJ, McFadden JJ, et al. Sequence and characteristics of IS900, an insertion identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res 1989;17:9063–73.
[14] Hulten K, El-Zimaity HM, Karttunen TJ, Almashhrawi A, Schwartz MR, Graham DY, et al. Detection of *Mycobacterium avium* subspecies *paratuberculosis* in Crohn's disease tissues by in situ hybridization. Am J Gastroenterol 2001;96:1529–35.

[15] Lennard-Jones JE. Classification of inflammatory bowel disease. Scand J Gastroenterol Suppl 1989;170:2–6.
[16] Maeda S, Hsu LC, Liu H, Bankston LA, Iimura M, Kagnoff MF, et al. NOD2 mutation in Crohn's disease potentiates NF-κB activity and IL-1β processing. Science 2005;307:734–8.
[17] Marks DJ, Harbord MW, MacAllister R, Rahman FZ, Young J, Al-Lazikani B, et al. Defective acute inflammation in Crohn's disease: a clinical investigation. Lancet 2006;367:668–78.
[18] Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. Lancet 2004;364:1039–44.
[19] Podolsky DK. Inflammatory bowel disease. N Engl J Med 2002;347:417–28.
[20] Reinecker HC, Steffen M, Witthoeft T, Pflueger I, Schreiber S, MacDermott RP, et al. Enhanced secretion of tumour necrosis factor-alpha, IL-6, and IL-1 beta by isolated lamina propria mononuclear cells from patients with ulcerative colitis and Crohn's disease. Clin Exp Immunol 1993;94:174–81.
[21] Ren Z, Pang G, Clancy R, Li LC, Lee CS, Batey R, et al. Shift of the gastric T-cell response in gastric carcinoma. J Gastroenterol Hepatol 2001;16:142–8.
[22] Ren Z, Turton J, Borody T, Pang G, Clancy R. Selective Th2 pattern of cytokine secretion in *Mycobacterium avium* subsp *paratuberculosis* infected Crohn's disease. J Gastroenterol Hepatol 2007;22, in press.
[23] Rowbotham DS, Mapstone NP, Trejdosiewicz LK, Howdle PD, Quirke P. *Mycobacterium paratuberculosis* DNA not detected in Crohn's disease tissue by fluorescent polymerase chain reaction. Gut 1995;35:660–7.
[24] Ryan P, Bennett MW, Aarons S, Lee G, Collins JK, O'Sullivan GC, et al. PCR detection of *Mycobacterium paratuberculosis* in Crohn's disease granuloma isolated by laser capture microdissection. Gut 2002;51:665–70.
[25] Sampaio EP, Sarno EN, Galilly R, Cohn ZA, Kaplan G. Thalidomide selectively inhibits tumor necrosis factor alpha production by stimulated human monocytes. J Exp Med 1991;173:699–703.
[26] Schwartz D, Shafran I, Romero C, Piromalli C, Biggerstaff J, Naser N, et al. Use of short-term culture for identification of *Mycobacterium avium* subsp. *paratuberculosis* in tissue from Crohn's disease patients. Clin Microbiol Infect 2000;6:303–7.
[27] Shanahan F, O'Mahony J. The mycobacteria story in Crohn's disease. Am J Gastroenterol 2005;100:1537–8.
[28] Sibartie S, Keohane J, Scully P, O'Neill S, O'Mahony J, O'Mahony L, et al. Disturbed cytokine response to *Mycobacterium avium* subspecies *paratuberculosis* is dysregulated in patients with Crohn's disease. Gastroenterology 2006;130:A-240.
[29] Tamaki H, Nakase H, Ueno S, Uza N, Kitamura H, Inoue S, et al. The first demonstration of the infectivity and immunological response of *Mycobacterium paratuberculosis* in human monocyte cell and novel specific antibodies against insertion element 900 in patients with Crohn's disease. Gut 2005;54(Suppl. 7):A154.
[30] Van Damme N, De Keyser F, Demetter P, Baeten D, Mielants H, Verbruggen G, et al. The proportion of Th1 cells, which prevail in gut mucosa, is decreased in inflammatory bowel syndrome. Clin Exp Immunol 2001;125:383–90.
[31] Van Dullemen HM, van Deventer SJ, Hommes DW, Bijl HA, Jansen J, Tytgat GN, et al. Treatment of Crohn's disease with anti-tumor necrosis factor chimeric monoclonal antibody (cA2). Gastroenterology 1995;109:129–35.
[32] Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat Immunol 2004;5:800–8.

Articles 2 and 3 have been removed for copyright reasons.

Citation details and access links are below.

Foell, D., Wittkowski, H., Ren, Z., Turton, J., Pang, G., Daebritz, J., Ehrchen, J., Heidemann, J., Borody, T., Roth, J. and Clancy, R. (2008), Phagocyte-specific S100 proteins are released from affected mucosa and promote immune responses during inflammatory bowel disease. J. Pathol., 216: 183–192.

http://dx.doi.org/10.1002/path.2394

Ren, Z., Turton, J., Borody, T., Pang, G. and Clancy, R. (2008), Selective Th2 pattern of cytokine secretion in Mycobacterium avium subsp. paratuberculosis infected Crohn's disease. Journal of Gastroenterology and Hepatology, 23: 310–314.

http://dx.doi.org/10.1111/j.1440-1746.2007.04865.x