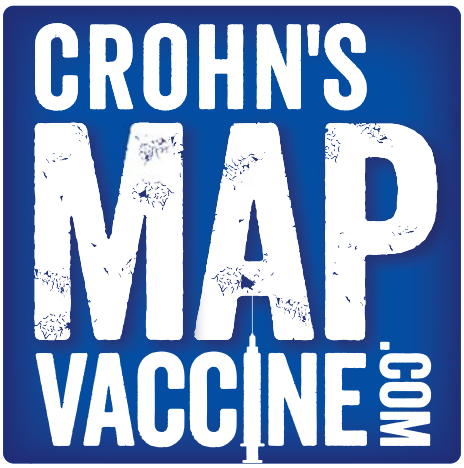


# Core Literature supporting the hypothesis that Mycobacterium avium subspecies paratuberculosis causes Crohn's Disease (2007-2015)

Compiled and summarised by
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## Review articles:



## Genetic data points to a mycobacterial cause for CD:



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*continued…*

## The anti-MAP action of conventional CD treatments:

Many have asked 'If MAP causes CD then why do anti-TNF agents and immunosuppressants, which would make TB disseminate, make CD better? Answer: (1) as Gitlin et al (2) describes well, the behaviour of MAP is not like TB in this respect; MAP is much more like its closer cousin leprosy which does not disseminate with anti-TNF agents or immunosuppressants and (2) these agents also have direct anti-MAP action; anti-TNFs, the immunosuppressants azathioprine, 6-mercaptopurine, cyclosporine, tacrolimus, methotrexate and the anti-inflammatory agent 5-ASA have ALL been shown to inhibit MAP growth in vitro in the following studies:

7 Treatment of Crohn's disease patients with infliximab is detrimental for the survival of Mycobacterium avium ssp. paratuberculosis within macrophages and shows a remarkable decrease in the immunogenicity of mycobacterial proteins
*Bach et al. Journal of Crohn's and Colitis 2012*

8 On the action of Methotrexate and 6-mercaptopurine on M. avium subspecies paratuberculosis
*Greenstein RJ et al. PloS One 2007*

9 On the action of cyclosporine A, rapamycin and tacrolimus on M. avium including subspecies paratuberculosis
*Greenstein RJ et al. PloS One 2008*

10 On the action of 5-amino-salicylic acid and sulfapyridine on M. avium including subspecies paratuberculosis
*Greenstein RJ et al. PloS One 2007*

11 Thiopurine drugs azathioprine and 6-mercaptopurine inhibit Mycobacterium paratuberculosis growth in vitro
*Shin SJ and Collins MT. Antimicrob Agents Chemother 2008*

## Difficulties in testing for MAP in humans have obscured the truth:

12 A report from the American Academy of Microbiology 2008: Mycobacterium avium paratuberculosis: Infrequent Human Pathogen or Public Health Threat?

A reliable test for MAP in humans remains a major unmet medical need. The American Academy of Microbiology report on MAP in 2008 made the development of such a test its number one recommendation, stating 'Virtually all the research topics that will clarify the role of MAP in CD rely on this'.

13 Mycobacterium avium subspecies paratuberculosis causes Crohn's disease in some inflammatory bowel disease patients
*Naser SA et al. World J Gastroenterol 2014*

Naser et al renewed the call for better MAP diagnostics in this 2014 review of 60 studies testing for MAP by culture and PCR, emphasizing that 'much of the controversy concerning MAP and CD stems from the inconsistent methodologies that have been used in the detection and isolation of MAP, which have questioned the causal relationship between this bacterium and CD'.

Several promising new diagnostics are currently being studied. The new test under development by Prof Hermon-Taylor is the only one that enables MAP to be seen within the tissues of people with CD, for the very first time.

*continued…*

## Immunological evidence of MAP in CD:

14 Isolation of Mycobacterium avium subspecies paratuberculosis reactive CD4 T Cells from intestinal biopsies of Crohn's disease patients
*Olsen I et al. PLoS One 2009*

In this study, researchers demonstrated the presence of MAP-reactive T-cells isolated from intestinal biopsies of patients with CD. Immunological evidence therefore also supports a role for MAP in the causation of CD.

## Therapeutic anti-MAP Vaccination:

15 Immunity, safety and protection of an Adenovirus 5 prime - Modified Vaccinia virus Ankara boost subunit vaccine against Mycobacterium avium subspecies paratuberculosis infection in calves
*Bull TJ et al. Veterinary Research 2014*

This paper provides evidence of the Vaccine's effectiveness as a therapeutic agent (and as a preventative agent) against MAP in cattle.

There are of course many more papers in this field... these are the highlights from amongst the recent literature.

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Future MICROBIOLOGY

EDITORIAL



# 'Global warming' to *Mycobacterium avium* subspecies *paratuberculosis*



Gaurav Agrawal*[,1], Thomas J Borody[1] & William Chamberlin[2]

**KEYWORDS**
• autophagy • Crohn's disease • Dietzia • fecal microbiota transplantation • microbiota • *Mycobacterium paratuberculosis*

There is a growing worldwide movement to investigate the relationship between Crohn's disease (CD) and microorganisms, especially for causality. Scientists and doctors are warming to this historical idea again, particularly with the advent of discoveries involving the gut microbiome, metagenomics and the revelations of the deficiencies of the innate immune system and autophagy. Looking back, early CD reports were already concerned with finding an infectious cause, which is being revisited by researchers around the globe.

## History

CD was described by the Scottish surgeon, TK Dalziel in 1913 [1], though it was probably described even earlier by a Polish surgeon Antoni Leśniowski in 1904 [1]. Subsequently CD was labeled as 'regional ileitis' by an American gastroenterologist, Burril Crohn [1] and so it became known as CD. Dr Burril Crohn commented initially upon its similarities to known mycobacterial infections of the gut, such as *Mycobacterium tuberculosis*, particularly in that it was of a 'granulomatous enteritis' nature. However, given mycobacteria were not cultured, alternative proposals were forwarded. Psychosomatic origins were discussed in the 1950s and a decade later the concept that CD was an autoimmune disorder came to the fore, and it included the involvement of dysfunctional T cells of the adaptive immune response. This explanation dominated the field for the next 40 years, but more recently, however, a newer concept has evolved which describes the inflammatory response occurring secondary to an aberrant reaction to the body's normal gut flora [2].

Recent work has led to the hypothesis that innate immune deficiencies are central to a dysregulated chronic inflammatory process. This proposal stresses the importance of dysfunctional macrophages and dendritic cells. These antigen-presenting cells are impaired in their ability to signal to the rest of the immune network and show diminished ability to kill intracellular infections. Along with other functional abnormalities, disturbances of cytokine signaling impair neutrophil chemotaxis resulting in significant aberrations throughout the innate and adaptive immune network.

"There is a growing worldwide movement to investigate the relationship between Crohn's disease and microorganisms, especially for causality."

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The concept of CD being triggered by a *Mycobacterium* is gaining momentum with the advent of new molecular techniques and cytogenetics. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is once again coming under the microscope due to the increased ability to detect it's genetic signature. This is the best contender for a causative microorganism of CD which infects macrophages and disrupts the microbicidal and immune signaling function of these immune cells. Indeed it is the accepted cause of a similar chronic inflammatory bowel disease in cattle, known as Johne's disease. This postulated zoonosis and its controversial mode of transmission shares similarities (clinically and histopathologically) with *M. tuberculosis* enteritis and CD in humans. CD is becoming an epidemic and has risen almost exponentially, with large increases being seen across the globe. Traditionally 'low-incident' countries, for example, China, India and Latin America now have to deal increasingly with this problem.

"Rational future therapies need to focus on reversing the ineffective autophagy and eradicating *Mycobacterium avium* subspecies *paratuberculosis*."

## Autophagy

Autophagy is essential for macrophages to eradicate intracellular pathogens, especially mycobacteria such as MAP. Dysfunctional macrophages are central players in the CD process characterized by intracellular infections or the converse where such successful intracellular pathogens downregulate autophagy as an evolved survival strategy, which pathogenic mycobacteria have been shown to adopt as a survival strategy. Genome-wide association studies identified over 70 single nucleotide polymorphisms associated with CD with the strongest linkages associated with genes involved in the recognition and response to intracellular infections – that is, *NOD2, IRGM* and *ATG16L1*. These peptides are all involved in macro-autophagy and implicated in the pathogenesis of CD. Even more striking was the discovery that these same mutant gene alleles are associated with leprosy, tuberculosis and 'other mycobacterial infections'. A weak autophagy response to microbial infections at the intracellular level results in all the findings of CD: innate immune deficiency, poor neutrophil chemotaxis, compromised macrophage microbicidal function impaired cytokine signaling, persistent intracellular infections, chronic inflammation and granulomas, among others [3].

## MAP & CD

MAP primarily targets the human intestine and causes similar inflammatory bowel disease in a wide range of mammals, including non-human primates. It infects dendritic cells and macrophages and when these cells are infected their molecular signaling pathways are impaired. The homeostatic balance of the entire immune system is disrupted and chronic inflammation occurs. Ineffective attempts by the immune system to destroy MAP results in tissue damage and the broad spectrum of clinical disease that occurs in patients with CD. Indeed this is similar to leprosy (which is caused my *Mycobacterium leprae*, and to which MAP is phenotypically similar) [4]. Bovine- and human-derived MAP isolates evoke the same immune responses. Similarities evoked by early MAP infection in cattle during the latent and early clinical period to those seen in human CD are striking. These similarities are found at the molecular, cellular and tissue levels [5].

The different species of the Mycobacteria family have similar general features but different characteristics and behavior. Literature suggests a qualitatively different role for TNF-α during infection with *M. tuberculosis* as compared with *M. avium* [6]. MAP has been shown to be associated with increased levels of TNF-α – more than other types of mycobacteria and that it thrives on high levels, as shown by Bach *et al.* [7]. TNF-α induces apoptosis of MAP infected cells and so drugs, such as infliximab, act as an antibiotic as well as an anti-inflammatory agent. Apoptosis is induced in MAP-infected macrophages, that secrete higher amounts of TNF-α on their surface and so the survival of the white cell is reduced along with the intracellular organism. In addition, it reduces the antibody titers of two mycobacterial proteins associated with MAP [7]. The authors propose that the combination of infliximab and anti-MAP antibiotics is likely to be synergistic in healing CD lesions and have noted this in their clinical practice to date.

A 2-year trial of anti-MAP therapy carried out by Selby *et al.* initially stated that it does not "find evidence of a sustained benefit and does not support a significant role for MAP in the pathogenesis of CD in the majority of patients" [8]. However, corrected analysis of the data by scientific peers revealed widespread and serious flaws and misinterpretations that led to inaccurate conclusions [9,10] and re-analyzed data showed differences between the two groups with a highly significant treatment response to antibiotics compared with the immunosuppressive arm [11].

## The future

Weak macro-autophagy response, either genetic, acquired or both, begets innate immune deficiency that predisposes to chronic intracellular macrophage infections and a dysregulated innate and adaptive immune response resulting in the CD. Rational future therapies need to focus on reversing the ineffective autophagy and eradicating MAP.

### • Targeting MAP using antibiotics

MAP has fulfilled Koch's postulates as the cause of CD [12], a set criteria used to prove causality of a disease by a microorganism. As such anti-MAP treatment is increasingly prescribed as a therapy for CD using a combination of antibiotics that targets MAP and has been shown to be quite effective [13]. To prevent development of resistance during long term therapy a combination of antibiotics is required to target the bacterium at all stages of the life cycle including reproduction and dormancy. A randomized controlled trial in CD using such a combination is currently in progress and this could prove the effectiveness of a therapy that targets the MAP organism [14].

### • Competitively inhibiting MAP

Dietzia subspecies C79793-74, previously known as *Mycobacterium gordonae*, is a potentially useful and novel step in treating MAP. Acting to displace MAP from the macrophage represents a novel therapeutic method of removing MAP from its niche so taking away its survival environment. By using an evolutionarily more 'adept and inert' member of the same family to replace its 'cousin', we could be utilizing a naturally occurring method in evolutionary competition. Data for this potential therapy are based on its effectiveness as a prophylactic therapy in cattle [15]. Dietzia is a nonpathogenic microorganism used to competitively displace and inhibit MAP infection. Some 40% of cattle with early Johne's disease – which is notoriously difficult to treat – were cured with this oral probiotic and the effect was long lasting compared with the use of antimycobacterial antibiotics. Hence, it could be used in the same manner for Crohn's patients [16].

### • Targeting the microbiome & modifying immunity

There have been reports of fecal microbiota transplantation reversing active CD by the implantation of normal donor gut microbiota into the bowel of CD individual [17,18]. Partial and complete disappearance of CD has been achieved and the authors have now three patients with complete remission off therapy between 12 months and 13 years after fecal microbiota transplantation. This is a sporadic result and not the norm but indicates potential for further research in restoration of the bowel microbiota as a future cotherapy in CD [19]. Furthermore it argues against the cause of inflammatory bowel disease being an "inappropriate and ongoing activation of the mucosal immune system driven by the presence of normal luminal flora" [2]. The mechanism(s) of action is not well understood but clearly the microbiome influences mucosal immune networks.

### • Developing therapeutic vaccination

One of the most exciting developments from John Hermon-Taylor's laboratory is the anti-MAP vaccine capable of driving MAP from infected tissues [20]. It is envisaged that the stimulation of immune responses in the CD host, contrary to current immune-suppression, will be another co-therapy in the eradication of the intracellular pathogen/s driving the chronic inflammation in CD.

## Conclusion

Current 'global warming' of scientific thought toward a microbial role in CD and seemingly unrelated therapeutic developments listed above augur well for patients with this chronic condition. The focus of therapy will now shift away from control of inflammation and toward control and eradication of the underlying pathogen/s, particularly MAP with restoration of defective immunity.

"The focus of therapy will now shift away from control of inflammation and toward control and eradication of the underlying pathogen/s, particularly MAP with restoration of defective immunity."

**Financial & competing interests disclosure**

*G Agrawal has filed patents in anti-*Mycobacterium avium *subspecies* paratuberculosis *antibiotics and infliximab combination therapy. TJ Borody has a financial interest in the Centre for Digestive Diseases, where fecal microbiota transplantation is a treatment option. In addition, he has filed patent applications in the field of fecal transplantation and anti-*Mycobacterium avium *subspecies* paratuberculosis *therapies. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

*No writing assistance was utilized in the production of this manuscript.*

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# *Mycobacterium avium ss paratuberculosis*-associated Diseases
## *Piecing the Crohn's Puzzle Together*

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*William Chamberlin, MD,‡ and Jordana Campbell, BSc†*

**Abstract:** The relation of *Mycobacterium avium ss paratuberculosis* (MAP) to Crohn's Disease (CD) and other MAP-associated conditions remains controversial. New data, coupled with the analogous *Helicobacter pylori* (*H. pylori*) story, has permitted us to piece together the MAP puzzle and move forward with a more scientific way of treating inflammatory bowel disease, particularly CD. As infection moves centre stage in inflammatory bowel disease, the dated "aberrant reaction" etiology has lost scientific credibility. Now, our growing understanding of MAP-associated diseases demands review and articulation. We focus here on (1) the concept of MAP-associated diseases; (2) causality, Johne Disease, the "aberrant reaction" hypothesis; and (3) responses to published misconceptions questioning MAP as a pathogen in CD.

**Key Words:** Crohn's Disease, *Mycobacterium avium paratuberculosis*, Koch's postulates

(*J Clin Gastroenterol* 2012;00:000–000)

*"There are three classes of people: Those who see. Those who see when they are shown. Those who do not see."*

*Leonardo da Vinci*

## *Mycobacterium avium ss paratuberculosis* (MAP)-ASSOCIATED DISEASES

Several reviews and editorials have summarized the Crohn's Disease (CD) controversies which need to be addressed.[1–5] One major advance that helps us understand CDs' numerous facets is the elucidation of a group of *H. pylori*-associated diseases including asymptomatic gastritis, duodenal ulcer, gastric ulcer, lymphoma, and gastric cancer.[6] We can draw on this analogy to gain insights into MAPs' variable presentations. In parallel with *H. pylori,* several expressions of MAP infection exist including an asymptomatic carrier state,[7] cervical lymphadenopathy,[8] irritable bowel syndrome,[9] CD,[9] and sarcoidosis.[10] Part of the controversy stems from the preconceived notion of a single CD etiology. Yet it is more plausible that the "CD syndrome"[11] is caused by more than a single agent, just as most, although not all, duodenal ulcers are caused by *H. pylori*.[6]

## PARADIGM SHIFT

Similar indoctrination to the "no acid no ulcer" *H. pylori* dictum[12] again prevents us from accepting an infectious CD etiology, and instead blindly accepting a nebulous, unproven "aberrant immune reaction theory."[13] Are we again "repeating the mistake of *H. pylori*?"[1]

## VARIABLE DISEASE CHARACTERISTICS

Although it is plausible that several pathogens cause CD-like pathology,[11] Prantera[2] suggested "variable disease locations and severity" disprove a single pathogen etiology. Yet a number of pathogens cause "variable disease locations and severity." Tuberculosis (TB) is a classic example. Around 90% of patients with TB have latent infection.[14] In the remaining 10%, there can be lymph node TB,[15] miliary TB,[16] pulmonary TB,[17] central nervous system TB,[18] cervical TB,[19] adrenal TB,[20] or joint TB.[21] *H. pylori*, another versatile pathogen can cause an asymptomatic carrier state, mild nonulcer dyspepsia, duodenal or gastric ulcers, mucosa-associated lymphoid tissue lymphoma, and gastric carcinoma.[6] Hence, variable presentations can be the result of a single pathogen. The reverse can also be true; with different etiologic agents causing similar presentations, for example. *H. pylori*, *H. heilmanii*, anti-inflammatory drugs, and pancreatic cancer can all cause "duodenal ulcers."[22]

## KOCH'S POSTULATES AND CAUSALITY

Koch's Postulates, the gold standard for establishing microbial causality of disease, was first described by Robert Koch in 1884 when he established a bacterial cause of *Mycobacterium tuberculosis* (*M. tuberculosis*)-associated diseases.[23] The Postulates included the following.

1. Growing the bacteria from infected tissue in pure culture.
2. Introducing these bacteria into an animal model to reproduce the disease.
3. Recovering the same bacteria from the diseased animal model.

In 1986, Van Kruiningen et al[24] isolated MAP in pure culture from a CD patient, infecting infant goats with this pathogen which resulted in humoral and cell-mediated responses at 3 weeks and terminal ileitis at 5 months. MAP was recovered thereby fulfilling Koch's Postulates. Fredricks and Relman (1996) updated these Postulates to incorporate molecular methods to deal with evolving infections, which have also been met for MAP in CD.[25,26] It is vital to note

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that antibiotic trials play no part in establishing Koch's Postulates of causality.

Dr Steven Hanauer, an internationally recognized and respected inflammatory bowel disease (IBD) expert, agreed in his editorial that Koch's Postulates remain crucial in proving disease causality.[27] Prantera[2] also confirmed the Postulates had been fulfilled for MAP in CD. We now must question why "leaders in the field" are unwilling to accept evidence-based findings and continue to request treatment trials[28] which do not determine causality. By this token, Koch himself would not have proven *M. tuberculosis* as the cause of TB in 1882,[29] some 62 years before the advent of antibiotics. In 1984, Marshall et al[30] fulfilled Koch's Postulates for *H. pylori* after swallowing a pure culture that resulted in histologic gastritis and mild illness. The bacteria were recovered proving gastritis causality, not ulcer disease, yet antibiotics are routinely employed as *H. pylori* eradication correlates with ulcer cure. It should also be noted that MAP infection remains incurable in animals with Johne Disease (JD) treated with antibiotics[31] despite MAP being the causative agents. Not only have Koch's Postulates been fulfilled for CD, but anti-MAP therapy induces remission in CD, yet MAP is not routinely treated as *H. pylori* is in ulcer disease.

## CURRENT IBD ETIOLOGY HYPOTHESIS

The dominant IBD hypothesis states that "dysregulation of the mucosal immune system in a genetically predisposed individual leads to an exaggerated and ongoing activation of immunologic responses to the person's own normal microflora."[13] Although genetic interactions play some predisposing role in CD causation as they do in TB,[32] the rapidly rising incidence of CD worldwide cannot be explained by genetic mutations.[33] Epidemiologic studies have shown substantial overlaps of CD in regions that have high levels of environmental MAP, with a recent Japanese study finding a statistical correlation between increased CD incidence and increasing consumption of animal proteins, particularly milk, a known source of viable MAP.[34] Another significant finding is that of isolated duodenal CD.[35,36] An "aberrant response to normal colonic flora" simply cannot exist in the duodenum, an area devoid of colonic bacteria, and is enough alone to disprove the current hypothesis. If we are to advance our understanding of IBD causality, these glaring anomalies can no longer be ignored, suggesting a timely reexamination of the current IBD hypothesis with the same scrutiny afforded to MAP.

It is critical that we be cognizant of what we know and what we merely assume, of what we submit as a plausible hypothesis, and what has accumulated sufficient evidence to be regarded as substantive theory. The real danger lies in repeating plausible hypotheses long and often enough for the medical community to regard them as accepted theories. The longstanding acceptance of the CD "Autoimmune Hypothesis" speaks to this danger. Currently, the same danger exists for the unquestioned, accepted hypothesis that the commensal microbiota in genetically susceptible hosts is responsible for CD development—to the exclusion of specific bacterial infections in these same genetically predisposed individuals.

## JD

JD, the animal equivalent of CD, is irrefutably caused by MAP.[37] As early as 1913, Dalziel,[38] a surgeon familiar with JD, observed the striking resemblance between the chronic diarrhea, wasting, and mucosal appearance in cattle JD and human CD, and the search for a mycobacterial cause of CD began. Numerous anti-TB drug trials were undertaken however the results showed a disappointing lack of efficacy,[39] leading scientists away from an infective cause and toward the "autoimmunity" hypothesis—a popular concept at the time. We now know the older anti-TB drugs employed were largely ineffective against MAP.[40] It was only with the availability of novel intracellularly active macrolides to combat *Mycobacterium avium* Complex in acquired immune deficiency syndrome that significant reductions in CD severity, with profound healing of severe inflammation,[41,42] complete mucosal healing, and scarring were achieved.[41]

## CONTENTIOUS ISSUES

Despite satisfying both Koch's and Relman's causality Postulates,[25,26,29] some contentious issues remain. To date, no comprehensive review has been published to counter published arguments against MAP causality in CD. These issues are addressed below.

### Issue 1: MAP Organisms "Are Absent or Very Rare by Immunohistochemistry and In-Situ Hybridization"[5]

Because of MAP's extremely slow growth, intracellular location, negative Ziehl Neelsen staining, and lack of detection using light microscopy, it has been extremely difficult to identify MAP in CD tissues until recently. However, technological advancements, particularly polymerase chain reaction, have allowed MAP DNA identification in up to 92% of CD tissues versus 26% of controls.[43] Another study examining resected bowel tissue in 300 subjects identified MAP DNA in 52% of CD patients versus only 2% of ulcerative colitis (UC) patients and 5% of controls.[44] A further study identified MAP DNA in 6/7 (86%) resected CD tissue versus 2/36 (5.6%) in control specimens.[45] Incidentally, the much lower prevalence of MAP in UC tissue versus CD discounts the notion that MAP is a mere bystander organism which "innocently lodges in ulcerated mucosa."[1] The presence of MAP, a known pathogen in diseased human tissue in individuals with CD suggests that until MAP zoonosis is disproven, its presence must be treated as pathogenic.

### Issue 2: "The Strongest Negative Evidence is the Lack of Exacerbating Disease by Use of Immunosuppression Such as Corticosteroids and Antitumor Necrosis Factor-(TNF)-α Antibodies" That Disseminate Mycobacterial Infection[2,5]

The lack of MAP dissemination during immunosuppressive therapy has baffled CD experts, with 1 expert stating "the strongest negative evidence" of a" mycobacterial etiology" is the lack of exacerbating disease during immunosuppressive therapy.[5] We must first emphasize that this has no bearing on Koch's Postulates and causality. Numerous studies have confirmed the presence of MAP in CD tissues without a single recorded case of dissemination in the presence of concomitant immunosuppression.[7,43,46] The above author suggests that as immunosuppressive therapies reproducibly potentiate and disseminate TB infection[5] leading to military TB,[47] other members of the Mycobacterium family such as MAP should also disseminate.

To address these issues 2 fundamental points need to be considered:

- *M. tuberculosis* is a unique pathogen, even among mycobacteria, in that it is adapted to replicate both in the vacuole and cytosol allowing the pathogen to escape from the phagosome to produce disseminated disease.[48] To understand the nondissemination of MAP, one must look to the "traditional mycobacterial infection"—leprosy, caused by *Mycobacterium leprae*, a pathogen that does not disseminate despite known treatment with azathioprine, steroids and TNF-α inhibitors.[49]
- The intracellular obligatory spheroplast form of MAP is incapable of replicating in the extracellular environment[50] and is therefore incapable of disseminated disease.

In addition, commonly used anti-inflammatory and immunosuppressive agents (eg, 5-aminosalicylic acid, 6-mercaptopurine, methotrexate, cyclosporine, and tacrolimus) inhibit MAP activity.[51,52] Mendoza et al, [7] despite detecting MAP DNA in the blood of 100% (30/30) of CD patients, did not report any incidence of MAP dissemination in those 14 of 30 patients (46.6%) who had concomitant infliximab and immunosuppressant use. Anti-TNF-α antibodies have additionally been shown to induce apoptosis of TNF receptor-positive lymphocytes and macrophages.[53,54] Naser et al[46] in his 2004 *Lancet* publication, reported the isolation of viable MAP from circulating white cells in 50% of Crohn's patients, pointing to a credible mechanism of anti-TNF-α destruction of MAP-infected cells leading to the release of intracellular dwelling MAP into the extracellular environment where they are destroyed. These findings were confirmed more recently by Bach et al[55] who demonstrated that anti-TNF-α antibodies destroy MAP explaining its usefulness in CD as an antibiotic.

## Issue 3: "Mycobacterial Antigen-specific T-Cell Responses have not Been Documented"[5]

This argument was made several years ago and has since been disproven by work performed in several laboratories. Olsen et al[56] isolated MAP-reactive CD4 T cells from the mucosa of CD patients convincingly disproving this argument. Several other groups have since demonstrated MAP-specific T-cell responses in CD patients.[57–60] A positive association between MAP T-cell response and CD was obtained when MAP-activated peripheral blood lymphocytes from CD patients showed an increased suppression of a mitogen-stimulated lymphoproliferative response in an antigen-specific manner.[61] Gut mucosal inflammation involves either Th1, Th2, or Th17 cytokine patterns with an excessive Th1/Th17 response believed to drive CD disease activity. However, a recent study reported a diminished MAP-specific Th1 response as determined by an interferon-γ response in MAP-positive CD patients when compared with MAP-positive control subjects.[58] Using a whole blood culture system to determine the relationship between T-cell responses and MAP in CD patients revealed interleukin (IL)-2 and IL-4 levels in plasma supernatants that were significantly higher in MAP-positive patients compared with MAP-negative patients, normal controls, and UC patients.[59] The findings of a selective Th2 cytokine response to MAP was supported by in-vitro MAP stimulation of peripheral blood mononuclear cells (PBMNCs) from CD patients that produced higher levels of Th2 cytokines than those produced in cultures stimulated with *Salmonella typhimurium* (*S. typhimurium*).[60] In addition, Th1/Th2 cytokine ratios were lower for CD patients than those observed for healthy control subjects when PBMNC were stimulated with MAP but not *S. typhimurium*. The skewed MAP-specific Th2 response in CD patients is consistent with that observed in cultures of chronically MAP-infected goat and cattle PBMNCs stimulated with MAP antigen in vitro under the influence of regulatory T cells[61,62] and favors intracellular MAP survival in macrophages.

## Issue 4: No Evidence of Transmission of Disease by Epidemiological Studies[2,5]

The rising IBD incidence worldwide cannot be accounted for by a genetic immune system dysregulation, incapable of altering over a few decades.[33] Some contend that if MAP were the cause, a higher CD incidence in veterinarians and farmers exposed to MAP-infected animals would occur. Yet US data suggests that these occupations protect against IBD.[63] In addition, children exposed to farm animals, particularly cattle, in early life have a lower CD incidence.[64] Raised antibodies to MAP lysates have been detected in adults with occupational exposure to MAP.[65] Hermon-Taylor[66] has contended that exposure to the extracellular Ziehl Neelsen-positive MAP phenotype excreted by shedding animals is not the human-susceptible form. MAP passaging through bovine macrophages in milk and cheese, and various protists in wet environmental locations have been hypothesized as completing the transformation.[67,68] In contrast, continual exposure to animal environmental MAP likely results in partial immunity against the human-susceptible MAP,[65] akin to the subclinical infection seen in hepatitis B where long-term exposure results in the development of protective immunity.[69] Chronic low-dose *H. pylori* exposure induces a similar immunity, with Radcliff and Ferrero[70] reporting significantly lower *H. pylori* bacterial loads in mice preexposed to a subinfectious dose.

However, a progressive increase in CD prevalence has been reported in MAP-endemic areas. In Iceland, JD incidence increased from 0% to 30% over an 18-year period after introduction of MAP-infected sheep into the local sheep population in 1938.[71] A subsequent study reported increased IBD incidence in Iceland occurring over a combined period of 40 years (1950 to 1989).[72] In another study the mean CD incidence was seen to rise from 0.5 to 5.5/100,000 between 1950 and 1994, a 10-fold increase.[73] With MAP the slowest known growing bacterial species it would seem that epidemiological studies spanning 40 to 50 years are required to show MAP trends. In a Sardinian island community where JD and MAP infection are widespread in cattle and sheep, 83.3% with CD and 10.3% control subjects tested positive for MAP DNA, consistent with widespread human exposure from contaminated dairy supplies and the environment.[74] Of these, MAP was successfully cultured from 63.3% CD subjects and 10% of control subjects. Another study in an Indian rural community in Agra District, north India, reported that 5 of 5 (100%) CD subjects; 6 of 8 (75%) goat herders working with JD-affected goats versus 27 of 71 (38%) normal controls tested positive for MAP by polymerase chain reaction, culture, and enzyme-linked immunosorbent assay.[75] The MAP isolates from patients had a "bison" genotype indigenous to the region and was shared between wildlife antelopes and livestock (goats and sheep) through interspecies transmission.[76]

Multiple reports of CD clustering among unrelated individuals, family members, and close friends have been reported in the literature.[77–84] One study of graduates from

the Mankato West High School was conducted after reports of CD clustering in students.[82] Seven CD cases of 285 students were documented and 2 additional students reported symptoms of CD. Bearing in mind CD incidence in the general population is approximately 7 per 100,000 cases, this is a remarkable prevalence of 2456 per 100,000 cases, a 350-fold increase. One of the richest agricultural regions in the nation, producing abundant cattle, swine, corn, and soybeans, the river flows through Mankato delivering surface water from a wide fan-shaped watershed to the south. Fecal coliform counts of the river are in excess of 200/dL year after year, regarded as unsafe for recreational use. All the 7 CD students reported prior recreational swimming in the lakes. In a more recent study, Pierce et al[83] reported an IBD outbreak in 15 unrelated children and teenagers living in close proximity to dairy farms in Forest, Virginia. Two of the children lived immediately adjacent to dairy farms and the others were close to Ivy Lake, Otter River, Elk Creek, and Ivy Creek and their tributaries, all of which receive water runoff from 4 of the 6 farms currently located in Forest. Five of the 7 CD samples tested strongly positive for MAP antibodies.

Similarly the statement that "no evidence of increased frequency of Crohn's Disease in parents and offspring"[1] is false. Van Kruiningen et al[84] reported a French family whose 6 members developed CD, including a daughter-in-law. The son first developed CD in 1974 before meeting his wife, whom he married in 1983. After the marriage she then went on to develop CD in 1991. Another study reported CD clustering among 3 unrelated, unmarried men who shared a sustained friendship and who, all within a decade of their initial contact, developed IBD.[85]

## Issue 5: "Antibiotic Studies do not Show any Long-Lasting Benefit"[5]

To understand the difficulty in treating MAP we must look to anti-MAP therapy in the treatment of JD in cattle of considerable genetic worth. St Jean, conducting the most comprehensive review of anti-MAP in JD, concluded that "therapy for clinical paratuberculosis…requires daily medication for long periods and does not provide a definitive cure, only achieving remission and palliation of the disease."[31]

As in *Mycobacterium avium* Complex infection anti-MAP therapy should be employed for 1 year or longer and comprise a multidrug regimen with intracellularly active antibiotics.[86] Numerous published drug studies examining the use of multidrug regimens containing macrolides in CD have shown long-term benefits.[87,28,41,42] The largest of these was a randomized controlled trial of triple antibiotic therapy (clarithromycin, rifabutin, clofazimine).[28] Despite the major study design faults, deficient drug dosing, drug delivery failure, and incorrect results analysis[87–90] the trial demonstrated a 66% remission rate in CD at 16 weeks versus 50% in placebo with no further benefit beyond this time point. However, using the statistically appropriate intention-to-treat reanalysis, it was found that the benefit at 16 weeks persisted at 52 and 104 weeks, with significant differences between anti-MAP therapy and placebo at these time points,[91] demonstrating long-lasting benefit. The results observed in this controlled trial are consistent with those reported in earlier open-label studies. Gui et al[87] reported 52 patients treated with a therapy containing macrolides for periods of 6 to 35 months (mean 18.7 mo). A reduction in inflammation scores were observed in 49 of 52 (89%) patients with only 2 of 19 steroid-dependent patients remaining on steroids. A sustained clinical improvement in 21 of 29 patients (72%) who tolerated triple antibiotic therapy was achieved in a follow-up study for 4 to 17 months.[92] Another study using triple antibiotic therapy containing macrolides over a period of 52 to 54 months in 12 CD patients achieved complete clinical, endoscopic, and histologic remission in 6 of 12 patients (50%) for up to 10 years.[93] In an open-label retrospective study, 52 patients with active CD were followed up for 6 months to 9 years.[94] The triple antibiotic therapy achieved clinical, colonoscopic, and histologic remission in 32 of these patients (61.5%). In addition, 22 of 39 patients (56.4%) who achieved complete clinical remission exhibited mucosal healing.[41] Given the poor response of MAP treatment in JD, the results of these trials are surprisingly good and certainly remain a far more effective therapy with fewer side effects than published results for the widely marketed infliximab in CD.[95–98]

## Issue 6: "Abundant Clinical Evidence Implicates the Commensal Enteric Flora in the Pathogenesis of CD"[5]

The "abundant clinical evidence" implicating the commensal enteric flora in CD pathogenesis comes from the results of several uncontrolled studies of diminutive sample size.[99–101] That being said, the enteric flora clearly plays a role as a potential reservoir of chronic infection. Normal uninfected flora causes no mucosal inflammation and can be used therapeutically to cure *Clostridium difficile* (*C. difficile*) colitis[102–109] and idiopathic colitis.[110–112] In contrast, flora infected with S*almonella*, *Shigella*, *Campylobacter*, *C. difficile*, and other pathogens induces enteric/mucosal inflammation until the pathogen is eradicated. There is currently no abundant clinical evidence which implicates the *commensal* flora in the pathogenesis of CD. Indeed the evidence implicates *infected commensal flora*. This is quite clear given the following.

1. Rutgeerts et al[100] himself indicated that a "factor" in the fecal stream was the culprit.
2. He demonstrated that when the fecal was restored, the "factor" reinitiated the CD.[100] However a "commensal" flora control group, that is an infusion of commensal flora from an unrelated individual without CD into CD patients, was not used to show that commensal flora can restart CD. However, this is known not to be the case,[110] as healthy commensal flora successfully reverses UC inflammation and 1 case of CD, likely by recolonization as in *C. difficile* colitis.[110]
3. Given the lack of a control group in D'haens and colleagues study, histologic inflammation due to repeated Foley catheter trauma may well be the published finding.[99,113]

## CONCLUSIONS

The "aberrant mucosal reaction" versus "MAP" debate is likely to continue among opinion leaders. Several published "issues" have divided the medical community and this review counters these widely held and often inaccurate beliefs. The causality debate is by no means trivial and carries important consequences for patients and physicians seeking evidence-based rationale to treat often desperate, nonresponsive CD patients. The peril is that we become so enamored with discovering the role of the multitude of microbes and cytokines and their influence on immune pathways that we disregard the basic infectious disease tenets and attribute CD to commensal bacteria

while some patients continue to harbor a proven and untreated pathogen.

It is time for us who previously could not "see," to become that Da Vinci group that has been shown the evidence and now can "see."

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Review



# *Mycobacterium avium* subspecies *paratuberculosis*, Crohn's disease and the Doomsday Scenario

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## Abstract

Johne's disease is chronic inflammation of the intestine caused by *Mycobacterium avium* subspecies *paratuberculosis*. Infection and disease are mainly in domestic livestock but can affect many species including primates. Johne's is a new disease which emerged at the turn of the 19th and 20th centuries and principally involved Europe and North America. It has since spread to former low incidence regions to become a global problem. Crohn's disease is a chronic inflammation of the intestine in humans which emerged in Europe and North America mid 20th century and increased to become a major healthcare problem. It has now spread to former low incidence regions. Infected animals shed *Mycobacterium avium* subspecies *paratuberculosis* in milk and into the environment. Human populations are widely exposed. Outcomes maybe influenced by microbial phenotype. Exposure to extracellular forms of these pathogens may confer some natural protection; exposure to intracellular forms which have passaged through milk macrophages or environmental protists may pose a greater threat to humans particularly individuals with an inherited or acquired susceptibility. Hot spots of human disease such as in Winnipeg which sits on rock at the junction of two rivers may result from local exposure to high levels of waterborne pathogens brought down from farmland. When appropriate methods are used most people with Crohn's disease are found to be infected. There are no data which demonstrate that these pathogens are harmless to humans. An overwhelming balance of probability and Public health risk favours the conclusion that *Mycobacterium avium* subspecies *paratuberculosis* is also pathogenic for people. A two tier co-operative pathogenic mechanism is proposed in Crohn's disease. Intracellular infection with the primary pathogen widely distributed throughout the gut causes an immune dysregulation and a specific chronic enteric neuropathy with loss of mucosal integrity. Segments of gross inflammatory disease result from the perturbed neuroimmune response to penetration into the gut wall of secondary pathogens from the lumen. These include both normal gut organisms and educated members of the enteric microbiome such as more aggressive *E. coli*. More new diseases may arise from failure to apply a range of remedial measures to this longstanding zoonotic problem.

## Review

### *MAP and the emergence of Johne's and Crohn's diseases*

Johne's disease (JD) is a systemic infection and chronic inflammation of the intestine in animals caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). It is most common in ruminants but can affect many other species including primates. It was first seen to emerge in Europe and North America at the end of the 19th and

beginning of the 20th centuries. Crohn's disease (CD) in humans is a systemic disorder whose principal clinicopathological manifestation is also chronic inflammation of the intestine. It is also a new disease which was first seen to emerge in the same continents 40–50 years after JD and increased in frequency steadily until it has become a major healthcare problem. In some areas in the USA in recent years the incidence of CD has seemed to plateau around 7–8 per $10^5$ population per year [1,2]. In Europe the incidence of CD in adults continues to grow [3-5]. Studies in Stockholm, Czech Republic, and Australia supported by data from Finland suggest that the incidence of CD in children in these areas in recent years has been rising in some cases as high as about 5 fold per decade [6-9]. These rapid changes in incidence rule out a primary genetic causation of CD. The data from recent genome wide scans which has identified 32 significant genomic loci related to susceptibility to CD are consistent with the involvement of intracellular bacterial pathogens including mycobacteria, in disease causation [10].

The rising incidence of CD reported from several former low incidence countries in Asia shows that, as with JD, CD is spreading worldwide [11,12]. Recent work from New Zealand reported a high incidence of CD of 16.5 per $10^5$ per year affecting the Canterbury region of South Island with Christchurch as its principal city [13]. Mountains are to the northwest and rivers from them run across rich agricultural pastures and either side of Christchurch before entering the sea. A small river meanders through the city itself. Some of these features are reminiscent of the situation in Cardiff, South Wales UK where a high incidence of CD in city wards bordering the river Taff draining the upland pastures of the Brecons and running through the city was consistent with exposure of the local population to aerosols from the river [14].

A conspicuously anomalous distribution in the incidence of CD exists in North America either side of the Canadian border between Minnesota and Manitoba. In Minnesota to the south the well documented population-based incidence of CD in Olmsted County is 7.9 per $10^5$/year [1] whereas in some areas of the city of Winnipeg little more than 400 miles to the north the incidence reaches a maximum 3.5 fold greater at 28.07 per $10^5$/year [15]. Winnipeg lies astride the junction of the Red River of the North running up from the south and the Assiniboine River coming in from the west. The city sits on bedrock which was once the floor of the immense prehistoric glacial lake Agassiz, with scant run-off in permeable sand and gravel aquifers [16]. The 'hot spot' of CD in the city of Winnipeg we see now is probably due to local exposure of the human population to high levels of waterborne MAP brought down from the agricultural river catchments of the US Midwest, meeting those from the provinces of Manitoba, Saskatchewan and Alberta. Waterborne MAP under these conditions would almost certainly include organisms which have adopted the intracellular phenotype having been taken up by abundant environmental protists [17].

### *Movement of people and pathogen*

Migrant studies show that the incidence of CD in people moving from a low CD and JD incidence area to a high incidence area subsequently rises to that of the host population. The inverse situation is that in which MAP is introduced into an isolated community usually by importation of infected animals. This happened in Iceland in 1933 [reviewed in [18]]. After a latent period following introduction of the pathogen there were at intervals successive epidemics of JD in the island sheep, then in the cattle, then CD in the human population. From 1960 to a peak in 1992 the incidence of CD increased 18 fold. Thus in either case, if people move in amongst MAP or if MAP is moved in amongst people the result is the same namely a steep rise in the incidence of CD. The time interval between the emergence and rise of JD in animals and CD in humans in Iceland was again about 40–50 years. With the almost unlimited opportunity for MAP to spread and evolve in intensively farmed domestic livestock and associated contaminated environments over more than a half century, an evolving virulence and species adaptation of the pathogen would be reflected in the JD to CD interval becoming shorter. A recent example of this happening maybe the steep 4.5 fold increase in CD in the Czech Republic 1995–2007 following the rise in JD caused by the unimpeded importation of subclinically infected cattle from Western Europe after independence in 1990.

### *Natural Immunity to MAP from environmental and occupational exposure*

Why don't dairy farmers and veterinarians exposed to MAP-infected animals get a much higher incidence of CD? Data from the US show that these occupations are in fact associated with a significantly reduced death rate from Inflammatory Bowel Disease [19]. Children exposed to farm animals, particularly cattle, in early life also subsequently have a lower incidence of CD [20]. Occupational exposure to MAP is associated with raised antibody levels to MAP lysates [21]. An answer to the question consistent with these observations is that the extracellular classical ZN-positive phenotype of MAP excreted in trillions by heavily infected animals is not one to which humans are most susceptible. Exposure to this form of the organism may result in the acquisition of some natural immunity to disease. A good example of this happening as a result of purposeful exposure is the approximate 10 fold reduction in clinical Johne's disease achieved subsequently by vaccination of calves using conventional whole killed MAP vaccines with the organisms in this form [22]. The well

described urban preponderance of CD may not be that townsfolk have an increased susceptibility to CD, rather that country folk have some natural protection. Passage of MAP through bovine macrophages in milk and cheese or through environmental protists would result in a switch to an intracellular phenotype of MAP likely to have an enhanced virulence for humans [23,24].

### *MAP and the convergence of candidate pathogens*

With the new 21st century, a steadily increasing volume of parallel research has identified three principal sets of bacteria as candidates for the causation of the gross inflammatory disease of the intestine in CD. These are the community of normal gut flora [25], abnormal gut flora such as adherent invasive *E. coli* (AIEC) [26], and MAP. Because of the global advance of CD and the serious implications for Public Health as well as cumulative individual suffering, there is a need for researchers and clinicians in the field to recognise that the reliable evidence obtained from each of the three lines of inquiry is convergent and that there is actually no conflict between them.

From experimental as well as clinical evidence there is no doubt that bacteria from the normal intestinal microbial community can infect and inflame the gut wall and that they do so in CD. However, the spontaneous emergence and rise of CD in human populations across the globe due to an epidemic of normal gut flora, in the absence of another specific initiating cause, seems rather improbable. The enteric microbiome is a fertile environment for horizontal gene transfer [27]. Advancement of pathogenicity in bacteria may follow the acquisition and mutation of genes and changes in their regulation [28-30]. We already have examples of the pathological consequences of such adaptation in common gut bacteria such as *E. coli* which can be enteropathogenic, enterohaemorrhagic, enterotoxigenic, enteroaggregative and recently enteroadherent and invasive AIEC [31]. Such adaptations usually arise due to the imposition of some external selection pressure. Recent evidence also suggests that common enteric bacteria like *E. coli* may display predictive behaviour [32].

The principal property of MAP which distinguishes it from all other candidate pathogens in the primary causation of CD is that it is an established multi-host chronic enteric pathogen. MAP has the proven specific ability to initiate and maintain chronic inflammation of the intestine of a range of different histopathological types in many species including primates. MAP infection in animals causes a local and systemic immune dysregulation. It is also specifically neuropathogenic especially for non-myelinated neurones and intestinal disease is accompanied by a chronic enteric neuropathy [33]. Despite its broad pathogenicity, MAP infection can persist in animals for years without necessarily progressing to clinical disease. Clinical disease in animals when it occurs is commonly of the pluribacillary type but paucimicrobial disease with the pathogens in a Ziehl Neelsen (ZN)-negative phenotype is well described.

The overall prevalence of MAP infection in US dairy herds is reported by a USDA survey to be 68.1% [34]. A range of broadly similar data shows that MAP infection in farm animals is widespread in many areas of Western Europe and elsewhere. MAP contaminates and persists in water and the environment, is in dairy products, can survive milk pasteurisation, and is present in meat from infected animals. It is inevitable that human populations are widely exposed.

### *MAP in humans*

MAP infection in humans is difficult to detect. The organisms are present in low abundance in a robust ZN-negative phenotype. They are intracellular and minimise their own immune recognition. They are extremely difficult to isolate and propagate in culture and are relatively resistant to chemical and enzymatic lysis. Reliable access to their DNA is only achieved during sample processing by combining exposure to stringent lysis buffers with an additional optimised mechanical disruption step. Freezing samples and tissue extracts especially at -20°C substantially reduces the PCR detection rate of their GC-rich DNA. The organisms have been cultured and detected in blood showing that, as in animals, the infection in humans is systemic [35-37]. At present, the benchmark diagnostic test for MAP infection in humans is nested PCR applied to single ~20 mg fresh endoscopic mucosal biopsies [38]. When validated methodologies have been used most people with CD have been found to be infected with MAP [39]. In simple words, most people with chronic inflammation of the intestine (of the CD type) are infected with a mycobacterium which is a proven specific cause of chronic inflammation of the intestine. There are no data which demonstrate that MAP are harmless to humans. The overwhelming balance of probability and public health risk favours the conclusion that MAP are also pathogenic for people.

### *Inflammation in Crohn's disease caused by a two tier co-operative pathogenic mechanism*

MAP infects the gut widely in CD and is found both in the more normal looking intestine and the grossly inflamed and diseased segments of intestine [33]. MAP antigens have appeared to dominate the immunological responses of intestinal CD4 T cell lines from patients with CD [40]. Mannans released by MAP inhibit intracellular killing of internalised bacteria [41].

The MAP infection causes a *primary* microscopic inflammation accompanied by a specific immune dysregulation and enteric neuropathy [33]. Mucosal integrity and other critical functions of the intestine are impaired. The visible segments of gross inflammatory disease result from the perturbed neuroimmune response to the *secondary* penetration into the gut wall of gut flora containing both normal intestinal bacteria and those which have undergone transformations leading to a more invasive phenotype like AIEC. It is important to note that genomic loci in the host conferring genetic susceptibility to Crohn's disease have the potential to operate at the levels of both primary and secondary pathogens. The entry of food residues into the gut wall contributes an allergic component to the inflammatory mess. Although MAP has been found in intestinal granulomas in humans [42], the presence or absence of these and other features of the variable histopathological picture of CD are principally determined by the large scale response to the secondary co-pathogens including especially other granulomatous species like *M. avium* subspecies *avium* which are frequently recovered in culture from CD tissues [38]. Thus the three lines of contemporary research inquiry come together in a two tiered co-operative pathogenic mechanism.

### *MAP doomsday*

Imagine the collective human enteric microbiome in, say, a crowded Europe. A vast composite structure made up of millions of individual highly mobile microbial reservoirs variably interconnected in time and space and degree. A dynamic structure possessing an inherent self governing order and stability not easily displaced. Into this cellular system is progressively introduced a slowly growing specific mycobacterial pathogen which has acquired the genetic machinery necessary to cloak itself with a predicted fucosylated surface [43] so that it conforms with the familiar molecular environment particularly of the host's epithelial cells and mucosal compartment [44]. It has come from the parallel universe of the collective enteric microbiome of human food animals and before that from the soil. It causes a microscopic inflammation and perturbs the microenvironment of the mucosa and gut wall. To survive and prosper it minimises its confrontation with the human immune system. It causes a variable immune dysregulation but it also inflames the fine structure and function of the enteric nervous system.

More than a hundred years go by. Both animal and human total microbiomes swell with increasing population density. The mycobacterial pathogen acquires additional properties resulting in an evolution in its behaviour with an increase in pathogenicity and species range. Some normal inhabitants of the enteric microbiome adapt to the disturbed intestinal microenvironment and they too acquire characteristics which make them more invasive. Chronic enteric disease emerges and spreads particularly in individuals with an inherited or acquired susceptibility. Humans responsible for controlling and managing these diseases, blind to what is really happening are distracted by detail and dismissive. The required remedial measures are not designed and applied and the problems get worse. Left undisturbed, maybe the education in hostility already received by increasingly aggressive members of the former normal gut flora will progress to the point where they too can emerge from background to become primary independent pathogens in their own right. When they do so more new diseases will emerge.

### *Can anti-MAP treatment heal Crohn's disease?*

The answer to this question supported by a correct interpretation of data both from open label studies [45-48] and the Australian controlled clinical trial is a qualified yes [49-51]. It can in some people with CD some of the time. When it does so in 'responders' receiving treatment with drug combinations including rifabutin and clarithromycin the clinical and pathological improvement can be dramatic and has been associated with the conversion of pre-treatment MAP positive tests in blood [52] and gut mucosa (my own unpublished observations) to negative. Furthermore, some of the clinical benefit resulting from treatment of CD with conventional 'immunosuppressive' agents such as 6-mercaptopurine or methotrexate may actually be a consequence of their demonstrable direct anti-MAP action [53-55]. But MAP infections are difficult to eradicate. The organisms are generally resistant in vivo to drugs conventionally used in the treatment of tuberculosis. Treatment is prone to all the problems of microbial drug resistance and latency encountered in the management of chronic lung disease caused by other members of the *M. Avium* Complex.

New clinical trials are needed of anti-MAP treatment in CD particularly of agents developed for the treatment of *M. tuberculosis* which are active against mycobacteria in the non-replicative state [56] and where the gene encoding the molecular target is shared by MAP. Rich clinical and commercial rewards are out there for those who do so successfully.

## Conclusion

Recognition and acceptance of the true nature of the expanding long term threat to human health posed by widespread exposure to MAP, based upon a perceptive understanding of the problem and the overwhelming balance of reliable scientific evidence, is a matter of urgency. The solutions lie in the identification and incremental introduction of a range of remedial measures which are both scientific and regulatory whose effective application on a global scale requires close international cooperation.

## Abbreviations

MAP: *Mycobacterium avium* subspecies *paratuberculosis*; JD: Johne's disease; CD: Crohn's disease; AIEC: adherent Invasive *E. coli*; ZN: Ziehl Neelsen; US: United States of America; USDA: US Department of Agriculture.

## Competing interests

The author currently owns the patents to a virally vectored vaccine against *Mycobacterium avium* subspecies *paratuberculosis* intended as a treatment for MAP infection in humans.

## Authors' contributions

I confirm that this is my work.

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LETTER

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# Host–microbe interactions have shaped the genetic architecture of inflammatory bowel disease

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**Crohn's disease and ulcerative colitis, the two common forms of inflammatory bowel disease (IBD), affect over 2.5 million people of European ancestry, with rising prevalence in other populations[1]. Genome-wide association studies and subsequent meta-analyses of these two diseases[2,3] as separate phenotypes have implicated previously unsuspected mechanisms, such as autophagy[4], in their pathogenesis and showed that some IBD loci are shared with other inflammatory diseases[5]. Here we expand on the knowledge of relevant pathways by undertaking a meta-analysis of Crohn's disease and ulcerative colitis genome-wide association scans, followed by extensive validation of significant findings, with a combined total of more than 75,000 cases and controls. We identify 71 new associations, for a total of 163 IBD loci, that meet genome-wide significance thresholds. Most loci contribute to both phenotypes, and both directional (consistently favouring one allele over the course of human history) and balancing (favouring the retention of both alleles within populations) selection effects are evident. Many IBD loci are also implicated in other immune-mediated disorders, most notably with ankylosing spondylitis and psoriasis. We also observe considerable overlap between susceptibility loci for IBD and mycobacterial infection. Gene co-expression network analysis emphasizes this relationship, with pathways shared between host responses to mycobacteria and those predisposing to IBD.**

We conducted an imputation-based association analysis using autosomal genotype-level data from 15 genome-wide association studies (GWAS) of Crohn's disease and/or ulcerative colitis (Supplementary Fig. 1 and Supplementary Table 1). We imputed 1.23 million single-nucleotide polymorphisms (SNPs) from the HapMap3 reference set (Supplementary Methods 1a), resulting in a high-quality data set with reduced genome-wide inflation (Supplementary Figs 2 and 3) compared with previous meta-analyses of subsets of these data[2,3]. The imputed GWAS data identified 25,075 SNPs that were associated ($P < 0.01$) with at least one of the Crohn's disease, ulcerative colitis, or combined IBD analyses. A meta-analysis of GWAS data with Immunochip[6] validation genotypes from an independent, newly genotyped set of 14,763 Crohn's disease cases, 10,920 ulcerative colitis cases and 15,977 controls was performed (Supplementary Fig. 1 and Supplementary Table 1). Principal-components analysis resolved geographic stratification, as well as Jewish and non-Jewish ancestry (Supplementary Fig. 4), and reduced inflation to a level consistent with residual polygenic risk, rather than other confounding effects (from a median test statistic inflation ($\lambda_{GC}$) = 2.00 to $\lambda_{GC}$ = 1.23 when analysing all IBD samples; Supplementary Fig. 5 and Supplementary Methods 1b).

Our meta-analysis of the GWAS and Immunochip data identified 193 statistically independent signals of association at genome-wide significance ($P < 5 \times 10^{-8}$) in at least one of the three analyses (Crohn's disease, ulcerative colitis, IBD). Because some of these signals (Supplementary Fig. 6) probably represent associations to the same underlying functional unit, we merged these signals (Supplementary Methods 1b) into 163 regions, 71 of which are reported here for the first time (Table 1 and Supplementary Table 2). Fig. 1a shows the relative contributions of each locus to the total variance explained in ulcerative colitis and Crohn's disease. We have increased the total disease variance explained (variance being subject to fewer assumptions than heritability[7]) from 8.2% to 13.6% in Crohn's disease and from 4.1% to 7.5% in ulcerative colitis (Supplementary Methods 1c). Consistent with previous studies, our IBD risk loci seem to act independently, with no significant evidence of deviation from an additive combination of log odds ratios.

Our combined genome-wide analysis of Crohn's disease and ulcerative colitis enables a more comprehensive analysis of disease specificity than was previously possible. A model-selection analysis (Supplementary Methods 1c) showed that 110 out of 163 loci are associated with both disease phenotypes; 50 of these have an indistinguishable effect size in ulcerative colitis and Crohn's disease, whereas 60 show evidence of heterogeneous effects (Table 1). Of the remaining loci, 30 are classified as Crohn's-disease-specific and 23 as ulcerative-colitis-specific. However, 43 of these 53 loci show the same direction of effect in the non-associated disease (Fig. 1b; overall $P = 2.8 \times 10^{-6}$). Risk alleles at two Crohn's disease loci, *PTPN22* and *NOD2*, show significant ($P < 0.005$) protective effects in ulcerative colitis, exceptions that may reflect biological differences between the two diseases. This degree of sharing of genetic risk suggests that nearly all of the biological mechanisms involved in one disease have some role in the other.

The large number of IBD associations, far more than reported for any other complex disease, increases the power of network-based analyses to prioritize genes within loci. We investigated the IBD loci using functional annotation and empirical gene network tools (Supplementary Table 2). Compared with previous analyses that identified candidate genes in 35% of loci[2,3] our updated GRAIL[8] -connectivity network identifies candidates in 53% of loci, including increased statistical significance for 58 of the 73 candidates from previous analyses. The new candidates come not only from genes within newly identified loci, but also integrate additional genes from previously established loci (Fig. 1c). Only 29 IBD-associated SNPs are in strong linkage disequilibrium ($r^2 > 0.8$) with a missense variant in the 1000 Genomes Project data, which reinforces previous evidence that a large fraction of risk for complex disease is driven by non-coding variation. By contrast, 64 IBD-associated SNPs are in linkage disequilibrium with variants known to regulate gene expression (Supplementary Table 2). Overall, we highlighted a total of 300 candidate genes in 125 loci, of which 39 contained a single gene supported by two or more methods.

Seventy per cent (113 out of 163) of the IBD loci are shared with other complex diseases or traits, including 66 among the 154 loci previously associated with other immune-mediated diseases[9], which is 8.6-times the number that would be expected by chance ($P < 10^{-16}$; Fig. 2a and Supplementary Fig. 7). Such enrichment cannot be attributed to the immune-mediated focus of the Immunochip (Supplementary Methods 4 and Supplementary Fig. 8), as the analysis is based on our combined GWAS–Immunochip data. Comparing overlaps with specific diseases is confounded by the variable power in studies of different diseases. For instance, although type 1 diabetes shares the largest number of loci (20 out of 39; tenfold enrichment) with IBD, this is partially driven by the large number of known type 1 diabetes associations. Indeed, seven other immune-mediated diseases

**Table 1 | Crohn's disease-specific, ulcerative colitis-specific and IBD general loci**

Crohn's disease

| Chr. | Position (Mb) | SNP | Key genes (+ no. of additional genes in locus) |
|---|---|---|---|
| 1 | 78.62 | rs17391694 | (5) |
| 1 | 114.3 | **rs6679677**‖ | ***PTPN22*** ¶ (8) |
| 1 | 120.45 | rs3897478 | *ADAM30* (5) |
| 1 | 172.85 | **rs9286879** | ***FASLG***,***TNFSF18*** (0) |
| 2 | 27.63 | **rs1728918** | *UCN* (23) |
| 2 | 62.55 | rs10865331 | (3) |
| 2 | 231.09 | **rs6716753** | *SP140* (5) |
| 2 | 234.15 | **rs12994997** | ***ATG16L1*** ¶ (8) |
| 4 | 48.36 | rs6837335 | (6) |
| 4 | 102.86 | rs13126505 | (1) |
| 5 | 55.43 | rs10065637 | ***IL6ST***, ***IL31RA*** (1) |
| 5 | 72.54 | rs7702331 | (4) |
| 5 | 173.34 | rs17695092 | *CPEB4* (2) |
| 6 | 21.42 | rs12663356 | (3) |
| 6 | 31.27 | **rs9264942** | (22) |
| 6 | 127.45 | rs9491697 | (3) |
| 6 | 128.24 | **rs13204742** | (2) |
| 6 | 159.49 | **rs212388** | *TAGAP* (5) |
| 7 | 26.88‡ | rs10486483 | (2) |
| 7 | 28.17 | rs864745 | *CREB5*, *JAZF1* (1) |
| 8 | 90.87 | rs7015630 | *RIPK2* (4) |
| 8 | 129.56 | **rs6651252** | 0 |
| 13 | 44.45 | **rs3764147** | *LACC1* (3) |
| 15 | 38.89 | rs16967103 | ***RASGRP1***, ***SPRED1*** (2) |
| 16 | 50.66† | **rs2066847**‖ | ***NOD2*** ¶ (6) |
| 17 | 25.84 | **rs2945412** | ***LGALS9***, ***NOS2*** (3) |
| 19 | 1.12 | **rs2024092** | *GPX4*, *HMHA1* (20) |
| 19 | 46.85‡ | rs4802307 | (9) |
| 19 | 49.2 | **rs516246** | *FUT2*, (25) |
| 21 | 34.77 | **rs2284553** | ***IFNGR2***, ***IFNAR1*** (10) |

Ulcerative colitis

| Chr. | Position (Mb) | SNP | Key genes (+ no. of additional genes in locus) |
|---|---|---|---|
| 1 | 2.5 | rs10797432 | ***TNFRSF14*** (10) |
| 1 | 20.15† | **rs6426833** | (9) |
| 1 | 200.09 | **rs2816958** | (3) |
| 2 | 198.65 | rs1016883 | *RFTN2*, *PLCL1* (7) |
| 2 | 199.70* | **rs17229285** | 0 |
| 3 | 53.05 | rs9847710 | *PRKCD*, *ITIH4* (8) |
| 4 | 103.51 | rs3774959 | ***NFKB1***, ***MANBA*** (2) |
| 5 | 0.59 | rs11739663 | *SLC9A3* (8) |
| 5 | 134.44 | rs254560 | (6) |
| 6 | 32.595 | **rs6927022** | (15) |
| 7 | 2.78 | **rs798502** | ***CARD11***, ***GNA12*** (5) |
| 7 | 27.22‡ | rs4722672 | (14) |
| 7 | 107.45* | **rs4380874** | *DLD* (9) |
| 7 | 128.57 | **rs4728142** | ***IRF5*** ¶ (13) |
| 11 | 96.02 | rs483905 | *JRKL*, *MAML2* (2) |
| 11 | 114.38 | **rs561722** | *NXPE1*, *NXPE4* (5) |
| 15 | 41.55 | rs28374715 | (11) |
| 16 | 30.47 | rs11150589 | ***ITGAL*** (20) |
| 16 | 68.58 | rs1728785 | *ZFP90* (6) |
| 17 | 70.64 | rs7210086 | (3) |
| 19 | 47.12‡ | rs1126510 | *CALM3* (14) |
| 20 | 33.8 | rs6088765 | (11) |
| 20 | 43.06 | **rs6017342** | *ADA*, *HNF4A* (9) |

IBD

| Chr. | Position (Mb) | SNP | Key genes (+ no. of additional genes in locus) |
|---|---|---|---|
| 1 | 1.24 | **rs12103** | *TNFRSF18*, *TNFRSF4* (30) |
| 1 | 8.02 | **rs35675666** | *TNFRSF9* (6) |
| 1 | 22.7 | **rs12568930**§ | (3) |
| 1 | 67.68† | **rs11209026**§ | ***IL23R*** ¶ (5) |
| 1 | 70.99 | rs2651244§ | (3) |
| 1 | 151.79 | **rs4845604**§ | *RORC* (14) |
| 1 | 155.67 | rs670523§ | (31) |
| 1 | 160.85 | rs4656958§ | ***CD48*** (15) |
| 1 | 161.47 | **rs1801274**§ | ***FCGR2A***, ***FCGR2B*** & ***FCGR3A*** (13) |
| 1 | 197.6 | **rs2488389** | C1orf53 (2) |
| 1 | 200.87 | **rs7554511** | *KIF21B* (6) |
| 1 | 206.93 | **rs3024505**§ | *IL10* (10) |
| 2 | 25.12 | **rs6545800**§ | *ADCY3* (6) |
| 2 | 28.61 | **rs925255**§ | *FOSL2*, *BRE* (1) |
| 2 | 43.81 | rs10495903§ | (5) |
| 2 | 61.2 | **rs7608910** | ***REL*** (9) |
| 2 | 65.67 | rs6740462 | *SPRED2* (1) |
| 2 | 102.86* | **rs917997**§ | *IL18RAP*, *IL1R1* (7) |
| 2 | 163.1 | rs2111485 | *IFIH1* (5) |
| 2 | 191.92 | rs1517352 | ***STAT1***, ***STAT4*** (2) |
| 2 | 219.14 | rs2382817 | (15) |
| 2 | 241.57* | **rs3749171**§ | ***GPR35*** (12) |
| 3 | 18.76 | **rs4256159**§ | 0 |
| 3 | 48.96† | **rs3197999** | ***MST1***, ***PFKB4*** (63) |
| 4 | 74.85 | rs2472649§ | (11) |
| 4 | 123.22 | **rs7657746** | ***IL2***, ***IL21*** (2) |
| 5 | 10.69 | rs2930047 | ***DAP*** (2) |
| 5 | 40.38† | **rs11742570**§ | *PTGER4* (1) |
| 5 | 96.24 | **rs1363907** | *ERAP2*, *ERAP1* (3) |
| 5 | 130.01 | rs4836519§ | (1) |
| 5 | 131.19* | **rs2188962**§ | *IBD5* locus (18) |
| 5 | 141.51 | **rs6863411**§ | *SPRY4*, *NDFIP1* (5) |
| 5 | 150.27 | **rs11741861**§ | ***IRGM*** ¶ (10) |
| 5 | 158.8† | **rs6871626**§ | ***IL12B*** (3) |
| 5 | 176.79 | rs12654812 | ***DOK3*** (17) |
| 6 | 14.71 | rs17119 | 0 |
| 6 | 20.77* | **rs9358372**§ | (2) |
| 6 | 90.96 | rs1847472 | (1) |
| 6 | 106.43 | **rs6568421**§ | (2) |
| 6 | 111.82 | **rs3851228** | ***TRAF3IP2*** (4) |
| 6 | 138 | **rs6920220**§ | *TNFAIP3* (1) |

IBD

| Chr. | Position (Mb) | SNP | Key genes (+ no. of additional genes in locus) |
|---|---|---|---|
| 10 | 35.3 | **rs11010067**§ | ***CREM*** (3) |
| 10 | 59.99 | rs2790216 | *CISD1*, *IPMK* (2) |
| 10 | 64.51† | **rs10761659**§ | (3) |
| 10 | 75.67 | rs2227564§ | (13) |
| 10 | 81.03 | **rs1250546**§ | (5) |
| 10 | 82.25 | **rs6586030**§ | *TSPAN14*, C10orf58 (4) |
| 10 | 94.43 | rs7911264 | (4) |
| 10 | 101.28 | **rs4409764** | *NKX2-3* (6) |
| 11 | 1.87 | rs907611 | ***TNNI2***, ***LSP1*** (17) |
| 11 | 58.33 | rs10896794 | *CNTF*, *LPXN* (8) |
| 11 | 60.77 | **rs11230563** | ***CD6*** (14) |
| 11 | 61.56 | **rs4246215**§ | (15) |
| 11 | 64.12 | rs559928 | ***CCDC88B*** (23) |
| 11 | 65.65 | rs2231884§ | *RELA* (25) |
| 11 | 76.29 | **rs2155219**§ | (5) |
| 11 | 87.12 | rs6592362 | (1) |
| 11 | 118.74 | rs630923§ | *CXCR5* (17) |
| 12 | 12.65 | rs116125085§ | *LOH12CR1* (8) |
| 12 | 40.77* | **rs11564258**§ | *MUC19* (1) |
| 12 | 48.2 | rs11168249§ | *VDR* (8) |
| 12 | 68.49 | **rs7134599**§ | ***IFNG*** (3) |
| 13 | 27.52 | **rs17085007**§ | (2) |
| 13 | 40.86† | **rs941823**§ | (3) |
| 13 | 99.95 | **rs9557195** | *GPR183*, *GPR18* (6) |
| 14 | 69.27 | rs1947495§ | *ZFP36L1* (4) |
| 14 | 75.7 | rs4899554§ | ***FOS***, ***MLH3*** (6) |
| 14 | 88.47 | **rs8005161** | ***GPR65***, ***GALC*** (1) |
| 15 | 67.43 | **rs17293632**§ | *SMAD3* (2) |
| 15 | 91.17 | rs7495132 | *CRTC3* (3) |
| 16 | 11.54* | **rs529866**§ | ***SOCS1***, ***LITAF*** (11) |
| 16 | 23.86 | rs7404095 | ***PRKCB*** (5) |
| 16 | 28.6 | **rs26528**§ | *IL27* (14) |
| 16 | 86 | rs10521318§ | *IRF8* (4) |
| 17 | 32.59 | **rs3091316**§ | ***CCL13***, ***CCL2*** (5) |
| 17 | 37.91 | **rs12946510** | *ORMDL3* (16) |
| 17 | 40.53 | **rs12942547**§ | ***STAT3*** (15) |
| 17 | 57.96 | **rs1292053**§ | *TUBD1*, *RPS6KB1* (9) |
| 18 | 12.8 | **rs1893217**§ | (6) |
| 18 | 46.39 | rs72400048§ | *SMAD7* (2) |
| 18 | 67.53 | rs727088 | ***CD226*** (2) |
| 19 | 10.49* | **rs11879191** | ***TYK2*** (27) |

**Table 1 | Continued**

| IBD | | | | IBD | | | |
|---|---|---|---|---|---|---|---|
| Chr. | Position (Mb) | SNP | Key genes (+ no. of additional genes in locus) | Chr. | Position (Mb) | SNP | Key genes (+ no. of additional genes in locus) |
| 6 | 143.9 | rs12199775 | *PHACTR2* (5) | 19 | 33.73 | **rs17694108** | *CEBPG* (8) |
| 6 | 167.37 | **rs1819333**§ | ***CCR6***, ***RPS6KA2*** (4) | 19 | 55.38 | rs11672983 | (19) |
| 7 | 50.245* | **rs1456896** | *ZPBP*, *IKZF1* (4) | 20 | 30.75 | rs6142618§ | *HCK* (10) |
| 7 | 98.75 | rs9297145 | *SMURF1* (6) | 20 | 31.37 | rs4911259 | *DNMT3B* (8) |
| 7 | 100.34 | **rs1734907**§ | ***EPO*** (21) | 20 | 44.74 | **rs1569723**§ | ***CD40*** (13) |
| 7 | 116.89 | rs38904§ | (6) | 20 | 48.95 | rs913678 | *CEBPB* (5) |
| 8 | 126.53 | **rs921720**§ | *TRIB1* (1) | 20 | 57.82 | rs259964 | *ZNF831*, *CTSZ* (5) |
| 8 | 130.62 | rs1991866 | (2) | 20 | 62.34 | **rs6062504** | *TNFRSF6B* (26) |
| 9 | 4.98 | **rs10758669** | ***JAK2*** (4) | 21 | 16.81 | **rs2823286**§ | 0 |
| 9 | 93.92 | rs4743820§ | *NFIL3* (2) | 21 | 40.46 | **rs2836878**§ | (3) |
| 9 | 117.60† | **rs4246905** | *TNFSF15* (4) | 21 | 45.62 | **rs7282490** | *ICOSLG* (9) |
| 9 | 139.32* | **rs10781499**§ | ***CARD9*** (22) | 22 | 21.92 | **rs2266959** | (13) |
| 10 | 6.08 | rs12722515§ | ***IL2RA***, ***IL15RA*** (6) | 22 | 30.43 | **rs2412970** | ***LIF***, ***OSM*** (9) |
| 10 | 30.72 | rs10420588§ | ***MAP3K8*** (3) | 22 | 39.69* | **rs2413583**§ | *TAB1* (18) |

The position given is the middle of the locus window, with all positions relative to human reference genome GRCh37. Bolded rs numbers indicate SNPs with *P* values less than $1 \times 10^{-13}$. Grey shading indicates newly discovered loci. Listed are genes implicated by one or more candidate gene approaches. Bolded genes have been implicated by two or more candidate gene approaches. For each locus, the top two candidate genes are listed. A complete listing of gene prioritization is provided in Supplementary Table 2. *Additional genome-wide significant associated SNP in the region. †Two or more additional genome-wide significant SNPs in the region. ‡These regions have overlapping but distinct ulcerative colitis and Crohn's disease signals. §Heterogeneity of odds ratios. ||Crohn's disease risk allele is significantly protective in ulcerative colitis. ¶Gene for which functional studies of associated alleles have been reported. Chr., chromosome; Mb, megabase.

show stronger enrichment of overlap, with the largest being ankylosing spondylitis (8 out of 11; 13-fold) and psoriasis (14 out of 17; 14-fold).

IBD loci are also markedly enriched (4.9-fold; $P < 10^{-4}$) in genes involved in primary immunodeficiencies (PIDs; Fig. 2a), which are characterized by a dysfunctional immune system resulting in severe infections[10]. Genes implicated in this overlap correlate with reduced levels of circulating T cells (*ADA*, *CD40*, *TAP1*, *TAP2*, *NBN*, *BLM*, *DNMT3B*) or of specific subsets, such as T-helper cells producing IL-17 ($T_H17$ cells) (*STAT3*), memory (*SP110*) or regulatory T cells (*STAT5B*). The subset of PID genes leading to Mendelian susceptibility to mycobacterial disease (MSMD)[10–12] is enriched still further; six of the eight known autosomal genes linked to MSMD are located within IBD loci (*IL12B*, *IFNGR2*, *STAT1*, *IRF8*, *TYK2*, *STAT3*; 46-fold enrichment; $P = 1.3 \times 10^{-6}$), and a seventh, *IFNGR1*, narrowly missed genome-wide significance ($P = 6 \times 10^{-8}$). Overlap with IBD is also seen in complex mycobacterial disease; we find IBD associations in seven out of eight loci identified by leprosy GWAS[13], including six cases in which the same SNP is implicated. Furthermore, genetic defects in *STAT3* (refs 14, 15) and *CARD9* (ref. 16), also within IBD loci, lead to PIDs involving skin infections with *Staphylococcus* and candidiasis, respectively. The comparative effects of IBD and infectious-disease-susceptibility-risk alleles on gene function and expression are summarized in Supplementary Table 3, and include both opposite (for example, *NOD2* and *STAT3*; Supplementary Fig. 9) and similar (for example, *IFNGR2*) directional effects.

To extend our understanding of the fundamental biology of IBD pathogenesis we conducted searches across the IBD locus list: (1) for enrichment of specific Gene Ontology terms and canonical pathways; (2) for evidence of selective pressure acting on specific variants and pathways; and (3) for enrichment of differentially expressed genes across immune-cell types. We tested the 300 prioritized genes (see above) for enrichment in Gene Ontology terms (Supplementary Methods 4a) and identified 286 Gene Ontology terms and 56 pathways demonstrating significant enrichment in genes contained within IBD loci (Supplementary Figs 10 and 11 and Supplementary Table 4). Excluding high-level Gene Ontology categories such as 'immune system processes' ($P = 3.5 \times 10^{-26}$), the most significantly enriched term is regulation of cytokine production ($P = 2.7 \times 10^{-24}$), specifically

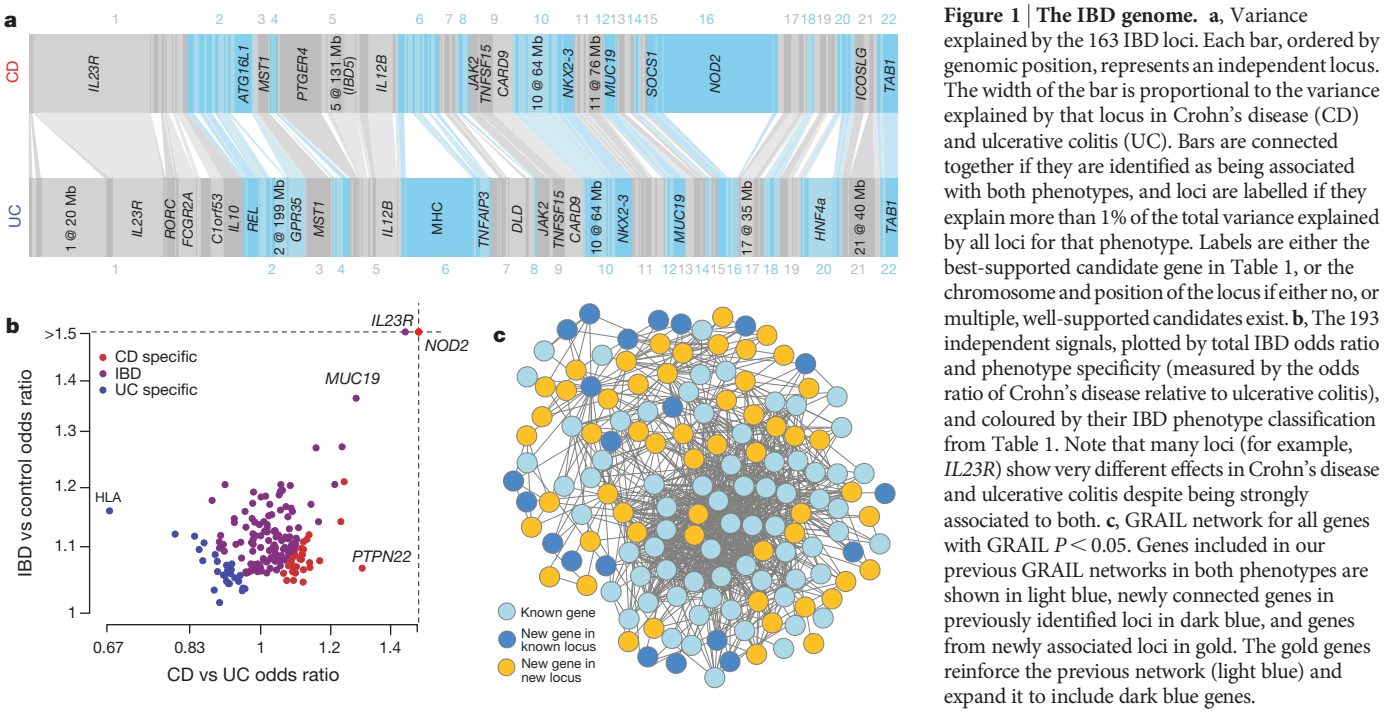


**Figure 1 | The IBD genome. a**, Variance explained by the 163 IBD loci. Each bar, ordered by genomic position, represents an independent locus. The width of the bar is proportional to the variance explained by that locus in Crohn's disease (CD) and ulcerative colitis (UC). Bars are connected together if they are identified as being associated with both phenotypes, and loci are labelled if they explain more than 1% of the total variance explained by all loci for that phenotype. Labels are either the best-supported candidate gene in Table 1, or the chromosome and position of the locus if either no, or multiple, well-supported candidates exist. **b**, The 193 independent signals, plotted by total IBD odds ratio and phenotype specificity (measured by the odds ratio of Crohn's disease relative to ulcerative colitis), and coloured by their IBD phenotype classification from Table 1. Note that many loci (for example, *IL23R*) show very different effects in Crohn's disease and ulcerative colitis despite being strongly associated to both. **c**, GRAIL network for all genes with GRAIL $P < 0.05$. Genes included in our previous GRAIL networks in both phenotypes are shown in light blue, newly connected genes in previously identified loci in dark blue, and genes from newly associated loci in gold. The gold genes reinforce the previous network (light blue) and expand it to include dark blue genes.

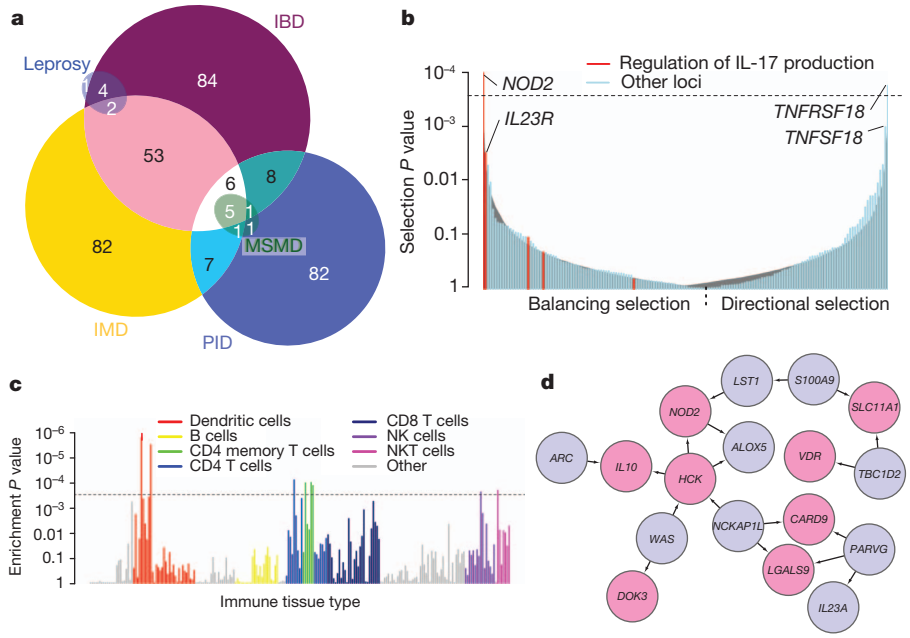


**Figure 2 | Dissecting the biology of IBD.** **a**, Number of overlapping IBD loci with other immune-mediated diseases (IMD), leprosy and Mendelian PIDs. Within PID, we highlight MSMD. **b**, Signals of selection at IBD SNPs, from strongest balancing on the left to strongest directional on the right. The grey curve shows the 95% confidence interval for randomly chosen frequency-matched SNPs, illustrating our overall enrichment ($P = 5.5 \times 10^{-6}$), and the dashed line represents the Bonferroni significance threshold. SNPs highlighted in red are annotated as being involved in the regulation of IL-17 production, a key IBD functional term related to bacterial defence, and are enriched for balancing selection. **c**, Evidence of enrichment in IBD loci of differentially expressed genes from various immune tissues. Each bar represents the empirical *P* value in a single tissue, and the colours represent different cell type groupings. The dashed line is Bonferroni-corrected significance for the number of tissues tested. **d**, *NOD2*-focused cluster of the IBD causal sub-network. Pink genes are in IBD-associated loci, blue are not. Arrows indicate inferred causal direction of regulation of expression.

interferon-γ, interleukin (IL)-12, tumour-necrosis factor-α and IL-10 signalling. Lymphocyte activation was the next most significant ($P = 1.8 \times 10^{-23}$), with activation of T cells, B cells and natural killer (NK) cells being the strongest contributors to this signal. Strong enrichment was also seen for response to molecules of bacterial origin ($P = 2.4 \times 10^{-20}$), and for the Kyoto Encyclopedia of Genes and Genomes (KEGG) JAK-STAT signalling pathway ($P = 4.8 \times 10^{-15}$). We note that no enriched terms or pathways showed specific evidence of Crohn's disease or ulcerative colitis specificity.

As infectious organisms are known to be among the strongest agents of natural selection, we investigated whether the IBD-associated variants are subject to selective pressures (Supplementary Table 5 and Supplementary Methods 4c). Directional selection would imply that the balance between these forces shifted in one direction over the course of human history, whereas balancing selection would suggest an allele-frequency-dependent scenario typified by host–microbe co-evolution, as can be observed with parasites. Two SNPs show Bonferroni-significant selection: the most significant signal, in *NOD2*, is under balancing selection ($P = 5.2 \times 10^{-5}$), and the second most significant, in the receptor *TNFRSF18*, showed directional selection ($P = 8.9 \times 10^{-5}$). The next most significant variants were in the ligand of that receptor, *TNFSF18* (directional; $P = 5.2 \times 10^{-4}$), and *IL23R* (balancing; $P = 1.5 \times 10^{-3}$). As a group, the IBD variants show significant enrichment in selection (Fig. 2b) of both types ($P = 5.5 \times 10^{-6}$). We discovered an enrichment of balancing selection (Fig. 2b) in genes annotated with the Gene Ontology term 'regulation of interleukin-17 production' ($P = 1.4 \times 10^{-4}$). The important role of IL-17 in both bacterial defence and autoimmunity suggests a key role for balancing selection in maintaining the genetic relationship between inflammation and infection, and this is reinforced by a nominal enrichment of balancing selection in loci annotated with the broader Gene Ontology term 'defense response to bacterium' ($P = 0.007$).

We tested for enrichment of cell-type expression specificity of genes in IBD loci in 223 distinct sets of sorted, mouse-derived immune cells from the Immunological Genome Consortium[17]. Dendritic cells showed the strongest enrichment, followed by weaker signals that support the Gene Ontology analysis, including $CD4^+$ T cells, NK cells and NKT cells (Fig. 2c). Notably, several of these cell types express genes near our IBD associations much more specifically when stimulated; our strongest signal, a lung-derived dendritic cell, had $P_{\text{stimulated}} < 1 \times 10^{-6}$ compared with $P_{\text{unstimulated}} = 0.0015$, consistent with an important role for cell activation.

To further our goal of identifying likely causal genes within our susceptibility loci and to elucidate networks underlying IBD pathogenesis, we screened the associated genes against 211 co-expression modules identified from weighted gene co-expression network analyses[18], conducted with large gene-expression data sets from multiple tissues[19–21]. The most significantly enriched module comprised 523 genes from omental adipose tissue collected from morbidly obese patients[19], which was found to be 2.9-fold enriched for genes in the IBD-associated loci ($P = 1.1 \times 10^{-13}$; Supplementary Fig. 12 and Supplementary Table 6). We constructed a probabilistic causal gene network using an integrative Bayesian network-reconstruction algorithm[22–24], which combines expression and genotype data to infer the direction of causality between genes with correlated expression. The intersection of this network and the genes in the IBD-enriched module defined a sub-network of genes enriched in bone marrow-derived macrophages ($P < 10^{-16}$) and is suggestive of dynamic interactions relevant to IBD pathogenesis. In particular, this sub-network featured close proximity among genes connected to host interaction with bacteria, notably *NOD2*, *IL10* and *CARD9*.

A *NOD2*-focused inspection of the sub-network prioritizes multiple additional candidate genes within IBD-associated regions. For example, a cluster near *NOD2* (Fig. 2d) contains multiple IBD genes implicated in the *Mycobacterium tuberculosis* response, including *SLC11A1*, *VDR* and *LGALS9*. Furthermore, both *SLC11A1* (also known as *NRAMP1*) and *VDR* have been associated with *M. tuberculosis* infection by candidate gene studies[25,26], and *LGALS9* modulates mycobacteriosis[27]. Of interest, *HCK* (located in our new locus on chromosome 20 at 30.75 megabases) is predicted to upregulate expression of both *NOD2* and *IL10*, an anti-inflammatory cytokine associated with Mendelian[28] and non-Mendelian [29] IBD. *HCK* has been linked to alternative, anti-inflammatory activation of monocytes (M2-group macrophages)[30]; although not identified in our aforementioned analyses, these data implicate *HCK* as the causal gene in this new IBD locus.

We report one of the largest genetic experiments involving a complex disease undertaken to date. This has increased the number of confirmed IBD susceptibility loci to 163, most of which are associated with both Crohn's disease and ulcerative colitis, and is substantially

more than reported for any other complex disease. Even this large number of loci explains only a minority of the variance in disease risk, which suggests that other factors—such as rarer genetic variation not captured by GWAS or environmental exposures—make substantial contributions to pathogenesis. Most of the evidence relating to possible causal genes points to an essential role for host defence against infection in IBD. In this regard, the current results focus ever-closer attention on the interaction between the host mucosal immune system and microbes, both at the epithelial cell surface and within the gut lumen. In particular, they raise the question, in the context of this burden of IBD-susceptibility genes, of what triggers components of the commensal microbiota to switch from a symbiotic to a pathogenic relationship with the host. Collectively, our findings begin to shed light on these questions and provide a rich source of clues to the pathogenic mechanisms underlying this archetypal complex disease.

## METHODS SUMMARY

We conducted a meta-analysis of GWAS data sets after imputation to the HapMap3 reference set, and aimed to replicate in the Immunochip data any SNPs with $P < 0.01$. We compared likelihoods of different disease models to assess whether each locus was associated with Crohn's disease, ulcerative colitis, or both. We used databases of expression quantitative trait loci SNPs and coding SNPs in linkage disequilibrium with our hit SNPs, as well as the network tools GRAIL and DAPPLE, and a co-expression network analysis to prioritize candidate genes in our loci. Gene Ontology, the Immunological Genome Project (ImmGen) mouse immune-cell-expression resource, the TreeMix selection software and a Bayesian causal network analysis were used to functionally annotate these genes.

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**Author Information** Data have been deposited in the NCBI database of Genotypes and Phenotypes under accession numbers phs000130.v1.p1 and phs000345.v1.p1. Summary statistics for imputed GWAS are available at http://www.broadinstitute.org/mpg/ricopili/. Summary statistics for the meta-analysis markers are available at http://www.ibdgenetics.org/. The 523 causal gene network cytoscape file is available on request. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.H.C. (judy.cho@yale.edu).

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EXPERT REVIEWS

# Primary treatment of Crohn's disease: combined antibiotics taking center stage

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Although controversial, the use of properly chosen antibiotics in Crohn's disease appears beneficial. Evidence supporting the use of targeted antibiotic therapy comes in two forms: statistical evidence derived from meta-analyses of multiple formal studies and the documented clinical and endoscopic responses in patients treated with antibiotic combinations outside of formal clinical studies. This article reviews evidence from both categories that support the use of properly chosen antibiotic regimens in treating Crohn's disease, comments on the advantages and disadvantages of antibiotic therapy, and attempts to present a unifying hypothesis related to the role of enteric bacteria, mucosal immunity and antibiotic therapy. Relevant studies identified through a Medline search from 1976 to 2011 were assessed for inclusion by two independent observers who resolved any disagreements by consensus. References from all identified articles and recent review articles were cross-checked to ensure a thorough search. Papers were selected based on scientific merit as to which presented original contributions to the results.

**Keywords:** anti-MAP therapy • Crohn's disease • *Mycobacterium avium paratuberculosis*

## Antibiotics in Crohn's disease

The rationale for using antibiotics as primary treatment of Crohn's disease (CD) is based on increasing evidence implicating gut bacteria in the pathogenesis of the disease [1]. Two hypotheses involving the role of bacteria in the etiology and pathogenesis of CD have been proposed. Both hypotheses support a role for antibiotic therapy to correct the disease process and shift the involved networks toward a healthier dynamic.

One hypothesis proposes that genetically determined defects of innate immunity reacting to nonspecific antigens from commensal gut bacteria result in the dysregulated inflammatory disease phenotype [2]. In this scenario antibiotics may affect the microbial ecosystem of the gut and influence the continual cross-talk between the microbiota and the mucosal immune system in such a way that a beneficial response is obtained. This hypothesis does not explain why patients develop the disease later in life and it does not readily explain Crohn's lesions found in the esophagus [3], duodenum [4], liver [5] and other organs [6].

Another hypothesis proposes that specific bacterial species infect dendritic cells and macrophages in genetically predisposed individuals [7]. Defects in the recognition and response to specific bacteria result in chronic intracellular infection of dendritic cells and macrophages. As with the first hypothesis, disturbances in autophagy, antigen presentation and downstream immune signaling are compromised. Defects of innate immune signaling result from persistent macrophage bacterial infection that occurs due to genetic defects.

As a result of these hypotheses, numerous antibiotic regimens have been employed as primary therapy to attempt to modify the gut microflora milieu and minimize colonization and invasion of harmful bacteria in CD.

Early studies (1978–1991) comparing metronidazole, a nitroimidazole with a broad-spectrum of anaerobic activity, with placebo or conventional treatment were the first to suggest a beneficial effect of antibiotics in CD. Blichfeldt *et al.* comparing metronidazole (1 g/day or placebo) with salazosulfapyridin versus prednisone in 22 CD patients in a double-blind crossover study found no significant clinical benefit, although six patients with colonic involvement showed improvement in symptoms and biochemical indices [8]. The Swedish Cooperative Crohn's Disease Study (1982) compared metronidazole

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(800 mg/day) alone versus sulfasalazine in 78 CD patients in a crossover design (1.5 g/day) for 2-month periods [9]. Reductions in Crohn's Disease Activity Index (CDAI) scores were similar for both groups at 4 months. Both metronidazole and sulfasalazine were equally effective in patients with colitis or ileocolitis but were not effective in ileitis. Patients who switched from sulfasalazine to metronidazole showed significant reductions in CDAI. This was not seen in patients who switched from metronidazole to sulfasalazine. The investigators deemed metronidazole slightly more effective than sulfasalazine in CD. Ambrose *et al.* compared metronidazole and cotrimoxazole in combination or alone, against placebo in 72 CD patients and found no benefit in any of the drug combinations after 1 month of treatment [10]. In 1991, Sutherland *et al.* trialed two metronidazole regimens – 10 and 20 mg/kg/day – versus placebo in 105 CD patients [11]. A greater proportion of patients receiving metronidazole had improved CDAI scores compared with placebo. No significant difference was observed between the 10 and 20 mg/kg/day groups. Metronidazole therapy was more effective in patients with colitis or ileocolitis versus ileitis alone, suggesting that gut flora differences between the ileum and colon may help explain the differing metronidazole effectiveness. De'Haens *et al.* compared metronidazole with azathioprine versus metronidazole alone to reduce recurrence of postoperative CD in high-risk patients [12]. In total, 81 patients were randomized and 19 patients discontinued the study early. Significant endoscopic recurrence was observed in 43.7% in the metronidazole/azathioprine group versus 69% of the placebo group. The authors concluded that despite the enhanced risk of recurrence, the overall incidence was rather low and probably attributable to the metronidazole treatment that all patients received. More recently, Feller and colleagues from the University of Bern, Switzerland, pooled data from 16 randomized, placebo-controlled trials involving a total of 865 patients in order to assess the effectiveness of long-term antibiotic treatment for CD [13]. The outcomes were remission in patients with active disease, or relapse in patients with inactive disease. Three trials of nitroimidazoles (206 patients) showed a significant benefit (odds ratio [OR]: 3.54). The number of patients needed to treat with nitroimidazoles to keep one additional patient in remission was 3.4 for patients with active disease and 6.1 for inactive disease.

Ciprofloxacin has also been used, in combination with metronidazole and as a monotherapy in CD, with good results (Table 1) [14–20]. A meta-analysis of three trials evaluating either ciprofloxacin or metronidazole in 123 patients with perianal CD fistula, reported a statistically significant effect in reducing fistula drainage (relative risk [RR]: 0.8; 95% CI: 0.66–0.98) with no heterogeneity ($I^2$ = 0%) and a number needed to treat of five (95% CI: 3–20) [21]. Despite current European Crohn's and Colitis Organisation guidelines stating that "at present antibiotics are only considered appropriate for septic complications, symptoms attributable to bacterial overgrowth, or perineal disease" [22] additional studies in CD patients without perianal disease have also shown positive results. Peppercorn *et al.*, treating four patients with Crohn's ileitis using ciprofloxacin for 6 weeks, reported a dramatic improvement in all patients with complete resolution of symptoms after 1 week of treatment; however, a control group was not used [14]. Three patients remained asymptomatic 3 and 6 months after treatment. One patient suffered a recurrence after 6 months and again responded to ciprofloxacin. Arnold *et al.* also reported benefit, treating 47 patients with moderately active, refractory CD for 6 months with ciprofloxacin [15]. Mean CDAI scores at the completion of study were 112 for the ciprofloxacin group (n = 25) and 205 for the placebo group (n = 12; p < 0.001) with an OR of 11.3, but wide confidence intervals (95% CI: 2.60–48.8). Prantera *et al.* comparing ciprofloxacin alone versus metronidazole alone versus ciprofloxacin and metronidazole in combination in 41 patients with active CD reported similar remission rates between the three groups (69, 73 and 71%, respectively) [16]. Colombel *et al.* demonstrated that ciprofloxacin was as effective as mesalazine for inducing remission, reporting remission rates of 56 and 55%, respectively [17]. However, Steinhart *et al.* reported no significant improvement in clinical remission using budesonide, metronidazole and ciprofloxacin versus budesonide alone in 130 patients with active CD of the ileum, right colon, or both [18]. Despite the lack of improvement, a greater proportion of patients with colonic disease achieved remission in the antibiotic group (53%) versus placebo (25%).

Several recent studies have also reported on the therapeutic benefit of rifaximin, the nonabsorbed broad-spectrum antibacterial antibiotic with an excellent safety profile, in mild to moderate CD. Shafran *et al.* reported endoscopic and clinical improvements in treatment-naive CD patients who received rifaximin (800 mg/day) as first-line therapy for 12 weeks [23]. The same investigators found in an open trial that 43% of 29 patients with active CD achieved clinical remission with rifaximin (600 mg/day). By the end of the trial, 60% of patients were in remission [24]. Shafran *et al.* retrospectively reported on 68 patients with CD treated with rifaximin over a 4-year (mean: 16.6 weeks) period [25]. Most patients (94%) received rifaximin 600 mg/day; 18 patients received rifaximin monotherapy; and 31 received rifaximin with concomitant steroids. Overall, 65% achieved remission. The remission rate was greater – 70% – in patients not receiving steroids versus 58% of those who received steroids. Of note is the remission rate of 67% achieved in patients on rifaximin monotherapy, suggesting that rifaximin alone is effective in maintaining remission. Remission rates were 65% for the small intestine, 66% for multiple locations and 55% for the large intestine. A meta-analysis by Khan *et al.*, reporting on two clinical trials involving 485 patients treated with rifaximin in CD, found rifaximin to be effective at inducing remission (RR: 0.81; 95% CI: 0.68–0.97) with no statistically significant heterogeneity between the two trials (x = 0%; Cochran Q = 0.17; df = 1; p = 0.68) [21].

## Anti-tuberculous therapy in CD

Preliminary case reports detailing improvements in CD during treatment for pulmonary tuberculosis were the first to suggest a mycobacterial etiology in CD. Several controlled and uncontrolled studies were subsequently performed. In 1986 Warren *et al.* first reported on a patient treated for intercurrent pulmonary tuberculosis treated with a combination of isoniazid, rifampicin,

**Table 1. Summary of ciprofloxacin studies in Crohn's disease.**

| Author (year) | Design | Treatment groups | Patients (n) | Results | Ref. |
|---|---|---|---|---|---|
| Peppercorn (1993) | Small, uncontrolled study | Ciprofloxacin | 4 | Dramatic improvement in clinical response | [14] |
| Colombel *et al.* (1999) | Randomized, controlled trial | Ciprofloxacin (500 mg/b.i.d.) vs mesalazine (4 g/day) | 40 | Remission achieved in 10 patients (56%) treated with ciprofloxacin vs 12 patients (55%) in the mesalazine group | [17] |
| Arnold *et al.* (2002) | Randomized, placebo-controlled trial | Ciprofloxacin (500 mg/b.i.d.) vs placebo (conventional therapy maintained) | 47 | Both groups showed a significant decrease in mean CDAI scores at 3 months<br>At study conclusion, the mean CDAI score in the placebo group increased to 205, resulting in a statistically significant difference between the groups | [15] |
| Prantera *et al.* (1996) | Randomized, controlled trial | Combination ciprofloxacin (500 mg/b.i.d.) and metronidazole (250 mg/q.i.d.) vs methylprednisone (0.7–1 mg/kg/day) | 22 | Remission achieved in 10 out of 22 (46%) patients in the ciprofloxacin/metronidazole group and 12 out of 19 (63%) patients in the methylprednisone group<br>The difference was not significant | [16] |
| Prantera *et al.* (1998) | Retrospective study | Combination ciprofloxacin (1 g/day) and metronidazole (1 g/day) vs ciprofloxacin (1 g/day) alone vs metronidazole (1 g/day) alone | 233 | Remission achieved in 71% in the combination group, 73% treated with metronidazole alone, 69% treated with ciprofloxacin alone | [19] |
| Greenbloom *et al.* (1998) | Uncontrolled study | Combination ciprofloxacin (500 mg/b.i.d.) and metronidazole (250 mg/t.i.d.) vs combination ciprofloxacin (500 mg/b.i.d.), metronidazole (250 mg/t.i.d.) and prednisone (mean dose 15 mg/day) | 72 | Remission achieved in:<br>68% in the ciprofloxacin/metronidazole group<br>A clinical response occurred in a greater proportion of patients with colonic disease (84%) vs ileal disease (64%) | [20] |
| Steinhart *et al.* (2002) | Prospective, multicenter, double-blind, randomized, controlled trial | Budesonide (9 mg/day), metronidazole (1 g/day) and ciprofloxacin (1 g/day) vs budesonide (9 mg/day) plus placebo | 134 | No significant improvement in clinical remission in the antibiotic group<br>By disease site, a greater proportion of patients with colonic disease achieved a higher remission rate (53%) in the antibiotic group than placebo (25%) | [18] |

b.i.d.: Twice a day; CDAI: Crohn's Disease Activity Index; q.i.d.: Four times a day; t.i.d.: Three times a day.

pyrazinamide, and ethambutol [26]. The patient was treated for 9 months which resulted in induction of remission of his CD. Schultz *et al.* further backed this claim with a *Lancet* report of a patient who achieved clinical remission and resolution of abnormal findings at 6 months using the same anti-tuberculous treatment [27]. Buoyed by these findings a number of investigators trialed various anti-tuberculous therapies in CD, with mixed results.

Early studies reporting the use of dapsone in CD appeared promising. Ward and McManus reported benefit from dapsone treatment in four out of six (66.7%) patients with resolution of symptoms, healing of fistulae and macroscopic and microscopic improvements [28]. Prantera *et al.* treating a patient with dapsone therapy (75 mg) for over 12 months reported a similarly dramatic improvement with complete CD symptom resolution after 2 weeks of treatment [29]. At 6 months, cutaneous lesions were healed and colonoscopy, histology and small bowel x-rays were normal. Prantera *et al.* followed up with a case study of five patients treated with dapsone with less impressive results, being effective in only two out of five patients (40%) [30]. Nevertheless, these patients achieved a reduction in CDAI by one month, with one patient experiencing complete healing of all cutaneous and rectal ulcers. Higher antimycobacterial antibody levels were observed in the two responders compared with the nonresponders, and may help to explain the results.

Despite these early studies reporting results rarely attained with other CD treatments, such as mucosal and fistula healing, later trials using heterogenous treatment regimes revealed a discordance of response. Shaffer *et al.* reported no significant benefit of rifampicin and ethambutol versus placebo in a 2-year, randomized trial of 27 patients [31]. Jarnerot *et al.* using the same regime in five patients reported an initial improvement in all, with one patient even experiencing mucosal and fistula healing [32]. However, the efficacy was lost over time. Swift *et al.* using a combination of rifampicin (450 mg/day for patients <50 kg or 600 mg/day for patients >50 kg), isoniazid (300 mg/day),

and ethambutol (15 mg/kg/day) versus placebo in 120 patients found no significant difference between the groups [33]. Rutgeerts *et al.* studied the effect of rifabutin and ethambutol in 16 complicated surgical patients who had undergone ileocolonic resection and those with severe neoterminal ileal disease [34]. In total, 11 patients were treated for >6 months and five were treated for 12 months without benefit.

## Anti-*Mycobacterium avium paratuberculosis* therapy in CD

Given the poor activity of classic anti-tuberculous agents against *Mycobacterium avium paratuberculosis* (MAP), and their lack of intracellular access, where MAP is located, new antimycobacterial agents with intracellular activity against MAP, alone and in combination, were slowly introduced with improving results. Employing a combination of rifampicin, ethambutol, isoniazid and pyrazinamide or clofazimine for 9 months, Hampson *et al.* reported that 10 out of 20 CD patients (50%) achieved remission by 9 months [35]. Of the remaining ten, three had achieved remission by 6 months but had relapsed. Nine out of ten patients (90%) on steroids were weaned off by 9 months. Five patients required surgery for stricture formation, although no evidence of CD was noted at colonoscopy. Three patients with severe CD facing total colectomy were spared surgery. Similarly Prantera *et al.* compared a combination of ethambutol, clofazimine, dapsone and a 1 day dose of rifampicin versus placebo for 9 months in 40 patients with refractory, steroid dependent CD [36]. In the active group, 16 out of 19 patients (84.2%) achieved clinical remission versus six out of 17 (35.3%) in the placebo group. Additionally nine patients who relapsed on placebo were crossed over to anti-MAP therapy where five out of nine (55.5%) were then able to achieve sustained clinical remission.

Afdhal *et al.* employed either 100 mg/day clofazimine with corticosteroids or corticosteroids alone in 49 CD patients [37]. A total of 18 patients achieved remission (36.7%) with 12 out of 18 (66%) in the clofazimine group versus six out of 18 (33%) in the corticosteroid group. The modified disease activity score at the end of the trial was 1.4 ± 1.6 for clofazimine patients versus 4 ± 5.3 for placebo.

In 1995, Graham *et al.* trialed clarithromycin alone in 15 severe CD patients, achieving prolonged remission after 3 months of treatment in approximately 40% of patients [38]. Leiper *et al.* also used clarithromycin alone in 25 patients for a 4-week period, continuing to 12 weeks in responding patients [39]. In total, 11 of the 25 patients (44%) continued after 12 weeks for a median of 28 weeks (20–60 weeks). Two patients were withdrawn owing to nonserious side-effects. Inoue *et al.* also reported positive findings, treating 14 patients with active CD using clarithromycin for 4 weeks [40]. Patients who showed a clinical response within 4 weeks continued the therapy for up to 24 weeks. The mean CDAI score at entry was 343.5. Within 4 weeks, eight (57.1%) of the 14 patients showed clinical improvement, and five (35.7%) of the eight patients achieved remission. Each of the eight patients continued clarithromycin therapy after 4 weeks, and six (42.9%) were in clinical remission by 12 weeks. Out of the 14 total patients, four (28.6%) continued clarithromycin for more than 24 weeks and have remained in remission.

We now know that effective MAP treatment should consist of at least two different drugs, including a macrolide, and be administered for >6 months in a dosage similar to that used in *Mycobacterium avium* complex infection [13,41–44]. Monotherapy with a macrolide such as clarithromycin, while achieving good initial results in studies by Graham *et al.* [38] and the recent pilot study with Japanese CD patients [40], is likely to result in the development of antibiotic resistance as is seen in tuberculosis therapy [45–47]. Combination regimens of three to four agents containing macrolides with known intracellular drug activity against MAP, such as rifabutin combined with the macrolide clarithromycin and/or clofazimine, have therefore been recommended for periods >6 months to minimize antibiotic resistance [13]. The favorable response of this treatment regimen, first evidenced in early studies by Hampson *et al.* [35] and Prantera *et al.* [36], has since resulted in clinical remission in seven clinical trials with a success rate ranging from 44 to 89%, the results of which have been summarized (Table 2) [48–55].

Of the previously summarized studies, perhaps the most well-known and widely criticized study of anti-MAP therapy (AMAT) in CD was that conducted by Selby *et al.* [54]. Touted as a 'landmark study' [56], the controversial trial used anti-mycobacterial drugs against atypical mycobacteria (rifabutin, clarithromycin, clofazimine) with prednisolone to induce remission followed by maintenance therapy with AMAT (n = 102). At 16 weeks, an unprecedented 66% of patients (n = 102) on AMAT were in remission without further benefit beyond this point by per-protocol analysis. However as surmised by Feller *et al.* the results of the Australian trial "were not based on an intention-to-treat analysis and may have underestimated the beneficial effects of the drug" [13]. By an intention-to-treat (ITT) analysis the remission rate was significantly higher in the AMAT group compared with placebo (Table 2) with a p-value of <0.005 at 52 weeks and <0.008 at 104 weeks. As expected, after cessation of medication at 102 weeks the difference between treatment and placebo at 156 weeks was not significant (p = 0.19). Had the trial been based on an ITT analysis, as is the case with a number of other CD drugs on the market, the remission rate for AMAT would probably have been much higher [13,55]. The drop in efficacy over time is uncharacteristic compared with previous studies and may be attributed to two problems identified in the trial. First, the authors used "a suboptimal dose of clofazamine (50 mg/day) and other antibiotics" [57], and second, by the author's own admission, the clofazamine capsule "failed to rupture due to hardening of the outer gelatine capsule shell, resulting in a period of approximately 10 months where patients were likely not exposed to the correct dosage of clofazamine" [54]; however, these patients were not replaced. Taken together, the results of the Selby trial were surprisingly good in spite of the shortcomings of the Australian Phase III trial. To demonstrate the superior efficacy of the treatment in CD it is essential to compare the results of the Selby trial with those of other CD treatments currently on the market, such as the anti-TNF therapy infliximab. AMAT

**Table 2. Summary of outcomes of anti-*Mycobacterium avium paratuberculosis* therapies in Crohn's disease.**

| Author (year) | Patients (n) | Antibiotics used | Duration (months) | Results | Ref. |
|---|---|---|---|---|---|
| Gui *et al.* (1997) | 52 | Clarithromcyin and rifabutin or azithromycin | 6–35 | Complete clinical remission in 46 out of 52 (88.5%) patients at 24 months<br>17 out of 19 (89.5%) steroid-dependent patients were able to be weaned off steroids | [48] |
| Douglas *et al.* (2000) | 28 | Rifabutin, clarithromycin and clofazimine | 12 | Complete clinical remission achieved in 10 out of 20 (50%) patients for 12 months<br>Symptomatic improvement in 20 out of 28 (71.4%) patients at 1–5 weeks of treatment | [49] |
| Borody *et al.* (2002) | 12 | Rifabutin, clarithromycin and clofazimine | 6–54 | Clinical response in 8 out of 12 (66.6%) patients<br>Complete clinical and endoscopic remission achieved in 6 out of 12 (50%) patients which lasted for 10 years | [50] |
| Shafran *et al.* (2002) | 36 | Rifabutin and clarithromycin | 4–17 | Sustained improvement in 21 out of 29 (58.3%) patients with reduction in CDAI score ≥70 points<br>7 patients were unable to tolerate the therapy (19.4%)<br>5 patients were nonresponders (13.8%) | [51] |
| Borody *et al.* (2005) | 52 | Rifabutin, clarithromycin and clofazimine | 6–108 | Complete clinical, colonoscopic and histological remission in 32 out of 52 (61.5%) patients | [52] |
| Borody *et al.* (2007) | 39 | Rifabutin, clarithromycin and clofazimine | 6–108 | Mucosal healing occurred in 22 out of 39 (56.4%) patients with unusual longitudinal scarring<br>Histologically, 15 out of 39 (38.5%) patients experienced a marked reduction in inflammation with 6 of these (40%) displayed restoration of normal mucosa<br>Improved histology coincided with longitudinal scarring in 12 out of 15 patients (80%)<br>2 out of 6 (33.3%) patients who were on treatment for >3 years after initial scarring had complete resolution of scarring and healing | [53] |
| Selby *et al.* (2007)<br>Behr and Kapur (2008) | 213 randomized<br>Active (n = 102)<br>Placebo (n = 111)<br>91 were withdrawn | Rifabutin, clarithromycin and clofazimine (AMAT) | 16–104 | ITT analysis:<br>Remission sustained in 66 out of 102 (65.7%) patients on AMAT vs 55 out of 111 (49.5%) on placebo ($p < 0.02$) at 16 weeks<br>Effects of AMAT after 16 weeks:<br>At 52 weeks: remission sustained in 41 out of 102 (40%) patients vs 24 out of 111 (21.6%), $p < 0.005$<br>At 104 weeks: remission sustained in 31 out of 102 (30%) patients vs 16 out of 111(14%), $p < 0.008$<br>At 152 weeks: remission sustained in 14 out of 102 (13%) patients vs 10 out of 111 (13%), p < NS | [54,55] |

AMAT: Anti-*Mycobacterium avium paratuberculosis* therapy; CDAI: Crohn's Disease Activity Index; ITT: Intention-to-treat; NS: Not significant.

remains a far more effective treatment than published results for infliximab, which reported remission rates of 39% at 12 weeks in the ACCENT I trial [58,59]. For example, on an ITT analysis, the AMAT remission rate at 16 weeks was 66% compared with 39% at 12 week for infliximab. Although no data is available for the trial of infliximab at 52 weeks, the ITT AMAT remission rate at 52 weeks was 41% compared with 26% at 26 weeks for the highest dose of infliximab used. The AMAT remission rate was also higher than that achieved with the recently US FDA-approved humanized anti-TNF antibody (adalimumab) in the CHARM trial, with the ITT remission rate of 24% for adalimumab at 52 weeks calculated for the highest dose [60]. The demonstrated superior results of anti-MAP therapy over other treatments currently on the market for CD support their use as a preferred primary treatment. This view was echoed by Feller *et al.* in their recent meta-analysis of antibiotic therapy in CD, who reported on the "potentially more favorable adverse effect profile and lower costs" of antibiotic therapy compared with infliximab [13]. With the serious, and at times fatal side effects encountered with anti-TNF therapy [61,62], coupled with an efficacy below 39% [57], it could readily be argued that failure to

inform patients of this safe and effective antibiotic CD treatment may expose physicians to the question of 'duty of care' with its legal implications.

## Case reports of anti-MAP therapy in CD

The results of the aforementioned anti-MAP trials are consistent with clinical observations reported by both Chamberlin *et al.* [63] and Borody *et al.* [53] that prolonged anti-MAP therapy is effective in CD. In 2007, Chamberlin *et al.* reported a patient with longstanding active CD who achieved clinical remission using a combination of clarithromycin, rifabutin and levofloxacin [63]. Prior to treatment the patient had severe CD with ulcerated, erythematous mucosa and exudates (Figure 1A & B). Buffy coat analysis by PCR and culture revealed circulating MAP DNA in blood. After 3 weeks of treatment the patient experienced complete resolution of abdominal pain, diarrhea and fatigue. At 6 months he was in complete remission, with no active inflammation on colonoscopy (Figure 2A). Residual mucosal pseudopolyps were viewed in the areas of previously severe involvement (Figure 2B). MAP DNA by *IS900* PCR and blood culture were now also negative. The patient remains in clinical remission 5 years later.

This is the first case report demonstrating the resolution of CD that correlated with suppression of MAP markers. These findings are similar to those reported by Borody *et al.* in a retrospective review of 39 severe CD patients treated with rifabutin, clofazimine and clarithromycin for 6 months to 9 years [53]. A total of 22 patients (56.4%) healed with unusual longitudinal scarring. Two out of six (33.3%) patients who were on treatment for >3 years after initial scarring presented had complete resolution of scarring and healing. Histologically, 15 out of 39 (38.5%) patients experienced a marked reduction in inflammation with six of these (40%) displaying restoration of normal mucosa. Improved histology coincided with longitudinal scarring in 12 out of 15 patients (80%). Of particular importance was one patient with severe CD treated with anti-MAP alone as primary therapy who healed completely without any immunosuppressive treatment. The patient initially presented with severe abdominal pain, perianal disease, six bloody stools daily and a 10 kg weight loss. Colonoscopy revealed extensive anorectal fissures and apthoid erosions progressing to very deep, destructive ulcers with pseudopolyps proximal to the rectum throughout the rest of the colon (Figure 3A). The patient was initiated only on anti-MAP therapy. At 6 weeks, the patient's symptoms had resolved, passing one to two formed stools daily with the absence of blood and abdominal pain. A colonoscopy was performed at 1 year while on therapy showing normal mucosa with no pseudopolyps, no ulcerations and no visible inflammation. Some scarring was observed in areas of previously severe disease (Figure 3B).

A number of patients have followed with similar treatment and results, indicating CD in these patients is infection-driven and immunosuppression has played no role.

## Meta-analyses of antibiotic trials in CD

Several meta-analyses have since summarized the results of these trials, and also pointed to the positive effect of antibiotics in CD. Feller and colleagues pooled data from 16 randomized, placebo-controlled trials involving a total of 865 patients in order to assess the effectiveness of long-term antibiotic treatment for CD [13]. The outcomes were remission in patients with active disease, or relapse in patients with inactive disease. Three trials of nitroimidazoles showed benefit, with a combined OR of 3.54 (95% CI: 1.94–6.47). Similarly, the combined OR from four trials of clofazamine was 2.86 (95% CI: 1.67–4.88). For patients with active disease, the number needed to treat was 3.4 (95% CI: 2.3–7.0) for nitroimidazoles and 4.2 (95% CI: 2.7–9.3) for clofazamine. The numbers needed to treat for inactive disease were 6.1 (95% CI: 5.0–9.7) for nitroimidazoles and 6.9 (95% CI: 5.4–12.0) for clofazamine. No benefit for classic drugs against tuberculosis was found (OR: 0.58; 95% CI: 0.29–1.18). Similarly, Rahimi *et al.* in a meta-analysis of six randomized, placebo-controlled trials of antibiotics in CD, found an OR of 2.257 (95% CI: 1.678–3.036; $p < 0.001$) in favor of antimicrobial therapy versus placebo in patients with CD [64].

More recently, Khan *et al.* published his meta-analysis of numerous randomized controlled trials (RCTs) employing different antibiotic regimens in CD [21]. A statistically significant superior effect of antibiotics compared with placebo was found (RR of active CD not in remission: 0.85; 95% CI: 0.73–0.99; $p = 0.03$). Rifamycin derivatives either alone or in combination with other antibiotics also appeared to have a significant effect at inducing remission in active CD. Ciprofloxacin or metronidazole were found to have a statistically significant effect in reducing fistula drainage (RR: 0.8; 95% CI: 0.66–0.98) from three trials evaluating 123 patients. For quiescent CD, a statistically significant effect in favor of antibiotics versus placebo (RR of relapse: 0.62; 95% CI: 0.46–0.84) was found from three RCTs involving 186 patients treated with different antibiotics combinations (all including antimycobacterials) versus placebo.

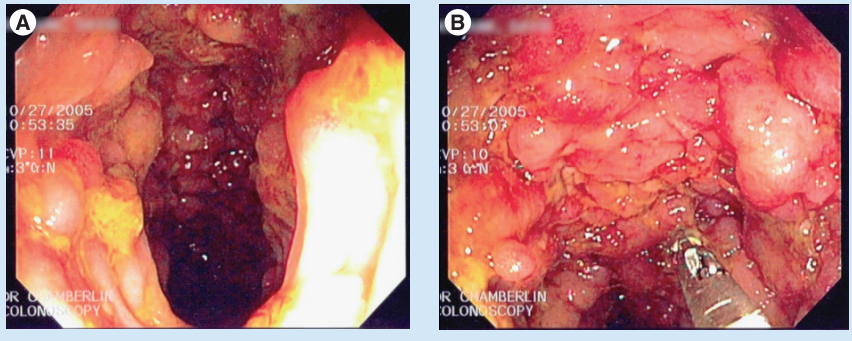


**Figure 1. Before anti-*Mycobacterium avium paratuberculosis* therapy. (A)** Inflamed, edematous, erythematous mucosa. **(B)** Severe mucosal ulceration, bleeding and exudates. Reproduced with permission from [59].

## Expert commentary

In CD an environmental 'trigger' acts upon an immune system in genetically predisposed individuals that disrupts immune signaling resulting in inappropriate, chronic inflammation. A few key predisposing genetic variables have been identified. The strongest variables are loss-of-function mutations of the *NOD2* genes involved in the recognition of intracellular bacteria and loss-of-function mutations of *IRGM* and *ATG16L1* genes involved in the process of autophagy [65]. The autophagocytic process is important in a cell's ability to kill intracellular bacteria, process antigenic information and deliver appropriate downstream signaling that instructs the innate and adaptive immune systems to effectively respond to an invading microbe. Abnormalities in this system may result in the survival of intracellular bacteria and/or an inappropriate dysregulated immune inflammatory response.

Evidence now clearly points to the use of antibiotics as potential modifying agents in the CD disease process [13,21]. Current concepts associate defects of innate immunity with CD; immune deficiency and infections go hand-in-hand. Theoretically, therapy targeted against the infecting microbe can be employed or therapy that restores immune function can be administered. Common sense suggests that the most effective therapy would combine both approaches. Any therapy that enhances innate immunity along with properly chosen antibiotics should provide a superior result. GM-CSF is an immune modulating cytokine that enhances innate immunity and was tested against CD. Although not achieving statistical significance in the primary objective, GM-CSF resulted in statistically significant responses in secondary criteria suggesting that enhancing innate immunity is beneficial in CD. The current popular approach to control inflammation is to molecularly resect key cytokines or receptors using monoclonal antibodies. This approach alters the immune network to produce profound anti-inflammatory effects but unfortunately it also severely impairs immunity such that serious, and sometimes fatal, side effects occur [60]. If CD is really a disease of innate immune deficiency leading to chronic intracellular infection and inflammation then all efforts should be directed at enhancing innate immunity, not further compromising immunity as is done with current 'anti-inflammatory' therapies.

In summary, properly chosen antibiotics are beneficial [13,21]. Whether the effect is on the commensal ecosystem or whether the positive clinical responses are due to effects on specific pathogenic species is controversial and requires additional study. However, the fact remains that properly chosen antibiotics are beneficial in treating CD.

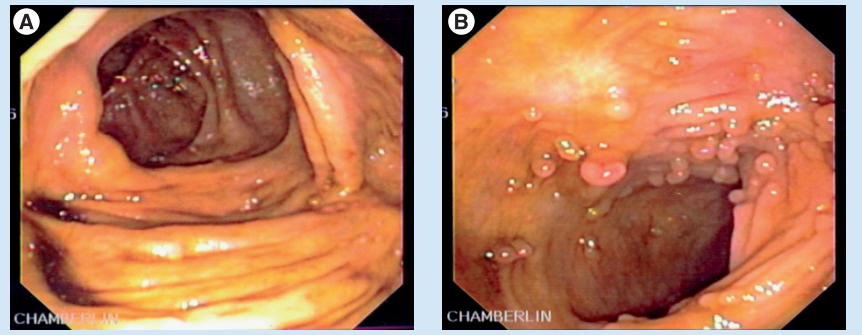


**Figure 2. During anti-*Mycobacterium avium paratuberculosis* therapy.** **(A)** Complete normalization of mucosa. **(B)** Residual pseudopolyps in areas of previous severe Crohn's disease.
Reproduced with permission from [59].

## Five-year view

The possibility for major advances in understanding the processes involved in CD has never been brighter. These advances should lead to better therapies. It will probably be said that the conceptual breakthroughs began with the work performed in Anthony Segal's laboratory that linked CD with deficiencies of innate immunity [66]. Innate and adaptive immunity evolved to combat threats from the microbial world. Evolutionary pressure from this 'arms race' shapes our immune systems and influences which bacteria are ultimately able to establish commensal or pathologic relationships within us. Immune deficiency is defined by an inability to control microbial infections – whether the infections are caused by a wide spectrum of species or just one pathogenic species. We predict that the growing realization that CD is a disorder of innate immune deficiency will drive research towards finding which microbial species are involved in the disease process. CD will once again be viewed through the infectious disease paradigm although the concept will be that immune deficiency, persistent infections and chronic inflammation are all intimately related.

Research directed at finding more effective therapies for CD will explore agents that enhance innate immunity, re-establish

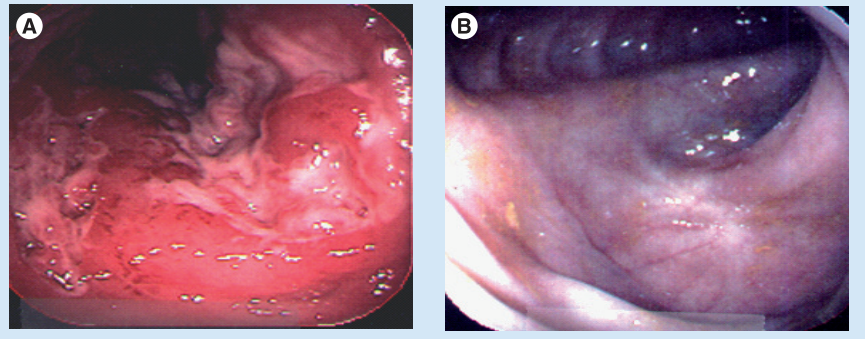


**Figure 3. The transverse colon pre- and post-treatment. (A)** Pretreatment: severe inflammation. **(B)** Post-treatment: no inflammation visible.
Reproduced with permission from [53].

appropriate immune signaling and restore a healthy Th1, Th2, Th17 and Treg homeostatic balance. Current therapies are based on the hypothesis that CD is an autoimmune disease and that an overactive adaptive response involving effector T cells is central to an excessive inflammatory disease process [2]. Treatment is directed at ameliorating symptoms by suppressing inflammation. Unfortunately, current therapies also suppress immunity. Contrary to this view, recent findings point to aberrant signaling from macrophages as central to the disease process. The future should see a movement away from therapies that suppress immunity and towards treatments that enhance innate immunity, restore appropriate immune signaling and allow the global immune system to properly handle whichever microbes are involved.

Potential approaches to influence immune networks include:

- Ligating pattern recognition receptors on immune cells
- Administering immune signaling cytokines
- Regulating immunity with steroid hormone derivatives

Clinical trials with peptide cytokines have already been performed with the innate immune enhancing cytokine GM-CSF. The raw data from these trials were very promising [67]. Immune modulation using steroid analogs is also very promising. There are 46 steroid receptors in the human genome, most of which are orphan receptors. Small changes of hydroxyl attachment to the steroid nucleus can have profound phenotypic effects – a statement best exemplified by androgens and estrogens. The ability of corticosteroids, androgens, estrogens and vitamin D to influence immunity is well recognized. The steroid metabalome interacting with pathways involving the 46 steroid receptors offers great potential in modulating immunity [68].

Phage therapy represents another promising therapeutic approach in the treatment of CD. Despite the discovery of bacteriophages more than a century ago, the therapy was quickly sidelined following the discovery and widespread application of broad-spectrum antibiotics in medicine [69]. Given the emerging threat of antibiotic resistance and our growing understanding of the effect broad-spectrum antibiotics can exert on the gastrointestinal microbiota, the use of phage therapy presents a promising treatment strategy aimed at targeting specific pathogens within the gastrointestinal microbiota to eradicate them with precision. Although in its infancy, phage therapy represents a potentially safer alternative while leaving the underlying gastrointestinal microbiota intact. However, much remains to be evaluated regarding its therapeutic effectiveness in CD.

Research in the aforementioned areas will meet resistance from institutions heavily committed to treatments that molecularly resect key molecules involved in immune networks. It will be interesting to see whether other idiopathic chronic inflammatory diseases are in fact immune deficiency disorders involving unknown infectious agents.

**Financial & competing interests disclosure**

*William Chamberlin owns shares in Harbor Biosciences, the biotech company with patent rights to the immune-enhancing hormone, HE2000. Thomas J Borody has a pecuniary interest in the Centre for Digestive Diseases and Giaconda Ltd, the licensor of Myoconda™, an anti-MAP therapy. Jordana Campbell has no financial interest or affiliation with any institution, organization, or company relating to the manuscript. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.*

*No writing assistance was utilized in the production of this manuscript.*

## Key issues

- Evidence from clinical trials and recent meta-analyses suggest that properly chosen antibiotics are effective in Crohn's disease (CD).
- Antibiotics with antimycobacterial properties report clinical improvements rarely seen with other treatments.
- The effectiveness of antibiotics, coupled with their more favorable adverse effect profile and lower costs compared with infliximab present a more attractive option in the treatment of CD.
- The optimum antibiotic regimens are not yet known.
- Much remains unknown about which bacterial species are involved in the disease and how they interact with mucosal immune systems. Further therapies aimed at enhancing rather than suppressing innate immunity represent an exciting new therapeutic strategy. Combining antibiotics with immune enhancing therapy may represent a promising therapeutic strategy in the treatment of CD.

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**[1712] Table 1. Results of cost per clinical remission and clinical response analyses**

| | 8 weeks (GLM Adjusted Linearly to 8 Week Estimate) | | | | | 6 weeks (ADA & IFX Adjusted Linearly to 6 Week Estimate) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ADA ULTRA 1 | ADA ULTRA 2 | IFX ACT 1 | IFX ACT 2 | GLM PURSUIT SC | ADA ULTRA 1 | ADA ULTRA 2 | IFX ACT 1 | IFX ACT 2 | GLM PURSUIT SC |
| Cost-per- clinical remission | $94,193 | $85,048 | $32,371 | $27,435 | $57,861 | $109,499 | $99,545 | $37,462 | $31,631 | $65,993 |
| Cost-per-clinical response | $87,599 | $42,318 | $24,027 | $21,979 | $32,363 | $102,199 | $49,451 | $27,710 | $25,401 | $37,028 |
| | 8 weeks (GLM adjusted with a Concave Function to 8 Week Estimate) | | | | | 6 weeks (ADA & IFX Adjusted with a Concave Function to 6 Week Estimate) | | | | |
| Cost-per- clinical remission | $94,193 | $85,048 | $32,371 | $27,435 | $43,396 | $147,403 | $132,154 | $50,043 | $42,175 | $65,993 |
| Cost-per-clinical response | $87,599 | $42,318 | $24,027 | $21,979 | $24,293 | $136,874 | $66,077 | $37,048 | $33,867 | $37,028 |
| | 8 weeks (GLM adjusted with a Convex Function to 8 Week Estimate) | | | | | 6 weeks (ADA & IFX Adjusted with a Convex Function to 6 Week Estimate) | | | | |
| Cost-per- clinical remission | $94,193 | $85,048 | $32,371 | $27,435 | $66,763 | $94,629 | $86,123 | $32,395 | $27,483 | $65,993 |
| Cost-per-clinical response | $87,599 | $42,318 | $24,027 | $21,979 | $37,439 | $88,103 | $42,821 | $24,035 | $21,986 | $37,028 |

**Results:** The CPR for GLM was $57,861, assuming that GLM would increase linearly, compared to $32,371 and $27,435 for IFX (ACT 1 and 2) and $94,193 and $85,048 for ADA (ULTRA 1 and 2). The CPR for GLM was $43,396 and $66,763, assuming concave and convex adjustment, respectively. The CPRes results were similar (Table 1). The results remained consistent for ADA and IFX when examining a 6-week, as opposed to an 8-week, induction period.
**Conclusion:** IFX and GLM had lower CPR and CPRes compared to ADA in the treatment of ulcerative colitis after adjusting for the different lengths of induction periods.
*Disclosure - Dr. Peter Mallow-Consultant: Janssen Scientific Affairs, LLC; Dr. John Rizzo-Consultant: Janssen Scientific Affairs, LLC; Dr. Mary Kay Queener-Employee: Janssen Scientific Affairs, LLC; Timothy Gathany-Employee: Janssen Research and Development; Dr. Jennifer Lofland-Employee: Janssen Scientific Affairs, LLC.*
*This research was supported by an industry grant from Janssen Scientific Affairs, LLC.*

## 1713

**Anti-MAP Therapy for Pediatric Crohn's Disease**

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**Purpose:** *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is associated with Crohn's disease (CD)[1], although its etiological capacity remains highly controversial. Previous studies in adults suggest anti-MAP, a combination antibiotic therapy, is a safe and effective treatment for CD, with remission rates ranging from 50-88.5%[2]. No work exists, however, regarding the use of anti-MAP in a pediatric population. Purpose: to evaluate the safety and efficacy of anti-MAP therapy for CD in pediatric patients.
**Methods:** A retrospective review was performed on 10 pediatric CD patients (five male, five female; 8-17 years old, male: 14.4±2.7years) treated with anti-MAP therapy. Patients underwent a ramp-up dosing regimen to final daily doses of 8.78±2.93 mg/kg rifabutin (max 600mg/d); 15.87±3.41mg/kg clarithromycin (max 1000mg/d), and 2.10±0.59 mg/kg clofazimine (100mg/d). Treatment duration ranged from 4.5-114 months (9.5 years). LFTs and FBC were measured regularly throughout treatment, with clinical state recorded at scheduled visits. Remission was defined as ≥ 3 month improvement in disease state with CDAI<150, and stable or reducing CD medication (with the exception of anti-MAP dose escalation).
**Results:** Eight patients (80%) achieved clinical remission, ranging from 3-60 months (5 years). Four patients have maintained remission to date (mdn: 22.5 mo), with an average time-to-relapse of 14 months. Of the two apparent treatment failures, one improved for 2 months before contracting a secondary infection, while the other was non-compliant. Additionally, the only time-to-relapse <12 months coincided with a reduction in anti-MAP dose and concurrent *C. difficile* infection. Red/brown skin discoloration occurred in all patients, and was the most common adverse effect. Raised LFTs were observed in three patients; however, one pre-dated the commencement of therapy. An isolated raised ALT was observed in a second patient, which resolved without dose adjustment. ALP was raised in a third patient at 4 months, and persisted for 5 months after the unrelated cessation of treatment. Isolated observations of mild neutropenia (ANC<1.5 x $10^9$/L) were seen in two patients; however, both resolved without dose adjustment. No adverse ocular effects were observed.
**Conclusion:** Anti-MAP is an effective therapy for pediatric CD, with 8/10 patients achieving clinical remission of ≥3 months. Treatment failures were associated with insufficient dosing and/or secondary infection. Furthermore, in contrast to the safety profiles of other CD therapeutics, adverse effects were mild and transient in nature, with none necessitating dose adjustment. As such, this review supports the use of anti-MAP therapy for CD in a pediatric population.
**References:** [1] Feller M *et al. Lancet Infect Dis* 2007; 7:607-13. [2] Chamberlin W *et al. Expert Rev Clin Immunol* 2011; 7:751-60.
*Disclosure - Dr Borody - Unpaid member of Scientific Advisory Board: RedHill BioPharma Ms Finlayson - no Disclosures Ms Nowak - no Disclosures Ms Lin - no Disclosures.*

## 1714

**Risk of Non-Melanoma Skin Cancer in Ulcerative Colitis Patients Treated with Thiopurines: A Nationwide Retrospective Cohort**

**ACG IBD Research Award**

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**Purpose:** There is controversy and limited data on the risk of non-melanoma skin cancer (NMSC) among thiopurine-treated ulcerative colitis (UC) patients. Our aim is to assess the rate of NMSC by ongoing, residual, and cumulative exposure to thiopurines.
**Methods:** Nationwide data were obtained from the Veterans Affairs (VA) healthcare system 2001-2011. We performed a retrospective cohort study following patients from UC diagnosis to development of NMSC. Cox regression was used to determine the association between thiopurine use

**[1714] Table 1. Results of multivariate cox regression analysis with exposure to thiopurine as time dependent variable**

| | N | P_Y | Events | IR | HR | UCI | LCI | P |
|---|---|---|---|---|---|---|---|---|
| *Age Group* | | | | | | | | |
| <40 | 1997 | 13036 | 5 | 0.4 | | | | |
| 40–65 | 7605 | 52522 | 215 | 4.1 | 7.4 | 3.0 | 18.0 | <.0001 |
| >65 | 4925 | 37494 | 201 | 5.4 | 14.2 | 5.8 | 34.7 | <.0001 |
| *Sex* | | | | | | | | |
| F | 853 | 5695 | 9 | 1.6 | | | | |
| M | 13674 | 97358 | 412 | 4.2 | 2.0 | 1.1 | 4.0 | 0.0349 |
| *Race* | | | | | | | | |
| Non-Caucasian/ Unknown | 3287 | 23195 | 31 | 1.3 | | | | |
| Caucasian | 11240 | 79857 | 390 | 4.9 | 3.0 | 2.1 | 4.4 | <.0001 |
| *UV Zones* | | | | | | | | |
| Low - Medium Exposure | 6850 | 49721 | 169 | 3.4 | | | | |
| High Exposure | 7596 | 53332 | 252 | 4.7 | 1.4 | 1.2 | 1.7 | 0.0004 |
| *Rate VA Visits Categories* | | | | | | | | |
| <6/y | 5092 | 39646 | 38 | 1.0 | | | | |
| 6–12/y | 3818 | 27055 | 124 | 4.6 | 5.2 | 3.6 | 7.4 | <.0001 |
| >12/y | 5617 | 36351 | 259 | 7.1 | 8.4 | 6.0 | 11.9 | <.0001 |
| *Thiopurine* | | | | | | | | |
| Never Used, Before Using | 14527 | 84980 | 317 | 3.7 | | | | |
| During Using | 3346 | 9198 | 77 | 8.4 | 2.1 | 1.6 | 2.6 | <.0001 |
| After Stopping | 2152 | 8874 | 27 | 3.0 | 0.7 | 0.5 | 1.0 | 0.0666 |

Notes: Rate is per 1000 person-year, P-Y: Person-Years of follow-up, HR: Hazard Ratio, UCI and LCI: Upper and Lower limits of the 95% confidence interval respectively.*Total number of patients who contributed person-years to the Unexposed period were 11,181 patients who never used thiopurine and 3,346 patients who later on used thiopurine during the follow-up period. **only 2152 of the total thiopurine users (3346) had follow up time after stopping.

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## LETTER TO THE EDITOR

# Treatment of Crohn's disease patients with infliximab is detrimental for the survival of *Mycobacterium avium* ssp. *paratuberculosis* within macrophages and shows a remarkable decrease in the immunogenicity of mycobacterial proteins

*Dear Sir,*

The association between *Mycobacterium avium* ssp. *paratuberculosis* (MAP) and Crohn's disease (CD) although debatable, is supported by several studies[1] which have reported the detection or isolation of MAP from human tissues[2] including serum,[3] body fluids (breast milk),[4] and high levels of TNF-α was found secreted by the gut mucosa in MAP-associated CD patients.[5] Infliximab is a monoclonal antibody that specifically inhibits TNF-α and is used as a current therapy for CD.

Recently, Nakase et al.[6] demonstrated that THP-1 cells infected with MAP induced the production of a higher amount of TNF-α when compared to macrophages infected with either *Mycobacterium avium* or *Mycobacterium smegmatis*, suggesting that MAP is directly involved in the upregulation of this cytokine.

Previously, we have reported that MAP is able to infect, reside, and multiply intracellularly in human macrophages,[7,8] suggesting that the pathogen is able to subvert the host's immune response to avoid its own demise even at the earliest stage of the infection. Moreover, we have reported that CD patients,[8] but not healthy controls, have a significantly higher level of antibodies against two MAP proteins, a Protein tyrosine phosphatase (PtpA), and a Protein kinase (PknG). Both proteins are part of the signal transduction system of the bacterium, have been shown to be secreted within the host, and are essential for the intracellular survival and the establishment of a successful infection of the MAP's close relative *Mycobacterium tuberculosis*.[9,10] Therefore, to persist within the host, both proteins have to be secreted in a regular manner by the pathogen, in order to manipulate the immunological response elicited by macrophages. Recently, we have shown that CD patients possess a higher titer of antibodies against PtpA and PknG when compared to healthy controls,[8] and we suggested that both proteins can be used to determine the status of MAP in CD patients.

We report our analysis of the impact that infliximab upon the presence of antibodies against PtpA and PknG in sera of CD patients. A cohort of 20 CD patients treated with infliximab, 20 CD patients not treated with infliximab, and 20 healthy controls were enrolled in this study. 43.3% of the subjects were male with average ages for CD, infliximab, and healthy control groups being 41 ± 14, 33 ± 12, and 46 ± 18 years, respectively. In the CD and infliximab groups, the mean Harvey Bradshaw Index score was 5.2 ± 5.0 and 4.6 ± 4.5 and the mean time since diagnosis of CD was 10.4 ± 10.2 and 10.4 ± 8.2 years, respectively. The Research Ethics Board of the University of British Columbia, Vancouver, Canada, approved the protocol for this study. Protocols for blood collection, serum processing, antigen production and ELISA were followed as published.[8]

Patients treated with infliximab show a significant decrease in the level of antibodies against both MAP proteins, and had levels similar to the negative control (Fig. 1), suggesting that inhibition of TNF-α has an effect in the secretion of both mycobacterial proteins within CD patients. Next, we evaluated the survival of MAP in THP-1 cells treated with infliximab. Prior to the infection with MAP,[8] THP-1 cells were differentiated with phorbol 12-myristate 13-acetate and exposed to infliximab (5 μg/mL) for 4 and

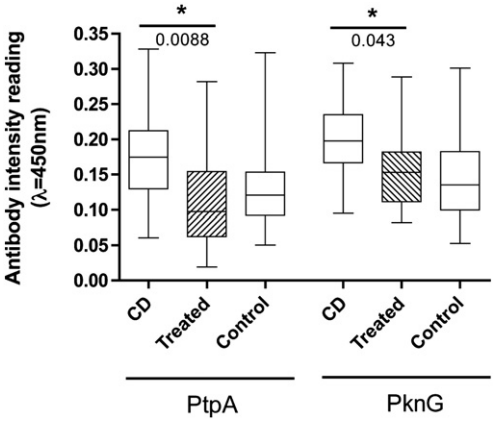


**Figure 1** Distribution of the antibody intensity readings. A boxplot analysis shows the distribution of the antibody intensity readings obtained by ELISA in CD patients, CD patients under treatment with infliximab, and healthy controls ($n$ = 20 individuals in each category). *P-value obtained using Wilconox rank sum test. CD = Crohn's disease; PtpA = protein tyrosine phosphatase; PknG = protein kinase G. Experiments were performed in triplicate.

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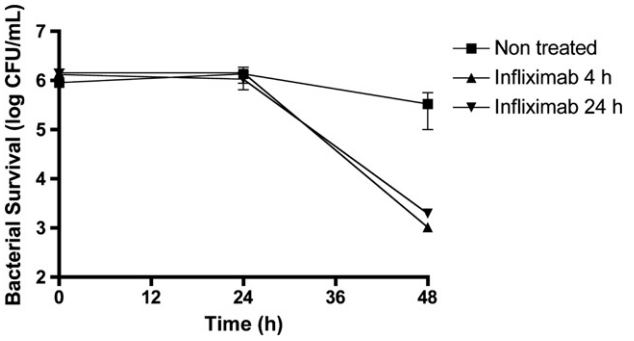


**Figure 2** Survival of MAP in infected human macrophages THP-1 cell lines treated with infliximab (5 μg/mL) for 4 h (triangle) and 24 h (inverted triangle). Non-treated cells (square) were used as control. Shown are the mean values (±SD) of three independent experiments. CFU = Colony Forming Unit.

24 h. Macrophages infected with MAP were collected and processed as published.[8] Interestingly, we observed a significant decrease in the survival of MAP when macrophages were exposed to infliximab (Fig. 2), suggesting that the suppression of TNF-α was detrimental to the intracellular survival of MAP. We found no difference in the survival of MAP between different times of infliximab exposure to macrophages prior to the infection.

In conclusion, our findings suggest that infliximab treatment results in: (i) a decrease in the antibody titer of two mycobacterial antigens that are essential for the establishment of an infection, and (ii) a decrease in the survival of the bacterium within human macrophages. Both observations imply that in CD patients, a suppression of TNF-α leads to the activation of other immunological pathways in macrophages, which suppresses the growth of MAP in these patients. Our findings together with the study reported by Nakase et al.[6] demonstrate that the regulation of specific cytokines, such as TNF-α, is critical for the survival of MAP within macrophages. Based on the accumulated information related to the increase of TNF-α production in infections associated with MAP, a remaining question is: why is it beneficial for MAP to induce TNF-α production upon infection? More studies related to the balance of cytokines and their link to MAP survival are necessary to understand the pathophysiology of CD.

## Conflict of interest

None.

## Acknowledgments

We thank Jeffrey Helm for helpful discussions.

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# On the Action of Methotrexate and 6-Mercaptopurine on *M. avium* Subspecies *paratuberculosis*

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***Background.*** **Clinical improvement in inflammatory bowel disease (IBD) treated with methotrexate and 6-mercaptopurine (6-MP) is associated with a decrease in pro-inflammatory cytokines. This has been presumed to indicate the mechanism of action of methotrexate and 6-MP. Although controversial, there are increasingly compelling data that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) may be an etiological agent in some or all of IBD. We hypothesized that the clinical efficacy of methotrexate and 6-MP in IBD may be to simply inhibit the growth of MAP. *Methodology*. The effect on MAP growth kinetics by methotrexate and 6-MP were evaluated in cell culture of two strains each of MAP and *M. avium* using a radiometric ($^{14}CO_2$ BACTEC®) detection system that quantifies mycobacterial growth as arbitrary "growth index units" (GI). Efficacy data are presented as "percent decrease in cumulative GI" (% $-\Delta$cGI). *Principal Findings*. The positive control antibiotic (clarithromycin) has ≥85% $-\Delta$cGI at a concentration of 0.5 μg/ml. The negative control (ampicillin) has minimal inhibition at 64 μg/ml. MAP ATCC 19698 shows ≥80% $-\Delta$cGI for both agents by 4 μg/ml. With the other three isolates, although more effective than ampicillin, 6-MP is consistently less effective than methotrexate. *Conclusions*. We show that methotrexate and 6-MP inhibit MAP growth *in vitro*. Each of the four isolates manifests different % $-\Delta$cGI. These data are compatible with the hypothesis that the clinical improvement in patients with IBD treated with methotrexate and 6-MP could be due to treating a MAP infection. The decrease in pro-inflammatory cytokines, thought to be the primary mechanism of action, may simply be a normal, secondary, physiological response. We conclude that henceforth, in clinical studies that evaluate the effect of anti-MAP agents in IBD, the use of methotrexate and 6-MP should be excluded from any control groups.**

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## INTRODUCTION

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes Johne's disease [1] in cattle worldwide. Johne's disease is clinically evocative of inflammatory bowel disease (IBD) in humans. The possibility that MAP may be zoonotic [2] is the subject of much interest [3] [4] (& see [5] for review.) Since first seeing our MAP RNA data [6], we have posited in private, in peer reviewing manuscripts [3,7,8], and at professional congresses that MAP is the primary and most culpable, potential etiological agent for some or all of IBD.

In the therapy of IBD, (and several other inflammatory diseases) both methotrexate and 6-MP are used because of empirical efficacy, even though their precise mechanism of action is unknown. [9] [10,11] Their use is attended by clinical improvement that is associated with a decrease in pro-inflammatory cytokines. Consequently, prevailing medical dogma posits that the mode of action of methotrexate and 6-MP, is to decrease the production of pro-inflammatory cytokines, and as a consequence the "inflammatory" response that is the consequence of these cytokines is diminished. This results in a clinical improvement in diseases that are conventionally conceptualized as being primarily "inflammatory."

Both methotrexate and 6-MP interfere with DNA replication. Methotrexate acts by inhibiting dihydrofolate reductase, folate generation and the consequent production of adenine.[12] The mechanism of action of 6-MP is to substitute for guanine in DNA replication.[12] Because prokaryotes must synthesize their own folic acid, they should be more susceptible to folate inhibition than eukaryotes. It is noteworthy that there are two distinct doses in human clinical use for both methotrexate and 6-MP. Each agent has a "high" dose, (used in to treat reticuloendothelial malignancies [13,14]) and a "low" dose (used to treat "inflammatory" diseases. [15] [16])

We hypothesized that the clinically relevant mechanism of action of "low" dose methotrexate and 6-MP in the therapy of IBD, may, in whole or part, be due to the inhibition of MAP growth. If this hypothesis is correct, the decrease in the pro-inflammatory cytokines, heretofore considered the primary mechanism of action of these two agents, could simply represent a secondary phenomenon. The observed decrease in pro-inflammatory cytokines could be ascribed to the treatment of the instigating MAP infection. To evaluate this hypothesis we have studied the effect of methotrexate and 6-MP on MAP and other *M. avium* isolates in culture. The effect of methotrexate has been evaluated on *E. coli* [17], 6-MP on *Salmonella typhimurium* [18] and

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**Competing Interests:** The corresponding author (RJG) has submitted provisional patent applications based on the hypotheses that were tested in this study. The final author (STB) was a member of the National Academy of Sciences panel that issued the 2003 report entitled "Diagnosis and Control of Johne's Disease".

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**Table 1.** Differences in growth kinetics and consequent length of experiment for each isolate.

| | MAP | | | *M. avium* | |
|---|---|---|---|---|---|
| | ATCC 19698 (Fig 1) | ATCC 19698 (Fig 2) | Dominic (Fig 2) | ATCC 25291 (Fig 2) | 101 (Fig 2) |
| GI at harvest | 526 | 523 | 548 | 669 | 267 |
| Harvested # CFU's/ml | $8.1\times10^5$ | $8.2\times10^5$ | $6.3\times10^5$ | $9.1\times10^6$ | $1.2\times10^6$ |
| # CFU's Inoculated | 20,250 | 20,500 | 15,750 | 910 | 120 |
| Days to reach GI "999" | 12 | 13 | 17 | 7 | 5 |

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both agents on *M. tb*. [19] To our knowledge, however, this is the first time that these two agents have been evaluated for their effect on MAP.

## METHODS

This study was approved by the Research & Development Committee at the VAMC Bronx NY (0720-06-038) and was conducted under the Institutional Radioactive Materials Permit (#31-00636-07).

### Culture

In this study, we used four well-characterized strains of mycobacteria. Two were MAP, a bovine isolate, ATCC 19698 (ATCC Rockville MD) and "Dominic" a human isolate from an individual with Crohn's disease (originally isolated by R. Chiodini [20].) The *M. Avium* subspecies *avium* strains (hereinafter called *M. avium*) were ATCC 25291 (veterinary source) and *M. avium* 101 [21]. Primary cultures were performed in Middlebrook 7H9 medium supplemented 9:1 with ADC (Both Difco. Detroit MI). All media for MAP (liquid and agar plates) were supplemented with 1 μg/ml Mycobactin J (Allied Monitor. Fayette MO.)

The detergent Tween 80 (recommended to prevent mycobacterial clumping) renders clinically resistant strains of MAP inappropriately susceptible to antimicrobials in cell culture. [22] Accordingly in our experiments, Tween 80 was not used. To minimize mycobacterial clumping, one ml of the log phase bacterial culture (~GI of 500) in 7H12 medium in the BACTEC vial was passaged ≥20 times through a 25 gauge needle [23] on a one ml syringe (Becton-Dickerson Franklin Lakes NJ), added to five ml 7H9 medium supplemented with Mycobactin J, vortexed and left standing at ambient temperature for 30 minutes. Subsequently, only the upper three of the six ml. were used for inoculations. The number of CFU's inoculated was determined by plating serial dilutions of the inoculum onto 7H10 plates (Difco) supplemented for MAP with Mycobactin J (1 μg/ml) and counted when colonies were easily visualized (two to six weeks). At the time of passage, additional aliquots were plated onto blood agar plates to ensure that inocula were not contaminated with non-mycobacterial organisms.

To confirm the identity of the species studied, DNA was obtained from the isolates (High Pure Template Prep. Kit Roche Diagnostics Indianapolis IN), PCR amplified using primers for IS 900 (MAP) [24]and IS 901/2 [25] (*M. avium* & specific subspecies including *silveticum*) as described. [6] Amplicon sizes were determined using 1% agarose gel electrophoresis. DNA sequence analysis was performed commercially, (Genewiz North Brunswick NJ) with sequence comparison performed using BLAST (NLM).

The positive antibiotic control clarithromycin (gift of Abbott Chicago IL) was dissolved in methanol. The negative control

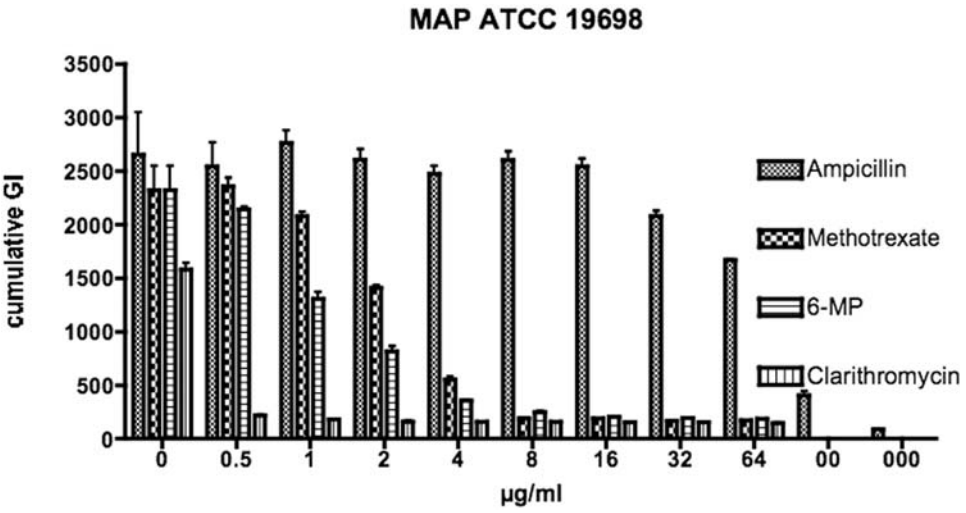


**Figure 1.** Shown is a bar graph of the cumulative GI data evaluating MAP ATCC 19698. Each drug dilution was studied in duplicate. Error bars are SD. There are three control inoculations, labeled on the abscissa as "0, 00 & 000. The left hand "0" had an equal number of CFU's as in each drug studied. "00" = 10 & "000" = 100 fold dilutions. In each control the maximum concentration of diluent used for each agent (methanol for Clarithromycin, water for ampicillin and NaOH for methotrexate and 6-MP) was added. Clarithromycin is most effective and ampicillin is the least effective at decreasing MAP growth. Both methotrexate and 6-MP are as effective as clarithromycin in MAP %−ΔcGI at a dose of 4 μg/ml.
doi:10.1371/journal.pone.0000161.g001

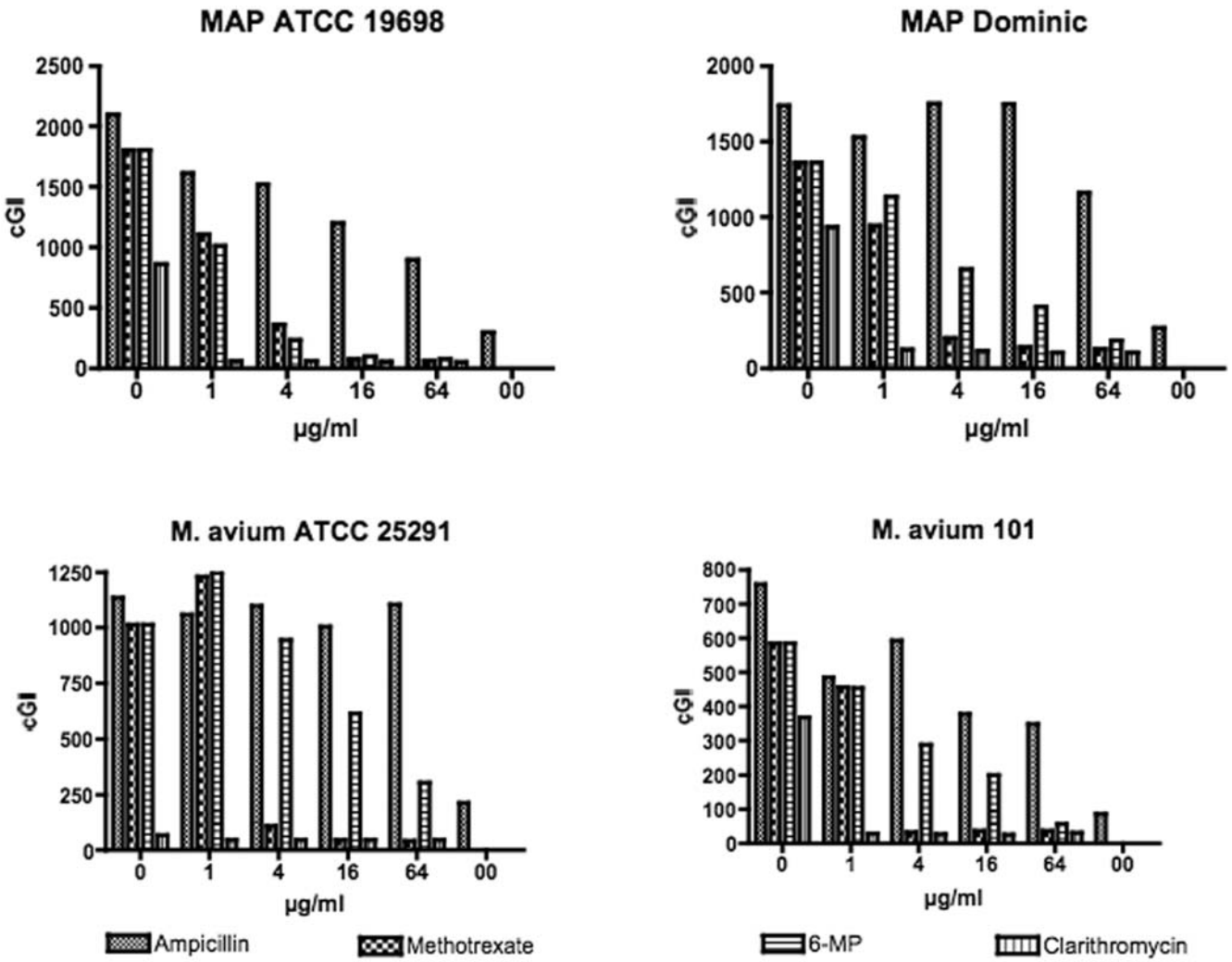


**Figure 2.** A composite graph of the four bacterial strains studied. "0" is diluent control with an equal CFU inoculum and "00" is a 1:10 dilution of the water control inoculum. Drug concentrations are indicated on the abscissa. For each isolate, drug dose was studied in singlicate. For all four bacteria, clarithromycin has achieved maximal inhibition by 1 µg/ml. For MAP ATCC 19698, replicating data presented in Figure 1, both methotrexate and 6-MP %−ΔGI is the same as for clarithromycin by 4 µg/ml. Note that with the other MAP isolate (Dominic) and both *M. avium* isolates, methotrexate is more effective at a lower dose than is 6-MP. The lower cumulative GI (seen on the ordinate) for the *M. avium* isolates is ascribable to their more rapid growth and earlier reaching the off scale BACTEC GI values of "999."
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antibiotic was ampicillin (Sigma St Louis MO) which was dissolved in water. Methotrexate and 6-mercaptopurine (both Sigma) were dissolved in NaOH at a maximal final concentration of 50 mM (Sigma). Control inocula were performed using the maximum concentration of each diluent. Agents were tested in serial dilutions from a minimum of 0.05 µg/ml to a maximum of 64 µg/ml (see individual Figures). Aliquots of chemicals being evaluated were prediluted, stored at −80°C, thawed, used once and discarded.

Data are presented as cumulative growth index (cGI) units±SD (when necessary, see individual figures). The effect (or lack thereof) of each agent is presented as the percent decrease in cGI units (% −ΔcGI), compared to the control cGI of that isolate in the diluent (e.g. methanol or NaOH) that was used for the specific chemical being evaluated. cGI data for each experiment is presented until the day prior to any GI reading exceeding the assay limit of "999" (Table 1). Raw data was archived onto Excel, and the cumulative results transferred to Prism (Graphpad, San Diego CA) for final graphing.

## RESULTS

Bacterial quantification must be performed retrospectively. Accordingly, for experimental reproducibility, bacterial passage and harvesting were performed when the GI was ~500. Quantification show that the CFU's of the *M. avium* isolates are approximately 10 fold higher ($\sim 1\times10^7$ CFU's/ml), compared to MAP ($\sim 1\times10^6$ CFU's/ml) (Table 1). Because of the difference in growth kinetics, *M. avium* CFU #'s inoculated were ≥10 fold lower than MAP (Table 1).

Both of our MAP isolates (ATCC 19698 & Dominic) were Mycobactin J dependant (data not presented), were IS 900 positive and had ≥99% homology with the GenBank accession NC_002944 of MAP (data not presented). *M. avium* ATCC 25 291 was positive for IS 902 and *M. avium* 101 was negative for both (Data not presented).

In our pivotal study (Figure 1) the positive control antibiotic, clarithromycin exhibits ≥86% −ΔcGI at the lowest concentration evaluated (0.5 µg/ml). The negative control antibiotic (the

$\beta$−lactam, ampicillin) has a minimal effect (21% −ΔcGI) at the 32 μg/ml. In contrast, 6-MP has an initial ≥43% −ΔcGI starting at 1 μg/ml increasing to ≥84% −ΔcGI at 4 μg/ml. Methotrexate has 40% −ΔcGI inhibition at 2 μg /ml and ≥75% −ΔcGI at 4 μg/ml. (Figure 1.)

We additionally evaluated the effect of methotrexate and 6-MP against two MAP and two *M. avium* isolates (Figure 2). In these studies, the MAP 19698 results replicate the data presented in Figure 1 showing ~80% −ΔcGI inhibition at 4 μg/ml for both 6-MP and clarithromycin. In contrast, MAP Dominic shows less susceptibility to 6-MP (41% −ΔcGI at 4 μg/ml) compared to MAP 19698 (84%−ΔGI at 4 μg/ml). Both *M. avium* isolates show less susceptibility to 6-MP than to methotrexate (Figure 2). The diluent control inoculum for the *M. avium* ATCC 25291 appears to exhibit completely inhibited growth (Figure 2: Bottom left graph, left hand column.) However, over the following two days this methanol control entered log phase growth, whereas the vials at every clarithromycin dose continued to show no evidence of growth (data not presented.)

## DISCUSSION

The efficacy of both methotrexate and 6-MP in the therapy of IBD is uncontested. Prevailing dogma accepts that the decrease in pro-inflammatory cytokines that attends their use is responsible for their beneficial effect. In this study we show that both methotrexate and 6-MP inhibit the growth kinetics of MAP. In the event that IBD is eventually accepted as being due to a MAP infection, our data are compatible with our hypothesis that methotrexate and 6-MP may be impairing MAP growth. If so, the decrease in pro-inflammatory cytokines could simply be an appropriate physiological response to their antibiotic-like activity.

We additionally show that there is a variation in response of the four different isolates to our tested agents. Three of the four isolates are more sensitive to methotrexate than to 6-MP. These observations need to be further evaluated in multiple isolates from a variety of individuals and clinical settings where development of MAP resistance may be responsible for a clinical deterioration. We conclude that antibiotic susceptibility testing will probably be indicated for putative MAP infections, just as they are for other (myco) bacterial infections.

As is conventional with antibiotic susceptibility studies, we compared agents on an equal weight basis. However, methotrexate (MW 450) is a much larger molecule than 6-MP (MW 170) with a molar ratio of ~3:1. Thus, in comparison to 6-MP on a molar basis, methotrexate is an even more potent inhibitor of growth than our data indicate. Additionally, a simple extrapolation of our data to a comparison with conventional "antibiotics" therapy is difficult. The dosages of methotrexate and 6-MP in clinical use have not been titrated according to standard antibiotic conventions. Dosage has been individualized, influenced by such factors as hematological toxicity and patient tolerance.

There is a remarkable discrepancy between the doses of methotrexate and 6-MP, used to treat different diseases, that merit discussion. Each agent has a "high" dose, (used in to treat reticuloendothelial malignancies [13,14]) and a "low" dose (used to treat "inflammatory" diseases. [15] [16]) For methotrexate the antineoplastic dose may be 1500–5000 mg $M^2$ by IV infusion over 2–24 hours (for a 70 kg man this could equate to 7500 mg in 24 hours.)[26] In contrast, for IBD a typical dose is 25 mg PO or IM weekly. We suggest that this 300-fold disparity may reflect the difference between treating a eukaryotic reticuloendothelial malignancy and a prokaryotic mycobacterial (specifically we hypothesize in the case of IBD a MAP) infection.

Our data are compatible with the thesis, but do not conclusively prove, that MAP may be zoonotic. Corroboration of our data will be necessary and multiple additional studies, both basic and clinical need to be performed. However, we suggest that, as a consequence of our observations antecedent clinical studies that have evaluated anti-MAP agents need to be reevaluated and that henceforth such studies will need to exclude methotrexate and 6-MP from "control" or placebo subjects.

### Conclusions and recommendations

We show that both methotrexate, as well as 6-MP, interfere with the growth of MAP, an organism that may be the etiological factor for some, or all of IBD. Some of the implications of these observations are discussed.

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### Author Contributions

Conceived and designed the experiments: RG SB. Performed the experiments: RG LS. Analyzed the data: RG LS VH AS SB. Contributed reagents/materials/analysis tools: RG VH AS SB. Wrote the paper: RG SB.

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# On the Action of Cyclosporine A, Rapamycin and Tacrolimus on *M. avium* Including Subspecies *paratuberculosis*

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## Abstract

***Background:*** *Mycobacterium avium* subspecies *paratuberculosis* (MAP) may be zoonotic. Recently the "immuno-modulators" methotrexate, azathioprine and 6-MP and the "anti-inflammatory" 5-ASA have been shown to inhibit MAP growth *in vitro*. We concluded that their most plausible mechanism of action is as antiMAP antibiotics. The "immunosuppressants" Cyclosporine A, Rapamycin and Tacrolimus (FK 506) treat a variety of "autoimmune" and "inflammatory" diseases. Rapamycin and Tacrolimus are macrolides. We hypothesized that their mode of action may simply be to inhibit MAP growth.

***Methodology:*** The effect on radiometric MAP $^{14}CO_2$ growth kinetics of Cyclosporine A, Rapamycin and Tacrolimus on MAP cultured from humans (Dominic & UCF 4) or ruminants (ATCC 19698 & 303) and *M. avium* subspecies *avium* (ATCC 25291 & 101) are presented as "percent decrease in cumulative GI" (%-ΔcGI.)

***Principal Findings:*** The positive control clofazimine has 99%-ΔcGI at 0.5 μg/ml (Dominic). Phthalimide, a negative control has no dose dependent inhibition on any strain. Against MAP there is dose dependent inhibition by the immunosuppressants. Cyclosporine has 97%-ΔcGI by 32 μg/ml (Dominic), Rapamycin has 74%-ΔcGI by 64 μg/ml (UCF 4) and Tacrolimus 43%-ΔcGI by 64 μg/ml (UCF 4)

***Conclusions:*** We show heretofore-undescribed inhibition of MAP growth *in vitro* by "immunosuppressants;" the cyclic undecapeptide Cyclosporine A, and the macrolides Rapamycin and Tacrolimus. These data are compatible with our thesis that, unknowingly, the medical profession has been treating MAP infections since 1942 when 5-ASA and subsequently azathioprine, 6-MP and methotrexate were introduced in the therapy of some "autoimmune" and "inflammatory" diseases.

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**Competing Interests:** STB was a member of the National Academy of Sciences of the USA pannel that issued the Report "Diagosis and Control of Jhone's Disease ISBN 0-309-08611-6. RJG has submitted provisional patents based on the hypotheses tested in this and prior publications. RJ is President of the International Paratuberculosis Association.

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## Introduction

The "immunosuppressants" Cyclosporine A [1], Rapamycin [2] and Tacrolimus (FK 506) [3] have conventionally been used to prevent or treat the rejection of transplanted organs.[4–8] They have well described mechanisms of actions [9,10] including calcineurin phosphatase inhibition by Cyclosporine and Tacrolimus and cell cycle inhibition by Rapamycin.[11] These agents are also used in the therapy of a variety of "autoimmune" and "inflammatory" diseases including inflammatory bowl disease (IBD) [12–19], skin diseases [20], asthma [21] and rheumatoid arthritis.[22,23] Generally, the effect of these immunosuppressants has been studied on intact animals or eukaryotic cells, although the effect on viruses has been addressed.[24]

*M. avium* subspecies *paratuberculosis* (MAP) causes a chronic wasting enteritis in ruminants called Johne's disease [25] that is highly evocative of Crohn's disease (CD.) [26] MAP has been cultured from USA chlorinated potable municipal water [27], pasteurized milk in the USA [28], and Europe [29] [30], breast milk of mothers with CD [31] and from the blood of patients with IBD. [32] Although controversial, there are increasingly compelling data [27,32–35] (& see [36] for review) that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) may be zoonotic. [33]

Until recently, it was unrecognized that the "anti-inflammatory" 5 amino salicylic acid (5-ASA) [37] and the "immune modulators" methotrexate [34], azathioprine [38] and its metabolite 6-mercapto-purine (6-MP) [34], [38] are antiMAP antibiotics. Antecedent studies evaluating the potential zoonotic character of MAP had permitted these "anti-inflammatory" and "immune-modulating" agents to be used in the control groups, as their antiMAP activity was not appreciated. We therefore concluded that all those prior studies now need to be reevaluated,

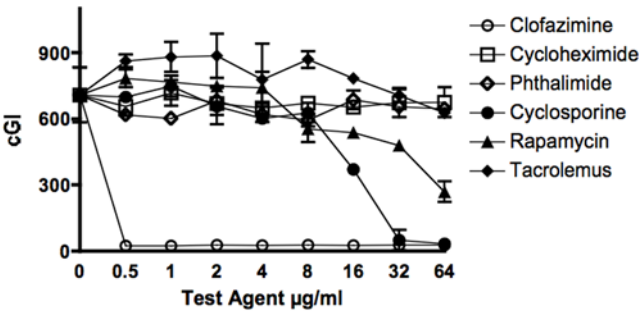


**Figure 1. Shown are the inhibition data for a study employing MAP Dominic.** The agents evaluated are Cyclosporine, Rapamycin and Tacrolimus. Of these three agents, the most pronounced inhibition is observed with Cyclosporine (see also Table 4.) Error bars are ±SD. cGI = cumulative Growth Index (Bactec®) The GI was 240 at the time of passage.
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as their control groups were not placebo. [34,37] Nevertheless, prevailing medical dogma [39] considers that MAP is not zoonotic.

It is of considerable interest that all three "immunosuppressants" were isolated from fungi, the source of multiple antibacterial antibiotics. Cyclosporine A, a cyclic undecapeptide, has immunosuppressant, anti-rheumatic [40,41], dermatological [42] and anti-asthmatic [21] activity. Tacrolimus [3,20] and Rapamycin [2,20] are from the macrolide antibiotic family of medications, amongst the most potent anti *M. avium* antibiotic families. [43]

We hypothesized that in addition to their protean effects on eukaryotes [9–11,44–46], and fungi [47], Cyclosporine A, Rapamycin and Tacrolimus, may also effect prokaryotes. Specifically we hypothesized that they would have antiMAP antibiotic activity. Accordingly, in bacterial culture we evaluate the effect of Cyclosporine A, Rapamycin and Tacrolimus on *M. avium*, including its subspecies MAP.

## Methods

This study was approved by the Research & Development Committee at the VAMC Bronx NY (0720-06-038) and was conducted under the Institutional Radioactive Materials Permit (#31-00636-07).

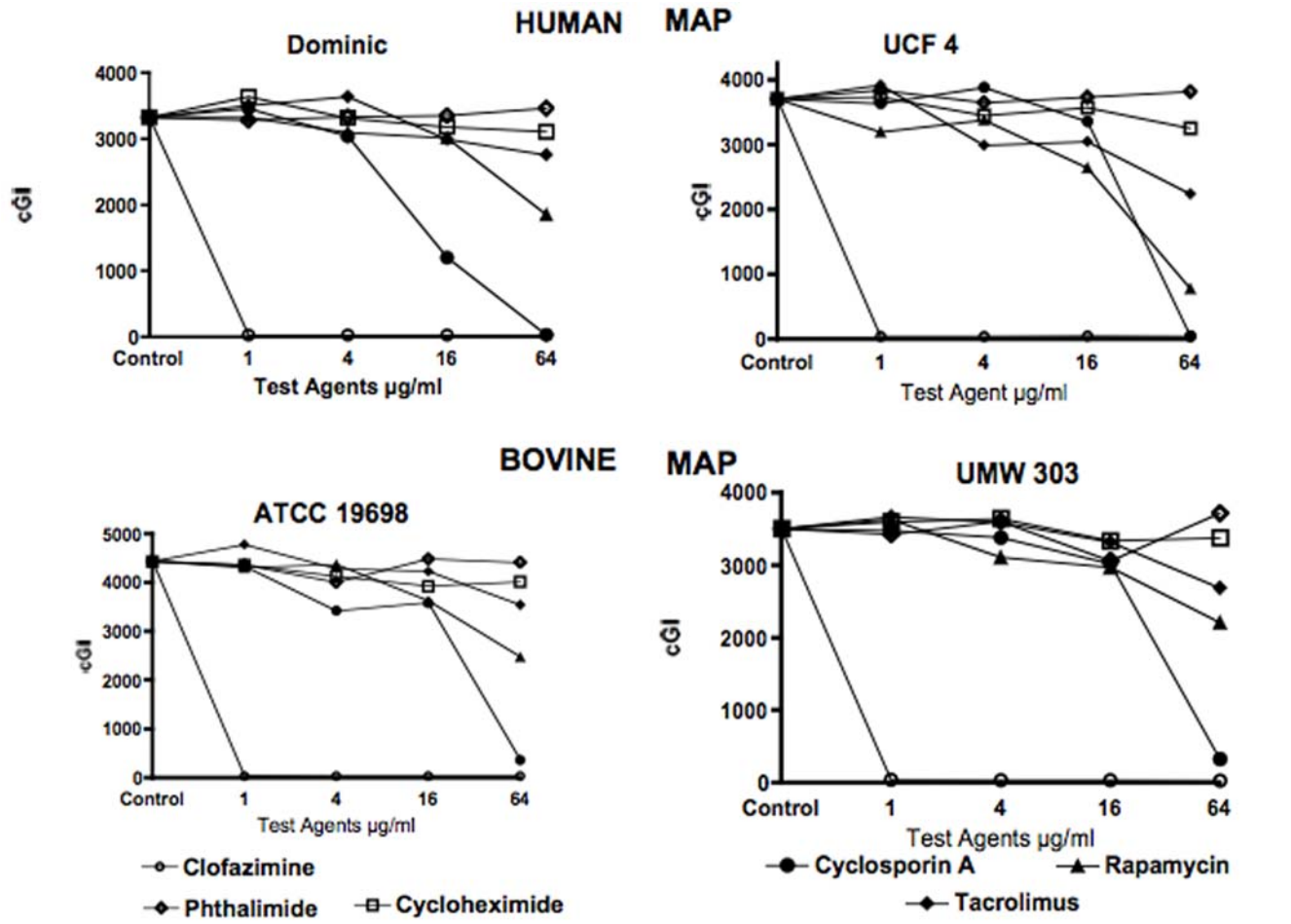


**Figure 2. Shown is a composite of four MAP strains.** The upper two are MAP isolated from humans, the lower two, MAP isolated from ruminants. "UMW 303" is University of Madison Wisconsin. UCF -4 is University of Central Florida. Note how cyclosporine is consistently the most effective of the three "immunosuppressants" tested (see also Table 5) followed by Rapamycin. The least effective of the three macrolides is Tacrolimus. cGI = cumulative Growth Index (Bactec®) For Dominic the GI was 331 at the time of passage.
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## Bacterial Culture

In this study we studied six strains of mycobacteria, four of which were MAP. Two MAP strains had been isolated from humans with Crohn's disease. Dominic (ATCC 43545, originally isolated by R. Chiodini from the intestine of a patient with Crohn's disease [48]) and UCF 4 (gift of Saleh Naser UCF Orlando FL., originally cultured from the blood of a patient with Crohn's disease.)[32] The other two MAP strains were from ruminants with Johne's disease ATCC 19698 (ATCC Rockville MD) and 303 (gift of Michael Collins Madison WI.) The *M. avium* subspecies *avium* strains (hereinafter called *M. avium*) were ATCC 25291 (veterinary source) and *M. avium* 101. [49]

Because it renders clinically resistant strains of MAP inappropriately susceptible to antimicrobials in cell culture, [50] we did not use the detergent Tween 80 (recommended to prevent mycobacterial clumping) in culture. Prior to inoculation, cultures were processed as described. [34,37,51]

In this study, for experimental comparability we used chemicals that could be solubilized with DMSO (Sigma St Louis MO.) The positive control antibiotic was clofazimine (an antibiotic used to treat leprosy [52] and now in clinical trials against Crohn's disease [39,53].) The two negative controls are the gluterimide antibiotics, cycloheximide and phthalimide.

The tested agents Cyclosporine A, Rapamycin and Tacrolimus (Sigma & LC Labs. Woburn MA) were solubilized in 100% DMSO. Aliquots were prediluted, stored at −80°C in 50% DMSO (Sigma) & 50% water, thawed, used once and discarded. Volumes of DMSO were adjusted so that final concentration in every Bactec vial used was always 3.2% DMSO. Agents were tested in serial dilutions from a minimum of 0.5 μg/ml to a maximum of 64 μg/ml (see individual Figures & Tables). Inhibition of mycobacterial growth is expressed as % -ΔcGI, and enhancement as % +ΔcGI compared to 3.2% DMSO controls. [37]

Data are presented in two ways: For individual mycobacterial strains as graphs (MAP in Figures 1& 2, and *M. avium* in Figure 3.) For individual chemical agents data are presented in tabular form. The positive experimental control is clofazimine (Table 1.) The "negative" controls are cycloheximide (Table 2) and phthalimide (Table 3.) Data for the "immunosuppressives" are Cyclosporine A (Table 4), Rapamycin (Table 5) and Tacrolimus (Table 6.)

In Table 7 we present the "High" trough doses of the three immunosuppressives that are used to treat organ transplant rejection in eukaryotes. These are compared with the "Low" dose that are used to treat "inflammatory" diseases and that we posit are actually treating a prokaryote (specifically we suggest a MAP) infection.

## Results

The most potent positive control is clofazimine, 97% −ΔcGI at 0.5 (Dominic; Figure 1 & Table 1.) The negative controls chemical agents are the gluterimide antibiotics cycloheximide and phthalimide. Cycloheximide has no dose dependent inhibition on any MAP strain (Figures 1 & 2 & Table 2.) Cycloheximide has dose dependent inhibition on *M. avium* ATCC 25291, (57% −ΔcGI at 64 μg/ml) but no effect on *M. avium* 101 (Figure 3 & Table 2.) Phthalimide, has no dose dependent effect on any strain tested (Figures 1–3 and Table 3.)

The three "Immunosuppressants" tested were Cyclosporine A, Rapamycin and Tacrolimus. There are differing amounts of inhibition depending on the agent and strain.

The control mycobacterial strains are *M. avium* subspecies *avium* ATCC 25291 and 101. Of the three "Immunosuppressants,"

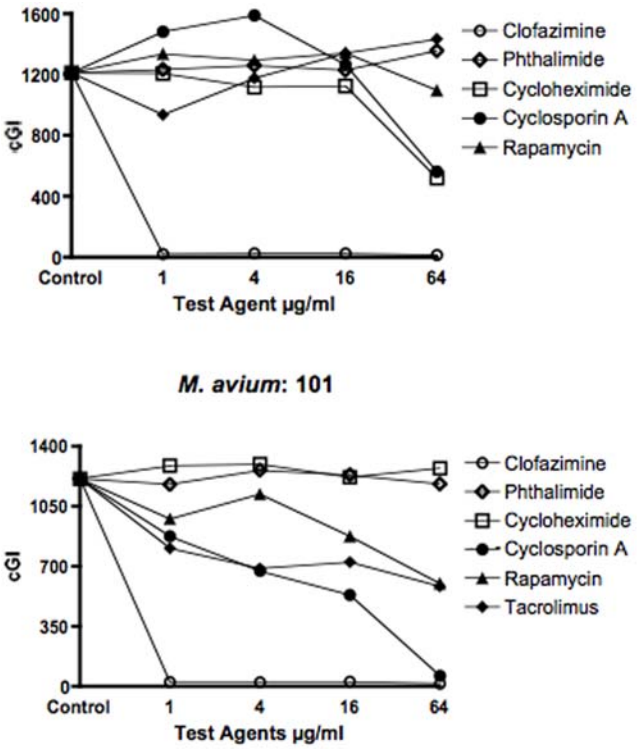


**Figure 3. Shown is a composite of two *M. avium* subspecies *avium* strains ATCC 25291 & 101.** Tacrolimus has most inhibition on *M. avium* 101 but enhances growth on *M. avium* ATCC 25291 cGI = cumulative Growth Index (Bactec®).
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Cyclosporine A has dose dependent inhibition on *M. avium* subspecies *avium* 101 (95% −ΔcGI at 64 μg/ml) (Figure 3 and Table 4.) There is no inhibition with Rapamycin or Tacrolimus on the control *M. avium* 25291 (Figure 3 and Table 5 & 6.)

Against MAP, Cyclosporine A is the most effective of the three "immunosuppressants" studied. On MAP isolated from humans, (Dominic and UCF 4), Cyclosporine has 97% −ΔcGI at 32 μg/ml against Dominic (Figure 1) and 99% −ΔcGI at 64 μg/ml on Dominic and UCF 4 (Figure 2 & Table 4.) On MAP isolated from ruminants, Cyclosporine A has slightly less dose dependent

**Table 1.** %-ΔcGI Clofazimine.

| μg/ml | Mycobacterial strain | | | | | | |
|---|---|---|---|---|---|---|---|
| | M. avium subspecies paratuberculosis (MAP) | | | | | M. avium | |
| | Human MAP | | | Bovine MAP | | Bovine | |
| | Dominic | | UCF 4 | 303 | 19698 | 25291 | 101 |
| | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 3 | Fig. 3 |
| 1 | −99% | −99% | −99% | −99% | −99% | −98% | −98% |
| 4 | −99% | −99% | −99% | −99% | −99% | −98% | −98% |
| 16 | −99% | −99% | −99% | −99% | −99% | −98% | −98% |
| 64 | −99% | −99% | −99% | −99% | −99% | −99% | −99% |

%-ΔcGI = percent decrease in cumulative GI compared to control inoculation.
doi:10.1371/journal.pone.0002496.t001

**Table 2.** %-ΔcGI Cycloheximide.

| μg/ml | Mycobacterial strain | | | | | | |
|---|---|---|---|---|---|---|---|
| | M. avium subspecies paratuberculosis (MAP) | | | | | M. avium | |
| | Human MAP | | | Bovine MAP | | Bovine | |
| | Dominic | | UCF 4 | 303 | 19698 | 25291 | 101 |
| | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 3 | Fig. 3 |
| 1 | 15% | 9% | 1% | 3% | −2% | 0% | 6% |
| 4 | −4% | 0% | −7% | 4% | −7% | −8% | 7% |
| 16 | −8% | −4% | −4% | −5% | −12% | −7% | 1% |
| 64 | −1% | −7% | −12% | −4% | −9% | −57% | 5% |

%-ΔcGI = percent decrease in cumulative GI compared to control inoculation.
doi:10.1371/journal.pone.0002496.t002

**Table 3.** %-ΔcGI Phthalimide.

| μg/ml | Mycobacterial strain | | | | | | |
|---|---|---|---|---|---|---|---|
| | M. avium subspecies paratuberculosis (MAP) | | | | | M. avium | |
| | Human MAP | | | Bovine MAP | | Bovine | |
| | Dominic | | UCF 4 | 303 | 19698 | 25291 | 101 |
| | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 3 | Fig. 3 |
| 1 | −3% | −1% | 4% | −2% | −2% | 2% | −3% |
| 4 | 7% | 0% | −2% | 3% | −10% | 4% | 4% |
| 16 | 0% | 1% | 1% | −13% | 1% | 1% | 2% |
| 64 | 2% | 4% | 3% | 6% | 0% | 12% | −2% |

%-ΔcGI = percent decrease in cumulative GI compared to control inoculation.
doi:10.1371/journal.pone.0002496.t003

**Table 4.** %-ΔcGI Cyclosporine A.

| μg/ml | Mycobacterial strain | | | | | | |
|---|---|---|---|---|---|---|---|
| | M. avium subspecies paratuberculosis (MAP) | | | | | M. avium | |
| | Human MAP | | | Bovine MAP | | Bovine | |
| | Dominic | | UCF 4 | 303 | 19698 | 25291 | 101 |
| | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 3 | Fig. 3 |
| 1 | 15% | 4% | −2% | −1% | −2% | 22% | −28% |
| 4 | −10% | −9% | 5% | −3% | −23% | 31% | −44% |
| 16 | −43% | −64% | −9% | −14% | −19% | 4% | −56% |
| 64 | −98% | −99% | −99% | −91% | −92% | −54% | −95% |

%-ΔcGI = percent decrease in cumulative GI compared to control inoculation.
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inhibition (ATCC 19698: 92% −ΔcGI at 64 μg/ml) than against MAP isolated from humans (Figure 2 & Table 4.)

Rapamycin is the second most effective "immunosuppressant" studied. At lower concentrations (1 & 16 μg/ml) Rapamycin has no inhibition and by 64 μg it has 76% −ΔcGI on UCF 4, a MAP isolated from humans (Figure 2 & Table 5). Rapamycin is less effective against MAP isolated from ruminants and has no effect on *M. avium* ATCC 25291 (Figure 3 & Table 5.)

**Table 5.** %-ΔcGI Rapamycin.

| μg/ml | Mycobacterial strain | | | | | | |
|---|---|---|---|---|---|---|---|
| | M. avium subspecies paratuberculosis (MAP) | | | | | M. avium | |
| | Human MAP | | | Bovine MAP | | Bovine | |
| | Dominic | | UCF 4 | 303 | 19698 | 25291 | 101 |
| | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 3 | Fig. 3 |
| 1 | 21% | 0% | −14% | 4% | −3% | 10% | −19% |
| 4 | 13% | −7% | −9% | −11% | −1% | 7% | −7% |
| 16 | −10% | −9% | −29% | −15% | −18% | 11% | −28% |
| 64 | −58% | −44% | −76% | −39% | −43% | −18% | −39% |

%-ΔcGI = percent decrease in cumulative GI compared to control inoculation.
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**Table 6.** %-ΔcGI Tacrolimus.

| μg/ml | Mycobacterial strain | | | | | | |
|---|---|---|---|---|---|---|---|
| | M. avium subspecies paratuberculosis (MAP) | | | | | M. avium | |
| | Human MAP | | | Bovine MAP | | Bovine | |
| | Dominic | | UCF 4 | 303 | 19698 | 25291 | 101 |
| | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 3 | Fig. 3 |
| 1 | 28% | 5% | 6% | 5% | 8% | −23% | −34% |
| 4 | 9% | 9% | −19% | 3% | −3% | −3% | −43% |
| 16 | 21% | −10% | −18% | −5% | −5% | 11% | −40% |
| 64 | 0% | −21% | −43% | −27% | −26% | 53% | −52% |

%-ΔcGI = percent decrease in cumulative GI compared to control inoculation.
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**Table 7.** Immunosuppressant Therapeutic Trough levels used in "High" dose for transplantation rejection and "Low" dose in "Inflammatory" Diseases.

| Medication | Targeted Trough levels (ng/ml) | | | |
|---|---|---|---|---|
| | "High" level for Organ Transplant Rejection | | "Low" level in "Inflammatory" Diseases | |
| | ng/ml | Citation | ng/ml | Citation |
| Cyclosporine A | 100–400 | [63] | 70–130 | [14] |
| | 396 | [64] | 100–200 | [12] |
| | 350–400 | [65] | | |
| Tacrolimus (FK 506) | 5–20 | [63] | 4–8 | [67] |
| | 17–18 | [66] | 5–10 | [68] |
| Rapamycin | 5–15 | [63] | No data available (PubMed) | |

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Tacrolimus has the least inhibition of the three "immunosuppressants" studied. Against MAP, Tacrolimus is most inhibitory against UCF 4 (43% −ΔcGI at 64 μg/ml) and ATCC 19698: 26% −ΔcGI at 64 μg/ml) (Figures 1 & 2 and Table 6.) Paradoxically, Tacrolimus exhibits the most inhibition on *M. avium* 101 of all six strains studied, yet actually enhances growth on *M. avium* ATCC 25291. (Figure 3 and Table 6.)

## Discussion

Rapamycin was initially evaluated as an anti-fungal agent. [54] To our knowledge however, this is the first time that antiMAP activity has been demonstrated for the "immunosuppressant" agents Cyclosporine, Rapamycin and Tacrolimus. These observations are therefore compatible with our thesis that MAP may be responsible for multiple "autoimmune" and "inflammatory" diseases, and that the action of these three "immunosuppressant" agents may simply be to inhibit MAP growth.

We have observed that methotrexate and 6-MP are used in "high" doses to treat human malignancies and at "low" doses in "autoimmune" and "inflammatory" conditions. [34] Similarly, there are "high" and "low" doses of the three "immunosuppressants" we now study (See Table 7.) The "high" doses are used to prevent or treat transplanted organ rejection. The "low" doses are used to treat "autoimmune" and "inflammatory" diseases. These data are compatible with our hypothesis that Cyclosporine, a cyclic undecapeptide, as well as Rapamycin and Tacrolimus, from the macrolide family of antibiotics, may have "low" dose prokaryotic antibiotic action in addition to "high" dose eukaryotic immunosuppressant activity.

Our observations are subtle and the negative controls are critical. For those not conversant with quantifying mycobacterial growth and determining the inhibitory effect of various agents, it must be emphasized that these data were obtained using the exquisitely sensitive radiometric $^{14}$C Bactec system®. Just as with 5-ASA [37,38], these effects may not be detectable using the more convenient, fluorescent based MIGT system®.

The chronic use of antibiotics, even for complex mycobacterial diseases, is not advocated. With leprosy the WHO recommends that MDT be limited to ≤2 years [52] and for tuberculosis ≤18 months and preferably six months. [55] The "immunosuppressant, " "antiinflammatory" and "immunomodulatory" agents that we show are antiMAP antibiotics have been administered indefinitely.

In the event that MAP is accepted as being zoonotic, there will need to be a reevaluation of how best to manage MAP infections in humans. There will be multiple factors that will then need to be taken into consideration. These include the fact that successfully treated leprosy and tuberculosis infections do not lead to mycobacterial eradication. Often the bacteria merely enter into a quiescent or "latent" phase and clinical symptoms progress [56] despite apparently "adequate" therapy. It will also be necessary to prevent reinfection, by removing MAP from the water supply [27], and food chain.[28] Genetic defects [57–59] that predispose to MAP infections will need to be identified, as affected individuals may need life long antiMAP therapy. Optimal MAP antibiotic combinations will need to be established. Designing clinical trial that consider the recently described antiMAP activity of "antiinflammatories", "immunomodulators" and "immunosuppressants" will need to be performed. Finally, the role of MAP pre and post exposure vaccination will need to be addressed. [60–62]

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## Author Contributions

Conceived and designed the experiments: RG. Performed the experiments: RG LS. Analyzed the data: RG LS SB RJ. Contributed reagents/materials/analysis tools: RG SB. Wrote the paper: RG.

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# On the Action of 5-Amino-Salicylic Acid and Sulfapyridine on *M. avium* including Subspecies *paratuberculosis*

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***Background.*** **Introduced in 1942, sulfasalazine (a conjugate of 5-aminosalicylic acid (5-ASA) and sulfapyridine) is the most prescribed medication used to treat ''inflammatory'' bowel disease (IBD.) Although controversial, there are increasingly compelling data that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) may be an etiological agent in some or all of IBD. We have shown that two other agents used in the therapy of IBD (methotrexate and 6-MP) profoundly inhibit MAP growth. We concluded that their most plausible mechanism of action is as antiMAP antibiotics. We herein hypothesize that the mechanism of action of 5-ASA and/or sulfapyridine may also simply be to inhibit MAP growth.** ***Methodology.*** **The effect on MAP growth kinetics by sulfasalazine and its components were evaluated in bacterial culture of two strains each of MAP and *M. avium*, using a radiometric ($^{14}CO_2$ BACTEC®) detection system that quantifies mycobacterial growth as arbitrary ''growth index units'' (GI). Efficacy data are presented as ''percent decrease in cumulative GI'' (%−*Δ*cGI).** ***Principal Findings.*** **There are disparate responses to 5-ASA and sulfapyridine in the two subspecies. Against MAP, 5-ASA is inhibitory in a dose-dependent manner (MAP ATCC 19698 46%−*Δ*cGI at 64 μg/ml), whereas sulfapyridine has virtually no effect. In contrast, against *M. avium* ATCC 25291, 5-ASA has no effect, whereas sulfapyridine (88%−*Δ*cGI at 4 μg/ml) is as effective as methotrexate, our positive control (88%−*Δ*cGI at 4 μg/ml).** ***Conclusions.*** **5-ASA inhibits MAP growth in culture. We posit that, unknowingly, the medical profession has been treating MAP infections since sulfasalazine's introduction in 1942. These observations may explain, in part, why MAP has not previously been identified as a human pathogen. We conclude that henceforth in clinical trials evaluating antiMAP agents in IBD, if considered ethical, the use of 5-ASA (as well as methotrexate and 6-MP) should be excluded from control groups.**

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## INTRODUCTION

In 1942 sulfasalazine (''Salazopyrin'') was introduced into clinical practice for ulcerative colitis. [1] Sulfasalazine has become, because of empirically observed clinical efficacy, ''the most common medicine used to treat ''Inflammatory'' Bowel Disease (IBD) [2] with greatest efficacy in ulcerative colitis. [3–5]

Sulfasalazine is a conjugate of sulfapyridine and 5- aminosalicylic acid (5-ASA.) It is cleaved into its two component molecules following ingestion. [2] The sulfapyridine moiety [(2-(p amino-benzenesulphonamido) pyridine] is an acknowledged antibiotic. [6,7] However, prevailing medical dogma concludes that ''it is unlikely that (sulfasalazine's) antibacterial activity accounts for its clinical efficacy.'' [2] In 1977, a two-week study on ulcerative proctitis, compared 5-ASA to sulfapyridine. Because of a decrease in inflammation in the 5-ASA group, the authors concluded, that in the therapy of ulcerative colitis the active moiety of sulfasalazine was 5-ASA. [8] As a consequence therapy with 5-ASA is called ''anti-inflammatory'' although ''the mechanism of action of 5-ASA in IBD is uncertain.'' [2]

Although controversial, there are increasingly compelling data that all [9,10] or some of IBD may be caused by a single infectious agent *Mycobacterium avium* subspecies *paratuberculosis* (MAP.) [9–13] (& See [14] for review.) We have shown that two agents, methotrexate and 6-mercaptopurine (6-MP) [10], presumed to have ''immunomodulatory'' actions in IBD, [15,16] are potent antiMAP antibiotics. We suggested that the decreases in pro-inflammatory cytokines in IBD ''immunomodulatory'' therapy might simply reflect a normal, secondary, physiological response, as the instigating MAP infection was effectively treated. We concluded that henceforth methotrexate and 6-MP should be excluded from the placebo group when evaluating antiMAP therapies in IBD. [10]

In this study we test the hypothesis that the ''anti-inflammatory'' action of 5-ASA and/or sulfapyridine could simply be due to one or both of sulfasalazine's components acting as an antiMAP antibiotic(s.) If correct the ''anti-inflammatory'' effect would simply represent a normal, secondary, physiological response as the causative MAP infection was controlled by antiMAP antibiotic action. Accordingly, in bacterial culture, we have evaluated the effect of sulfasalazine and individually and in combination its cleavage products 5-ASA and sulfapyridine, on *M. avium* including its subspecies MAP.

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**Competing Interests:** RJG has submitted a patent application based on the hypotheses that were subsequently tested in this study. STB was a member of the panel of the report issued by the National Academies of Science entitled, ''The Diagnosis and Control of Johne's Disease.''

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## METHODS

This study was approved by the Research & Development Committee at the VAMC Bronx NY (0720-06-038) and was conducted under the Institutional Radioactive Materials Permit (#31-00636-07).

### Bacterial Culture:

In this study (as previously [10]) we use four well-characterized strains of mycobacteria. Two are MAP, a bovine isolate, ATCC 19698 (ATCC Rockville MD) and "Dominic" a human isolate from an individual with Crohn's disease (originally isolated by R. Chiodini [17].) The *M. avium* subspecies *avium* strains (hereinafter called *M. avium*) were ATCC 25291 (veterinary source) and *M. avium* 101. [18] Because it renders clinically resistant strains of MAP inappropriately susceptible to antimicrobials in cell culture, [19] we did not use the detergent Tween 80 (recommended to prevent mycobacterial clumping) in culture. Prior to inoculation cultures were processed as described. [10] [20]

Our negative control was intact sulfasalazine (a conjugate of sulfapyridine & 5-ASA) and the positive control was methotrexate. [10] Sulfapyridine and 5-ASA were evaluated individually and in combination (All from Sigma St Louis MO.) Aliquots of chemicals being evaluated were prediluted, stored at $-80^{\circ}$C in 50 mM NaOH, thawed, used once and discarded. Volumes of NaOH were adjusted so that final concentration in each Bactec vial was always 3.2 mM NaOH. Agents were tested in serial dilutions from a minimum of 0.05 μg/ml to a maximum of 64 μg/ml (see individual Figures). When the individual molecules 5-ASA and sulfapyridine were studied in combination, the same weight for each was used as when tested individually (see appropriate Table.)

Data for the Bactec ® System (Becton-Dickinson Franklin Lakes NJ) are presented as cumulative growth index (cGI) units±SD (when necessary, see individual figures). cGI data for each experiment is presented until the day prior to any GI reading exceeding the assay limit of "999." The effect (or lack thereof) of each agent is presented as the percent decrease in cGI units (%$-\Delta$cGI), The calculation of %$-\Delta$cGI is performed in two stages (using Excel) according to the formula:

$$\text{Step one} = [(A-B)/A] = C \quad \text{Step two} = -C \times \% =$$

$$\text{Final result of } \% - \Delta\text{cGI}$$

Where A = the cGI of the control inoculum for the given diluent (usually in these experiments NaOH); B = the cGI for the particular chemical at a particular dose being tested, incubated for the same number of days as A. C = the product of [(A−B)/A]. Raw data were archived onto Excel, collated and the cumulative results transferred to Prism (Graphpad, San Diego CA) for final graphing.

## RESULTS

Data for the effect of test agents on bacterial growth are presented in two ways. Either as results for an individual mycobacterial strain (Figures 1 & 2) or as a comparison of the effect of each agent tested on all four mycobacterial strains in Tabular form, where inhibition is expressed as %$-\Delta$cGI, and enhancement as %$+\Delta$cGI (Tables 1–5.) Tables 1 & 2 are the experimental controls; Table 1 = the positive control methotrexate & Table 2 = the negative control sulfasalazine. Tables 3–5 have the agents tested, 5-ASA (Table 3), sulfapyridine (Table 4) and the combination of 5-ASA+sulfapyridine (Table 5.)

The positive control is methotrexate, previously shown to be almost as effective as the acknowledged antiMAP antibiotic clarithromycin. [10] There is≥80%$-\Delta$cGI at 4 μg/ml for all four species (Figures 1 & 2 and Table 1). In the MAP Dominic study (Figure 2), the positive control was clarithromycin, which because it was diluted in methanol was off scale, but showed maximal inhibition by 1 μg/ml (data not presented).

The negative control is the intact progenitor molecule sulfasalazine. [1] Sulfasalazine is manufactured by combining 5-ASA with sulfapyridine. Surprisingly, rather than having no effect or inhibiting *mycobacterial* growth we found that intact sulfasalazine actually enhances growth at high doses (Figures 1 and 2 & Table 2). By 64 μg/ml, sulfasalazine increases both MAP ATCC 19698 and Dominic by 27%+$\Delta$cGI, (Figures 1 & 2 and Table 2) and of *M. avium* ATCC 25291 by 160%+$\Delta$cGI (Figure 2 and Table 2.)

There are two different responses for MAP compared to *M. avium* from the two cleavage products of intact sulfasalazine, 5-ASA and sulfapyridine. Against MAP, 5-ASA has dose dependent inhibition. At 64 μg/ml of 5-ASA, inhibition against MAP ATCC 19608 is 46%-$\Delta$cGI (Figures 1 & 2 and Table 3), and against Dominic inhibition is 17%-$\Delta$cGI (Figure 2 and Table 3). In contrast, sulfapyridine alone has no inhibition against either MAP strain (Figures 1 and 2 and Table 4.)

In contrast to MAP, both *M. avium* strains are very susceptible to sulfapyridine. Inhibition is 88%-$\Delta$cGI at 4 μg/ml for ATCC 25291 and 92%-$\Delta$cGI at 4 μg/ml for M. avium 101 (Figure 2 and Table 4.) *M. avium* is not inhibited by 5-ASA, (Figure 2 and Table 4.)

Finally, the two components 5-ASA and sulfapyridine were studied in combination. For experimental comparability, equal doses of each agent were used (See Left Hand column in Table 5.) In two of the three MAP studies there is subtle evidence of synergy when the 5-ASA/sulfapyridine combinations are used (Figure 2 and Table 5.) This possible synergy is not seen in the MAP data presented in Figure 1. Different numbers of CFU's were inoculated in the two MAP ATCC 19698 studies. In the experiment from Figure 1 we inoculated $2.7\times10^4$ CFU's and the experiment lasted 11 days and in Figure 2, $6\times10^4$ CFU's were inoculated and the experiment lasted 10 days. There is no evidence of any 5-ASA/sulfapyridine synergy with either *M. avium* strain (Figure 2 and Tables 3–5.)

## DISCUSSION

Although its precise mechanism of action has never been established, the utility of sulfasalazine (or 5-ASA alone) in the therapy of IBD, is uncontested since Svartz's seminal publication in 1942. [1] Sulfasalazine has been used because of empirical efficacy and prevailing medical dogma accepts that it acts in a non-specific "anti-inflammatory" manner. In the event that IBD is eventually accepted as being due to a MAP infection, our data are compatible with our hypothesis that the efficacy of 5-ASA is due to impairment of MAP growth.

Results with our positive control methotrexate, replicate our previous findings against all the *M. avium* strains studied. [10] In this study, our negative control is the intact progenitor molecule sulfasalazine that comprises sulfapyridine linked to 5-ASA. We show it has virtually no inhibitory action, and at high doses intact sulfasalazine actually enhances growth in all the strains studied. The mechanism(s) whereby mycobacteria are able to utilize uncleaved sulfasalazine remain to be elucidated.

We show completely different responses to 5-ASA and sulfapyridine in the two *M. avium* subspecies studied. Against MAP, 5-ASA is inhibitory in a dose dependent manner, whereas sulfapyridine alone has minimal effect. Our data therefore offer a rational

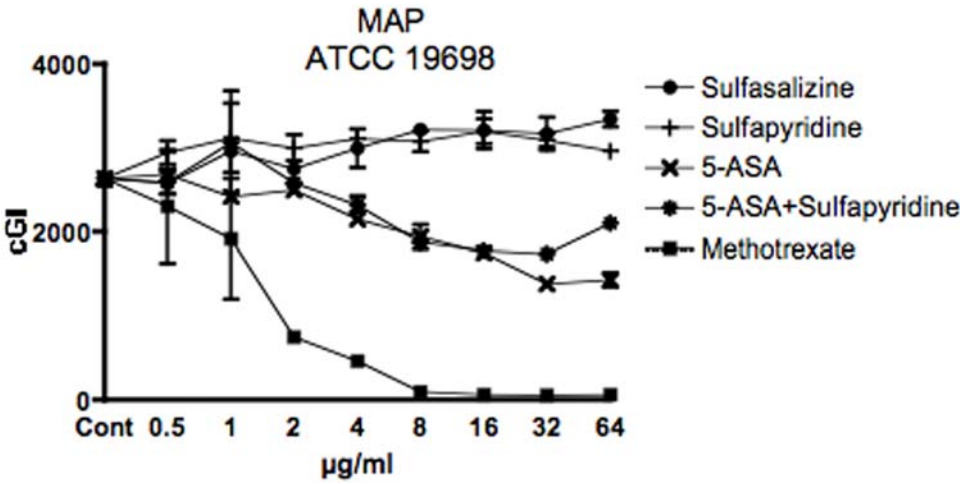


**Figure 1. Cumulative GI data when $2.7\times10^4$ CFU/vial of MAP ATCC 19698 was inoculated into each Bactec vial.** Each drug dilution was studied in duplicate. Error bars are SD. The positive control is methotrexate, with maximal inhibition by 8 mg/ml. Neither the negative control sulfasalazine, nor sulfapyridine, have any inhibition. Both and 5-ASA alone and in combination with sulfapyridine have dose dependent inhibition.
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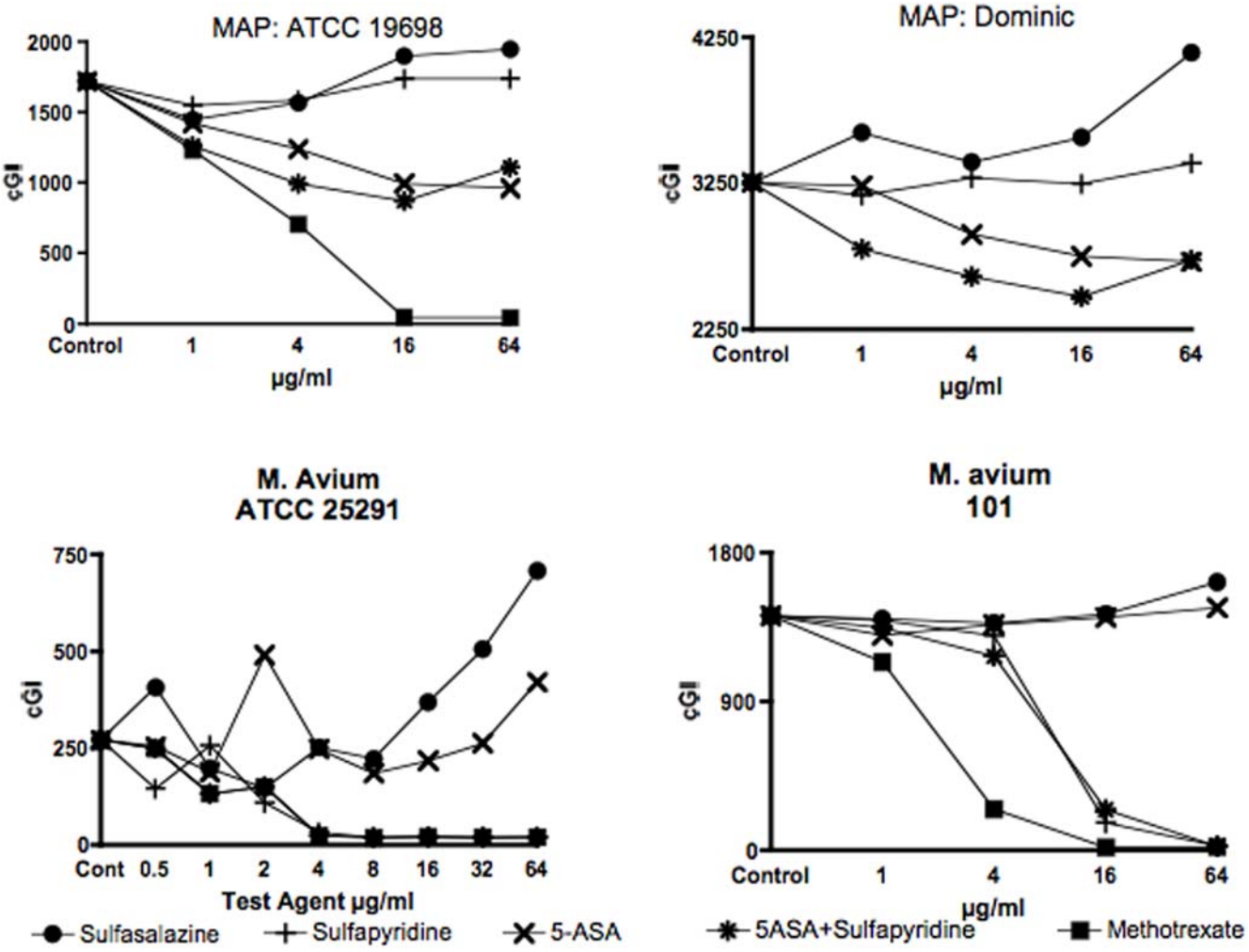


**Figure 2. A composite graph of the two MAP and two M. avium bacterial strains studied.** Each dose was studied in singlicate. For MAP ATCC 19698, $6\times10^4$ CFU's were inoculated/vial. Sulfasalazine is the negative control for both MAP and *M. avium* strains. In all strains there is dose dependent increase in cGI. There is no inhibition by sulfapyridine alone with either MAP strain. 5-ASA has dose dependent inhibition on both MAP strains. Note the subtle synergy up to 16 µg/ml for both MAP strains for the 5-ASA+sulfapyridine group compared to 5-ASA alone. The positive control in the Dominic study was clarithromycin which, because it was diluted in methanol, is off scale. Accordingly, the clarithromycin data are not presented.
doi:10.1371/journal.pone.0000516.g002

**Table 1.** Positive Control: Methotrexate

| Methotrexate | MAP | | | M. avium | |
|---|---|---|---|---|---|
| | ATCC 19698 | | Dominic [10] | ATCC 25291 | 101 |
| µg/ml | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig 2 |
| 0.5 | −13% | | | −44% | |
| 1 | −27% | −28% | −52% | 52% | −20% |
| 2 | −72% | | | −72% | |
| 4 | −83% | −59% | −89% | −92% | −82% |
| 8 | −96% | | | −93% | |
| 16 | −98% | −97% | −93% | −92% | −99% |
| 32 | −98% | | | −94% | |
| 64 | −98% | −98% | −93% | −94% | −98% |

%−ΔcGI

doi:10.1371/journal.pone.0000516.t001

**Table 2.** Negative Control: Intact Sulfasalazine

| Sulfasalazine | MAP | | | M. avium | |
|---|---|---|---|---|---|
| | ATCC 19698 | Dominic | | ATCC 25291 | 101 |
| µg/ml | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig 2 |
| 0.5 | −2% | | | 50% | |
| 1 | 13% | −16% | 10% | −27% | −1% |
| 2 | 5% | | | −46% | |
| 4 | 14% | −9% | 4% | −7% | −3% |
| 8 | 22% | | | −18% | |
| 16 | 22% | 10% | 9% | 36% | 1% |
| 32 | 20% | | | 86% | |
| 64 | 27% | 13% | 27% | 160% | 15% |

%−ΔcGI

doi:10.1371/journal.pone.0000516.t002

**Table 3.** Test Agent: 5-ASA

| 5-ASA | MAP | | | M. avium | |
|---|---|---|---|---|---|
| | ATCC 19698 | Dominic | | ATCC 25291 | 101 |
| µg/ml | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 |
| 0.5 | 2% | | | −6% | |
| 1 | −8% | −17% | −1% | −31% | −8% |
| 2 | −5% | | | 80% | |
| 4 | −18% | −28% | −11% | −9% | −4% |
| 8 | −26% | | | −32% | |
| 16 | −33% | −42% | −16% | −20% | −1% |
| 32 | −47% | | | −3% | |
| 64 | −46% | −44% | −17% | 55% | 4% |

%−ΔcGI

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explanation for the empirical observation, made thirty years ago [8], showing that 5-ASA is more active than sulfapyridine in the therapy of ulcerative proctitis. As a consequence, we suggest that the "anti-inflammatory" effect of 5-ASA may simply represent a normal, physiological, secondary response as an instigating MAP infection is treated.

**Table 4.** Test Agent: Sulfapyridine

| Sulfapyridine | MAP | | | M. avium | |
|---|---|---|---|---|---|
| | ATCC 19698 | Dominic | | ATCC 25291 | 101 |
| µg/ml | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 |
| 0.5 | 12% | | | −47% | |
| 1 | 18% | −10% | −3% | −5% | −2% |
| 2 | 14% | | | −60% | |
| 4 | 18% | −8% | 1% | −88% | −8% |
| 8 | 17% | | | −93% | |
| 16 | 21% | 1% | 0% | −93% | −88% |
| 32 | 17% | | | −93% | |
| 64 | 13% | 1% | 4% | −93% | −98% |

%−ΔcGI

doi:10.1371/journal.pone.0000516.t004

**Table 5.** Test Agents: 5-ASA+Sulfapyridine

| 5-ASA+ Sulfapyridine | MAP | | | M. avium | |
|---|---|---|---|---|---|
| | ATCC 19698 | Dominic | | ATCC 25291 | 101 |
| µg/ml | Fig. 1 | Fig. 2 | Fig. 2 | Fig. 2 | Fig. 2 |
| 0.5+0.5 | −2% | | | −8% | |
| 1+1 | 16% | −27% | −14% | −51% | −5% |
| 2+2 | −2% | | | −44% | |
| 4+4 | −12% | −42% | −20% | −91% | −17% |
| 8+8 | −29% | | | −93% | |
| 16+16 | −32% | −49% | −24% | −92% | −83% |
| 32+32 | −34% | | | −93% | |
| 64+64 | −20% | −35% | −16% | −92% | −98% |

%−ΔcGI

doi:10.1371/journal.pone.0000516.t005

In contrast, we observe that sulfapyridine is as effective as the positive control methotrexate against *M. avium* subspecies *avium*, whereas 5-ASA has no effect. We conclude that unlike its utility in putative MAP infections, 5-ASA has no role to play in *M. avium* subspecies *avium* infections.

As is conventional in studies such as these, we evaluated each agent on an equal weight basis. The data show that against MAP, 5-ASA is not nearly as effective as methotrexate. Our data are therefore consistent with many decades of empirical clinical observation with 5-ASA and methotrexate. Neither 5-ASA nor methotrexate has traditionally been administered as an "antibiotic." Their dosage has been determined by titration to clinical efficacy and limited by side effects. For sulfasalazine, the recommended dosage is ≤4 gm/day, as tolerated. [3] In contrast, for methotrexate the usual "low dose" [10] that is used to treat "inflammatory" diseases such as IBD is 25 mg/week. [21] This >1000 fold difference (28,000 mg/wk sulfasalazine compared to 25 mg/wk methotrexate) is compatible with our data both in this manuscript and previously [10] showing that methotrexate is, on a weight/weight basis, far more effective than 5-ASA at inhibiting MAP growth.

In the clinical therapy of IBD, either sulfasalazine or one of its components, 5-ASA is used. Sulfapyridine is not used alone. Our culture inhibition data offer a rational explanation for these

empirically derived medication use patterns. We show a possible subtle synergy when sulfapyridine is added to 5-ASA at higher inoculation counts. These observations need to be replicated in more MAP strains, different CFU inoculation counts and other inhibition methods. [22] Additionally, to prevent the emergence of resistant strains, single antibiotic therapy is not acceptable in the therapy of other mycobacterial diseases such as leprosy. [23] We conclude that, unless there is a sulfapyridine allergy, it may be preferable to treat with the combination medication sulfasalazine in preference to 5-ASA alone.

Previously we documented the antiMAP action of the "immune-modulators" methotrexate and 6-MP. We now show that the "anti-inflammatory" 5-ASA likewise has antiMAP action. These medications are bedrock "immuno-modulatory" and "anti-inflammatory" therapies in IBD and other "inflammatory" and "auto-immune" diseases. Our data raise the reasonable concern that an infectious cause of IBD has been overlooked, simply because the infectious agent has unknowingly been treated since 1942. It is therefore possible that the "I" in "I"BD could stand for "Infectious" and that the causative organism may be MAP.

We conclude that, since the placebo groups were receiving active antiMAP agents, prior studies that evaluated antiMAP agents need to be revaluated. Clinicians who continue to opine that MAP is not zoonotic will now need to substantiate their position. To do so they will need to conduct trials that exclude agents that we show have antiMAP activity from the placebo group. However, the ethical implications of excluding these agents [24] (see particularly Note of Clarification on Paragraph 29 added by the World Medical Association General Assembly, Washington 2002) will need to be fully addressed when they design their protocols.

## Conclusions and Recommendations

We show that the "anti-inflammatory" agent 5-ASA, interferes with the growth of MAP, an organism that may be the etiological factor for some, or all of IBD. Some of the implications of these observations are discussed.

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## Author Contributions

Conceived and designed the experiments: RG. Performed the experiments: RG LS. Analyzed the data: RG LS SB. Contributed reagents/materials/analysis tools: RG AS SB. Wrote the paper: RG.

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# Thiopurine Drugs Azathioprine and 6-Mercaptopurine Inhibit *Mycobacterium paratuberculosis* Growth In Vitro▽

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**The in vitro susceptibility of human- and bovine-origin *Mycobacterium paratuberculosis* to the thioupurine drugs 6-mercaptopurine (6-MP) and azathioprine (AZA) was established using conventional plate counting methods and the MGIT 960 ParaTB culture system. Both 6-MP and AZA had antibacterial activity against *M. paratuberculosis*; isolates from Crohn's disease patients tended to be more susceptible than were bovine-origin isolates. Isolates of *Mycobacterium avium*, used as controls, were generally resistant to both AZA and 6-MP, even at high concentrations (≥64.0 μg/ml). Among rapidly growing mycobacteria, *Mycobacterium phlei* was susceptible to 6-MP and AZA whereas *Mycobacterium smegmatis* strains were not. AZA and 6-MP limited the growth of, but did not kill, *M. paratuberculosis* in a dose-dependent manner. Anti-inflammatory drugs in the sulfonamide family (sulfapyridine, sulfasalazine, and 5-aminosalycilic acid [mesalamine]) had little or no antibacterial activity against *M. paratuberculosis*. The conventional antibiotics azithromycin and ciprofloxacin, used as control drugs, were bactericidal for *M. paratuberculosis*, exerting their killing effects on the organism relatively quickly. Simultaneous exposure of *M. paratuberculosis* to 6-MP and ciprofloxacin resulted in significantly higher CFU than use of ciprofloxacin alone. These data may partially explain the paradoxical response of Crohn's disease patients infected with *M. paratuberculosis* to treatment with immunosuppressive thiopurine drugs, i.e., they do not worsen with anti-inflammatory treatment as would be expected with a microbiological etiologic pathogen. These findings also should influence the design of therapeutic trials to evaluate antibiotic treatments of Crohn's disease: AZA drugs may confound interpretation of data on therapeutic responses for both antibiotic-treated and control groups.**

The etiology of Crohn's disease remains elusive, but current consensus opinion is that Crohn's disease results from the interplay of host genetics and one or more environmental triggers (7). Genetic markers for susceptibility to Crohn's disease have been discovered, notably the CARD15 (15, 21, 28) gene and most recently the interleukin-23r (10) and ATG16L1 (16, 32) genes. The increased incidence seen in many industrialized countries supports the existence of an environmental trigger for Crohn's disease (11, 23, 37, 39, 43). Bacterial pathogens fit a pathobiology model involving abnormal host response to infection resulting from genetic defects in gastrointestinal antigen processing.

One trigger for Crohn's disease may be *Mycobacterium paratuberculosis*, also referred to as *Mycobacterium avium* subsp. *paratuberculosis*, with or without host genetic influences that increase infection susceptibility or alter response to infection (9, 40). *M. paratuberculosis* causes a type of chronic inflammatory bowel disease in a wide array of ruminant species, as well as in nonhuman primates (5, 18). The infection is prevalent in domestic agriculture ruminants, and it has been diagnosed in wildlife species as well. Human and animal *M. paratuberculosis* isolates share common genotypes (12, 29). Exposure of humans to *M. paratuberculosis* could occur by both direct and indirect contamination of food and water (3, 4, 44).

The thiopurine drugs azathioprine (AZA; Imuran) and its metabolite 6-mercaptopurine (6-MP) are among the most commonly used immunosuppressive drugs used to treat Crohn's disease symptoms (42). They both induce and maintain Crohn's disease remission. Compounds related to 5-aminosalicyclates (sulfasalazine [SS] and mesalamine) are other drugs used to suppress inflammation in Crohn's disease patients (17). One observation arguing against an involvement of *M. paratuberculosis*, or any other infectious agent, as a primary cause of Crohn's disease is that patients treated with immunosuppressive drugs do not clinically worsen (35).

Greenstein et al. demonstrated, however, that thiopurine drugs inhibit the growth of *M. paratuberculosis* (13). The goal of this study was to characterize 6-MP's effect on *M. paratuberculosis* growth in culture and contrast it with the antibacterial effects of conventional antimycobacterial antibiotics.

## MATERIALS AND METHODS

**Bacterial strains and inoculum preparation.** A total of 11 bovine- and human-origin *M. paratuberculosis* strains were used in this study (Table 1). All strains were initially cultured in 7H9 broth supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson, Sparks, MD) and 2 μg/ml of mycobactin J (Allied Monitor, Fayette, MO) for 1 month at 37°C. Seven *Mycobacterium avium* strains were used for comparison. These were cultured in 7H9 broth supplemented with 10% OADC (Becton Dickinson, Sparks, MD) for 2 weeks to 1 month at 37°C (Table 1).

Three representatives of rapidly growing mycobacteria were tested: *Mycobacterium phlei* ATCC 11758, *Mycobacterium smegmatis* ATCC 14468, and *M. smegmatis* mc$^2$155. Two nonmycobacterial strains, *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212, were included as commonly used drug susceptibility control organisms (Table 1).

The identity of all mycobacteria was verified by multiplex PCR for insertion

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TABLE 1. Bacterial strains tested in this study

| Bacterial strain | Isolate source |
|---|---|
| *M. paratuberculosis* | |
| ATCC 19698 | Bovine, clinical case of paratuberculosis, type strain |
| JTC303 | Bovine, clinical case of paratuberculosis, JTC[a] |
| UCF-3 | Human, Crohn's disease patient ileum, UCF[b] |
| UCF-4 | Human, Crohn's disease patient ileum, UCF[b] |
| UCF-5 | Human, Crohn's disease patient ileum, UCF[b] |
| UCF-7 | Human, Crohn's disease patient ileum, UCF[b] |
| UCF-8 | Human, Crohn's disease patient ileum, UCF[b] |
| B213 | Bovine, clinical case of paratuberculosis, UCF[b] |
| B236 | Bovine, clinical case of paratuberculosis, UCF[b] |
| B238 | Bovine, clinical case of paratuberculosis, UCF[b] |
| B244 | Bovine, clinical case of paratuberculosis, UCF[b] |
| *M. avium* | |
| ATCC 35712 | Chicken, TMC701,[c] serotype 2 |
| ATCC 25291 | Chicken, liver, TMC724, serotype 2, type strain |
| 104 | Human, AIDS patient, serotype 1[d] |
| JTC48627 | Bison, fecal sample |
| JTC981 | Bongo, fecal sample |
| EPA3 | Water, WSLH[e] |
| WSLH1544 | Water, WSLH |
| Rapidly growing mycobacteria | |
| *M. phlei* ATCC11758 | TMC1458, type strain |
| *M. smegmatis* ATCC14468 | TMC1546, suggested neotype |
| *M. smegmatis* mc$^2$155[f] | Transformably competent isolate of mc$^2$6 |
| Other bacterial species[g] | |
| *E. coli* ATCC 25922 | Clinical isolate, FDA strain |
| *E. faecalis* ATCC 29212 | Human urine |

[a] JTC, Johne's Testing Center, Madison, WI.
[b] Saleh Naser, University of Central Florida (UCF), Orlando, FL.
[c] Trudeau Mycobacterial Culture Collection.
[d] Obtained from A. M. Talaat (University of Wisconsin-Madison; originally from Raul Barletta at the University of Nebraska).
[e] WSHL, Wisconsin State Hygiene Laboratory.
[f] Obtained from the American Type Culture Collection.
[g] Quality control strains used in antibiotic susceptibility testing.

elements IS*900*, IS*901*, IS*1311*, and IS*1245* (Johne's Testing Center, Madison, WI) as well as by high-pressure liquid chromatography (HPLC) of cell wall mycolic acids by a reference laboratory (Wisconsin State Laboratory of Hygiene, Madison, WI).

Single-cell suspensions of each strain were prepared as previously described with slight modifications (41). Tenfold serial dilutions from seed lots of each strain were plated on 7H10 agar, and single colonies were inoculated into MGIT ParaTB tubes (Becton Dickinson, Sparks, MD) to quantify the number of organisms per ml (38). Seed lots of each strain were then kept in small aliquots at −80°C until use. After plate counting, $10^4$ to $10^6$ CFU of each strain were inoculated to tubes of MGIT ParaTB medium to determine the average time to detection (TTD) in the absence of drugs. For *M. paratuberculosis* in MGIT ParaTB medium, the TTD is directly related to the number of organisms inoculated into each tube (38).

**Drugs tested.** All test drugs were obtained from Sigma-Aldrich Co., St. Louis, MO, in a chemically pure form (purity > 99.0%). The drugs were SS, 5-aminosalicylic acid (5-ASA), sulfapyridine (SP), rifampin (RIF), nalidixic acid (NAL), 6-MP, AZA, azithromycin (AZM), and ciprofloxacin (CIP). Lyophilized drugs were dissolved in appropriate diluents according to the manufacturer's instructions. The drugs SS, 5-ASA, SP, 6-MP, and AZA were dissolved in 0.05 M NaOH in 10 mM phosphate-buffered saline (PBS), RIF and NAL were dissolved in 10 mM PBS (pH 7.2), AZM was dissolved in 50% ethanol, and CIP was dissolved in 0.5 N HCl in 10 mM PBS. All stock drug solutions were freshly prepared for each experiment and filter sterilized using a 0.22-μm polycarbonate syringe filter (Millipore Corp., Bedford, MA).

**MGIT 960 drug susceptibility testing.** Methods published for *M. tuberculosis* and *M. avium* susceptibility testing (1, 2, 20, 22, 31, 34) were adapted as closely as possible; however, the significantly longer generation time of *M. paratuberculosis* compared to *M. tuberculosis* made this challenging. For this reason, we performed drug susceptibility studies in three phases. First, we evaluated our MGIT 960 antibacterial susceptibility testing method for *M. paratuberculosis* ATCC 19698 by comparing MGIT 960 results with those of conventional agar plate methods using drugs well characterized for antimycobacterial activity (phase I). Second, we evaluated the effects of thiopurine drugs and 5-aminosalicylates on *M. paratuberculosis* and *M. avium* growth in MGIT ParaTB medium in comparison to conventional bactericidal antibiotics (phase II). Next, we tested the reproducibility of the findings and expanded the study to include more strains and mycobacterial species (phase III). Lastly, we tested the effect of a combination of CIP and 6-MP over a range of achievable concentrations in tissue on the viability of *M. paratuberculosis* (phase IV). The methods for each of the four phases of study were as follows.

**(i) Phase I.** A single type strain of *M. paratuberculosis*, ATCC 19698, was tested at three MGIT ParaTB medium inoculum levels ($10^4$, $10^5$, and $10^6$ CFU per MGIT ParaTB tube). The final concentrations of each drug tested ranged from 1.0 to 64.0 μg/ml.

**(ii) Phase II.** Phase II, designed based on the results of phase I, used seven clinical strains of *M. paratuberculosis* of bovine or human origin and one *M. avium* strain, ATCC 35712, as a control. Some drugs to which *M. paratuberculosis* was not susceptible in phase I, specifically the SS drug family (SS, 5-ASA, and SP), were excluded, and AZA, a prodrug of 6-MP, was added to the trial (19, 27).

**(iii) Phase III.** The reproducibility of findings in phase II was determined. Additional strains of *M. paratuberculosis* also were tested, along with three other species of mycobacteria. The thiopurine drug concentrations tested were the same as in phase II, but one higher concentration (128.0 μg/ml) was added for some strains (see Table 4).

**(iv) Phase IV.** The effect of combined CIP and 6-MP on *M. paratuberculosis* strain UCF-7 viability was determined over a range of concentrations (0, 2, 4, and 6 μg/ml) and drug exposure times (0, 3, 6, 9, and 12 days). The results for this drug combination for *M. paratuberculosis* counts were compared to those for each drug individually, the drug-free control, and the 1% (1:100 original inoculum dilution) drug-free control.

**MGIT 960 methodology details.** Serial dilutions of single-cell suspensions of each mycobacterial strain were prepared, and 100 μl was inoculated into MGIT ParaTB medium (Becton Dickinson, Sparks, MD). Each tube contained 7 ml of medium and a fluorescent indicator embedded in silicone on the bottom of the tube. To each tube was added 800 μl of MGIT ParaTB supplement (Becton Dickinson, Sparks, MD), 500 μl of egg yolk suspension (Becton Dickinson, Sparks, MD), and 100 μl of test drug, resulting in final concentrations of 0.5 to 64.0 μg/ml. Tubes were incubated at 37°C in a MGIT 960 instrument and removed when the instrument signaled them as being positive. Samples from all signal-positive tubes were subcultured on Trypticase soy agar plates with 5% sheep blood (Becton Dickinson, Sparks, MD) to check for contamination. Acid-fast staining (Ziehl-Neelsen) was also performed on smears made from each signal-positive tube to confirm the presence of mycobacteria. For *E. coli* and *E. faecalis*, the standard broth microdilution method was used for susceptibility tests (8).

**Interpretation of susceptibility results.** There are no interpretive criteria of MGIT 960 algorithms for antibacterial susceptibility test interpretation for mycobacteria other than *M. tuberculosis* and *M. avium.* We used a similar interpretation system with slight modifications (1, 2, 22, 33, 45). Briefly, positive control MGIT ParaTB tubes were inoculated with only the test organism and the relevant drug solvent (solvent control). For comparison, these drug-free solvent control vials were inoculated with a 1:100 dilution of the normal organism inoculum (designed to represent growth of 1% of the original bacterial population). All MGIT ParaTB medium tubes were inoculated in duplicate with specified numbers of test organisms. In phase I trials, three *M. paratuberculosis*

inoculum levels were tested ($10^4$, $10^5$, and $10^6$ CFU), and in phases II, III, and IV only $10^5$ to $10^6$ CFU were inoculated to each MGIT ParaTB tube.

The bacterial growth rate in each MGIT ParaTB tube was defined by TTD, i.e., the number of days the MGIT ParaTB tubes were incubated until they were determined to be signal positive by the MGIT 960 instrument (38). The baseline for *M. paratuberculosis* inhibition was defined as the day that the 1:100 dilution inoculum control became signal positive. Thus the lowest concentration for each test drug that inhibited growth was the concentration that produced a TTD greater than that of the 1:100 dilution control tube. The minimum concentrations of each drug that completely suppressed growth of the test organism in the MGIT 960 system, i.e., the tubes were never signal positive by the end of the experiment at 56 days postinoculation, were also reported. For MGIT ParaTB tubes inoculated with $10^6$ CFU *M. paratuberculosis*, this usually occurred between days 5 and 7 of incubation.

If the positive control tube became signal positive earlier than incubation day 4, the *M. paratuberculosis* inoculum was considered too high. Similarly, if the 1:100 dilution inoculum control tube did not become signal positive within 8 days after the positive control, the tube was considered underinoculated. If either control tube criterion was not met, the test was considered invalid and the test was repeated. An uninoculated MGIT ParaTB tube was used as the negative control for every trial.

**Agar plate counting method.** Agar plate counting methods for drug susceptibility testing were performed with 7H10 medium supplemented with 10% OADC (Becton Dickinson, Sparks, MD) and 2 μg/ml of mycobactin J (Allied Monitor, Fayette, MO) for phase I, phase III, and phase IV trials (26). At selected times after exposure to drugs, surviving *M. paratuberculosis* cells were quantified by conventional plate counting for all controls and every drug concentration for the $10^6$-CFU *M. paratuberculosis* inoculum in phase I. In phase III, plate counts were done for only two concentrations of each drug, 2.0 μg/ml (low concentration) and 16.0 μg/ml (high concentration).

Bacteria were mixed with each specific drug and drug concentration in MGIT ParaTB medium and incubated at 37°C. At 3 and 5 days postinoculation an aliquot (0.1 ml) was removed for plate count determinations (note that MGIT 960 instrument readings were not taken on these tubes; the tubes merely provided the medium in which the bacterium-drug interaction took place). Bacterial cells were harvested from MGIT tubes by centrifugation, resuspended in PBS, and homogenized to break up bacterial cell clumps by vortexing with glass beads (30). Then, 10-fold serial dilutions ($10^0$ to $10^{-6}$) were made in PBS, and 100 μl of each dilution was plated in quadruplicate on 7H10 agar supplemented 10% OADC (Becton Dickinson, Sparks, MD) and 2 μg/ml of mycobactin J (Allied Monitor, Fayette, MO). The numbers of CFU were determined by visual inspection after incubation of plates at 37°C for 8 weeks. The lowest concentration of each drug tested by the agar counting method that inhibited growth was defined as the lowest concentration of the drug that produced a 99% ($2 \log_{10}$) reduction in CFU. These concentrations were compared to those determined by MGIT 960 drug susceptibility testing.

**Quality control.** *M. paratuberculosis* strain ATCC 19698 was included in all experiments and used to test the ability of each new lot of MGIT ParaTB medium, growth supplement, egg yolk, and mycobatin J to support *M. paratuberculosis* growth. Also, *E. coli* ATCC 25922 and *E. faecalis* ATCC 29212 were used as controls to assure drug efficacy, as these agents have predictable antibiotic susceptibility patterns. Solvent controls were used with each drug to distinguish anti-*M. paratuberculosis* activity due to solvents themselves from the activity of the drugs, and uninoculated MGIT ParaTB medium controls served to detect any contamination by extraneous organisms.

## RESULTS

**Phase I.** Inoculum size is a critical factor in drug susceptibility testing of slowly growing mycobacteria (31). Three *M. paratuberculosis* ATCC 19698 inocula initially tested were $10^4$, $10^5$, and $10^6$ CFU/tube. The average TTDs for inoculum controls (drug solvent only) were 4.97, 7.89, and 10.76 days for $10^6$, $10^5$, and $10^4$ CFU/tube, respectively. The 1:100 dilution inoculum controls became signal positive at 10.76, 14.32, 21.74 days on average, respectively. The drug solvents had no effect on TTD if $>10^2$ CFU *M. paratuberculosis* were inoculated (multiple comparison test, $P = 0.83$; data not shown). However, the $10^4$-CFU inoculum was excluded from subsequent analyses because of the long incubation time required for the 1:100 dilution control to become signal positive, especially in the face of drug solvents, notably 50% ethanol.

TABLE 2. Tested drugs in phase I experiment and their effect on *M. paratuberculosis* ATCC 19698, as determined by MGIT 960[a] and agar plate counting methods

| Drug | Drug family/type | Lowest concn (μg/ml) producing inhibition in comparison with 1:100 dilution control by: | | Minimum drug concn resulting in no detectable growth of *M. paratuberculosis*[b] |
|---|---|---|---|---|
| | | MGIT 960 | Agar counting method | |
| AZM | Macrolide | 4.0 | 4.0 | 16.0 |
| CIP | Quinolone | 1.0 | 1.0 | 32.0 |
| NAL | Quinolone | >64.0 | >64.0 | >64.0 |
| RIF | First-line tuberculosis drug | 2.0 | 2.0 | >64.0 |
| SP | Sulfonamide | >64.0 | >64.0 | >64.0 |
| SS | Sulfonamide | >64.0 | >64.0 | >64.0 |
| 5-ASA | Anti-inflammatory | >64.0 | >64.0 | >64.0 |
| 6-MP | Anti-inflammatory | 1.0 | 1.0 | 32.0 |

[a] Each tube was inoculated with $10^6$ CFU bacteria.
[b] Signal negative by the BACTEC MGIT 960 instrument up to 56 days postinoculation.

When the *M. paratuberculosis* inoculum was $\geq 10^5$ CFU/tube, there was good agreement between MGIT 960- and agar plate-determined drug effects, with previously reported mycobacterium MIC ranges for all drugs tested (Table 2). An *M. paratuberculosis* inoculum amount of $10^5$ versus $10^6$ CFU did not affect the drug effects for any compound with the exception of RIF (data not shown). While the concentration of RIF needed to inhibit *M. paratuberculosis* was low, the organism was able to grow to some extent even in the face of the highest RIF concentration tested, 64.0 μg/ml.

Growth inhibition was seen at the lowest concentration with CIP (1.0 μg/ml). For AZM the lowest growth-inhibitory concentration was 4.0 μg/ml, and *M. paratuberculosis* was highly resistant to NAL (inhibitory concentration > 64.0 μg/ml) (Table 2).

Among the immunomodulatory drugs, the SS drug family (SS, 5-ASA, and SP) showed no significant anti-*M. paratuberculosis* activity even at high concentrations (64.0 μg/ml). Unexpectedly, 6-MP showed anti-*M. paratuberculosis* activity similar in potency to that of CIP (lowest inhibitory concentration = 1.0 μg/ml) by the MGIT 960 drug susceptibility test (Table 2).

**Phase II.** The susceptibility of *M. paratuberculosis* to 6-MP was confirmed, and similar results were found for its precursor drug, AZA (Table 3). Across the seven *M. paratuberculosis* strains tested, growth inhibition for 6-MP was comparable to that found for CIP in concentrations ranging from 1.0 to 4.0 μg/ml. It required twofold-larger amounts of AZA to inhibit growth, with concentrations ranging from 2.0 to 8.0 μg/ml. *Mycobacterium avium* (ATCC 35712) was resistant to both thiopurine drugs, even at the 64.0 μg/ml (Table 3).

In general, *M. paratuberculosis* isolates originating from Crohn's disease patients were more susceptible to thiopurine drugs than were bovine-origin isolates among tested strains. The patterns of drug susceptibility to CIP were the same regardless of isolate origin. One human isolate, UCF-7, failed to grow after 56 days of incubation in MGIT ParaTB medium

TABLE 3. Lowest concentration to inhibit[a] and concentration to kill[b] seven *M. paratuberculosis* strains and one *M. avium* type strain by the MGIT 960 drug susceptibility test

| Drug | Concn (μg/ml) required to inhibit (kill) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *M. paratuberculosis* strain: | | | | | | | *M. avium* ATCC 35712 |
| | JTC303 | UCF-4 | UCF-5 | UCF-7 | B213 | B236 | B238 | |
| 6-MP | 1.0 (32.0) | 2.0 (>32.0) | 1.0 (>32.0) | <1.0 (8.0) | 4.0 (>32.0) | 4.0 (>32.0) | 4.0 (>32.0) | >64.0 |
| AZA | 2.0 (>32.0) | 4.0 (>32.0) | 2.0 (>32.0) | 1.0 (16.0) | 8.0 (>32.0) | 8.0 (>32.0) | 16.0 (>32.0) | >64.0 |
| AZM | 1.0 (4.0) | 1.0 (2.0) | 0.5 (2.0) | 0.5 (1.0) | 1.0 (2.0) | 0.5 (4.0) | >0.5 (1.0) | 1.0 (16.0) |
| CIP | 2.0 (8.0) | 1.0 (4.0) | 2.0 (4.0) | 1.0 (4.0) | 4.0 (16.0) | 4.0 (16.0) | 4.0 (16.0) | 16.0 (64.0) |

[a] The lowest drug concentration producing a TTD greater than that for the 1:100 inoculum dilution control tube.
[b] Signal negative by the BACTEC MGIT 960 instrument for up to 56 days postinoculation.

containing 8.0 μg/ml 6-MP or 16.0 μg/ml AZA, while the growth of the other strains was eventually detected even in the presence of 32.0 μg/ml 6-MP or AZA.

**Phase III.** Phase III verified the susceptibility of *M. paratuberculosis* to thiopurine drugs and showed that the effect was relatively specific for this mycobacterial species. Both 6-MP and AZA showed a stronger in vitro antimicrobial activity against 11 *M. paratuberculosis* strains than 7 *M. avium* strains; however growth inhibition patterns for thiopurine drugs against *M. avium* strains varied widely. The mean 6-MP concentrations for *M. paratuberculosis* growth inhibition ranged from <1.0 to 4.0 μg/ml, while those for *M. avium* ranged 8.0 to >128.0 μg/ml. Among *M. avium* strains, lower concentrations of the test compounds were needed to inhibit growth for isolates originating from water compared to clinical samples. The 6-MP initial inhibition range for human-origin *M. paratuberculosis* was <1.0 to 2.0 μg/ml, while for bovine-origin strains it ranged from 2.0 to 4.0 μg/ml. Again, 6-MP was found to inhibit *M. paratuberculosis* growth at one-half the concentration of AZA regardless of *M. paratuberculosis* strain origin. This finding was observed in other susceptible mycobacteria as well. No growth in MGIT ParaTB medium with 8.0 to 16.0 μg/ml of 6-MP was observed for three of the five Crohn's disease patient *M. paratuberculosis* isolates by 56 days, while growth of all *M. avium* strains was detected within 15 days at these same drug concentrations (Table 4).

The mycobacterial control *M. phlei* was as susceptible to thiopurine drugs as *M. paratuberculosis*, but *M. smegmatis* growth was completely resistant at 128 μg/ml of both thiopurine drugs. No antibacterial activity of thiopurine drugs against *E. coli* or *E. faecalis* was found, even at the highest concentration tested (128.0 μg/ml) (Table 4).

**Comparison of drug actions against *M. paratuberculosis*.** The drugs studied were either bacteriostatic or bactericidal or had no effect on *M. paratuberculosis*. Those with no effect, as evidenced by both plate count and MGIT 960 TTD data after either 3 or 5 days of bacterial exposure to the drugs, included the sulfonamide family (SS, SP, and 5-ASA; data not shown) and NAL (Fig. 1A).

Antimicrobial drugs AZM, CIP, and RIF were bactericidal for *M. paratuberculosis.* The minimum concentration for each drug needed to kill the organism was defined as the drug concentration producing fewer CFU than the non-drug-containing 1:100 inoculum dilution control (Fig. 1A and B). Killing concentrations for AZM and CIP by plate counting and MGIT methods were in agreement. In the presence of RIF at lower doses, viable *M. paratuberculosis* numbers initially declined but then the organism resumed growth (Fig. 2C). Bovine- and human-origin *M. paratuberculosis* strains were similarly affected by the compounds; *M. paratuberculosis* was more sus-

TABLE 4. Lowest concentrations of two thiopurine drugs to inhibit[a] and kill[b] strains of *M. paratuberculosis*, *M. avium*, *M. smegmatis*, and *M. phlei*

| Bacterial strain | Lowest concn of: | | | |
|---|---|---|---|---|
| | 6-MP for: | | AZA for: | |
| | Inhibition | Killing | Inhibition | Killing |
| *M. paratuberculosis* | | | | |
| ATCC 19698 | 1.0 | 16.0 | 2.0 | 16.0 |
| UCF-3 | <1.0 | 8.0 | 2.0 | 32.0 |
| UCF-4 | 2.0 | >32.0 | 4.0 | >32.0 |
| UCF-5 | 1.0 | >32.0 | 2.0 | >32.0 |
| UCF-7 | <1.0 | 8.0 | 2.0 | 16.0 |
| UCF-8 | <1.0 | 16.0 | 2.0 | 32.0 |
| B213 | 2.0 | >32.0 | 4.0 | >32.0 |
| B236 | 4.0 | >32.0 | 4.0 | >32.0 |
| B238 | 2.0 | >32.0 | 4.0 | >32.0 |
| B244 | 4.0 | >32.0 | 8.0 | >32.0 |
| *M. avium* | | | | |
| ATCC 35712 | >64.0 | >64.0 | >64.0 | >64.0 |
| ATCC 25291 | >64.0 | >64.0 | >64.0 | >64.0 |
| 104 | >64.0 | >64.0 | >64.0 | >64.0 |
| JTC4862 | 32.0 | >64.0 | 64.0 | >64.0 |
| JTC981 | >64.0 | >64.0 | >64.0 | >64.0 |
| EPA3 | 16.0 | >64.0 | 32.0 | >64.0 |
| WSLH1544 | 8.0 | >64.0 | 32.0 | >64.0 |
| Rapid growing mycobacteria | | | | |
| *M. phlei* ATCC 11758 | 1.0 | 16.0 | 2.0 | 32.0 |
| *M. smegmatis* ATCC 14468 | >128.0 | >128.0 | >128.0 | >128.0 |
| *M. smegmatis* $mc^2155$ | >128.0 | >128.0 | >128.0 | >128.0 |
| Nonmycobacterial controls | | | | |
| *E. coli* ATCC 25922 | >128.0 | >128.0 | >128.0 | >128.0 |
| *E. faecalis* ATCC 29212 | >128.0 | >128.0 | >128.0 | >128.0 |

[a] The lowest drug concentration producing a TTD greater than that for the 1:100 inoculum dilution control tube.
[b] Signal negative in the BACTEC MGIT 960 instrument for up to 56 days postinoculation.

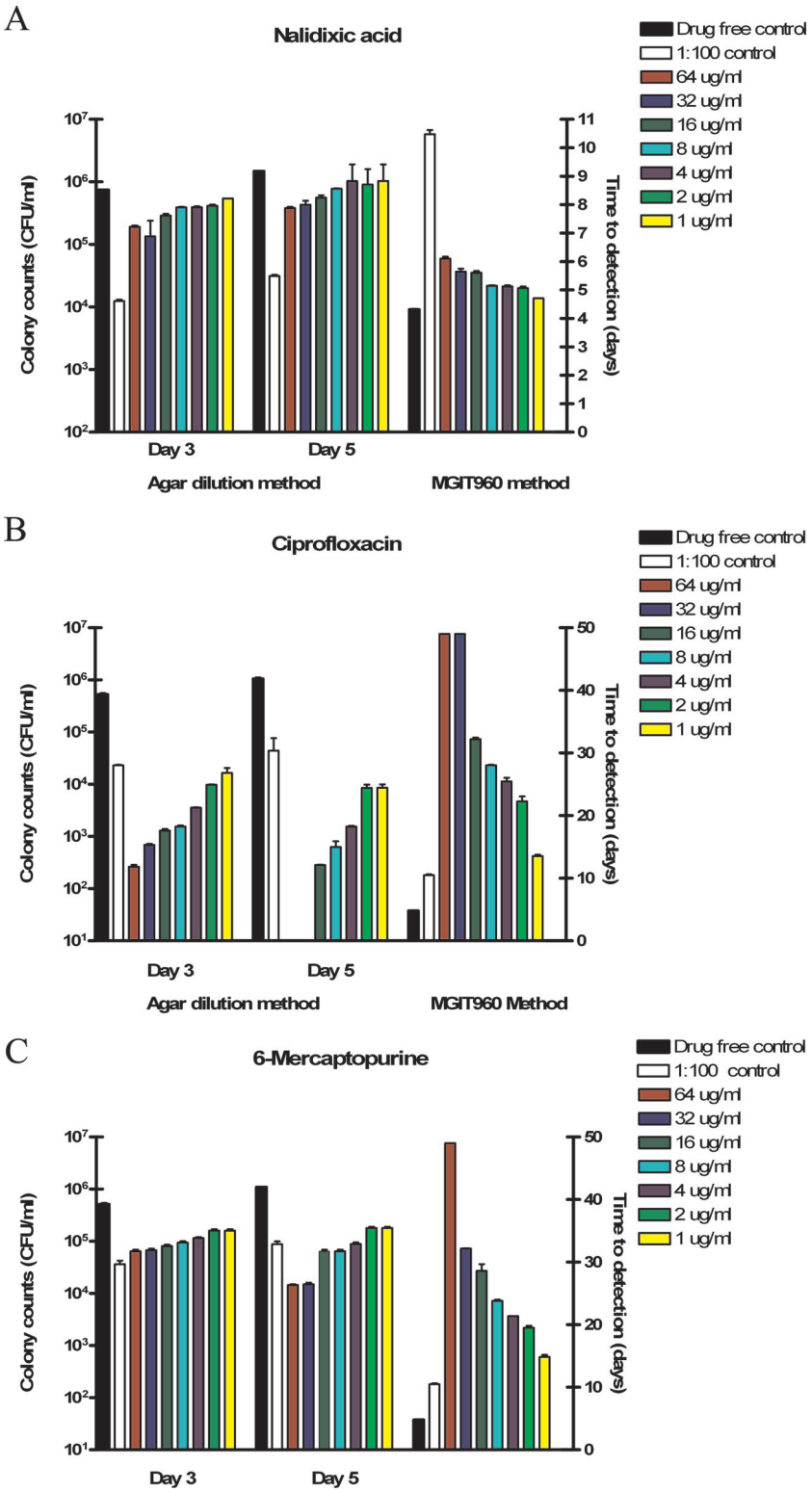


FIG. 1. Inhibition of *M. paratuberculosis* ATCC 19698 growth by exposure to NAL, CIP, and 6-MP at 1 to 64 μg/ml for 3 or 5 days as determined by agar plate counts, and the impact of the same drug concentrations on TTD in MGIT ParaTB medium, as monitored by the BACTEC MGIT 960 instrument.

ceptible to these antibiotics than was the control *M. avium* strain tested (Table 3). The concentration of CIP required for complete *M. paratuberculosis* growth inhibition after up to 56 days of incubation in MGIT ParaTB medium for human isolates was twofold lower than that for bovine isolates, i.e., 4.0 μg/ml and 16.0 μg/ml, respectively.

Thiopurine drugs AZA and 6-MP inhibited *M. paratuberculosis* growth in a dose-dependent fashion. Plate count CFU

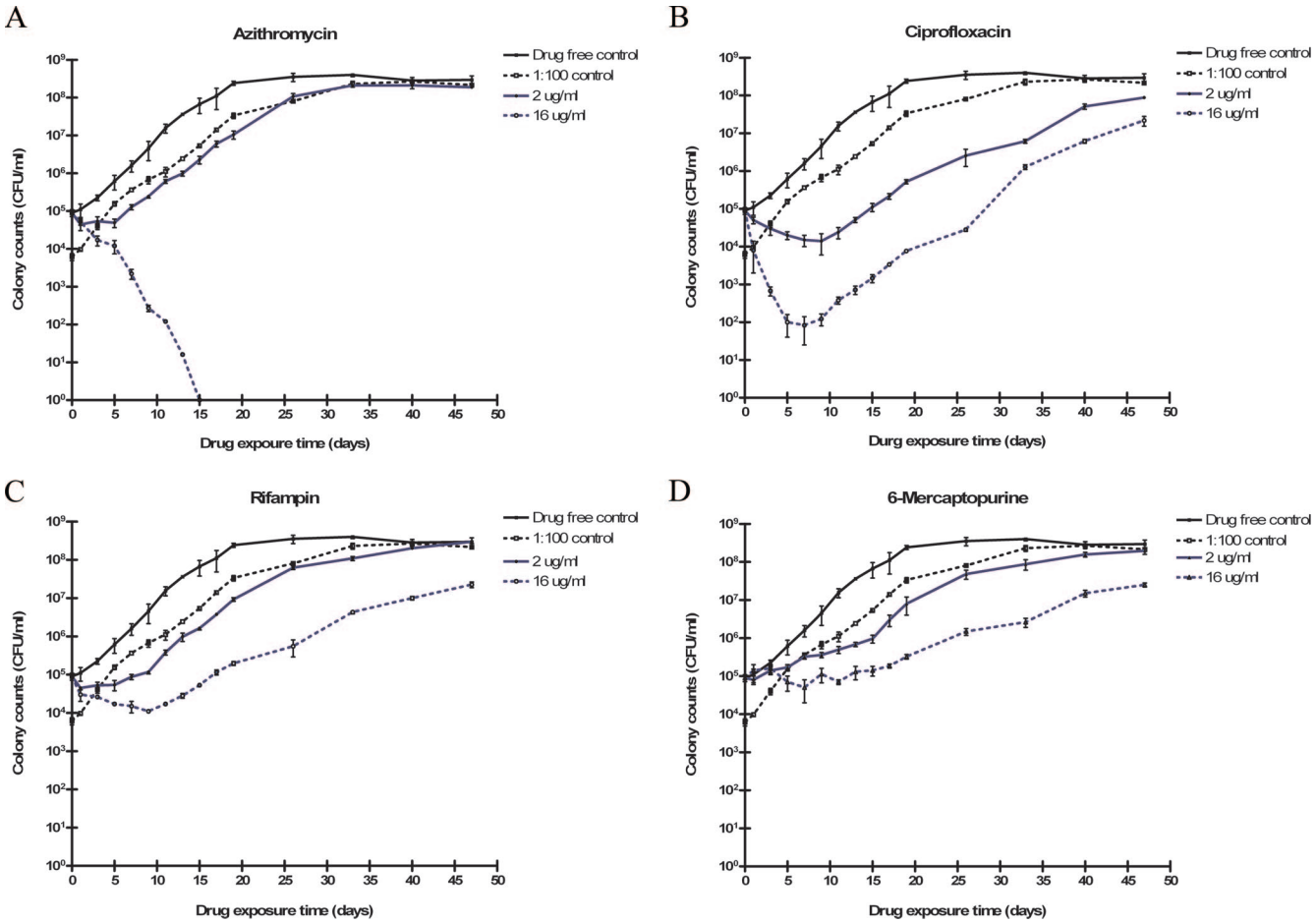


FIG. 2. Effect of AZM (A), CIP (B), RIF (C), and 6-MP (D) on the viability of *M. paratuberculosis* ATCC 19698 over time (up to 56 days), as determined by standard plate counts (CFU).

were lower than the number obtained by the MGIT 960 culture counting method but generally higher than that for the 1:100 dilution control after both 3 and 5 days of *M. paratuberculosis* exposure to the drugs (Fig. 1C). The effect of these drugs appeared more profound by the MGIT 960 drug susceptibility method, where there was continuous contact of the drug with *M. paratuberculosis* in broth, i.e., the TTD was greater than that for the 1:100 inoculum dilution control for all concentrations of drug tested (≥1.0 μg/ml). MGIT 960 analysis also showed a direct relationship between AZA or 6-MP concentration and suppression of *M. paratuberculosis* growth, i.e., longer TTD.

**Mycobacterial species specificity for thiopurine drugs.** Not all mycobacterial species were affected by thiopurine drugs to the same extent: *M. paratuberculosis* and *M. phlei* were susceptible to growth inhibition effects, but *M. avium* and *M. smegmatis* were not (Fig. 3).

**Phase IV.** Exposure to ≥2 μg/ml 6-MP resulted in lower *M. paratuberculosis* CFU than the CFU in the 1:100 inoculum dilution control at every drug exposure time beyond 6 days. Simultaneous exposure of *M. paratuberculosis* to 6-MP and CIP at the same concentrations resulted in significantly higher CFU than use of CIP alone (Fig. 4).

## DISCUSSION

Results for the antimicrobial susceptibility of *M. paratuberculosis* to standard drugs were consistent between plate counting and MGIT 960 methods (Fig. 1). When growth-suppressive effects were observed, they were drug concentration dependent. Additionally, the results were consistent with those previously reported (31, 45).

Immunosuppressive drugs in the sulfonamide family, SS, 5-AZA, and SP, had no effect on *M. paratuberculosis* growth, even at 64 μg/ml (Table 2). NAL was tolerated by *M. paratuberculosis* up to the maximum concentration tested, 64.0 μg/ml, supporting its use in primary *M. paratuberculosis* culture media for suppression of contaminating microflora (Table 1 and Fig. 1).

AZM was bactericidal for *M. paratuberculosis* at concentrations of 4.0 to 16.0 μg/ml (Fig. 2A). These findings are in agreement with reports of improved clinical status of Crohn's disease patients treated with macrolides (14). CIP, by contrast, killed 1 to 3 $\log_{10}$ of *M. paratuberculosis* ATCC 19698 cells over the first 5 to 8 days, and then a seemingly drug-resistant population of cells resumed growth at rates comparable to the that for the drug-free controls (Fig. 2B). (An alternative explanation for these results is that by day 8 the CIP was no longer

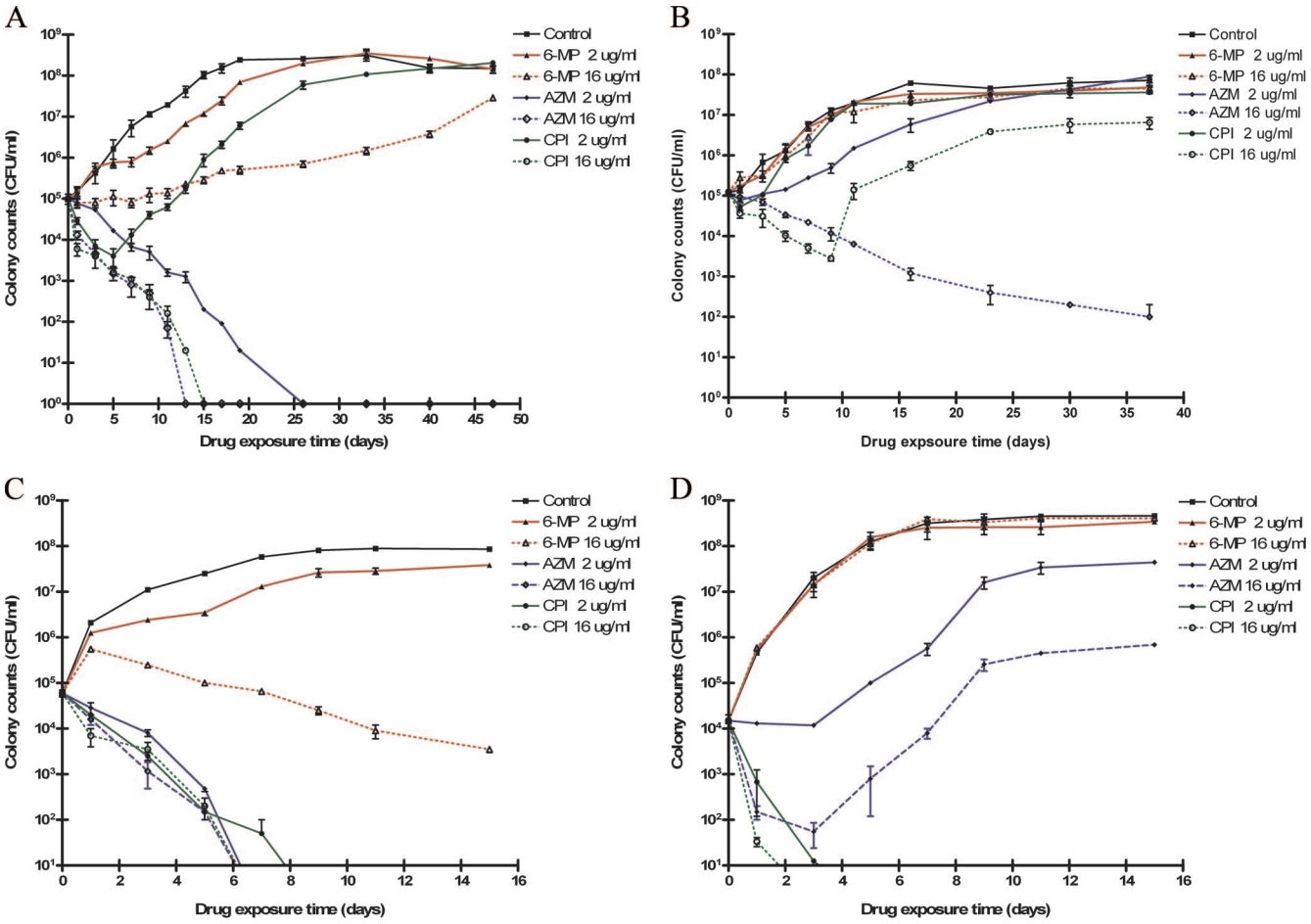


FIG. 3. Effect of AZM, CIP, and 6-MP on the viability of *M. paratuberculosis* strain UCF-5 (A), *M. avium* ATCC 35712 (B), *M. phlei* ATCC 11758 (C), and *M. smegmatis* $mc^2155$ (D) over time (up to 56 days), as determined by agar plate counts (CFU).

active, and future studies should evaluate residual drug activity.) Human-origin strains were more susceptible to CIP, with complete killing of $10^5$ CFU *M. paratuberculosis* in 12 to 26 days (Fig. 3A). RIF produced an *M. paratuberculosis* growth pattern similar to that produced by CIP, i.e., transitory decline in CFU followed by regrowth (Fig. 2B and C).

AZA drugs killed *M. paratuberculosis*. The thiopurine drug 6-MP suppressed its growth more than did AZA at the same

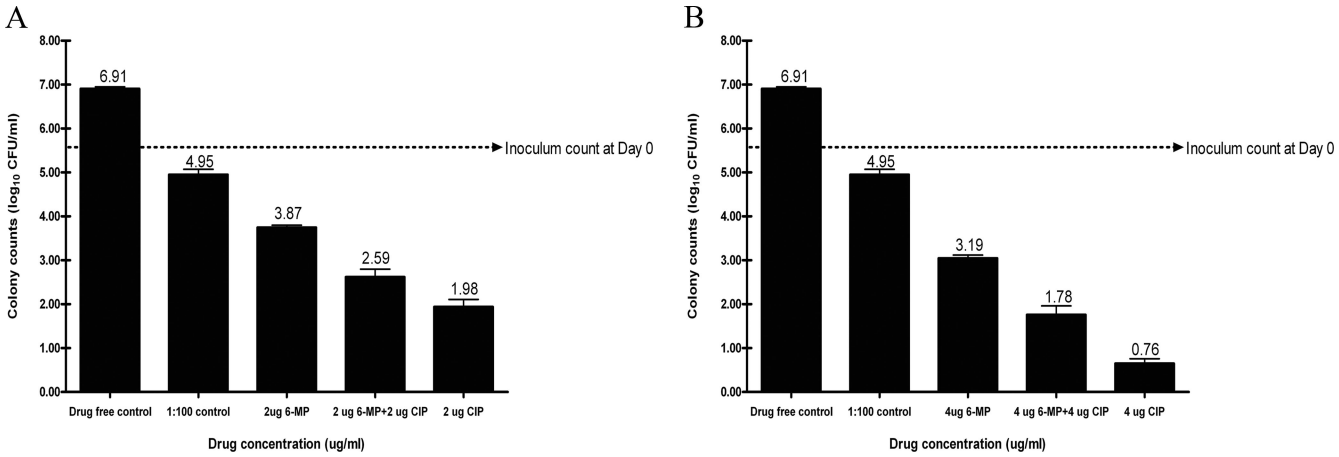


FIG. 4. Effect of CIP, 6-MP, and the combination of the two drugs both at 2 μg/ml (A) and 4 μg/ml (B) on the viability of *M. paratuberculosis* strain UCF-7 after 12 days of drug exposure as determined by MGIT 960 counting methods (38).

concentrations (μg/ml). If MGIT drug susceptibility standards for *M. tuberculosis* were used to interpret the data for 6-MP versus *M. paratuberculosis*, growth was suppressed at ≥2.0 μg/ml (potentially the MIC) compared with the 1:100 drug-free growth control (Fig. 2D). However, growth of *M. paratuberculosis* was not completely stopped, even at 16 μg/ml 6-MP, but was simply slowed in comparison to that of drug-free controls. Human-origin (Crohn's disease patient) isolates of *M. paratuberculosis* tended to be more susceptible to 6-MP than were bovine-origin isolates (Table 4). These findings are comparable to those reported by Greenstein et al. using the BACTEC 460 system for M. paratuberculosis in in vitro susceptibility studies (13, 45).

The growth-suppressive effects of 6-MP differed by mycobacterial species. These data suggest a possible mycobacterial-species-specific mechanism by which 6-MP interferes with replication or metabolism.

The antimycobacterial effect of thiopurine drugs is a novel and unexpected observation. The implications of the present study for the possible etiologic role of M. paratuberculosis in Crohn's disease and approaches to Crohn's disease therapy are important. Some investigators describe the failure of *M. paratuberculosis* to multiply in the face of immunosuppressive therapy with thiopurine drugs as evidence that Crohn's disease cannot be caused by *M. paratuberculosis* (35). Perhaps this pathogen, found in resected bowel tissue and peripheral blood leukocytes of some Crohn's disease patients (6, 25, 36), is held in check by thiopurine therapy since it has both immunosuppressive and anti-*M. paratuberculosis* activity.

These data also offer another perspective on data from clinical trials with Crohn's disease patients using antimycobacterial drugs. Since patients in the "control" group are maintained on standard therapy (which commonly employs thiopurine drugs), both the treatment group (antimycobacterial drugs) and control group (no antimycobacterial drugs but continued thiopurine drugs) are exposed to compounds with anti-*M. paratuberculosis* activity. If *M. paratuberculosis* is integral to Crohn's disease, then the opportunity to observe clear-cut therapeutic differences between the treatment and control groups in these trials is limited.

Antimycobacterial and thiopurine drugs used in concert may produce an interactive effect. The apparently bacteriostatic effects of 6-MP on *M. paratuberculosis* rendered the organism less susceptible to the bactericidal effects of CIP. This further complicates interpretation of many of the prior clinical trials with Crohn's disease patients that employed antimicrobials in addition to immunosuppressive drugs. Given the potential side effects of these medications, pursuit of therapeutic trials with patients in the absence of sound in vitro data is both premature and inappropriate.

A major challenge for antimicrobial susceptibility studies of *M. paratuberculosis* is its long generation time, i.e., roughly 2 days (24), and uncertainty about the stability of the test drugs in MGIT ParaTB medium at 37°C over the course of incubation. Without accepted standards for *M. paratuberculosis* antimicrobial susceptibility testing, it was vital that multiple methods and controls be employed and that results be descriptive and interpreted in relative rather than absolute terms such as "susceptible" or "resistant." Multiple mycobacterial species as well as nonmycobacterial species were needed as antibiotic susceptibility quality control standards. We also assessed whether drugs had an inhibitory or lethal effect on target organisms by subculture to drug-free media. More-extensive in vitro drug susceptibility trials with *M. paratuberculosis* are required to establish which drugs are most efficacious and which drugs, when used in combination, have a modulated effect on *M. paratuberculosis*. While the results of this research are provocative, expanded studies should include an assessment of the stability and activity of antimicrobial drugs in MGIT ParaTB medium at 37°C over the extended incubation period required for *M. paratuberculosis* drug susceptibility testing.

This work is hypothesis generating, not definitive. Methodological issues, in particular, that the mycobacteria were tested in an extracellular location, and the limited number of strains of each mycobacterial species tested are among just some of the caveats regarding extension of these findings to the clinical situation. However, this work highlights the complexity of studying *M. paratuberculosis* interactions with antibiotics and the possibility that anti-inflammatory drugs may exert antibacterial effects directly on this organism and/or have negative interactions with conventional antimicrobial drugs. These caveats argue against simple adoption of drug susceptibility testing methods used for *M. tuberculosis* or *M. avium* and for more-comprehensive characterization of the bactericidal or growth-inhibitory effects of antimicrobial and anti-inflammatory drugs alone and in combination on *M. paratuberculosis*.

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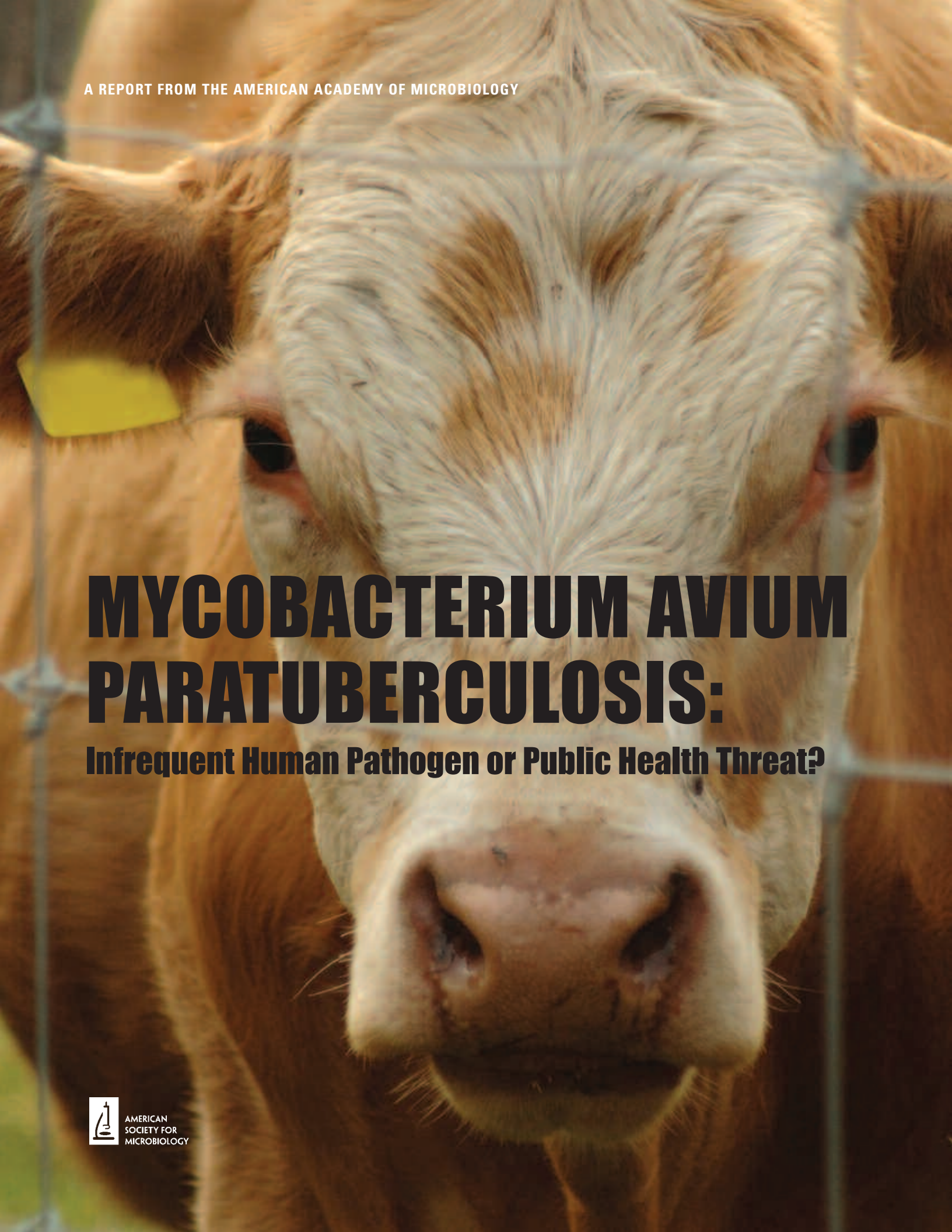

A REPORT FROM THE AMERICAN ACADEMY OF MICROBIOLOGY

# MYCOBACTERIUM AVIUM PARATUBERCULOSIS:

## Infrequent Human Pathogen or Public Health Threat?

AMERICAN SOCIETY FOR MICROBIOLOGY

# MYCOBACTERIUM AVIUM PARATUBERCULOSIS:
## Infrequent Human Pathogen or Public Health Threat?

**BY CAROL NACY, PH.D., AND MERRY BUCKLEY, PH.D.**

This report is based on a colloquium, sponsored by the American Academy of Microbiology, convened June 15-17, 2007, in Salem, Massachusetts.

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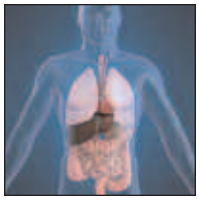
# EXECUTIVE SUMMARY



Crohn's Disease (CD) is a devastating illness in search of a cause and a cure. More than 800,000 people in North America suffer from CD, a gastrointestinal disorder characterized by severe abdominal pain, diarrhea, bleeding, bowel obstruction, and a variety of systemic symptoms that can impede the ability to lead a normal life during chronic episodes that span months to years. Researchers and clinicians agree that onset of CD requires a series of events; implicated are certain inherited genetic traits, an environmental stimulus, and an overzealous immune and inflammatory response. The combination of these factors contributes to a disease whose course is variable among patients and whose symptoms range from mild to devastating on any given day. The economic and social impact of this disease is substantial for the patient, the family, the community, and the healthcare system.

Long considered an autoimmune and chronic inflammatory disorder, current CD therapies are designed to treat symptoms of overactive inflammation in the gut. Chronic inflammation, however, does not generally induce itself. Inflammation is normally caused by a "foreign body," an inanimate object (i.e., splinter) or animate objects like rogue cells (i.e., cancer) or microorganisms (i.e., bacterium, virus, or fungus). Until the cause of inflammation is eliminated, the body continues to send in its clean-up crew, the white blood cells of inflammation whose job it is to expel the tissue invader. Inflammation only subsides when the causative agent is finally banished.

There is suspicion, supported by reports of genetic inability to interact appropriately with certain bacteria or bacterial products in some patients, that CD may have a currently unrecognized infectious origin, perhaps environmentally derived. That CD is a set of wide-ranging symptoms, more like a syndrome than a specific disease, suggests that if its origin is microbial, more than one etiologic agent may ultimately be identified. Bacterial suspects at the moment include a Mycobacterium and a variant of the normal bacterial flora of the gut, *Escherichia coli.* The possibility of more than one infectious cause that leads to a similar set of symptoms confounds the research agenda to find both a cause and a cure for CD.

One acknowledged potential microbial agent of CD is *Mycobacterium avium* subspecies *paratuberculosis* (MAP), a microorganism that causes a gastrointestinal disease similar to CD in ruminants, including dairy cattle, called Johne's disease (or paratuberculosis). People with CD have 7:1 odds of having a documented presence of MAP in blood or gut tissues than those who do not have CD, thus the association of MAP and CD is no longer in question (see Figure 1, page 11). The critical issue today is not whether MAP is associated with CD, but whether MAP causes CD or is only incidentally present, not an inciter or participant in the disease process.

If MAP is involved in the disease process of CD or other gastrointestinal disorders, then we need to determine how people are exposed to this microorganism, how to prevent that exposure, and how to treat the infection.

Despite its prevalence in the U.S. population in numbers that exceed most cancers, CD is not a focus of research attention in the same way as these other feared diseases. The American Academy of Microbiology convened a colloquium with experts in medicine, microbiology, veterinary pathology, epidemiology, infectious diseases, and food safety to describe the state of knowledge about the relationship between MAP and CD and to make recommendations for effective research that will move the field forward.

The general consensus of the assembled experts was that there are certainly reasons to suspect a role for MAP in CD:

- MAP persists in contaminated soil and water, which links the environmental factor of CD to the disease.

- MAP has a cell wall that contains muramyl dipeptide (MDP). One genetic trait that is affiliated in certain patients with CD is the NOD2 gene, which regulates ability to respond appropriately to MDP, thus the link between the genetic trait and MAP, or other bacteria.

- MAP causes Johne's disease, an illness of cattle and other ruminants that has many similarities with CD. The similarities of MAP disease in animals, for which the etiologic agent is known, and CD, for which the etiologic agent is unknown, provide a symptomatic link between agent and disease.

- MAP can survive standard milk pasteurization processes and has been identified in off-the-shelf milk in retail grocery stores in the U.S. and the European Union (E.U.). There is increasing concern that MAP can also be found in cheese made from the milk of MAP-infected cattle and meat from Johne's diseased animals. These observations could provide the exposure route of CD patients to MAP.

- Treatment of some CD patients with antibiotics that have activity on certain other Mycobacteria, although not specifically selected for their activity against MAP, provides short-term or long-term relief or remission of symptoms.

Circumstantially, these observations appear to make a compelling case for MAP as involved in CD. On the other hand, the ability to definitively identify MAP as the cause of CD, or the cause of a significant number of CD cases, has been stymied by the elusive characteristics the organism itself, the lack of broadly available and validated clinical tools to easily and definitively identify MAP in accessible tissues, and the late symptomatic stage at which CD is finally diagnosed, where the origin of the destructive inflammation could have

been years before the patient sought medical care. Most important, however, is the lack of resources, financial and scientific, to generate the tools that clinicians and patients need to determine whether MAP is involved in the disease process or not.

Several important clinical trials of antibiotics have been attempted in CD patients, with variable results. Treating CD patients with existing antibiotics with activity against other Mycobacteria (*M. tuberculosis*, which causes TB, and *M. avium* complex, or MAC, which is pathogenic in immune compromised persons) have either failed to provide relief (TB drugs) or produced promising outcomes for some patients, but not all (MAC drugs). Confounding these clinical results is the lack of information about which patients in the clinical trial population were actually infected with MAP, and whether any MAP organisms in infected patients were susceptible to the antibiotics used in the trials. Without sensitive and specific diagnostics that can detect early MAP infection, knowledge of how and where to isolate MAP for antibiotic susceptibility studies, and drugs that are known to be active against MAP itself, alone or in combination, the role of MAP in CD will remain circumstantial and the controversy over CD etiology will continue.

There is little known about where exactly viable MAP can be found in human tissues or, since most pathogenic Mycobacteria are intracellular, in which cells MAP can live and grow in humans. While the site of infection and tissue pathologies of MAP in animals can be assessed at necropsy, there is enough dissimilarity between digestive processes of ruminants and humans that this information may not necessarily inform studies in humans.

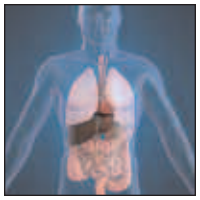
Of concern from a public health perspective is the ongoing presence of MAP disease in commercial livestock that supply the U.S. with dairy and meat products. If, in fact, CD is a zoonotic infection (one that is passed from animals to humans) and MAP is the (or one) cause of CD, then early identification of MAP disease in veterinary practice and appropriate management of these animals to safeguard the food supply will be critical to guard the public health.

Even in animals, it is nearly impossible to diagnose Johne's disease in the early stages of disease. Diagnosis is by a combination of clinical observation (wasting and reductions in milk production in dairy cattle, for instance) and microbiological, histopathological, and immunological testing of Johne's disease suspects. Although efforts to eliminate Johne's disease and MAP from livestock herds are ongoing, the lack of an accurate and easily-administered diagnostic for early disease onset is hampering these efforts. The results are mixed, and food products containing MAP or MAP DNA can be found on supermarket shelves. Veterinary diagnostics that are sensitive (detect MAP at early stages of infection) and specific (identify MAP and not other microorganisms) will be necessary to eliminate Johne's disease from the commercial food supply. Research to discover and validate these techniques may also shed light on diagnosis of human disease.

Colloquium participants agreed that research to elucidate the role of MAP in CD must address two major unknowns: (1) whether MAP from livestock and other animals is transmissible to humans and how it is transmitted and (2) whether humans are susceptible to infection and disease after exposure to MAP. No single study will fill all the gaps in our understanding of the possible relationship between MAP and CD. Furthermore, participants agreed that validated, reproducible biological markers confirming human MAP infection are desperately needed. If MAP can be causally associated with CD using reproducible analytical techniques, appropriate patient populations can be treated with antibiotics that are selected for their MAP activity. Then, at least MAP-infected CD patients will have both a cause and a cure.

# INTRODUCTION

## CROHN'S DISEASE (CD)



CD was named after Burrill B. Crohn, an American physician who published a paper in 1932 clearly distinguishing CD from intestinal TB (1). CD is a syndrome characterized by chronic and debilitating inflammation of the gastrointestinal tract that can be accompanied by mild to excruciating pain, frequent bouts of diarrhea, and malnutrition due to rapid passage of food through the inflamed intestinal tract. Some patients must be fed intravenously during the most difficult of their episodes. These devastating and episodic symptoms can force patients to maintain a limited work schedule or to refrain from working altogether, and the psychological effects of the disease are profound. The uncertainty of their condition and the ever-present possibility of symptomatic flare-ups often drive patients into anxiety, depression, and isolation. As of 2001, the Centers for Disease Control and Prevention estimated that over 500,000 people in the U.S. are living with CD (2), and more recent statistics show that 800,000 people in North America and 1 in 1000 persons in westernized countries have diagnosed CD. In the U.S., roughly 50% of CD patients are children. There is currently no cure for CD.

With the right anti-inflammatory drug or immunomodulatory biologic regimen, CD patients can experience temporary remission of symptoms, but long-term flare-ups inevitably follow, coming on suddenly or developing gradually over time. Surgery to remove inflamed sections of the bowel is the only option to alleviate the symptoms of many people living with CD. Many other patients endure endless regimens of drugs to alleviate some of their symptoms, but these drugs, too, have side effects, some that can even be life threatening.

## Table 1. Drugs for Therapy of Crohn's Disease, 2008[1]

| CLASS | DRUG | DOSE | TABLET SIZE | AV. WHOLESALE PRICE/TAB ($)[2] | AV. DOSE (70KG) | # TABLETS | COST/DAY | COST/MO | DURATION | NOTES |
|---|---|---|---|---|---|---|---|---|---|---|
| **GUT ANTI-INFLAMMATORY** | | | | | | | | | | |
| | sulfazalazine | 3g/day | 500mg | 0.50 | 3 grams | 6 | $3.00 | $90.00 | ongoing | |
| | olsalazine | 1g/day | 250mg | 1.66 | 1 gram | 4 | $7.00 | $210.00 | ongoing | |
| | balsalazide | 6.75 grams | 750mg | 4.45 | 6.75 grams | 9 | $40.00 | $1200.00 | ongoing | |
| | mesalamine | 3 grams | 400mg | 1.40 | 3 grams | 8 | $11.00 | $330.00 | ongoing | |
| | mesalamine suppository | 1 gram | 1gm | 5.41 | 1 gram | 2 | $10.82 | $325.00 | acute | |
| | mesalamine | 4 grams | enema 60cc | 25.86 | | 1 | $25.86 | $776.00 | acute | |
| | mesalamineXR | 4 grams | 500mg | 1.79 | 4 grams | 8 | $14.00 | $420.00 | ongoing | |
| **STEROIDS** | | | | | | | | | | |
| | prednisone | 20-60mg | 20mg | 0.10 | 40mg | 2 | $0.20 | $6.00 | acute, then taper | |
| | dexamethasone | 0.75-9.0mg | 4mg | 0.58 | 4mg | 1 | $0.58 | $17.00 | acute use only, taper | |
| | prednisolone | 5-60mg | 5mg | 0.03 | 40mg | 8 | $0.24 | $7.00 | acute increase then taper | |
| | hydrocortisone | 20-240mg | 10mg | 0.41 | 20mg | 2 | $0.82 | $25.00 | acute, then taper if possible | |
| | budesonide | 9mg | 3mg | 5.72 | 9mg | 3 | $17.00 | $510.00 | acute, taper | |
| **ANTIBIOTICS** | | | | | | | | | | |
| | ciprofloxacin | 1 gram | 500mg | 5.37 | 1 gram | 2 | $10.74 | $322.20 | 2-4 weeks | cost offset by medicare |
| | metronidazole | 1 gram | 250mg | 1.45 | 1 gram | 4 | $5.80 | $174.00 | 2-4 weeks | |
| | ethambutol | 15mg/kg | 100mg | 0.60 | 1 gram | 10 | $6.00 | $180.00 | ongoing | |
| | rifabutin | 600mg | 150mg | 10.07 | 600mg | 4 | $40.28 | $1208.00 | ongoing | |
| | clarithromycin | 1 gram | 500mg | 4.52 | 1 gram | 2 | $9.04 | $271.00 | ongoing | |
| | azithromycin | 500mg | 500mg | 15.55 | 500mg | 1 | $15.55 | $467.00 | ongoing | |
| | rifaximin | 600mg | 200mg | 3.72 | 600mg | 3 | $11.00 | $330.00 | ongoing | |
| | clofazimine | 100mg | 100mg | n/a | 100mg | 1 | | | ongoing | n/a in U.S. Free in rest of world |
| **BORODY COCKTAIL (CLARITHROMYCIN, RIFABUTIN, CLOFAZIMINE)** | | | | | | | | | | |
| | | | | | | | $49.32 | $1479.00 | ongoing | Assumes clofa-zimine is free |
| **IMMUNE SUPPRESSANTS** | | | | | | | | | | |
| | azathioprine | 2.5mg/kg/d | 50mg | 1.31 | 100mg | 2 | $2.62 | $79.00 | ongoing | Requires hepatic testing |
| | 6-mercaptopurine | 2mg/kg/d | 50mg | 4.08 | 100mg | 2 | $8.16 | $244.80 | ongoing | |
| | methotrexate | 25mg IM/wk | 2.5mg | 3.56 | 25mg | I0 units | $5.09 | $152.70 | ongoing | |
| | cyclosporine | 2.5-15 mg/kg/d | 100mg | 1.65 | 7mg | 7 | $49.50 | $1485.00 | ongoing | |
| | tacrolimus | 0.1-0.2 mg/kg/d | 5mg | 23.82 | 10.50mg | 5 | $119.10 | $3573.00 | ongoing | investigational |

## Table 1. Drugs for Therapy of Crohn's Disease, 2008[1]

| CLASS | DRUG | DOSE | TABLET SIZE | AV. WHOLESALE PRICE/TAB ($)[2] | AV. DOSE (70KG) | # TABLETS | COST/DAY | COST/MO | DURATION | NOTES |
|---|---|---|---|---|---|---|---|---|---|---|
| **BIOLOGICAL RESPONSE MODIFIERS** | | | | | | | | | | |
| | infliximab | 5mg/kg IV at wk 0,2,6 | | | | | | $2542.00 /infusion | | Cost of infusion ~ same as AWP Initial cost = $7,626.00 |
| | | 5mg/kg IV every 8 weeks | | 726.18/100mg | 350mg | | | $1271.00 /mo average | | Cost of infusion ~ same as AWP IV infusion total cost ~$6,000/2 mo |
| | adalimumab | 40mg/wk SQ | 40mg | | | 1 pen/wk | | Starter pack $5100.00 | | |
| | | 40mg/wk SQ | 40mg | 866/pen | | 1 pen/wk | | $3464.00 | | |
| | thalidomide | 50-300mg | 100mg | 190.74 | | 100mg | $190.74 | $5722.20 | investigational | Immune suppressant, now considered TNF inhibitor |
| | natalizumab | 300mg IV q 4 weeks | | n/a | | | | | investigational, for MS only | |
| | alicaforsen | n/a | | | | | | | investigational | |
| | sargromostim | 6mg/kg/d SQ | 500mg | 373.36 | | | | | investigational | |
| | cortolizumab | n/a | | | | | | | investigational | |
| **STEM CELLS** | | | | | | | | | | |
| | Stem Cell Transplant | n/a | | | | | | $100,000 | investigational | Risk: sepsis/death from infection |
| **ANTIDIARRHEAL AGENTS** | | | | | | | | | | |
| | diphenoxylate | 1 tab QID | | 0.42 | | 4 | $1.68 | $50.40 | | |
| | paregoric | 5-10cc up to QID | | 0.70 per 5cc | 10cc | | $1.40 | $42.00 | | Narcotic, not for long term use |
| **PROBIOTICS** | | | | | | | | | | |
| | Saccharomyces boulardii | 1-3 caps/d | | | | | | $12.80 | | Online cost |
| | VSL #3 | 1 pack/d | | | | | $2.65 | $79.50 | | Online cost |
| **IMMUNE STIMULANTS - PARASITES** | | | | | | | | | | |
| | hookworms | | | | | | | $4000 | lasts 5 yr | |
| | pig whipworms | | | 450.00 for 2500 eggs | | | | $900 | every 2 wk | |
| **UNKNOWN MECHANISM** | | | | | | | | | | |
| | Low dose naltrexone | 4.5mg/d | | Compounded Regular dose is 50mg | | | | | | Cost in Canada is 25.00/mo for 4.5mg tabs |
| **TOTAL PARENTERAL NUTRITION** | | | | | | | | | | |
| | | | | $5600 | weekly | Medicare allows $1400/wk | | | | |

[1] Table compiled by J. Lipton, M.D. with assistance from F. Cunningham (UC Berkeley, CA) and I. Barash, Ph.D., M.D. (UCSD).

[2] Average Wholesale Prices (AWP), provided B. Faulkner, Woodinville Medical Center Pharmacy, Woodinville, WA 98072 USA on February 29, 2008. NOTE: AWP change from day to day based on information from the drug manufacturers and/ or by calculations made by drug wholesalers. The AWP does not always accurately reflect the retail cost of a drug, especially in the case of many generic drugs which can be purchased and sold below the AWP in many markets. AWP is the price used in comparing prices of drugs, although for some drugs it is not an accurate reflection of cost to the patient.

## PROMINENT THEORIES ON THE ETIOLOGY OF CD

### AUTOIMMUNE DISEASE

For decades, CD was considered an autoimmune disease, where the immune system erroneously identifies tissues of the gastrointestinal tract, specifically the large intestine, as "altered" or "foreign" and begins a relentless attack. In recent years, however, the pathologies associated with CD have been observed in other conditions that affect the regulation of immune and inflammatory cells to cope with bacterial pathogens, such as Chronic Granulomatous Disease (CGD) and Chronic Variable Immunodeficiency (CVI). Moreover, studies of white blood cells present in tissue biopsies from CD patients demonstrate a dysfunction in the ability of these cells to ingest particles, a preliminary event for clearance of bacteria that invade tissues (3). These more recent studies call into question the autoimmune etiology of CD. This disease does not appear to be a result of immune activities directed specifically against human tissue.

### GENETIC DISORDER

Studies strongly suggest that CD has a genetic component (4, 5), one that controls an immune defect. Whole genome studies of CD patients identified an association of the disease with genes of the NOD2 family that influence the immune system (3, 5). Mutations in the NOD2 gene found in CD patients disrupt the ability of immune cells to recognize certain signals that are present in pathogenic bacteria. But it is also clear that heredity is not the full story, nor is an overactive immune system. The inflammation of CD may be due to a malfunction of the immune system that is infectious in origin, genetically controlled, and environmentally exacerbated.

### ENVIRONMENTAL TRIGGER

The incidence of CD has been rising over the last several decades, as observed in multiple countries by independent investigators. There is an association between rising economic conditions and increasing reports of CD in a population. This has been interpreted as evidence that an environmental trigger is as, if not more, important in the etiology of CD as human genetics.

## MICROORGANISMS

That NOD2 gene mutations are found in 25-35% of CD patients suggests a microbial factor in disease onset for at least a subset of patients. CD tissue pathology and symptoms could be due either to an inappropriate response to otherwise harmless bacteria, or to an appropriate (but ineffective) immune response to harmful bacteria that are introduced into the gut. In these scenarios, tissues of the gut become damaged and inflamed as ancillary events to the main battle, the attempt of the immune and inflammatory cells to eliminate the offending bacteria. The bacteria that have been implicated to date include certain soil Mycobacteria and variant of the normal flora of the gut, *Escherichia coli.*

## MULTI-FACTORIAL ETIOLOGY

The cause (or causes) of CD is not known, and the disease is complex. It is possible that CD is a syndrome with many different origins. Considering the diverse range of symptoms in different patients, CD could result from different infectious agents and/or different underlying genetic or immune factors.

## SOURCES OF MAP FOR ANIMALS AND HUMANS

Soil contains a wide variety of habitats and ecological niches that teem with life, playing host to as many as 10 million bacteria, fungi, and parasites per gram. MAP belongs to a large and diverse Mycobacteria family of bacteria, many of whose members, some 40 different species, are ubiquitous in the environment. Most of these Mycobacteria are not pathogenic for animals or humans, but live out their lives contributing to the richness and fertility of croplands or pastures. Some, like *M. kansasii*, *M. abscissium*, or *M scrofulaceum*, live primarily in the soil, but under the right circumstances can become "opportunistic pathogens" and cause disease if introduced into mammalian tissues inappropriately or inadvertently.

On the other end of the family spectrum are several mycobacterial species that are not generally found in soil and that are highly adapted to life inside mammalian hosts. These Mycobacteria are facultative intracellular pathogens that spend much of their life within mammalian white blood cells called macrophages, the very cells that should be the first line of defense against infection. They can cause serious, life-threatening chronic illnesses in both animals and humans and have been acknowledged public health threats for over a century. They belong to the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* and *M. canetti*) and are the etiologic agents of contagious and fatal tuberculosis (TB) disease in animals (from *M. bovis*) or humans (from *M. tuberculosis*). Human TB can be acquired from infected animals (a zoonosis) or from other infected humans through contaminated respiratory secretions. So pathogenic are these agents that 2 billion of the 6 billion people in the world are infected with *M. tuberculosis* (1 of every 3 people), and 2-3 million deaths from TB are recorded each year.

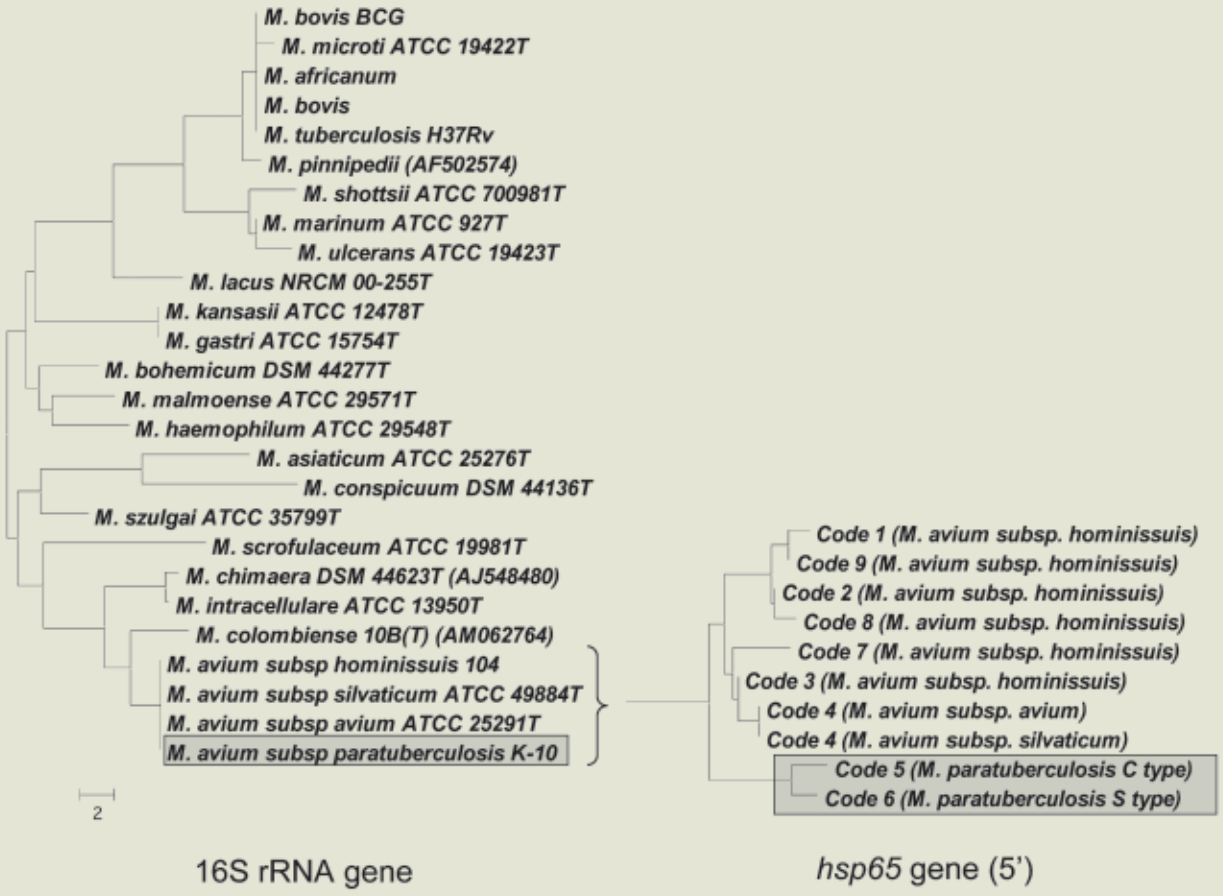
Species of another mycobacterial family, *M. avium* Complex (MAC), represent another threat to human health. These bacteria are found in the environment in soil and water, but certain MAC members have specifically adapted to life within particular mammalian or avian hosts. The MAC family evolved two distinct subfamilies that distinguish themselves both genetically and phenotypically (6) (see Figure 2, Page 22).

- *M. avium* subspecies avium (MAA) has evolved to be more like the soil Mycobacteria that can become opportunistic pathogens as described above. MAA strains cluster genetically into a group of free-living bacteria that replicate in the environment, especially in water. But MAA can also infect birds and human lungs, causing a transient low-pathology infection or triggering a chronic inflammation that is ultimately fatal. For example, MAA can cause a potentially fatal pneumonia in immunocompromised hosts, and is one of the more intractable opportunistic pathogens of Human Immunodeficiency Virus (HIV)-infected individuals.

- *Mycobacterium avium* subspecies paratuberculosis, or MAP, comprises a second genetic subgroup that can survive in soil or water for months to years. It is not clear whether MAP can actually replicate in the environment because it is unable to produce mycobactin, an essential iron transport chemical synthesized by every other Mycobacterium sp. Instead, MAP has learned to successfully infect and replicate inside the same white blood cells of mammals as *M. tuberculosis*, macrophages. Rather than targeting the respiratory tract, however, MAP has adapted to the gastrointestinal tract of ruminants and other animals and can cause a slowly developing, but eventually fatal, inflammatory disorder called Johne's disease.

**The question the colloquium addressed is whether MAP has also evolved to infect human gastrointestinal tissue, and whether, once there, it can cause disease.**

**Figure 1. Mycobacteria family genealogy. MAP is genetically linked to other MAC strains, but is a genetically distinct subspecies. (Courtesy of M. Behr, McGill University, Canada)**



## MAP AND JOHNE'S DISEASE

Johne's disease is a $1.5 billion a year cattle industry problem in the U.S. (7). Johne's disease is found in livestock herds around the globe, and though the condition is easy to identify once symptoms arise, it is challenging to diagnose in the early stages of infection. Healthy but infected animals transmit the organism through milk and manure, invisibly spreading the infection to young animals. As a result of this silent transmission, it is difficult to eliminate MAP from individual herds.

### Prevalence

Although MAP, the cause of Johne's disease, has a broad host range, the most commonly infected animals are ruminants, such as cattle, sheep, goats, or deer, both wild and domestic (8). Johne's disease is more common in the more closely confined dairy cattle than in beef cattle, however, where higher animal density and more extensive premise contamination means greater contact with the organism. While there is evidence for host predisposition for certain strains of MAP, it is believed that all strains are capable of causing infection and pathology.

MAP has a broad geographic distribution, and Johne's disease has been reported on every continent in virtually every country that has animal agriculture and laboratory diagnostic capability. In 1996, 3-10% of U.S. dairy cattle were infected with MAP, and 22% of U.S. dairy herds had a MAP prevalence of >10% (9). A similar survey in 2007 reported that 68% of U.S. dairy herds are infected with MAP. In Europe, MAP infection rates among dairy cattle range from ~0% now in Sweden and Norway, where new infections are actively culled, to 50% in the Netherlands and 80% in Denmark. Infection rates vary widely across the various cattle-growing regions of Australia. Few countries are free of MAP and Johne's disease, but the reported prevalence of infected animals in any country is only as good as the diligence with which the disease is surveyed. Although difficult early diagnosis certainly contributes to the uncertainty of the actual figures, a recent increase in the number of infected herds is noticeable, including in countries that previously had low infection rates. A study ending in the fall of 2008 is expected to reveal much higher U.S. domestic MAP infection rates than previously reported (10).

MAP is shed from infected animals in feces, and infection transmission between and among animals is similar to other fecal-orally-transmitted diseases. There is an age-dependent susceptibility to MAP infection, and most new infections happen early in the animal's life. Infection of newborns likely happens through transmission *in utero* (before birth), by contaminated colostrum or milk from fecal-contaminated teats or a MAP-contaminated birthing environment.

Infected animals are the only source of MAP in nature, since the pathogen does not generally replicate outside of mammalian cells. When found in soil or water samples, one can assume that the environment was contaminated by MAP in feces deposited by an infected animal (11). MAP shed by infected animals can persist in the environment for years, and soil is another possible source of new animal infec-

tions, although more research is needed to clarify the role of MAP-contaminated water and soil on animal infection rates. The second source of infection, one more amenable to experimentation, is the transmission of MAP from dam to offspring through nursing from contaminated teats or contaminated milk.

**RESEARCH:**

**The high variability in Johne's disease infection rates in various geographic areas presents an opportunity to learn more about the factors that maintain MAP infection and Johne's disease in cattle herds. Epidemiological studies should be designed to make use of data on infection rates and information regarding hygiene practices.**

### Symptoms

Johne's disease in cattle is characterized by chronic or intermittent diarrhea, weight loss, decline in milk production, and, eventually, death. Animals usually become infected with MAP prior to six months of age, but the signs of Johne's disease are not likely to be observed until two or more years after the initial infection. This extended delay between infection and onset of illness makes the disease difficult to manage in cattle herds, since infected non-symptomatic cattle can contaminate the livestock areas and spread the infection to other animals.

### Diagnosis

There are several types of diagnostic tests available for Johne's disease; some detect the presence of MAP-specific antibody (serology), some depend on identification of the organism itself (fecal, tissue or environmental sample culture; PCR to detect MAP-specific nucleic acids) (12). Additional support of a positive Johne's disease diagnosis can be obtained from assessing the clinical condition of the animal and from microscopically examining tissue samples for characteristic pathologic lesions and/or the presence of the organism itself, by culture or PCR.

In general, accuracy of diagnosis is affected by stage of the infection, host species, and MAP strain differences (6). Laboratory diagnostic tests for Johne's disease have high specificity (low false-positive results), but low sensitivity (percentage of MAP-infected animals that test positive). ELISA, a commonly used immunological technique to detect MAP-specific antibodies in blood, detects only about 30% of infected animals when they are still in the preclinical disease phase. In the very early stages of infection, before animals begin to shed MAP in feces or develop an immune response to infection, all animals usually test negative. As the infection progresses and clinical symptoms appear, most of the tests become positive. Diagnostics work best to confirm MAP infection in animals with clinical signs of disease, i.e., diarrhea and/or weight loss.

In the absence of sensitive and accurate diagnostics for early infection, animals are culled when they begin to exhibit decreased milk production, a practice that may result in unnecessary destruction of cattle and economic losses. The lack of tools for early diagnosis also cripples the ability to interrupt the chain of MAP transmission, critical for limiting loss of livestock and, if MAP is a human pathogen, for limiting human exposure.

### Control Measures

A number of different control measures are available to limit spread of MAP within herds of cattle and other livestock. Animals become infected with MAP by ingesting the organism through contaminated feed or water. Young animals can

acquire MAP by consuming tainted milk or colostrum. Newborns may become infected *in utero*, but the significance of this route of infection has been difficult to ascertain.

Most dairy cattle herds have frequent turnover (35-40% per year), so preventing the introduction of new animals with uncertain MAP credentials to an uninfected herd is critical. Control measures for MAP infection include (13):

- Segregating birthing facilities from the general herd premises and maintaining good hygiene,
- Bottle-feeding colostrum from test-negative cows,
- Using milk replacer (pasteurized) instead of herd milk (raw),
- Diagnostic test surveillance for the infection and culling test-positive animals,
- Certifying MAP-free herds to provide sources of her replacement, and
- Vaccinating against MAP.

Although anti-MAP vaccines attenuate severity of Johne's disease in domestic cattle, sheep, goats and deer, and may reduce MAP shedding by up to 90%, vaccination does not protect against all new cases of infection by MAP (7). It may, paradoxically, facilitate transmission of MAP within a herd and to other herds, by diminished culling of infected animals. Until otherwise certified as MAP-free, MAP-vaccinated animals should be regarded as potentially infected with MAP.

There is evidence that the physical control measures described above can reduce the prevalence of Johne's disease (14), but regional disparities in effectiveness do exist. This may be because most control programs are voluntary, in part due to the costs of replacing culled animals. Considering this, and the current limitations in testing and control measures, eradication of MAP and Johne's disease does not appear to be a practical target.

Unless MAP is demonstrated to cause disease in humans, however, there is no immediate imperative for widespread mandatory cattle culling. If MAP is not a pathogen of humans, but only animals, then economic considerations, the effectiveness of other transmission control measures, and local policy considerations will help inform the need for culling infected animals.

**RESEARCH:**

**A number of methodological and technical improvements are needed to refine existing veterinary diagnostic tests for Johne's disease and to develop new molecular and antigenic targets using MAP gene sequences or other approaches to improve speed, accuracy, and sensitivity of diagnosis in the earliest stages of infection. These improvements will facilitate cattle and other domestic animal disease management, interrupt the MAP transmission cycle, and stem the financial losses due to culling that the dairy industry currently experiences. If MAP is a human pathogen, then a better diagnostic for early-stage Johne's disease will help to reduce MAP in the food supply and environment and may help prevent development of human disease.**

## MAP IN THE FOOD SUPPLY

Cattle are the source of milk on the shelves of our supermarkets and the source of beef in our freezers. Cattle herds in the U.S. that are plagued with MAP infections and Johne's disease in increasing numbers should be a public health concern until the controversy over MAP as an etiologic agent of human disease is resolved.

### Prevalence

A recent study using culture-dependent methods detected viable MAP in 2.8% of homogenized milk cartons sampled from supermarket shelves in the U.S. Molecular detection methods, which detect the presence of MAP nucleic acids, detected MAP in 64% of samples (15). Other studies detected MAP in samples of soft cheese (16, 17). There is some evidence that MAP can be found in meat, but research in this area is scanty (18, 19). Overall, different levels of MAP contamination of milk and food products have been noted in the U.S. and a variety of countries around the world. MAP has also been identified in other environmental sources in addition to food, including river water and municipal water (20).

Prevention of MAP in the food chain could be assured by only sourcing raw products from animal herds free of MAP. A formal regulatory policy for mandatory screening for MAP in consumer foods should only be considered, however, when focused research studies determine the actual risks of human exposure from this source. Knowing the incidence of MAP in the food chain is of no use unless we also have a detailed understanding of the infectious MAP dose from these sources and an understanding of the consequences of infection.

### Control Measures

Standard HTST pasteurization reduces the number of culturable MAP cells in milk products, but does not eradicate MAP (21, 22). "Light pasteurization" (thermization), a process that is less rigorous than regular pasteurization, permits development of higher densities of MAP cells. Successful methods for reducing MAP in milk may include:

- Ultra-high temperature pasteurization (UHT milk),
- UV irradiation,
- Dehydration followed by rehydration,
- Freezing before pasteurization,
- Gamma irradiation,
- Centrifugation, and
- Mycobacteriophages (viruses that infect and kill mycobacteria).

**RESEARCH:**

**Better methods to determine the true prevalence of MAP in foods are needed, as well as methods to determine whether MAP in food is viable and infectious, the infectious dose for animals, if not humans, and the dose that can cause disease in animals and in humans. More research is needed to determine which processes, if any, are effective at killing MAP in food products. More research is also needed to identify whether MAP can be carried away from MAP-contaminated farms and pastures to potable water supplies via surface water and dust, and whether these MAP are sources of infection and disease in animals or humans.**

**Public Health Concerns**

Scientists and public health authorities do not know whether meat and milk products contaminated with MAP expose the public to any risk of illness. When seen on a global scale, the potential implications of such a scenario are staggering. Responsibility for managing exposure and risk from MAP in consumer goods in the U.S., in this case, would fall to the Department of Agriculture, the Food and Drug Administration, the Centers for Disease Control and Prevention, and other federal agencies.

The question of whether MAP-infected animals are fit for human consumption depends on whether MAP is a zoonotic pathogen. Although there is evidence for public exposure to MAP in consumer goods, the biological significance of MAP presence in milk, cheese, and meat is not known, and it is also not known whether eating these products leads to MAP infection, or whether MAP infection necessarily leads to disease.

The appropriateness of taking precautionary measures in the face of a plausible but uncertain risk to human health by exposure to MAP in food sources is a hotly contested debate. What is the level of evidence that must be met in order to take action to limit human exposure to MAP?

In the face of current evidence, at least minimal actions, like removing clinically diseased animals from the food chain, are considered appropriate.

## MAP AS A HUMAN PATHOGEN

At the heart of the controversy of MAP as an etiologic agent of CD is whether MAP can initiate human infection at all. MAP can be detected in the human body, and researchers and clinicians have published this fact in a number of case reports in the scientific literature. Moreover, persons with CD are seven-fold more likely than the general population to have MAP associated with their disease (23). However, there has been no definitive causal relationship established between MAP and a specific disease process in humans similar to that which has been established in animals (Johne's disease).

The current methods for isolating MAP from human intestinal tissue for authoritative identification are invasive and are generally obtained from individuals suffering the later stages of CD. The relationship between MAP and the initial stages of CD cannot be extrapolated from testing such late disease-stage tissue samples, and the role of MAP in the initiation of the infection is unclear. The question that needs to be answered is whether MAP is:

- A "professional" human pathogen (pathogenic to otherwise healthy individuals),

- A co-conspirator with other pathogenic bacteria (either one or both partners pathogenic only in the presence of the other, similar to the "enhancement effect" seen in certain virus pairs),

- An opportunistic pathogen (a pathogen in only diseased, genetically-susceptible or immune-compromised individuals), or

- A harmless commensal organism that is simply a bystander, with no role in any human disease process.

**RESEARCH:**

**Efficient and effective clinical methods for sub-speciation of MAP into ruminant-specific strains would enable the identification of sources of human MAP and assist in the epidemiology of MAP infection.**

MAP is unequivocally pathogenic in animals and may also be pathogenic in humans, but until sensitive and specific diagnostics are discovered and distributed for widespread use, the controversy over MAP and its role in any disease, including CD, will continue.

It is possible that MAP plays a role not only in CD and other gastrointestinal dysfunctions, but also in a variety of other diseases. MAP has been detected in tissues outside the gastrointestinal tract, in a patient with HIV, in lymph tissue, and in breast milk from a lactating mother.

Although the numbers of MAP organisms isolated from any tissue, gastrointestinal or other, has been invariably small, there is precedence for paucibacillary (exhibiting few bacteria) disease caused by other Mycobacteria. Tuberculoid leprosy, caused by *M. leprae*, is a very good example of a mycobacterial disease in which extensive tissue pathology, immunologic in nature, is observed in the absence (or sparse presence) of the inciting bacteria. Since MAP is not widely recognized as a pathogen, physicians do not consider MAP during differential diagnosis, and pathological tissues are seldom tested for the presence of MAP. Compounding this problem is the absence of non-invasive and specific diagnostics available to test for MAP in humans, even if it were suspected.

The noted differences between bovine MAP strains and human MAP strains pose another barrier to our understanding the possible role of MAP in human disease. If MAP is transmitted from infected livestock to humans through the consumption of contaminated food, then testing for the presence of bovine MAP strains in humans could be useful for determining the epidemiology of MAP disease. However, species-specific strain identification is infrequently performed, so it is not known whether ovine strains can lead to human illness.

**Prevalence**

The limited number of studies looking for MAP in CD tissue revealed the prevalence of MAP to be between 2% and 20% in various control groups, as well 25-35% in persons with CD (www.cdc.gov). The prevalence of MAP in the general population is currently unknown because the main method for isolation of MAP is intestinal tissue biopsy, and healthy people are not routinely subjected to this procedure. Prevalence and incidence of MAP infection in the general population

awaits the identification and development of specific and sensitive non-invasive diagnostics for this organism.

Infections with MAP must be distinguished from infections caused by other agents, since identifying the correct etiologic agent is the basis for understanding the epidemiology of the disease and its public health implications.

**RESEARCH:**
**Non-invasive screening diagnostics with high specificity for MAP will need to be developed for epidemiology studies in humans to assess incidence and prevalence of MAP infections and determine the public health importance of these infections.**

### Pathologies

For every condition MAP causes in animals, there may also be a parallel condition in humans. Moreover, it is possible that MAP can cause disease similar to that caused by its close mycobacterial family members or distant cousins, although evidence for MAP infections of other organs (i.e., lungs) is certainly more limited than for CD. MAP has been implicated in scrofula, ulcerative colitis, sarcoidosis, and type 1 diabetes, and there are also isolated reports of MAP disseminating outside the gastrointestinal tract, causing bacteremia (24).

### Diagnosis

Individual research laboratories have developed a number of different assays and techniques for diagnosing MAP disease in animals and humans, including bacterial culture, PCR techniques to identify cultured bacteria or to identify MAP DNA in tissues, immunological techniques (cell proliferation and serologic assay), and histopathological examination of tissue using special stains. Samples for diagnosing MAP infection include blood cells or serum, stool, breast milk, biopsies of bowel tissue, and tissue from bowel resection. Culture, stain, or DNA assays are most frequently performed on tissue samples, generally from patients with late-stage disease, and the ability to do these tests on other samples, such as blood, are limited to just a few research labs (24, 25). Immunologic assays have not been sufficiently sensitive for diagnosis of early Johne's disease, where MAP is the definitive causative agent, so it is not a surprise that serologic studies to identify MAP infection of healthy humans or patients with gastrointestinal or other disorders are not demonstrating differences in these populations.

**RESEARCH:**
**Scientists and clinicians in different labs should be encouraged to collaborate to establish standardized, reproducible assays, to make inter-laboratory comparisons, and to clarify the possible connection between the presence of MAP in tissues and disease.**

From a regulatory and commercial perspective, there are no approved tests for diagnosing MAP infection in humans. GenProbe's Amplified™ MTD test is often used for "diagnosing" MAP, but this product is not specific for MAP and will also be positive for other members of the broad Mycobacteria family. Unfortunately, the most definitive research techniques for diagnosing MAP infection are not widely used in clinical laboratories, so isolation and identification of MAP is spotty across a wide distribution of interested parties.

### Prophylaxis

Despite decades of research, there are no universally effective vaccines against other Mycobacteria sp., such as *M. tuberculosis* (TB) or *M. leprae* (leprosy). Like these well-known pathogens, it may be possible to develop a MAP vaccine using post-genomic techniques, but so far these efforts have not been fruitful for any of the Mycobacteria. *M. tuberculosis*, *M. leprae*, *M. avium*, and MAP (26) genomes

have been sequenced, and comparative analysis might provide direction for MAP vaccine design. Until MAP is decisively identified as a human pathogen, however, vaccine developers are likely to direct their efforts elsewhere.

## ANIMAL MODELS OF MAP INFECTION IN HUMANS

If MAP is a human pathogen and causes human disease, it is one of the few in which Koch's postulates were satisfied before the associated disease in humans was described. MAP can be isolated from ruminants with Johne's disease, identified genetically as MAP, and can be re-infected into the same ruminant class (or different ruminants) to cause the same disease. MAP unequivocally causes Johne's disease.

If any human gastrointestinal syndrome is equivalent to Johne's disease, then ruminants could help in the identification of diagnostics and drugs for MAP. However, the link between any gastrointestinal disease and Johne's disease is still highly contentious, and the pathologies of these diseases and Johne's disease, while sharing many characteristics, also have certain characteristics that are distinct. Veterinary experience with Johne's disease identified potential ways to diagnose of human MAP infections, but none of these tests have yet been validated, and they are not routinely available in clinical laboratories. Since it is impractical to treat MAP infections in animals, no guidance for treatment of human MAP infections is found in veterinary literature.

**RESEARCH:**

**Once MAP is definitively identified as a human pathogen, the research community must develop, characterize, and standardize appropriate animal models that can address critical research questions about protective anti-MAP immunological processes and can demonstrate candidate vaccine efficacy. These animal models will be necessary to power the vaccine development process.**

Small animal models of CD are available, but the extent of their relevance to human disease remains uncertain.

### Treatment

If MAP is an infectious cause of human disease, there is only one therapeutic regimen that could address cure: antibiotic therapy. The only antimicrobial therapies currently recommended for potential MAP infections use drugs presumed to have activity against MAP (macrolides, rifamycins, clofazimine, and ethambutol), all of which actually have limited potency against MAP. They were selected for clinical evaluation because they worked in a small number of CD patients whose disease was presumed to be initiated by MAP. To date, there are no antibiotics that have been specifically developed for treatment of MAP infections.

As is the case with other mycobacterial diseases (including *M. avium* pneumonias, TB, and leprosy), multi-drug therapy with agents from different antibiotic classes may be necessary to eliminate MAP infections and avoid development of MAP resistance. There is recent evidence that existing anti-inflammatory agents may also affect MAP directly. This possibility should be considered in clinical study design and analysis, since most patients with suspected MAP infections are treated for their inflammatory disorder before (and sometime during) treatment with antibiotics.

**RESEARCH:**

**Suitable *in vitro* techniques or animal models that can be used to accurately predict the effectiveness of prophylactics, diagnostics, and antimicrobials useful in MAP infections in humans will be an important research agenda. MAP can infect and cause disease in primates. Primate research has the potential to contribute substantially to the understanding of pathogenicity of MAP and risk of MAP exposure of humans, as well as development of new anti-MAP prophylaxis and therapies.**

**Table 2. Clinical similarities between CD and Johne's Disease[a]**

| FEATURE | CROHN'S DISEASE | PARATUBERCULOSIS |
|---|---|---|
| Diarrhea | Yes | Yes |
| Intermittent diarrhea | Yes | Yes |
| Abdominal pain | Yes | ---[b] |
| Weight loss | Yes | Yes |
| Obstruction | Yes | No |
| Ileac region mass | Yes | No |
| Blood in stool | Rare | Rare |
| Vomiting | Yes | No[c] |
| Quiescent periods | Yes | Yes |

[a] Table abstracted from Chiodini, 1989 (31)
[b] Domestic animals generally fail to display chronic pain
[c] Vomiting (regurgitation) is uncommon in ruminants, although they eructate (move ingesta from their stomach into their mouth for repeated mastication, commonly called "chewing their cud")

## MAP AS ETIOLOGIC AGENT OF CD

Researchers and physicians debate whether MAP, which causes the gastrointestinal inflammation, diarrhea, and weight loss of Johne's disease in animals, may also be to blame for CD. Since Johne's disease shares a number of similarities with CD, a connection between infected livestock and a disease in humans is conceivable. In fact, a correlation between CD and Johne's disease was first suggested in 1913, nearly 100 years ago. Dalziel (27) described several patients with chronic intestinal enteritis that, although very similar to intestinal TB, was believed to be a new disorder. He compared this new disease to a disease in cattle described first in 1894, now known as paratuberculosis, "...in which the histological characters and naked-eye appearances are as similar as may be to those we have found in man. In many cases the absence of acid-fast bacilli would suggest a clear distinction, but the histological characters are so similar as to justify a proposition that the disease may be the same."

Evidence of MAP as an etiologic agent includes the similarity of CD to Johne's disease caused by MAP (Table 2), the detection of MAP in the bowels, blood, or breast milk of some CD patients (24, 25, 28, 29), the effectiveness of antimicrobial drugs in certain MAP-positive and MAP-negative CD patients (30), and the seven-fold higher incidence of MAP in CD patients compared to the population in general (23).

Since MAP can be found in soil, it is not a mystery how grazing ruminants can be infected. But how are CD patients exposed? MAP has been traced to potable water from streams and rivers adjacent to MAP-infected dairy herds. MAP transmission could also occur by ingestion of contaminated milk or milk products or meat from Johne's-infected animals. Molecular analysis of MAP isolates from humans and animals show remarkable similarity. Exposure to a potential enteropathogenic bacterium through the commercial food supply is a troubling prospect.

### Genetic Susceptibility

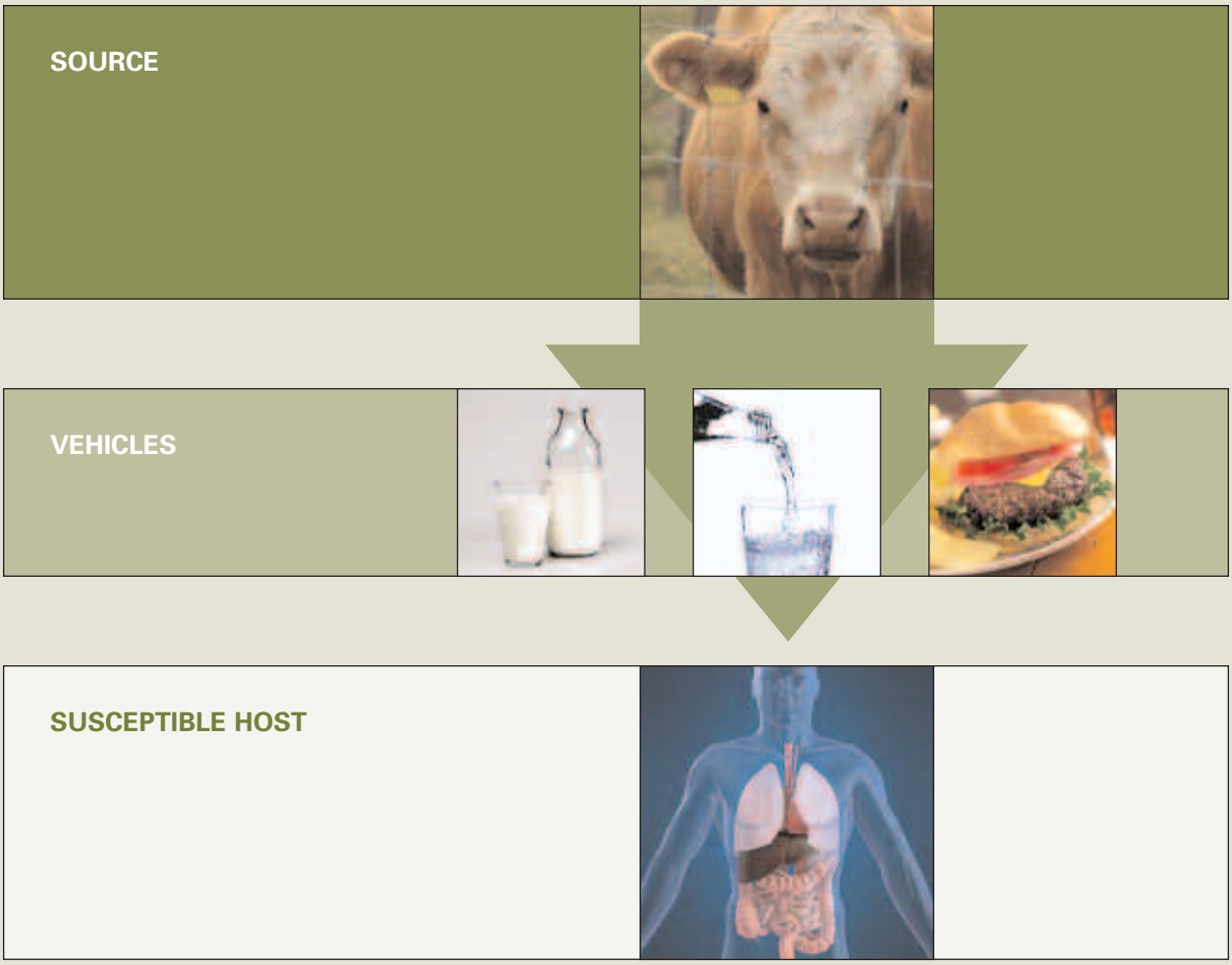
Susceptibility to CD is multigenetic and can be conferred by any of a number of different genes. A recent investigation (4, 5) compared the gene expression patterns of healthy individuals with those of CD patients and was able to identify nine genes linked to susceptibility to this disease. One follow-up study (3) implicated certain immune pathways as genetically deficient in CD patients and suggested that defects in the immune response to intracellular bacteria in particular may be responsible for CD. The pathways that are associated with increased susceptibility to intracellular pathogens have not, however, been specifically investigated for MAP.

Genetic susceptibility traits and exposure to bacteria, however, are not sufficient to predict the development of CD in an individual. Other non-genetic factors appear to be at work.

**RESEARCH:**

**Research should be targeted to identify novel anti-mycobacterials that act specifically and synergistically against MAP. This will require better methods to culture MAP, identification of drug targets that are specific for MAP, development of high throughput screens to evaluate existing drug compound libraries to establish structure-activity relationships, and creation of new chemical entities that are safe and effective for MAP disease.**

**Figure 2. Farm to Fork transmission of MAP: possible route of human exposure**




### Diagnosis

The principle hurdle in diagnosis of CD is simply recognizing the possibility that CD is in the differential, since disease symptoms vary from patient to patient, and CD symptoms are difficult to distinguish from other gastrointestinal disorders, such as Inflammatory Bowel Disease (IBD). Diagnosis of CD is currently made by combining clinical observations with radiologic, endoscopic, and histological findings. Because of the range of symptoms, CD can be a difficult condition to diagnose with certainty.

CD can be stratified according to age at the onset of symptoms, anatomical location of the diseased tissue, and the behavior of the disease (including inflammatory, structuring, or fistulizing forms).

**RESEARCH:**

**There is an urgent need to find the underlying cause of CD in order to design therapies that can cure this disease. Fully exploring the potential links between CD and MAP is critical to rule in or rule out MAP as a pathogen of humans and as a public health threat transmitted through the commercial food supply.**

The prevalence of CD varies from nation to nation and often varies even among the various ethnic groups within a country. The annual incidence of CD is ~6-10/100,000 and the prevalence of CD is about 20 times the incidence, or approximately 150/100,000 people in the U.S. (www.cdc.gov). Prevalence is roughly 250/100,000 in the Canadian province of Manitoba. CD appears to be increasing among Asian populations, and researchers note that individuals of Native American heritage experience lower rates of CD than other ethnic groups.

**RESEARCH:**

**Investigation of the genes responsible for CD susceptibility could enable scientists to understand the underlying host issues in development of CD, and could generate further research into therapies that address a specific genetic defect, resulting in individualized therapy and facilitating the current trend for personalized medicine.**

### Treatment

There are currently no treatments that cure CD. Medical treatment for CD can be divided into treatments to induce remission of clinical symptoms and treatments to maintain remission. Treatment approaches are also different for fistulizing and non-fistulizing CD. The primary goal of CD treatment today is to control the disease by increasing the length and frequency of disease-free remissions. Because the current CD therapies address only the symptoms of disease, at least 80-90% of CD patients have to undergo surgery at least once for their condition during their lifetimes, and 50% of CD patients undergo a second surgery.

Table 1 lists all the current anti-inflammatory agents, biologics, and other treatments for CD, duration of treatment, and wholesale costs for a 30-day supply. Retail costs will be substantially higher, and costs for administering IV infusions are higher yet because hospital or clinic costs must also be incorporated into the final cost. There are many treatments of varying efficacy, and the majority of these therapies treat the symptoms of a runaway inflammatory response, not the etiologic agent of the disease, if there is one.

**RESEARCH:**

**Off-label use of existing approved antibiotics for other conditions may be applicable to CD, and clinical research on these drugs and drug combinations should continue. Research should also, however, be targeted to identify novel antimycobacterials that act specifically against MAP and that, if MAP is discovered to be the (or one) etiologic agent, are efficacious for CD.**

Treatments for maintaining remission of CD symptoms include, in order of effectiveness (most effective to least effective): immunomodulatory agents (i.e., azathioprine, 6-mercaptopurine, and methotrexate); anti-tumor necrosis factor-alpha drugs (anti-TNF alpha); and the antibiotics ciprofloxacin and metronidazol. Steroids can rapidly induce remission of CD symptoms, but steroids are rarely able to maintain remission and can be quite toxic if administered for long periods of time. The long-term outcomes that could be achieved using newer approaches and treatments (anti-TNF alpha drugs) will require time to assess, as most of these biologics have been approved only in the last year or so. One major impediment to their widespread use is, however, cost (see Table 1, page 11).

If CD is caused by MAP or any other microorganism, appropriate antibiotic therapy will be critical for its cure. The only antibacterial drugs approved for use in CD in the U.S. were selected because they worked in a small number of patients (30). There are no antibiotics available today that have been developed specifically for treatment of MAP-induced CD. As described in MAP in Human Infections, it is anticipated that combination drug therapy will ultimately be recommended for CD, if MAP is an etiologic agent, to reduce the probability of MAP drug resistance.

Most drugs used for treating CD are associated with significant side effects and toxicities, some life-threatening, and all require long-term monitoring of the patient.

## EVIDENCE FOR AND AGAINST A ROLE FOR MAP IN CD

Evidence for a possible link between MAP and CD includes similarity of disease patterns with Johne's disease, immune response data, the results of tissue sample analysis, and the effectiveness of antimycobacterial drugs. Epidemiologic pattern similarities between Johne's disease (known to be caused by MAP) and CD include:

- Triggering event is in early in life.
- Prolonged period of time between trigger and clinical disease (incubation period).
- Clinical disease onset commonly after sexual maturity.
- Both diseases follow a normal distribution pattern for onset.
- Main target organ is the ileum.
- Host response for both is chronic granulomatous inflammation.

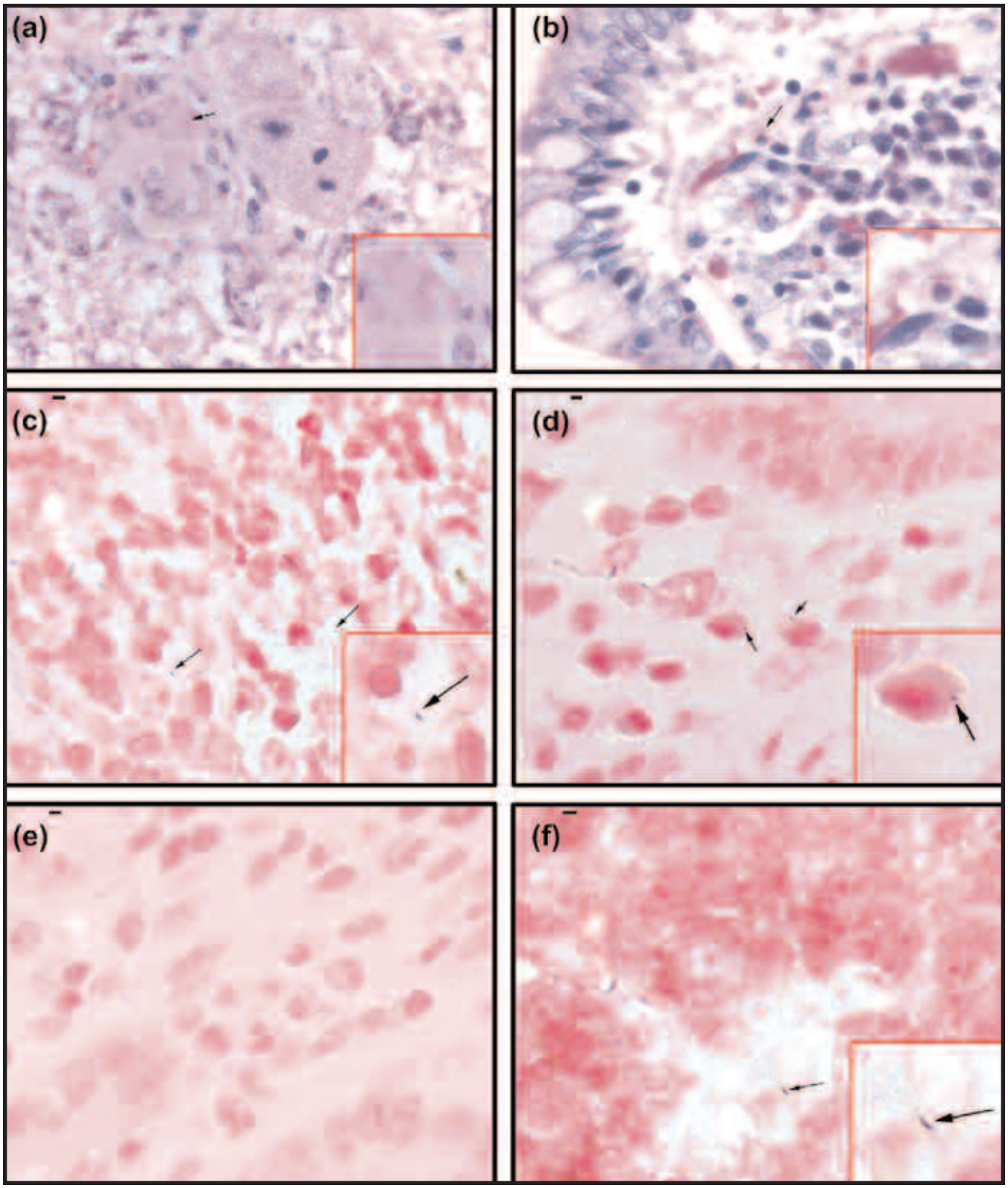
### Immune Responses to MAP in CD

The results of immunological testing of CD patients are mixed; some CD patients respond specifically to MAP antigens, while others do not (32). Consistent and reproducible immune responses to MAP by patients with CD would be a strong indication that MAP plays a direct role in this condition, but the lack of a consistent immune response would not necessarily mean the lack of an association. For example:

- If MAP causes CD and susceptibility to MAP depends upon diminished and dysfunctional immune responses, then immune cells of a CD patient could be insensitive to MAP. Immune responses to MAP in cattle are only evident at late stages of disease, when the clinical signs and symptoms of disease are overwhelming. So impaired early immune responses to MAP may be the norm.
- Testing of CD patients may be confounded by the use of immunomodulatory therapies to ameliorate symptoms of disease, which would also dampen the development of antigen-reactive T cells or production of antibodies.
- Since CD symptoms vary, it is also possible that not all CD, but only a portion of CD, is caused by MAP and the rest is caused by a different etiologic agent(s). An immunological survey of all CD patients in a geographic region looking for those who had immunologic responses to MAP might demonstrate that only a segment of the CD population had MAP antibodies. This would be expected with multiple etiologies, but could be misinterpreted as lack of

**RESEARCH:**

**Development of MAP-specific immunologic assays will be necessary to distinguish between MAP and other Mycobacteria family members, many of whom are present in soil and water, and many of which confound current screening techniques for such public health hazards as TB in broad populations in the US and around the world. Validation of immunological assays will be essential and could be problematic, since there is no gold standard technique against which a new method can be measured and current human MAP diagnostic tests are not standardized.**




This figure was published in Microbes and Infection, 9, Mangalakumari Jeyanathan, Odette Boutros-Tadros, Jasim Radhi, Makeda Semret, Alain Bitton, Marcel A. Behr, Visualization of Mycobacterium avium in Crohn's tissue by oil-immersion microscopy, page 1572, Copyright Elsvier (2007).

causality if the other agents are unknown. Moreover, antibodies are only an indirect indication of exposure and do not necessarily relate to disease.

- It also follows that, if CD is in part controlled by genetic susceptibility, randomly surveying a population for MAP antibodies could identify healthy individuals who had experienced and controlled MAP infection or (unless the antigen was specific only to MAP) any other Mycobacteria in the past. This would also lead (and has led) to confusion about a MAP etiology of CD.

**RESEARCH:**

**Investigators involved in MAP or CD research must develop and agree upon standards for proficiency testing in molecular methods of MAP detection, so that results from different labs can be compared meaningfully.**

### Tissue Samples from CD Patients and MAP

Nucleic acid amplification methods (which rely on the presence of DNA or RNA) can detect MAP in diseased tissues obtained from CD patients and patients with ulcerative colitis, but not consistently. These variable results could be due to at least four possibilities: (1) distribution and number of bacteria may vary among individuals experiencing CD symptoms or vary with the specific symptoms (differences in sensitivity to infective doses); (2) people may have different infection rates in different parts of the country (geographical differences); (3) different methods may be used by different investigators, some more sensitive or robust than others (methodological differences); or (4) only a portion of CD is caused by MAP (etiologic differences).

Due to the exacting requirements for growth of MAP for *in vitro* culture, it is difficult to detect MAP by culture-dependent techniques. It is critical to expand the successes achieved in some laboratories to other laboratories involved in determination of CD etiology. Until more consistent cultivation methods are developed, however, molecular methods are the most efficient surrogate for detecting MAP.

## ANTIMYCOBACTERIAL DRUGS AND CD

There are reports of the successful treatment of CD, including remission and sustained response, using drugs selected for their antimicrobial activity against *M. avium* organisms. The only reported controlled clinical trial of combination antibiotic treatment in CD, using these drugs, showed a significant short-term advantage of anti-MAP therapy over steroid therapy, but did not show long-term effects in the majority of patients (32).

Prior clinical trials with anti-tuberculous therapies used to combat *M. tuberculosis* infections (the front-line TB drugs rifampin, isoniazid, ethambutol, and pyrazinamide) did not show a benefit for CD patients. These drugs, however, are very specific for *M. tuberculosis,* and even *M. avium* is far less susceptible to any of these drugs than *M. tuberculosis*. So these results were not unexpected.

No studies have systematically tested for the presence of MAP in CD patients before and after treatment with anti-*M. avium* therapies, so it is not known whether these drugs actually reduce the numbers of MAP bacteria in CD patients. The reported success of these drugs in treating CD disease could be due to their effects on MAP, or could be due to other activities of the drugs, or to their effects on other bacteria.

**Table 3. Pros and Cons of the Association Between MAP and CD**

| Facts that SUPPORT the existence of a link between MAP and Crohn's disease: | Facts that DO NOT support the existence of a link between MAP and CD: |
|---|---|
| ■ MAP causes a severe and fatal gastrointestinal disease in ruminants (Johne's Disease) that has clinical and pathological similarities to CD (7).<br><br>■ MAP has been detected in milk, cheese, meat, and water targeted for human consumption, signifying a possible route of exposure to MAP for the general public (15-20).<br><br>■ Genes associated with CD suggest that inappropriate response to an intracellular pathogen may trigger this disease, and MAP is an intracellular pathogen (3, 5).<br><br>■ Increased serological responses to MAP have been detected in CD patients.<br><br>■ MAP has been detected using molecular and histopathological techniques in tissues from CD patients, including blood (24, 25).<br><br>■ MAP has been observed in tissues of CD patients by Ziehl-Neelsen staining (35).<br><br>■ MAP has been grown from various tissues and fluids taken from CD patients (24, 25).<br><br>■ One clinical study identified a significant and prolonged response to antimicrobial therapy in patients with CD, another study demonstrated a short-term benefit to antimicrobial therapy (30).<br><br>■ CD patients have a seven-fold more likely chance of having MAP in their tissues than the general population (23). | ■ Dairy farmers and others who may have greater exposure to MAP than the general population did not experience higher rates of CD in one study (33).<br><br>■ There are some dissimilarities in the clinical and pathological presentation of CD and Johne's disease (31).<br><br>■ Cell-mediated immune responses to MAP or MAP antigens have not been demonstrated in CD patients.<br><br>■ There have been no systematic studies, but CD has not yet been reported to worsen with progressive immunocompromise, such as happens with *M. tuberculosis* infections (exacerbated by HIV or anti-TNF therapy) (34).<br><br>■ In a controlled clinical trial, CD patients undergoing six months of antibiotic therapy did not maintain a sustained response to the drugs, and the relapse rate after two years was similar in treated and control groups (30). |

Considering reports of success in using antibiotic therapy to treat CD in some patients, it is perplexing that more physicians aren't actively pursuing antimicrobial drug therapies for their CD patients and more research is not happening to discover new and better (more specific) drugs to treat this disease. It is likely that the current lack of evidence-based studies on the MAP causality of CD is the basis for this reluctance. On the positive side of antibiotic therapy, most antibacterial drugs are generally well tolerated compared to the anti-inflammatory drugs used to suppress CD symptoms. Side effects are well characterized through trials for other infections and are not notably different in CD patients. On the other hand, use of antibiotics for a disease without a known infectious etiology could induce drug resistance in MAP or other bacteria, adding more antimicrobial resistance genes to the pool of genes circulating in the population. Despite these reservations, physicians are encouraged to continue helping CD patients with whatever tools they deem necessary.

There has been no systematic and widespread effort to explore possible links between exposure to MAP bacteria (in food or elsewhere) and the subsequent development of CD disease. In the U.S., CD is not reported to public health agencies, so public documents on the actual prevalence of CD over time are extrapolated from academic and clinical publications in various populations. There is both insufficient data on the occurrence of MAP in the environment and an absolute requirement for a widely available, sensitive, and specific human MAP diagnostic(s) to undertake an epidemiological study of sufficient scope to discover a possible link.

More research support and substantial additional research effort by both scientists and clinicians is necessary before we will know whether CD has an infectious etiology, and whether MAP is the culprit. It is possible that a subset of CD patients have MAP disease; future research must be directed toward establishing whether there is a strong and reproducible relationship between MAP and CD.

**RESEARCH:**

**Despite the difficult nature of MAP culture, attempts to develop consistent and reproducible methods to isolate and culture MAP from tissues of CD patients continues to be an important research activity, the results of which will eventually inform many of the outstanding questions regarding the diagnosis and treatment of CD.**

## RECOMMENDED RESEARCH TO CLARIFY MAP AS A HUMAN PATHOGEN

The prospect that MAP could play a role in the incitement or development of CD is a sobering one, and once the situation becomes clearer, there could be important changes in store for agriculture, food safety, and public health. It is in the best interest of the public that the possible connection between CD and MAP be explored exhaustively.

Colloquium participants strongly urge scientists to carry out rigorous, exacting research and epidemiological studies on MAP and CD and to develop reproducible diagnostics for MAP in order to provide the data needed to achieve consensus on this matter in the scientific and regulatory communities.

### RESEARCH PRIORITIES

There are no easy answers to the problem of identifying the role of MAP in CD or other human infections; no single "home run" study will fill the many gaps in the collective scientific knowledge. Instead, researchers must tackle the problem from multiple directions.

The two broad areas of uncertainty are:

- The transmissibility of MAP from food and the environment to humans, and
- The susceptibility of the human body to MAP infection and disease.

To clear up these uncertainties, a strong international research collaboration must be forged with a variety of stakeholders, all with common interests in solving the puzzle—microbiologists and mycobacteriologists, veterinarians, food microbiologists and regulators, epidemiologists, research scientists, product developers and regulators, domestic food-source animal industries, public health officials, clinicians, gastroenterologists, and patient populations. If MAP is associated with human disease and is in the U.S. food supply, this will be a public health concern to rival that of TB in the early part of the 20th century.

### RESEARCH ISSUES

#### Transmissibility of MAP to Humans

MAP exists in Johne's-infected livestock, in livestock waste, on farms, and possibly in the food and water supplies (see MAP in the Food Supply, above), but it is far from clear whether humans can become infected with MAP upon exposure to these sources. And if they become infected with MAP, do humans develop disease?

Of primary importance to the issue of MAP transmissibility is whether or not the MAP strains isolated from humans can be traced back to MAP strains isolated from animals. Are they related genetically? To determine this will require development of robust and highly specific MAP speciation techniques and the ability to

isolate MAP from human tissues. Genetic similarities and differences may also identify potential virulence factors in human MAP isolates.

Direct assessment of transmissibility will be difficult. A potential source of MAP exposure for humans is the consumption of contaminated milk from Johne's disease-infected cows, but it is unethical to conduct a prospective study to clarify this possibility (i.e., humans fed deliberately contaminated milk). Retrospective studies, which require subjects to think back on past exposure to milk, are not particularly illuminating, since subjects must recall when and how much milk was consumed, and researchers are unable to confirm whether or not the milk drunk in the past was contaminated at the time of consumption. The best direct answer to MAP transmission by contaminated milk may be to employ an animal model. Johne's disease in goats, whose pathophysiologic symptoms are the most similar of the ruminants to human CD, could be developed into a surrogate animal model for this purpose. The incidence of Johne's disease in groups of animals fed contaminated or MAP-free milk (or other foods or water) could then be compared, and the dose of MAP that is infective by this route could be established. Moreover, having a well-characterized animal model would allow researchers to determine the relative infectivity and pathogenicity of MAP strains isolated from humans.

MAP transmissibility to humans from environmental sources, particularly from MAP-infected livestock, could possibly be uncovered and evaluated by epidemiologic studies that look at populations living in geographical regions with a low rate of reported Johne's disease, including Sweden, Iceland, and the eastern regions of Australia, compared to geographic regions with a high rate of Johne's disease. For these studies to occur, sensitive and specific MAP diagnostics must first be developed and standardized, and for these studies to be informative, simultaneous (or contemporaneous) MAP surveys of cattle and other ruminants in the area must also be performed. MAP contamination of milk and water from the specific region will also be important to assess. If MAP infection rates are absent or significantly lower in low Johne's disease regions, and are high in high Johne's disease regions, then a strong inference could be made that humans contract MAP (directly or indirectly) from infected livestock. However, this will be complicated by the extensive regional and international trade of foods, making it challenging to assure that the foods consumed originated from the place where the CD patients live.

If it is established that humans exposed to MAP in their environments become infected (i.e., diagnostics detect MAP), all the stakeholders will need to know the health consequences of exposure in order to gauge the possible risks of disease from MAP. These studies will not be easy, because all measurements will be an indirect indication (or surrogate marker) of infection or disease—symptoms or immunological reactions to MAP. In some instances, new animal models may be needed to address questions of MAP pathogenesis and therapy.

**Human Immune Responses to MAP**

One way to assess exposure (but not necessarily infection or disease) is to determine human MAP immunological reactions in the general population or in specific disease populations like CD. Onset of immune responses to most Mycobacteria, especially the intracellular pathogens, takes months. Because of their intracellular habitat, these pathogens are nearly impossible to eradicate, although they can be immunologically contained. The immune response accelerates and expands to include all manner of reactions and immune factors over time, and tissue destruction in both TB and leprosy is largely due to the constant antigenic challenge of immune cells and resulting immunologic chaos.

To understand the interaction of human immune system following exposure to MAP, it would be helpful to first have an indication of the timing, type, and duration of inflammatory and immune events in an animal model that is undergoing disease reasonably similar to CD. That would at least narrow down the types of immune responses that should be investigated with priority in humans.

A basic understanding of the ongoing MAP-specific cellular and humoral immune interactions that constitute a response to MAP in humans could facilitate identification of specific immunological responses that occur in CD patients versus healthy individuals, or that occur in different stages or types of CD. Of course, it will not be useful to survey all CD patients for MAP responses if MAP causes only a portion of CD cases; in that event, sensitive and specific human diagnostics will need to be identified, developed, and standardized so that patients can be segregated in to MAP-infected CD and non-MAP CD. It may then be possible to identify MAP-specific human immune reactions that can be developed into additional diagnostics to detect early symptoms of relapse or verify cure. It may also be possible to correlate human MAP-specific immune reactions to bacillary load in the infected tissues or to other markers of disease.

What if MAP is not the sole agent of CD? What if the relevant aspect of MAP infection is its ability to modulate human immune response(s) to other pathogens? Or to divert the proper immune reaction for a different bacterium to an ineffective one, thus allowing the second pathogen to gain a foothold in tissues that it is not normally allowed to penetrate? Some understanding of the "typical" human immune reaction to MAP may enable scientists to recreate the more complicated CD induction scenario (more than one pathogen necessary to create disease) in appropriate animal models of CD.

There are reports that a percentage of healthy humans are positive for MAP serology. If screening diagnostic tests are highly specific for MAP, these healthy MAP-exposed individuals could provide a window into the "normal" MAP response. Research including these subjects could provide information on immuno-dominant MAP antigens, as well as a comparison with immune responses observed in CD patients.

At the moment, and unfortunately, human MAP infection is most obvious and easiest (although not easy) to identify in CD patients with symptoms of disease. These patients are generally being treated with powerful anti-inflammatory agents or immune suppressive therapies. There may be no way to assess a "typical" anti-MAP immune response in CD patients unless clinicians are able to identify MAP-infected persons when they first seek medical care and are not yet on therapeutics that suppress immune cells. Good MAP diagnostics will be essential. If diagnostics are not yet available, good animal models that mimic CD could be helpful, with the caveat that immune systems and immune reactivity differ between species. With an animal model, however, MAP can ethically be administered by itself or co-administered with other pathogens to determine outcomes.

**Therapeutics**

The antimicrobial agents available to treat MAP infections or CD were not developed specifically to treat MAP and appear inadequate to provide significant long-term results for patients. Clinical research to establish effective therapeutic regimens with existing antibiotics is encouraged. Further research should also be directed toward identifying new and effective antimicrobials for treating MAP infection.

**Other Research Priorities**

The National Research Council has made recommendations for research examining possible links between CD and MAP (2003). Recommendations 17 through 25 in the report are particularly compelling and require follow-up. In addition, two new reports will be released by agencies in the U.S. Federal Government in 2008, and these reports may contain additional Research Recommendations:

- Development of an Action Plan to Address Surveillance, Epidemiologic, Laboratory and Environmental Issues Related to Disease Caused by Nontuberculous Mycobacteria. Centers for Disease Control and Prevention External Consultation. May 2007 – April 2008.

- National Advisory Committee on Microbiological Criteria for Foods. Assessment of Food as a Source of Exposure to Mycobacterium avium subspecies paratuberculosis (MAP). FINAL DRAFT REPORT. September 24, 2007

**Development of Reproducible Analytical Techniques for MAP**

There is a conspicuous and fundamental barrier to accomplishing the research on MAP as an etiologic agent of CD: the lack of a reproducible, sensitive, and specific diagnostic(s) for MAP. New tools to identify and isolate MAP are critical to every recommended activity outlined in this document, and without them researchers cannot move forward. Public health laboratories and the U.S. Centers for Disease Control and Prevention laboratories have made it clear that they cannot grow MAP, which hinders diagnosis and reporting. Research would also benefit from more sensitive methods for identifying MAP when it is isolated, and for speciating MAP isolates from infected animals and humans.

A gold-standard test for MAP that researchers and clinicians alike can turn to may stratify testing results, clearly separating individuals with MAP infections from uninfected CD (or other) patients, and thus clarify the role of MAP in CD. Research that uses unreliable, inconsistent technologies for detecting MAP will always be open to criticism and negative scrutiny.

Inter-laboratory collaborations to establish reproducible, standardized techniques for detecting MAP are strongly encouraged. Research in this area often requires the comparison of assay results from different laboratories. Therefore, standardized techniques that produce the same results regardless of the service provider are critical.

Once better methods have been developed, a network of proficiency testing for laboratories that offer MAP diagnostics should be established. A strong, unbiased, external laboratory proficiency evaluation program or quality assessment program is critical to establishing and maintaining confidence in MAP research and clinical testing.

A task force should be established to move forward and develop more specific recommendations for developing improved methods for MAP detection.

Eventually, interested parties will be forced to address the problem of which specimen types are most appropriate for testing for MAP infections in humans. Blood, intestinal mucosal tissue, stool, and colonic exudate are all possibilities for testing purposes. Better assays are also needed for detecting MAP in environmental (water and soil) samples, as well as food.

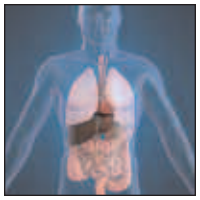
**A Note About Resolving Causality**

There are certain details to consider in attempting to find a causal link between MAP and CD. For example, if MAP is associated with CD-affected tissues, the possibility that MAP is an opportunist taking advantage of inflammation caused by another organism must be considered and investigated. In this scenario, anti-MAP therapies would alleviate the secondary MAP infection, but not the underlying inflammation due to a different pathogen, as yet identified.

Another imperative in defining causality is the use of appropriate controls. Whenever researchers test tissue for the presence of MAP, they should also check for the presence of other organisms in order to bolster confidence in the results.

CD is likely to be a multifactorial disease, created by the confluence of many different phenomena, and the dividing line between CD and other similar diseases, like ulcerative colitis and IBD, is sometimes difficult to discern. Researchers need to clearly define the conditions of study participants in order to make the conclusions of the work as unambiguous as possible.

# RECOMMENDATIONS



- Research to discover and standardize diagnostics that are both sensitive and specific for MAP in animals and in humans and can determine the source of MAP cultured from human tissue is imperative. Virtually all the research topics that will clarify the role of MAP in CD rely on this.

- Research must address the issue of MAP transmissibility and determine whether or not the MAP strains isolated from animals or food are genetically identical to the MAP strains isolated from humans.

- Research should also examine the potential virulence factors in human MAP isolates and determine whether MAP can be transmitted from human to human.

- Researchers must develop better animal models for evaluating MAP effects on human hosts and for evaluating the effectiveness of potential therapies for MAP infection.

- A policy for regularly screening foods for MAP should not be put into place until focused research studies can determine the actual risks of exposure and disease.

- Identifying novel MAP-specific antimicrobials and effective antibiotic treatment regimens for MAP infections is a research priority.

**A Word on the Precautionary Principle**

The Precautionary Principle (http://en.wikipedia.org) as a formal concept evolved from German social and legal tradition in the early 20th century. It is underpinned by common sense aphorisms that pre-date the term, such as "an ounce of prevention is worth a pound of cure," "better safe than sorry," and "look before you leap." The Precautionary Principle is also considered to have evolved from the ancient medical principle, "first, do no harm," as it applies to institutions and institutional decision-making processes, rather than individuals.

The 1998 Wingspread Statement on the Precautionary Principle summarizes the principle this way: "When an activity raises threats of harm to human health or the environment, precautionary measures should be taken, even if some cause and effect relationships are not fully established scientifically" (Science and Environmental Health Network). In deciding how to apply the principle, one uses cost-benefit analysis, assessing both the opportunity cost of not acting and the option value of waiting for further information before acting. In modern policy making, there is often an irreducible conflict between different interests, so the debate is necessarily political. This is no more evident than in the debate about MAP and CD.

When should policy makers evaluate the Precautionary Principle as it relates to the role of MAP in CD? There are indications that MAP is pathogenic for humans, and MAP is found in U.S. cattle that supply food for human consumption and in milk and perhaps other foods on the supermarket shelf. A significant number of U.S. citizens, more than 500,000, are affected by CD.

The colloquium participants were not prepared to recommend as a group that public health authorities move today to mitigate the public's exposure to Johne's disease-infected animals by enacting mandatory agriculture and food safety regulatory policies to eliminate potential routes of exposure to MAP.

It is important, however, for all regulatory bodies concerned with agriculture and public health to consider this report, and the other Federal reports that will be published later this year, in light of the Precautionary Principle. The science and clinical communities will continue to identify gaps in the current knowledge relevant to MAP exposure and, with appropriate funds and collaborations, begin to provide data that will inform these deliberations. But the decision to invoke the Precautionary Principle should be a political debate at the highest levels of our federal institutions charged with guarding the public health, and the timing of this debate should be now.

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REVIEW

# *Mycobacterium avium* subspecies *paratuberculosis* causes Crohn's disease in some inflammatory bowel disease patients

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Author contributions: Sagramsingh SR led the literature search, and writing of the manuscript; other co-authors contributed significantly to data analysis, and writing and revision of the manuscript.

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## Abstract

Crohn's disease (CD) is a chronic inflammatory condition that plagues millions all over the world. This debilitating bowel disease can start in early childhood and continue into late adulthood. Signs and symptoms are usually many and multiple tests are often required for the diagnosis and confirmation of this disease. However, little is still understood about the cause(s) of CD. As a result, several theories have been proposed over the years. One theory in particular is that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is intimately linked to the etiology of CD. This fastidious bacterium also known to cause Johne's disease in cattle has infected the intestines of animals for years. It is believed that due to the thick, waxy cell wall of MAP it is able to survive the process of pasteurization as well as chemical processes seen in irrigation purification systems. Subsequently meat, dairy products and water serve as key vehicles in the transmission of MAP infection to humans (from farm to fork) who have a genetic predisposition, thus leading to the development of CD. The challenges faced in culturing this bacterium from CD are many. Examples include its extreme slow growth, lack of cell wall, low abundance, and its mycobactin dependency. In this review article, data from 60 studies showing the detection and isolation of MAP by PCR and culture techniques have been reviewed. Although this review may not be 100% comprehensive of all studies, clearly the majority of the studies overwhelmingly and definitively support the role of MAP in at least 30%-50% of CD patients. It is very possible that lack of detection of MAP from some CD patients may be due to the absence of MAP role in these patients. The latter statement is conditional on utilization of methodology appropriate for detection of human MAP strains. Ultimately, stratification of CD and inflammatory bowel disease patients for the presence or absence of MAP is necessary for appropriate and effective treatment which may lead to a cure.



**Key words:** *Mycobacterium paratuberculosis*; Crohn's disease; Culture; PCR; Johne's disease; Inflammatory bowel disease

**Core tip:** The review manuscript describes the past, present and predicted future research accomplishments in the area of Crohn's disease and *Mycobacterium avium* subspecies paratuberculosis. This is a highly debated area and Dr. Naser's thoughts described in this review will fuel interest and discussions in inflammatory bowel disease research. The manuscript has been in preparation for a couple of years and it is of high quality.

Naser SA, Sagramsingh SR, Naser AS, Thanigachalam S. *Mycobacterium avium* subspecies *paratuberculosis* causes Crohn's disease in some inflammatory bowel disease patients. *World J Gastroenterol* 2014; 20(23): 7403-7415 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i23/7403.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i23.7403

## INTRODUCTION

One of the earliest documented descriptions of Crohn's disease (CD) was described in 1769 by Giovanni Battista, an Italian physician. He described the results of an autopsy of a man who had suffered from chronic bowel movements throughout his life and subsequently died from diarrhea and fever[1]. This may have been the first account of granulomatous inflammatory bowel disease (IBD). Several years later in 1813, Combe and Saunders observed a patient who suffered from an abnormally narrow and thickened ileum[1] and Abercrombie had reported a case in 1828 whereby a patient suffered from an inflamed ileum (ileitis) as well as skip lesions affecting certain segments of the ascending colon and cecum[1]. There were several medical publications made in the 20th century which provided further insight into the characteristics and features of CD. For example, some such cases were reported by Braun (1901), Koch (1903), Lesniowski (1903), Wilmanns (1905), Moynihan (1907), and Proust (1907)[1]. In 1913 a surgeon by the name of Dalziel (1861-1924) had reported the symptoms of several of his patients that closely resembled clinical manifestations in cattle suffering from Johne's disease[2]. He is credited as being the first scientist to hypothesize that the causative agent of Johne's disease, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), may in fact be responsible for chronic intestinal inflammation observed in the intestines of humans. In 1923 Moschcowitz and Wilensky[3] had reported four cases of young patients suffering from non-specific granulomata of the intestine. In these patients they observed what appeared to be tumor-like masses that were hard, thick, and associated with all four coats of the large intestine which caused stricture of the lumen. Based on these observations it was originally believed that these structural changes were confined to the colon. However, it was found that such lesions could also be located in the small intestine which was later seen in one patient[4]. In fact, it is interesting to note that all four of the patients had a history of appendicitis and appendectomy.

In 1932 acknowledgement of CD as an official medical entity was as a result of an article published by Drs. Burrill Crohn, Leon Ginzburg, and Gordon G. Oppenheimer, who all worked at Mount Sinai Hospital in New York at the time. Their article entitled "Regional ileitis: A Pathological and Clinical Entity" had appeared in the *Journal of the American Medical Association* in October 1932[5]. The title "Crohn's disease," has been coined after Dr. Burrill B. Crohn, a gastroenterologist who presented the above named paper at the annual American Medical Association in May 1932. The study described a disease that exclusively affected the terminal ileum of 14 patients from a pathological and clinical standpoint[5]. The patients were primarily young adults of ages ranging from 17 to 52 years, but only two of them were older than 40 years[5]. It was expressed that this was a moderately acute disease that was associated with inflammation characterized by rapid necrosis throughout the affected tissue, and by inflammation associated with scar tissue[5]. Furthermore, it was indicated that the disease is clinically represented by certain common symptoms similar to ulcerative colitis-fever, diarrhea, and even weight loss[5]. Dr. Crohn *et al*[5] also witnessed that the ulcers associated with the mucosa were accompanied by a non-uniform connective tissue reaction of the remaining walls of the involved intestine, a process which frequently leads to the narrowing of the lumen of the intestine, and this has been known to be associated with the formation of multiple fistulas. Other physicians reported of similar concurrent observations, but these reports cited the involvement of a number of different parts of the gastrointestinal (GI) tract. For instance, the first case reporting evidence of inflammation present in the colon and not just in the ileum was by Colp in 1934[6]. His report is considered as the first case detailing of ileocolitis which described that this inflammatory process could also extend to the cecum and the ascending colon[6]. In addition, several years later granulomatous lesions were also found in the skin[7]. As a result, it was becoming quickly apparent that CD is a chronic inflammatory disease that can affect any region of the GI tract ranging from the mouth to the anus, the ileum being the most commonly targeted site. In 1938, Penner and Crohn observed that 8 out of 50 analyzed patients suffering from regional ileitis displayed anal fistulae as a possible complication. They were initially unaware that anal and perianal fistulae could present such a complication of ileitis[8]. In 1952, Wells[9] introduced the term *segmental colitis* while delivering a lecture at the Royal College of Surgeons of England. According to Wells, this condition is associated with the formation of fibrous tissue on the bowel wall leading to its increased thickening as well as the presence of ulcers of the mucosa. These ulcers have a patchy pattern of spreading and are therefore known as "skip lesions"[9]. Most importantly this condition was observed in patients without lesions present in the ileum or jejunum. Wells presumed that segmental colitis is a form of colonic CD, but this was never acknowledged by Crohn himself[9].

At present, research on CD has partitioned it into three categories: inflammatory, obstructive, and fistulating[10]. The inflammatory and obstructive types tend to occur simultaneously and cause obstructions of the bowel due to thickening of the intestinal wall as a result of inflammation. The fistula types are commonly associated with erosion of the bowel walls including perianal fistulas and enteroenteric fistulas[10]. Depending on the locations of these erosions the disease is called Crohn's or granulomatous colitis if symptoms occur in the large intestine, Crohn's enteritis if symptoms occur in the small intestine, or Crohn's ileitis if symptoms occur in the ileum[11]. Furthermore, it has been documented that some patients suffer with inflammation of fat cells under the skin (erythema nodusum), or in large joints (peripheral arthritis)[10]. It is important to note that CD is quite similar to another IBD known as ulcerative colitis (UC). The latter affects only the colon whereas CD can affect any region of the GI tract[12].

## WHO IS AFFECTED BY IBD?

Statistical evidence has indicated that the highest prevalence of CD and UC is in North America, northern Europe, and the United Kingdom. These diseases are beginning to rise in southern Europe, Asia, Africa, and Latin America. In fact, as much as 1.4 million persons in the United States and approximately 2.2 million individuals worldwide cope with IBD on a daily basis[13]. However, in one epidemiological study of CD based on ethnicity, it was revealed that CD is least prevalent in African Americans, Asians, and Hispanics. The rate of prevalence for African Americans was 29.8 per 100000, for Hispanics it was 4.1 per 100000, and for Asians it was 5.6 per 100000[14]. It has also been found that the occurrence of IBD is higher in industrialized countries such as North America and Europe *vs* under developed or developing countries. Therefore, this suggests that the pathogenesis of IBD may be caused by certain environmental factors[15]. This indicates that genetic susceptibility alone cannot account for the prevalence of CD. In addition, the incidence and prevalence of CD is essentially equal among men and women. Unfortunately, CD is a lifelong debilitating disease which can start in early childhood and continue into late adulthood. Most cases of CD are usually reported or initially diagnosed when the patient is in his or her late teens or early twenties. Recent studies have indicated that in the last few decades the number of CD patients diagnosed before the age of 40 years has increased to 80%[16]. This therefore emphasizes the young adult and adolescent age group as a primary target of this disease. Understanding the etiology of CD may facilitate the development of rapid and cost effective methods for disease diagnosis.

## DIAGNOSIS OF CD

The most accurate and effective examination for the diagnosis of CD is a full colonoscopy along with intubation of the ileum[17]. This type of endoscopic examination allows the physician to clearly visualize the colon, ileum, and even certain parts of the lower regions of the small intestine. Physicians can also take multiple biopsies of all the segments of the colon as well as the terminal ileum[17]. Dye-based chromoendoscopy is an advanced imaging technique which allows for the visualization of subtle changes in the lining of the intestine. An alternative imaging method that can be utilized is capsule endoscopy, which is usually selected when there is no evidence of stricture or stenosis[18]. Other technology detects inflammation of the distal ileum such as enhanced gadolinium magnetic resonance imaging which has been proven to be very effective in distinguishing between inflammatory diseases of the GI tract, is non-invasive, and does not produce any radiation[19].

## WHAT IS THE ETIOLOGY OF CD?

Unfortunately, the etiology or cause(s) of CD are still unknown. However, there have been several theories that have been proposed to explain this phenomenon. For example, the leading theories suggest that CD can be caused by certain environmental factors or by a dysregulated immune response in a genetically susceptible host. Many believe a milieu of environmental factors such as diet and certain infectious agents may trigger this disease. For example, it has been found that a diet of refined sugars, fatty acids, fast foods, and minimal consumption of fruits, vegetables, and fibers can contribute to triggering the disease[20]. Certain foods play a pivotal role in influencing the microbiome composition of the human gut. In fact, a "Westernized" diet is believed to change the microbiological environment such that there is an increased susceptibility for the development of intestinal bowel disease[20]. Some of the infectious causative agents studied in connection with CD include viruses, yeast, and bacteria including *Escherichia coli, Listeria monocytogenes, Chlamydia trachomatis, Pseudomonas maltophilia, Bacteroides fragilis, Mycobacterium kansasii, and* MAP. Fortunately, it is almost universally accepted that a host genetic predisposition is critical for development of CD[21].

In recent years, the amount of interest and research data in support of a possible infectious etiology for CD has been well noted. More specifically, the forerunner of the proposed infectious causative agents is MAP. However, there are several critics and skeptics who still discredit this theory. Therefore the goal of this review article is to shed light on this current predicament with the intention to further clarify our understanding of the pathogenesis of CD from the perspective of an infectious agent such as MAP.

## MAP AND JOHNE'S DISEASE

It was in 1895 when Johne and Frothingham first identified MAP as the causative agent of chronic inflammation in the gut of a cow[22]. Johne's disease was later coined after Johne for his work in identifying this chronic inflammatory enteric disease in cattle, but this disease has also been observed over the years in several different animals such as sheep, goats, rabbits, monkeys, and even chimpanzees[22]. MAP belongs to the *Mycobacterium avium* complex (MAC) which consists of at least *M. avium* and *M. intracellulare*[23]. Through DNA sequence analysis it is possible to evaluate the similarities and differences among mycobacterial strains. It has been documented that MAP shares certain sequence similarities with other strains of MAC. For example there is a 16S-23S rDNA internal transcribed spacer (ITS) that is approximately a 280 base paired region located on the rRNA operon of mycobacteria[24]. It was found that ITS sequence analysis of MAP taken from 3 different mammalian species-bovine, primate, and human did not indicate much sequence variation between them and in 17 strains of MAC[24]. Thus, the connection between MAP and other mycobacterial strains is observed through this highly conserved sequence similarity. In addition, mycobacteria can be broadly classified as either an environmental or parasitic

species on the basis of their epidemiological and pathological nature[25]. Environmental mycobacteria such as the other strains of MAC can be considered as opportunistic bacteria that are found in a variety of habitats. Some of these environments include wet soil, rivers, agricultural slurry, the intestines of birds, ruminants, humans, and even within protists[26,27].

MAP is an obligate parasitic mycobacterium that causes chronic inflammation in the gut of several mammalian species and is considered to have three major genetic differences that serve to separate it from other non-pathogenic MAC. These differences are the presence of an insertion element designated as IS900, the presence of a genetic element known as "GS", and the presence of a unique MAP gene (*hsp*X) located in a specific genomic region. MAP contains a highly conserved insertion sequence (IS) or IS element referred to as IS900, which is repeatedly found in its genome approximately 15-20 times[28]. IS900 contains 1451 base pairs and harbors neither terminal inverted repeats nor flanking direct repeats normally found in other classical IS elements[28]. As a result, IS900 is grouped in a family of insertion elements that is specifically found in certain microorganisms. Some of these IS elements include IS901 and IS902 found in *Mycobacterium avium* subsp. *silvaticum*[29], IS116 present in *Streptomyces clavuligerus*[30], and IS1110 located in *M. avium*[31]. It has been documented that the pathogenic nature of several microorganisms has been linked to the presence of IS elements[32]. IS900 is able to take control of the translational machinery of MAP and thereby affects the expression of certain genes. It achieves this task by encoding for a putative transposase of 399 amino acids in size called p43 on one strand[33]. On the complementary strand IS900 encodes for a very unique gene called the *hed* (host-expression-dependent) gene[34]. This gene is quite unique in that upon entry into the MAP genome it requires a promoter, termination codon, and ribosome binding site (RBS). Previous studies have indicated that IS900 enters the genome of MAP at a specific consensus target sequence such that it is located between the RBS and start codon of the target gene in one specific direction[33]. As a result of this alignment, the *hed* ORF comes under the control of the mycobacterium host promoter thereby allowing for the translation of the Hed protein[33]. Thus, this is one probable explanation for how the insertion element IS900 may assist in the pathogenic phenotype of MAP compared with the other strains of MAC.

The second major genetic difference between MAP and other mycobacterial strains of MAC is that MAP contains a genetic element designated as "GS", which contains a low guanosine and cytosine (G + C) content[35]. GS is a 6496 bp element which possesses six genes-*gsa*, *gsb*A, *gsb*B, *gsc*, *gsd*, and *mpa*[36]. In addition, it has been found that the *mpa* gene of the GS element in MAP is a putative acetyltransferase, and has *mpa* homologues present in other microorganisms such as *oaf*A and *oac* of *Salmonella typhimurium* and *Shigella flexneri,* respectively[37-39]. Other virulence regions including "pathogenicity islands" or Pais have been reported on MAP chromosome[37] and have been found to be similar to a few protein-coding genes found in *Mycobacterium tuberculosis*. These protein-coding genes are *drr*A, *drr*B, and *drr*C, are located at Rv2936-Rv2938, and have been commonly associated with the pathogenic phenotype observed in *M. tuberculosis*[38].

## TRANSMISSION OF MAP TO HUMANS THROUGH COW'S MILK

The real concern for the transmission of MAP from cattle to humans is that MAP-infected cows remain asymptomatic in a lengthy subclinical phase[39]. As a result of this, infected cows are not removed and may continue to be harvested for milk and meat, and the spread of MAP can go unnoticed through fecal matters to the rest of the herd[40]. There have been several cases reporting the culture and isolation of MAP from the milk of subclinical or asymptomatic cows[39].

There has been a plethora of documentation about the number of cases in several countries reporting outbreaks of human illness due to improper 'heat-treated' milk and dairy products. It has been observed that certain pathogens such as *Campylobacter* species, *Salmonella* species, *L. monocytogenes,* and even *Y. enterocolitica* have been found in pasteurized milk, powdered milk, and even cheese, thereby contaminating these products and causing human illness[41]. Thus, it is apparent that milk can serve as a means of transmission of these pathogens. Similarly because MAP is found to a large extent in dairy herds and domestic livestock, it can be inferred that it may be present in raw milk. It is assumed that the pasteurization process will destroy any viable pathogens including MAP. However, there have been numerous case studies indicating the thermal-resistant characteristics of MAP thereby enabling its survival after pasteurization. Chiodini and Hermon-Taylor simulated pasteurization methodologies under laboratory conditions as defined by the Public Health Service, US Food and Drug Administration[42]. They performed the high-temperature, short-time (HTST) method of pasteurization in which the milk samples were heated to 72 ℃ for 15 s in accordance with commercial pasteurization techniques[43]. The results indicated that approximately 3%-5% of strains of MAP survived this process. Also, the pasteurization of MAP obtained from human tissues suspended in milk showed to have a higher survival rate (38.7% and 26.2%) than the bovine samples (8.7% and 9.0%)[43]. Grant *et al*[44] reported that MAP was not completely destroyed after pasteurization if it was already present in the milk at a concentration greater than $10^4$ cfu/mL. In other studies, Sung *et al*[45] were able to statistically determine the *D* values for various strains of MAP tested which estimated that MAP can survive HTST pasteurization methods when initially present at a concentration greater than $10^1$ organisms/mL of milk. However, there have been some critics who have dismissed the validity of the previous studies because they claimed that the HTST pasteuriza-

tion method performed in the laboratory setting cannot simulate commercial pasteurization conditions such as the turbulent flow of milk[44]. For example, Stabel *et al*[46] challenged the validity of these previous studies and reported that there was no evidence indicating the presence of viable MAP after the performance of HTST pasteurization simulated with an Armfield HTST laboratory pasteurizer. However, Grant *et al*[44] defended the studies previously performed in the field and criticized the methodology selected by Stabel *et al*[46]. She indicated that Stabel *et al*[46]. had frozen and sonicated MAP prior to its addition to raw milk. Grant *et al*[44] also expressed that MAP will not under normal circumstances naturally undergo such treatments prior to contaminating milk samples. Furthermore, freezing and sonicating MAP will only make it more susceptible to heat shock As already indicated by Richards *et al*[47] in 1977, freezing (-70 ℃) of bovine fecal samples contaminated with MAP substantially reduced the viability of MAP. Also, it was Sung *et al*[45] who reported the decreased thermal resistance of declumped MAP cells compared to clumped MAP cells, thereby highlighting the changes caused by the sonication of MAP cells. It is without a doubt that milk can serve as a vehicle for the transmission of MAP from animals to humans through consumption of diary and meat products from infected animals.

## PREVALENCE OF MAP IN THE ENVIRONMENT AND IN WATER

One of the major contributors to the spread of MAP in the environment is through the feces of infected cattle. Both subclinically and clinically infected cows excrete massive amounts of MAP through their feces on various pastures and farmlands[48,49]. This is a serious problem because it has already been documented that MAP can persist in the environment for long durations[50]. MAP is capable of surviving in fecal matter and in the soil for up to 12 wk[51]. Muskens *et al*[52] conducted a study investigating whether infected cattle could transmit MAP to other animals such as sheep grazing on the same pastures. They reported that 20% (10/50) of sheep showed evidence for the presence of MAP in their tissues. Subsequently, MAP can spread and infect other animals which come in contact with infected cattle. Furthermore, the prevalence of MAP in the environment is not only due to infected cattle, but can be due to other infected animals such as rabbits and deer which can also spread MAP abundantly through their feces[53]. Unfortunately, this is only part of the problem. In most cases the cow's fecal matter is used to make manure which is subsequently distributed across agricultural lands as fertilizer and thus contaminating ground water, rivers, and other surface bodies of water[36]. It will be just a matter of time before the accidental host (human population) is infected with MAP. MAP has been shown to resist chlorine disinfection treatment at concentrations similar to those used to disinfect public drinking water systems[54]. Clearly, it is apparent that water is a very potent vehicle for the transmission of MAP to humans.

## MAP CHALLENGES IN THE LABORATORY

From the outset, MAP is an obligate intracellular bacterium which presents multiple challenges in the laboratory with respect to its cultivation from tissue samples from both CD patients and even Johne's disease in animals. Unfortunately this fastidious bacterium is very slow-growing and often requires the cultures to be incubated for an extended period as much as 16 wk at a time[55]. As a result it has become quite problematic over the years to isolate and culture it through conventional means. Furthermore, MAP has very specific growth requirements which must be met for its survival. For example, this intracellular bacillus is unable to synthesize iron-chelating compounds, and therefore its host must provide iron for MAP to survive. Furthermore, due to its high mycolic content mycobacteria can easily adapt to intracellular growth in macrophages and may even become drug resistant[56]. In addition, MAP in CD assumes a cell-wall deficient spheroplast-like form which complicate culture requirement and void the use of the golden standard Ziehl-Neelsen mycobacterial staining test. For this reason, MAP in its spheroplastic form cannot be identified by light microscopy which adds to the challenges of confirming its presence in a laboratory setting[57]. Due to these difficulties, MAP-specialized scientists looked towards better techniques for the detection and characterization of microorganisms. This led them to the utilization of IS900 polymerase chain reaction (PCR) for the detection of MAP and later on the development of appropriate culture media. Nevertheless, some challenges remain including standardization of the methodology, and most importantly spreading the awareness to clinicians and scientists that standard methodology is not appropriate for the detection of MAP in humans[58].

## INVESTIGATING MAP ASSOCIATION WITH CD

It was in 1913, when Dalziel (1861-1924), a surgeon at Glasgow reportedly characterized 13 cases of chronic intestinal enteritis in humans[2]. Upon histological and clinical examination of nine patients, Dalziel specifically noticed that different parts of the gastrointestinal tract were affected: the jejunum, transverse and sigmoid colon, as well as the mid-ileum[2]. He reported that these symptoms closely paralleled clinical findings observed in cattle suffering from Johne's disease, a chronic inflammatory disease of the gut. As a result, Dalziel speculated that *paratuberculosis*, the then known causative agent of Johne's disease, could be a potential etiological agent responsible for the observed symptoms in his patients[2]. It was not until 1932 when Crohn's disease was first introduced as a clinical entity was it possible to connect the pathological and clinical findings described in CD to Dalziel's observations in 1913. However, much skepticism and uncertainty

**Table 1 Studies supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by Culture *n* (%)**

| Study | Crohn's disease | Control |
|---|---|---|
| Bull *et al*[63] | 14 (33) | 3 (33) |
| Chiodini *et al*[82] | 16 (26) | 13 (26) |
| Chiodini *et al*[59] | 3 (100) | NP |
| Collins *et al*[64] | 15 (19) | 3 (6.3) |
| Gitnick *et al*[65] | 4 (14.8) | 1 (1.8) |
| Kirkwood *et al*[66] | 4 (40) | 0 (0) |
| Markesich *et al*[61] | 12 (50) | 1 (7.7) |
| Mendoza *et al*[67] | 30 (100) | 0 (0) |
| Moss *et al*[68] | 6 (33.3) | 1 (16.7) |
| Naser *et al*[69] | 2 (100) | 0 (0) |
| Naser *et al*[58] | 14 (50) | 0 (0) |
| Schwartz *et al*[62] | 10 (37) | 2 (5.6) |
| Sechi *et al*[57] | 19 (63.3) | 3 (10.3) |
| Singh *et al*[70] | 4 (80) | 6 (27.3) |
| Singh *et al*[71] | 29 (50) | 5 (12.5) |
| Wall *et al*[72] | 6 (20) | 0 (0) |

NP: Not performed.

**Table 2 Studies not supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by Culture *n* (%)**

| Ref. | Crohn's disease | Control |
|---|---|---|
| Clarkston *et al*[83] | 0/21 (0) | NP |
| Dumonceau *et al*[105] | 0/31 (0) | 0/22 (0) |
| Graham *et al*[84] | 6/19 (31.5) | 7/17 (41) |
| Kallinowski *et al*[75] | 0/21 (0) | 0/24 (0) |
| Kreuzpaintner *et al*[85] | 0/23 (0) | 0/23 (0) |
| Parrish *et al*[73] | 0/130 (0) | 0/130 (0) |
| Ricanek *et al*[74] | 2/75 (2.7) | 2/135 (1.5) |

NP: Not performed.

persisted with respect to the etiology of Crohn's disease. Furthermore, confidence in this mycobacterial hypothesis over the years has suffered tremendously due to the substantial difficulty and failure in culturing mycobacteria from CD tissues and the reliance on methodology which were not appropriate for MAP from humans. MAP association with CD theory was revived when Chiodini *et al*[59] in 1984 reported the isolation of uncharacterized mycobacteria from tissues of three CD patients. They proposed that the bacterium existed in a cell-wall defective form which was later characterized as MAP[60]. Similar results were reported from studies out of David Graham and John Hermon-Taylor laboratories (discussed below). Advancements in cultural techniques and PCR assays unique to MAP by Naser's team (discussed below) fueled and renewed interest in investigating a possible etiological connection between MAP and CD.

## CULTURE OF MAP FROM CD PATIENTS

In this review, data from a total of 23 peer review studies which investigated the presence of MAP in CD specimens using culture techniques were reviewed. As shown in Table 1, the results from 16 (70%) studies supported the association between MAP and CD. Only 7 (30%) studies did not support such association (Table 2). Much of the difficulty in culturing or isolating MAP stems from the fact that this fastidious organism has very specific nutritional requirements and is a very slow growing bacterium[59,61,62]. Culture of MAP in liquid or agar-based media requires weeks to months of laboratory incubation[22]. The presence of MAP in a cell wall-deficient spheroplastic form in humans adds additional challenges to growing it in the laboratory. Many investigators reported the recovery of MAP in a cell wall-deficient form from the tissues of CD patients at a higher occurrence than control groups consisting of non-IBD patients[59,61-72]. Certainly, the advent of PCR, RT-PCR and nested PCR had facilitated the detection of MAP IS900 in cultures from CD patients[53,57,58,64,66,68,69,72]. Table 1 lists a total of 16 studies which strongly support the association between MAP and CD. The development of mycobacterial growth indicator tube (MGIT) sparked a new wave of interest led by Saleh Naser team who supplemented MGIT media with additives essential for survival of cell wall-deficient *in vitro* and restoration of the cell wall. Consequently, Schwartz *et al*[62] reported a higher frequency of MAP in CD patients at 37% (10/27) *vs* healthy controls at 5.6% (2/36). What is truly insightful in this study is the fact that MAP was found at a higher percentage (86%) in surgically resected tissue samples than in tissue biopsies (20%) taken from CD patients[62]. These results alluded to the supposition that MAP may in fact be located below the mucosal layer instead of found on the apical surface area[62]. Naser *et al*[69] further employed the same culture condition to study whether or not MAP is present in human milk. They reported the presence of MAP in 100% (2/2) of breast milk samples taken from lactating CD mothers who had just given birth, compared to 0% (0/5) of healthy lactating controls. Thus, this study provides critical evidence to support the similarity between Johne's disease and MAP infection in CD. MAP was later on detected from breast milk from additional CD patients (data not shown). Most interestingly, Naser *et al*[58] were able to culture viable MAP from the buffy coat of blood sampled from CD patients at a significant percentage 50% (14/28). These intriguing results are further substantiated based on the fact that there was no evidence for the culture of MAP from the blood of the healthy control groups 0% (0/15). Other scientists reported the presence of MAP in 14/33 (42%) bowel-pinch biopsies of CD patients (14/33) compared to 3/33 (9%) non-IBD controls. It was Kirkwood *et al*[66] who sought to investigate if there was an association between MAP and CD in children who were symptomatic of this disease at an early stage. They revealed that 40% (4/10) of the cultured mucosal biopsies from the CD patients contained viable MAP, whereas 0% (0/4) of the healthy non-IBD controls showed no evidence for the presence of MAP. Consequently, these findings clearly indicate the possible association between MAP and CD, and according to Kirkwood *et al*[66] these results

**Table 3 Studies supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by polymerase chain reaction *n* (%)**

| Ref. | Crohn's disease | Control |
|---|---|---|
| Autschbach *et al*[76] | 52 (100) | 5 (100) |
| Bentley *et al*[86] | 122 (33.8) | 43 (21.5) |
| Bull *et al*[63] | 34 (92) | 9 (26) |
| | 14 (42) | 3 (9) |
| Collins *et al*[64] | 15 (19) | 3 (6.3) |
| Dell'Isola *et al*[87] | 13 (72) | 7 (29.2) |
| Erasmus *et al*[88] | 10 (38) | 4 (11) |
| Fidler *et al*[89] | 4 (12.9) | 0 (0) |
| Gan *et al*[90] | 17 (47.2) | 3 (15) |
| Ikonomopoulos *et al*[91] | 7 (35) | NP |
| Kirkwood *et al*[66] | 22 (39) | 6 (15) |
| | 8 (16) | 0 (0) |
| Lisby *et al*[92] | 11 (46) | 3 (11) |
| Mendoza *et al*[67] | 18 (60) | 0 (0) |
| Mishina *et al*[78] | 8 (100) | 0 (0) |
| Moss *et al*[68] | 6 (33.3) | 1 (16.7) |
| Murray *et al*[93] | 2 (22) | 0 (0) |
| Naser *et al*[69] | 2 (100) | 0 (0) |
| Naser *et al*[58] | 13 (46) | 3 (20) |
| Romero *et al*[77] | 10 (83) | 1 (17) |
| Ryan *et al*[94] | 6 (50) | 0 (0) |
| Sanderson *et al*[95] | 26 (65) | 5 (12.5) |
| Scanu *et al*[96] | 20 (87) | 3 (15) |
| Sechi *et al*[57] | 25 (83.3) | 3 (10.3) |
| Singh *et al*[70] | 4 (80) | 5 (22.7) |
| Singh *et al*[71] | 28 (96.6) | NP |
| Tiveljung *et al*[97] | 3 (27) | 0 (0) |
| Tuci *et al*[98] | 21 (68) | 11 (48) |
| Wall *et al*[72] | 6 (20) | 0 (0) |

NP: Not performed.

imply that MAP maybe implicated with the early-onset of CD in children. Sechi *et al*[57] also reported a particularly strong association between MAP and CD based on their population study which involved the analysis of people in Sardinia diagnosed with and without CD. According to their results it was found that MAP DNA was detected in intestinal mucosal biopsies of approximately 63% (19/30) of CD patients compared to 10.3% (3/29) of control patients.

Contrary to the above data, there have been some studies providing evidence for the dismissal of MAP as a causative agent of CD (Table 2). For example, Parrish *et al*[73] conducted a study analyzing blood samples taken from 260 individuals who consisted of 130 CD patients and 130 healthy individuals. After culturing MAP, the results revealed that none of the CD patients 0% (0/130) as well as the healthy controls 0% (0/130) showed evidence for the presence of MAP[73]. Only one patient was reported having a positive result by PCR[73]. Due to the fact that MAP and MAP DNA are present in the food chain and the fact that MAP DNA has been detected in the blood of patients with CD and type I diabetes mellitus and in less frequency in the blood of healthy controls, most scientists in the field may question the protocol used in this study. In another study, Ricanek *et al*[74] collected bowel biopsies from 321 individuals, of which 75 of these biopsies were collected from CD patients and 135 were collected from non-IBD patients. After long-term culture of MAP it was reported that only 2.7% (2/75) of CD patients and 1.5% (2/135) of non-IBD patients showed the presence of MAP[74]. Similarly, Kallinowski *et al*[75] documented the inability to culture MAP from a variety of sources such as stool, sera, and even gut tissue samples. They reported that 0% (0/21) of CD patients and 0% (0/24) of healthy controls had MAP through culture[75]. The results from these studies should not be surprising since MAP is extremely fastidious and requires specialized culture media to grow which is contrary to culture media used in these studies. Other studies which failed to detect MAP in CD have depended on traditional standard methodology designed to culture and detect bacillary MAP from Johne's disease animals or other *Mycobacterium species*. It is important that investigators realize that *M. avium subspecies paratuberculosis* is not the same as *M. avium* or *M. tuberculosis*. Moreover, tissue and blood specimens collected from patients with active antibiotic treatment should be used for attempts to culture MAP in the laboratory. Rarely did the studies described in Table 2 allotted to whether the subjects used in their studies had antimicrobial agents prior to submission of the specimens.

## DETECTION OF MAP DNA BY PCR

A total of 52 studies investigating MAP DNA in CD have been reviewed. Table 3 lists a total of 27 studies providing evidence in support of MAP association with CD by PCR. On the contrary. Table 4 lists 25 studies which present data in contradiction of MAP-CD association.

One of the studies showing a strong connection between MAP and CD has been performed by Autschbach *et al*[76]. They reported that a staggering 52% (52/100) of tissue from CD patients were found positive for the presence of MAP DNA compared to only 5% (5/100) of the non-IBD patients. Similarly, Romero *et al*[77] had examined several surgical tissue samples from 20 individuals by performing nested PCR specific for the IS900 sequence. The results from Naser's lab indicated that a substantially high percentage 83% (10/12) of CD patients were positive for the presence of MAP, while a much smaller percentage 17% (1/6) of non-IBD patients were positive for MAP[77]. In addition, there was another compelling study conducted by Bull *et al*[63] in 2003 in John Hermon-Taylor's laboratory, which presented data in support of MAP as a causative agent for CD. Fresh ileocolonic mucosal biopsies were collected and analyzed for the presence of MAP by the performance of PCR specific for IS900. The results revealed that 92% (34/37) of CD patients were positive for the presence of MAP DNA compared to a significantly diminished number of healthy controls 26% (9/34)[63]. In this same study Bull *et al*[63] had cultivated MAP using MGIT cultures described by Naser *et al*[58] and Schwartz *et al*[62]. After twelve weeks of incubation, PCR was performed with these cultures which again indicated

**Table 4 Studies not supporting *mycobacterium avium* subspecies *paratuberculosis* association with Crohn's disease by polymerase chain reaction *n* (%)**

| Ref. | Crohn's disease | Control |
|---|---|---|
| Al-Shamali *et al*[99] | 0 (0) | 0 (0) |
| | 0 (0) | 0 (0) |
| Baksh *et al*[100] | 0 (0) | NP |
| Bernstein *et al*[101] | 0 (0) | 6 (21.4) |
| Cellier *et al*[102] | 2 (4) | 2 (10) |
| | 0 (0) | 0 (0) |
| Chiba *et al*[103] | 0 (0) | 0 (0) |
| Clarkston *et al*[83] | 1 (4.8) | 0 (0) |
| Dumonceau *et al*[104] | 17 (47) | 13 (57) |
| | 0 (0) | 0 (0) |
| Domonceau *et al*[105] | 0 (0) | 0 (0) |
| Ellingson *et al*[106] | 0 (0) | 0 (0) |
| Frank and Cook[81] | 0 (0) | 0 (0) |
| Gibson *et al*[107] | 0 (0) | 0 (0) |
| Kallinowski *et al*[75] | 0 (0) | 0 (0) |
| Kanazawa *et al*[108] | 0 (0) | 0 (0) |
| Kreuzpaintner *et al*[85] | 0 (0) | 0 (0) |
| Lozano-Leon *et al*[109] | 0 (0) | 0 (0) |
| Parrish *et al*[73] | 0 (0) | 1 (0.77) |
| Ricanek *et al*[74] | 0 (0) | 1 (0.28) |
| Riggio *et al*[110] | 0 (0) | 0 (0) |
| Quirke[21] | 0 (0) | 0 (0) |
| Rowbotham *et al*[80] | 0 (0) | 1 (3.8) |
| Sasikala *et al*[79] | 0 (0) | 0 (0) |
| Suenaga *et al*[111] | 10 (100) | 14 (87.5) |
| | 10 (100) | 14 (87.5) |
| Toracchio *et al*[112] | 1 (5) | NP |
| Tzen *et al*[113] | 0 (0) | 3 (27.3) |
| Wu *et al*[114] | 0 (0) | NP |

NP: Not performed.

a higher frequency of CD patients 42% (14/33) positive for MAP DNA *vs* only 9% (3/33) of healthy controls[63]. This data strengthens the support of MAP in connection with CD. Mishina *et al*[78] analyzed mucosal specimens using RT-PCR for the detection of MAP RNA where they found MAP in 100% (8/8) of CD patients and 0% (0/2) in non-IBD. This study is of particular importance because MAP RNA was amplified (without culture) adding more support to the presence of viable MAP in CD[78].

At the same time, many studies based on PCR techniques have failed to detect MAP DNA in CD and concluded the lack of association between MAP and CD (Table 4). For example, Sasikala *et al*[79] indicated that 0% (0/93) of CD patients showed the presence of MAP and 0% (0/97) of healthy controls were also negative for the presence of MAP. Similarly, Rowbotham *et al*[80] reported that none (0/68) of CD patients were positive for the presence of MAP and just 3.8% (1/26) of healthy controls had MAP. Lozano-Leon *et al*[109] indicated the absence of MAP in the blood of 73 CD patients and 73 healthy controls. Frank and Cook in 1996 also reported the absence of MAP in both CD and control subjects[81]. The investigators in these studies should be commended on their interest to question whether or not MAP is associated with CD, and for including impressive numbers of specimens in their studies. Due to the fact that MAP and MAP DNA are found in the food chain including dairy and meat products as well as in drinking water, it is difficult to accept that MAP or MAP DNA is not detected even accidently in some specimens. The methodology used in many of these studies must have lacked essential steps to recover the low abundance of MAP in CD specimens and must have not been able to reduce the laboratory loss of some MAP or MAP derivatives. Whether the loss of MAP occurred at the specimen collection level or during the analysis, it should be avoided. Tissue specimens must be collected appropriately and adequately from active ulcerated sites. Specimens should be transported promptly and appropriately by avoiding freezing and use of anti-microbial solutions. Blood should be withdrawn into tubes with anticoagulants, transported without freezing, and promptly, to avoid lysis of leukocytes and loss of MAP. DNA extraction conditions should be optimized to recover single MAP genome which is also free from PCR inhibitors such as hemoglobin. Earlier study in our laboratory suggested that MAP from CD patients contained limited IS900 copies compared to bovine MAP strains. Nested PCR consisting of two amplification rounds is necessary for sufficient detection of MAP DNA. For reasons mentioned above and other unknown factors, standard PCR based on a single amplification should not be used for detection of MAP in CD.

## CONCLUSION

In this review, data has been presented in the form of tables providing evidence for and against an association between MAP and CD by PCR and culture. It was revealed that MAP can be detected and isolated from the tissues, blood, and milk of many CD patients. Based on this information, MAP is definitively involved in the pathogenesis of some CD cases even though other studies have not acknowledged this association as represented in Tables 2 and 4. It must be emphasized that much of the controversy concerning MAP and CD stems from the inconsistent methodologies that have been used in the detection and isolation of MAP, which have questioned the causal relationship between this bacterium and CD. These observed discrepancies result from the fact that the methods that were designed for the detection of MAP in animals with Johne's disease are inappropriate for the detection of MAP in humans. Consequently, the need for more sophisticated and optimized methodologies are required so that there can be accurate detection and isolation of MAP in CD patients. One such methodology has been developed in our laboratory, and success has been achieved based on key principles shown in Figure 1. Other factors that may also limit the detection of MAP in clinical samples from some CD patients include the stage of the disease, and prior treatment with antibiotics or drugs with antimicrobial activity. For example, negative detection of MAP in peripheral blood samples could be correlated with a localized intestinal CD com-

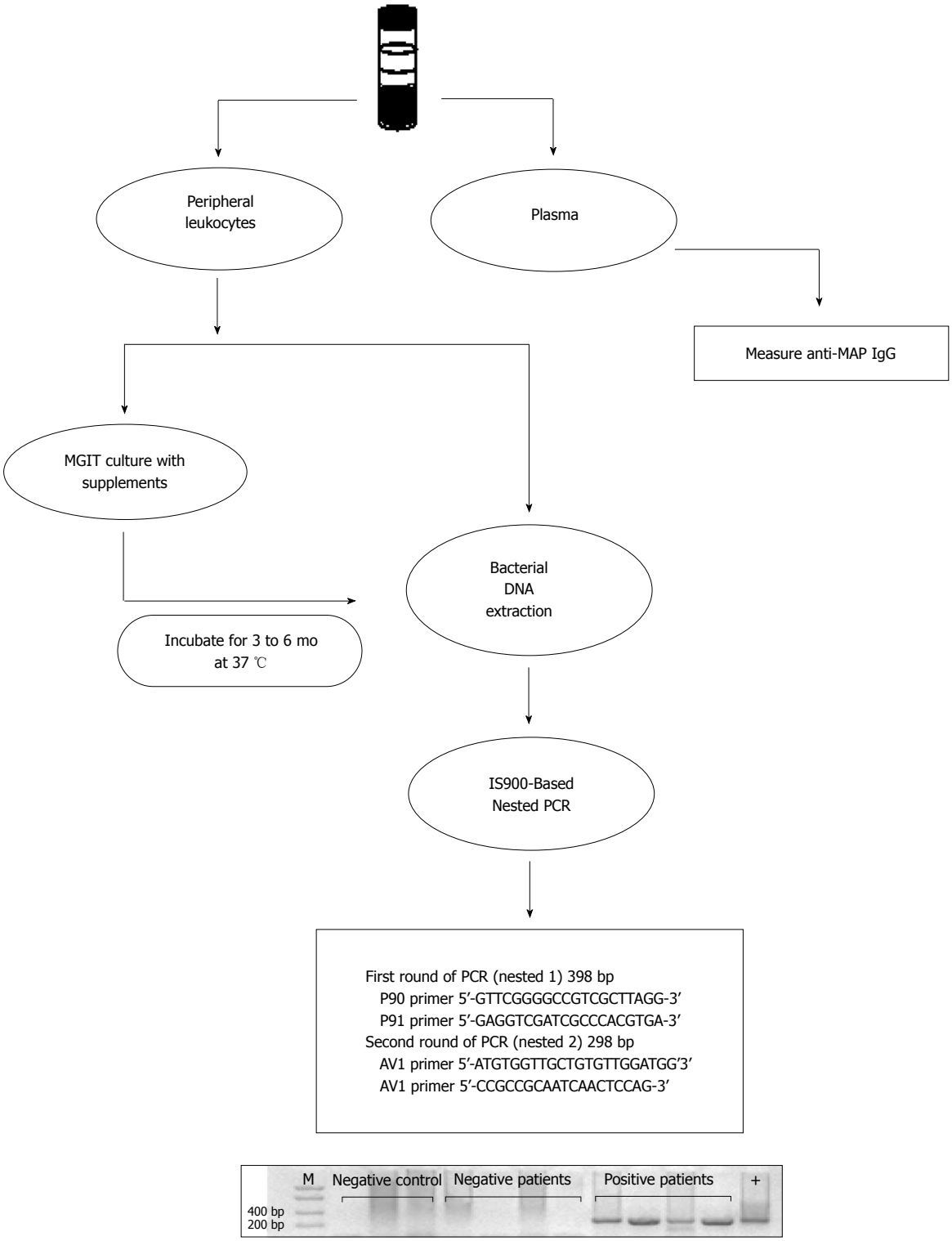


**Figure 1 Schematic illustration of successful *Mycobacterium avium* subspecies *paratuberculosis* detection in clinical samples.** Coded EDTA blood samples were collected from patients for investigating the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Blood plasma was analyzed by measuring the concentration of anti-MAP IgG antibodies. Peripheral leukocytes were analyzed for the presence of MAP. In the first method, DNA was extracted followed by IS900-based nested polymerase chain reaction (PCR) using MAP-specific primers. In the second method a mycobacterium growth indicator tube (MGIT) liquid culture system with supplements was used to culture MAP lacking cell wall followed by 3 to 6 mo incubation and IS900-based nested PCR analysis.

pared to cases with advanced disease associated with systemic complications. The latter is most likely to lead to the presence of MAP in circulation.

Finally, it is also worth noting that it is a fact that CD is a syndrome with multifactorial etiology. It is very possible that lack of detection of MAP in clinical samples from some CD patients may be due to the absence of MAP role in these patients. The latter statement is con-

ditional on utilization of methodology appropriate for detection of human MAP strains. Stratification of CD and IBD patients for the presence or absence of MAP is necessary for appropriate and effective treatment which may lead to a cure.

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# Isolation of *Mycobacterium avium* Subspecies *paratuberculosis* Reactive CD4 T Cells from Intestinal Biopsies of Crohn's Disease Patients

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## Abstract

***Background:*** Crohn's disease (CD) is a chronic granulomatous inflammation of the intestine. The etiology is unknown, but an excessive immune response to bacteria in genetically susceptible individuals is probably involved. The response is characterized by a strong Th1/Th17 response, but the relative importance of the various bacteria is not known.

***Methodology/Principal Findings:*** In an attempt to address this issue, we made T-cell lines from intestinal biopsies of patients with CD (n = 11), ulcerative colitis (UC) (n = 13) and controls (n = 10). The T-cell lines were tested for responses to various bacteria. A majority of the CD patients with active disease had a dominant response to *Mycobacterium avium* subspecies *paratuberculosis* (MAP). The T cells from CD patients also showed higher proliferation in response to MAP compared to UC patients ($p<0.025$). MAP reactive CD4 T-cell clones (n = 28) were isolated from four CD patients. The T-cell clones produced IL-17 and/or IFN-γ, while minimal amounts of IL-4 were detected. To further characterize the specificity, the responses to antigen preparations from different mycobacterial species were tested. One T-cell clone responded only to MAP and the very closely related *M. avium* subspecies *avium* (MAA) while another responded to MAP, MAA and *Mycobacterium intracellulare*. A more broadly reactive T-cell clone reacted to MAP1508 which belongs to the esx protein family.

***Conclusions/Significance:*** The presence of MAP reactive T cells with a Th1 or Th1/Th17 phenotype may suggest a possible role of mycobacteria in the inflammation seen in CD. The isolation of intestinal T cells followed by characterization of their specificity is a valuable tool to study the relative importance of different bacteria in CD.

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## Introduction

Crohn's disease (CD) is an intestinal disorder characterized by granulomatous inflammation. The etiology is still unknown, but it is generally believed that an inappropriate inflammatory response to the commensal bacteria is involved [1]. Lately it has become clear that the risk of developing CD is associated with polymorphisms in several genes that are involved in interaction with bacteria. In particular, NOD2 [2,3], which is an intracellular sensor of bacteria, and ATG16L1 [4] and IRGM [5], which are involved in autophagy, are genetic factors for CD. NOD2 activates an NF-κB signaling pathway upon binding of the bacterial peptidoglycan component muramyl dipeptide (MDP), but exactly how NOD2 is involved in CD has not been settled. There is evidence both for loss and gain of functions [6,7]. Autophagy, with involvement of ATG16L1 and IRGM, is an important constitutive cellular process involved in protein turnover and the removal of subcellular components. Recently ATG16L1 was shown to be important for the biology of intestinal Paneth cells [8], and interestingly the autophagy pathway is also important for resistance against intracellular bacteria [9]. Functional knock down of ATG16L1 abrogated autophagy of the intracellular pathogen *Salmonella typhimurium* [10]. Moreover, knockdown of IRGM leads to markedly prolonged survival of *Mycobacterium tuberculosis* in human macrophages [11]. It is notable that *NOD2*, *ATG16L1* and *IRGM* are all risk factors for CD but not ulcerative colitis (UC), while many other genes including the *IL-23 r* gene and the *IL-12B* gene [12] , coding for the common p40 subunit of IL-12 and IL-23, are susceptibility determinates for both conditions. This indicates that some of the inflammatory pathways are likely shared between the two conditions, while the importance of immune handling of bacteria differentiates CD pathophysiology from UC.

At this stage it is unclear whether the CD associated variants of *NOD2*, *ATG16L1* and *IRGM* influence the host response to

particular bacteria or whether they have more general effects to a wide range of gut bacteria. Several bacteria have been suggested to be involved in CD pathogenesis including *Escherichia coli* and *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Invasive *E. coli* have been found in higher frequencies in ileal CD [13]. The data on the presence of MAP are not uniform, but two meta-analysis of several published studies have concluded that MAP is more often present in CD patients than patients with UC and non-inflammatory bowl disease (non-IBD) [14,15]. However whether the bacterium can contribute to the inflammatory response is not known.

The CD lesions are transmural, and typically they have granulomas and lymphoid aggregates with abundance of CD4+ T cells that produce inflammatory cytokines like IL-17 and IFN-γ [16]. To get more information about the bacteria involved in CD pathogenesis one approach is to isolate intestinal T cells. Studies of the specificity of intestinal T-cells in CD are limited. A decade ago Duchman et al showed that both CD and ulcerative colitis (UC) patients had T cells with reactivity to various commensal bacteria, including *E. coli*, however no differences were found between the two groups [17,18]. To get information about the relative importance of various bacteria in the ability to elicit an inflammatory T cell response, we chose to characterize the specificity of intestinal T cells from CD patients. We subsequently isolated T cells from intestinal biopsies of CD, UC and non-IBD patients and detected responses to all the tested bacteria. However, CD patients had a higher frequency of MAP reactive T cells than the UC patients and also a higher frequency of response to MAP compared to other bacterial antigens. Furthermore these T cells produced inflammatory cytokines like IFN-γ and IL-17. Our data justify further studies into the possible role of mycobacteria in CD immunopathology.

## Methods

### Study subjects

Intestinal biopsies were obtained by colonoscopy from adult patients with CD (n = 11), UC (n = 13) and non-IBD (n = 10). The colonoscopy was performed as a part of the routine investigation. Patients with endoscopically active and inactive disease were included. Patients that had received, or were receiving anti-TNF-α treatment, were not included. Of the CD patients (2 men, 9 women), four had inactive while seven had active disease. The average age was 45 years (range 27–66) and the average time since diagnosis was 19 years (range 5–28). Of the UC patients (8 men, 5 women), five had inactive disease while eight had active disease. The average age was 41 years (range 19–61) and the average time since diagnosis was 12 years (range 2–30). The average age of non-IBD patients (3 men, 7 women) was 49 years (range 18–73). Information about disease localization, medication and diagnosis is given in table 1. All patients gave written informed consent before the colonoscopy. The study was approved by the Regional Committee for Medical Research Ethics, South Norway, and approval for storing of biological materials was obtained by the Norwegian Directorate for Health and Social Affairs.

### HLA-typing

The patients were genomically HLA typed using the Olerup SSP HLA kits for DQB1*, DRB1*, DPB1* (GenoVision/Qiagen) or serologically typed by a complement dependent cytotoxicity test with immunomagnetically separated cells (Dynabeads® HLA class II, Invitrogen).

### Strains and antigens

The following strains were used to prepare the antigens: *Bacterioides thetaiotaomicron* CCUG 12297, *Lactobacillus gasseri* CCUG 39972, *Bifidobacterium bifidum* CCUG 45217, *Escherichia coli* ATCC 43893 (enteroinvasive), *M. avium* subsp. *paratuberculosis* 2E, *Mycobacterium avium* subspecies *avium* D4, *Mycobacterium intracellulare* MNC72, *Mycobacterium gordonae* MNC 64, *Mycobacterium tuberculosis* clinical isolate. The bacteria were grown on standard medium under recommended conditions. The cells were scraped off the agar plates, sonicated (two cycles of 10 min) and centrifuged. The supernatants were sterile filtered (0.2 μm) before the protein concentration was assessed according to Lowry [19] using the Bio-Rad $D_C$ Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). The mycobacterial antigens were prepared as previously described [20]. In short, mycobacteria were grown as a surface pellicle on liquid Reids or Sauton medium. Proteins secreted by the bacteria into the culture medium were precipitated using ammonium sulphate, dissolved in PBS, dialysed and sterile filtrated (0.2 μm). Recombinant MAP antigens and pooled peptides from single MAP antigens used for testing are listed in Table S1. Synthetic peptides were purchased from Genscript, NJ, USA.

### Antibodies

The following antibodies were used for analyzing T cells by flow cytometry: anti-TCR αβ FITC (IgM, clone T10B9.1A-31), anti-CCR6 PE (IgG1κ, clone 11A9), anti-TCRγδ APC (IgG1κ, clone B1), anti- IFNγ FITC (IgG1κ, clone 4S.B3) isotype control FITC (IgM, clone G155-228) isotype control PE (IgG1κ, clone MOPC-21) (all BD Pharmingen); anti-CD4 PE (IgG2a, clone EDU-2), anti-CD8 FITC (IgG2a, clone UCHT-4), Isotype PE (IgG2A, clone BH1), Isotype FITC (IgG2A, clone BH1) (all Diatec) and anti-IL-17a Alexa fluor647 (IgG1κ, clone eBio64DEC17) (ebioscience). Anti-CD56 (IgG2a, clone MEM 188) and goat anti-mouse-IgG2a FITC (Southern Biotechnology).

The HLA restriction of the T cells was determined by testing inhibition of T-cell proliferation in the presence of monoclonal antibodies B8.11 (pan-DR), SPV-L3 (pan-DQ) or B7/21 (pan-DP) at a concentration of 20 μg/ml.

### Establishment of T-cell lines and clones

The protocol for establishment of T-cell lines was adapted from the protocol used for establishing T-cell lines from small intestinal biopsies of celiac disease patients [21,22] with some modifications. The biopsies were taken from the distal part of the small intestine, or upper part of colon. In patients with active disease, biopsies were taken from inflamed mucosa and from the surrounding non-inflamed areas. Separate, single biopsy specimens from each location were incubated with either complete medium (RPMI 1640 (Gibco) containing 10% human serum, β mercaptoethanol, penicillin, streptomycin and fungizone) or complete medium with MAP (100 μg/ml) overnight. After incubation the biopsies were homogenized for 120 seconds in a BD Medimachine Medicon (BD Medimachine™ Medicon, 35 μm Sterile). The single cells from each biopsy were centrifuged, dissolved in 1 ml complete medium containing $2\times10^6$ autologous, irradiated (25 Gy) PBMC, 10 U/mL human IL-2 (R&D Systems, Abingdon, UK), and 1 ng/mL human IL-15 (R&D Systems) and seeded into 8 wells on a U-bottomed 96-well plate. On day 8, cells from duplicate wells were restimulated separately with $1\times10^6$ allogenic, irradiated PBMC, 10 U/mL IL-2, 1 ng/mL IL-15, and 1 μg/mL phytohemagglutinin (Remel) in a 48-well plate and propagated as four separate T-cell lines. The four lines established from each biopsy were tested in triplicates on day 15.

T-cell clones were generated from MAP and *E. coli* reactive biopsy-derived T-cell lines. The T cells were diluted in irradiated feeders from three donors with IL-2, IL-15 and PHA as described

**Table 1.** Patient characteristics

| CD | Disease localisation and actvity | Medication |
|---|---|---|
| CD-6 | Small intestine (inactive) | Azathioprin |
| CD-9 | Small intestine and colon (active) | Topical steroids |
| CD-10 | Colon (active) | Prednisolone |
| CD-11 | Small intestine (inactive) | None |
| CD-15 | Colon, fistulas (active) | None |
| CD-18 | Small intestine and colon (active) | Mesalazine, Budesonide CR, Colestyramine |
| CD-33 | Colon (inactive) | Azathioprin |
| CD-36 | Colon (inactive) | None |
| CD-46 | Ileocecal and perianal (active) | None |
| CD-47 | Small intestine, ileocecal and perianal (active) | Azathioprin |
| CD-48 | Colon (active) | Balsalazide, Prednisolone |
| **UC** | **Disease localisation and actvity** | **Medication** |
| UC-14 | Pancolitis (active) | Azathioprin, Balsalazide, Budesonide CR |
| UC-16 | Proctitis (active) | Mesalazine topical |
| UC-17 | Pancolitis (inactive) | Mesalazine, Prednisolone, Tacrolimus |
| UC-19 | Pancolitis (inactive) | Mesalazine |
| UC-25 | Proctitis (inactive) | None |
| UC-27 | Pancolitis (active) | Prednisolone |
| UC-31 | Pancolitis (active) | None |
| UC-32 | Pancolitis (active) | Balsalazide |
| UC-35 | Pancolitis, inactive) | Balsalazide |
| UC-37 | Pancolitis (inactive) | None |
| UC-40 | Pancolitis (active) | Mesalazine |
| UC-44 | Proctitis (active) | Mesalazine topical |
| UC-45 | Pancolitis (active) | Mesalazine |
| **non IBD** | **Diagnosis** | **Medication** |
| non IBD-20 | Diarrhea | None |
| non IBD-23 | Healthy, family history of colon cancer | None |
| non IBD-24 | Cancer control | Levotyroksin, allergy medication |
| non IBD-26 | Familial adenomatous polyposis | None |
| non IBD-28 | Healthy, family history of colon cancer | None |
| non IBD-30 | Cancer control | None |
| non IBD-34 | Healthy, family history of colon cancer | None |
| non IBD-13 | Primary sclerosing cholangitis, no sign of UC or CD | None |
| non IBD-49 | Healthy, family history of colon cancer | None |
| non IBD-51 | Celiac disease | None |

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above and seeded on Terasaki plates (Greiner Bio-One) at a concentration of 0.3–3 cells/well. After 9 days, growing T-cell clones were transferred to 48 well plates and restimulated as before. Established T-cell clones were tested for reactivity to MAP or *E. coli*. T-cell clonality was tested by the IOTest® Beta Mark (Beckman Coulter) TCR Vβ staining kit covering about 70% of the normal human TCR Vβ repertoire of $CD3^+$ lymphocytes.

## APC and T cell proliferation assay

Testing of the reactivity of the T-cell lines was done by assessing proliferation in restimulation assays using irradiated adherent cells as APC. The APC were isolated by incubating PBMC (50 000 cells/well, 96 well plate) in medium containing 15% FCS for 1.5 hours. The wells were washed three times in medium with 15% FCS before medium with 10% human serum and antigens were added. The plates were irradiated (25 Gy) the next day before the T cells were added. Autologus adherent cells from frozen PBMC were used for all initial screenings while HLA-II matched donors or autologus APC were used for later testing. The cells were incubated for three days with the addition of $^3H$ thymidine for the last 20 hours. Proliferation was assessed by scintillation counting after harvesting of the cultures. Positive T-cell responses were defined as a stimulatory index (SI) above 5 ([T+APC+antigen] divided by [T+APC]).

The following homozygous B-lymphoblastoid cell lines derived from the $10^{th}$ and $11^{th}$ International Histocompatibility Workshop

**Table 2.** Response to various bacteria in T-cell lines from CD patients with active disease.

| | Number of positive[A] T-cell lines (n = 8) | | | | |
|---|---|---|---|---|---|
| | **MAP** | ***B. thetaiotaomicron*** | ***B. bifidum*** | ***L. gasseri*** | ***E. coli*** |
| CD-9 | 5 (2)[B] | 1 (1) | 2 (2) | 2 (2) | 1 (1) |
| CD-18 | 5 (2) | 1 (1) | 0 | 0 | 2 (2) |
| CD-15 | 7 (0) | 0 | 0 | 0 | 0 |
| CD-48 | 2 (0) | 0 | 1 (1) | 1 (1) | 0 |
| CD-46 | 2 (2) | 1 (1) | 1 (1) | ND[C] | 3 (2) |
| CD-47 | 0 | 1 (1) | 0 | 0 | 3 (1) |
| CD-10 | 0 | 0 | 0 | 0 | 0 |

[A]Positive lines are defined as SI>5
[B]Some lines reacted to several bacterial antigens. The total number of positive lines to each bacterium is shown. The number in brackets shows how many of these lines that were multi-reactive.
[C]ND = not done
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(IHWS) were used as APC for identification of HLA restriction of the MAP1508 specific T-cell clone: #9002/MZ070782; (DRB1*0102, DQA1*0101, DQB1*0501, DPB1*0401), #9003/KAS116; (DRB1*0101, DQA1*0101, DQB1*0501, DPB1*1301), #9042/TISI; (DRB1*1103, DQA1*0501, DQB1*0301, DPB1*0402), #9055/H0301; (DRB1*1302, DQA1*0102, DQB1*0605, DPB1*0501), #9063/WT47; (DRB1*1302, DQA1*0102, DQB1*0604, DPB1*1601) and YT (DRB1*0405, DQA1*03, DQB1*0401, DPB1*0501). The B-cell lines were all irradiated with 75 Gy before use.

### Staining of intracellular cytokines

T-cell clones (approximately 500 000 cells) were stimulated with PMA (10 ng/ml) and ionomycin (2 μM) or left unstimulated. Monensin was added and the cells were incubated for 18 hours followed by staining for intracellular IFN-γ and IL-17. Briefly the cells were fixed in 1% PFA for one hour and permabilized in PBS with 2% FCS and 0.2% saponin for 30 minutes. The cells were stained with antibodies against IFN-γ and IL-17a and analyzed on a FACS CALIBUR flow cytometer (Becton Dickinson), equipped with Cell-Quest software.

### Cytokine assays

The amount of cytokines was measured in supernatant from antigen stimulated T-cell clones and unstimulated controls. The stimulation was performed as for the T-cell proliferation assay, and the supernatant was removed after 48 hours and stored at −20C until tested. As a control for T-cell viability $^{3}$H thymidine was added and the T-cell proliferation measured after incubation for another 24 hours. The amount of cytokines was measured using the Bio-plex™ Cytokine Assay, (Bio-Rad) according to the manufacturer's instructions. Values above the detection limits defined by the standard curve were considered positive.

### Statistics

The Wilcoxon Mann-Whitney non-parametric test was used to compare patient groups and $p<0.05$ was considered significant.

## Results

### Reactivity of intestinal T cells to various bacterial antigens

The T-cell lines generated without *ex vivo* stimulation with any antigen, were tested for responses in a T-cell proliferation assay against antigen preparations from *B. thetaiotaomicron*, *L. gasseri*, *B. bifidum*, *E. coli* and MAP. Minimal responses were seen in patients with inactive disease and in non-IBD patients while the results from CD patients with active disease are summarized in Table 2. T-cell lines that reacted to MAP were detected in 5 of 7 (71%) patients in the CD group. Next to MAP, responses to *E. coli* were most frequently detected, and one CD patient (CD-47) had a strong response to *E. coli* with no response to MAP. More T-cell lines reacted to MAP than to the other bacterial antigens in the CD group, while in the UC group there were similar responses to MAP and the commensal bacteria (Table 3). Some of the T-cell lines, especially in the CD group, exhibited extensive multi-reactivity with response to several of the tested antigen preparation. Whether the multi-reactivity was due to cross reactive T cells or the presence of multiple specificities in the T-cell lines was not investigated. There were no systematic differences between T-cell lines obtained from biopsies from inflamed area and biopsies taken from the surrounding non-inflamed area.

Next we wanted to enrich for mycobacteria reactive T cells, and lines were therefore also generated from intestinal biopsies stimulated with MAP *ex vivo*. These lines were tested for response to MAP. The MAP stimulated biopsies from non-inflamed mucosa of one CD patient and one UC patient with active disease were contaminated and disregarded. The mean response in T-cell lines generated from non-inflamed mucosa of CD patients was significantly ($p<0.025$) higher than the responses in UC patients (Figure 1A). A similar tendency was seen in T cells from inflamed mucosa (not statistically significant, data not shown). The responses were strongest in CD patients with active disease compared to CD patients with inactive disease ($p<0.05$) (Figure 1B). Proliferation was however also detected in T-cell lines from some of the patients with inactive CD and in some control patients. These patients usually had responses in one single line T-cell line while patients with active CD had responses in several T-cell lines suggesting a higher frequency of MAP reactive T cells in the latter group. The three CD patients (CD-10, CD-33 and CD-36) with no detectable response to MAP in any of the tested T-cell lines had colon involvement only (Table 1).

### Cytokine response in MAP and E. coli responsive T-cell clones

In addition to MAP, *E. coli* was the bacterial antigen eliciting the strongest responses in CD patients.To further characterize the T

**Table 3.** Reactivity of T-cell lines to MAP and commensal bacteria in CD and UC patients with active disease

| | Number of positive[A] T-cell lines (n = 8) | | |
|---|---|---|---|
| | MAP[B] | Multi-reactive [C] | Commensal[D] |
| **CD patients** | | | |
| CD-9 | **3** | **2** | 0 |
| CD-18 | **3** | **2** | 0 |
| CD-15 | **7** | 0 | 0 |
| CD-48 | **2** | 0 | **1** |
| CD-46 | 0 | **2** | **1** |
| CD-47 | 0 | 0 | **3** |
| CD-10 | 0 | 0 | 0 |
| **UC patients** | | | |
| UC-27 | **4** | 0 | 0 |
| UC-40 | **1** | 0 | 0 |
| UC-14 | 0 | 0 | 0 |
| UC-31 | 0 | **1** | **3** |
| UC-32 | 0 | 0 | **1** |
| UC-45 | 0 | 0 | **1** |
| UC-44 | 0 | 0 | 0 |
| UC-16 | 0 | 0 | 0 |

[A]Positive lines are defined as SI>5
[B]Lines reacted only with MAP.
[C]Lines reactive with MAP and one or more commensal bacteria
[D]Lines reactive to one or more of the following commensal bacteria: *B. thetaiotaomicron, L. gasseri, B. bifidum, E. coli*
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cells in CD patients, we attempted to isolated single T-cell clones from three patients with strong MAP responses, one patient with a strong *E. coli* response and one patient with a mixed MAP and *E. coli* response. Altogether we obtained 28 T-cell clones (CD-46:17 clones, CD-11: 3 clones, CD-6: 2 clones and CD-9: 6 clones) that reacted to MAP antigens. From patient CD-46, who had a mixed response we also obtained eight *E. coli* reactive T-cell clones. From patient CD-47 with a strong *E. coli* response, a loss of reactivity was seen after expansion of the T-cell line and cloning was thus abandoned.

CD patients have a granulomatous inflammation with excessive production of IL-17 and IFN-γ in the intestine [16]. We thus wanted to see whether the MAP reactive and the *E. coli* reactive T-cell clones secreted any of these inflammatory cytokines. Two MAP reactive T-cell clones could not be expanded and thus excluded from further studies. The T-cell clones were incubated with MAP (n = 26) or *E. coli* (n = 8) antigens using HLA-II matched adherent cells as APCs and the supernatant was assayed for IFN-γ, IL-17 and IL-4. All the MAP reactive clones produced IFN-γ (ranging from 88 to 9786 pg/ml) and 23 of 26 clones produced IL-17 (ranging from 25 to 4320 pg/ml), while low levels of IL-4 (<17 pg/ml) was detected in three clones. The *E. coli* reactive clones produced some IL-17 (411 pg/ml±81) and lower, but detectable amounts of IFN-γ (117 pg/ml±25). In comparison the MAP reactive clones (n = 17) from the same patient produced a mean of 1593 pg/ml±328 of IL-17 and 1770 pg/ml±631 of IFN-γ (Figure 2A). Although most of the MAP reactive clones produced both IFN-γ and IL-17 they appeared to have either a dominant IL-17 secretion or a dominant IFN-γ production (Figure 2B). To see if polyclonal activation would give a different cytokine pattern, three clones producing only IFN-γ and two clones producing predominantly IL-17 but with detectable IFN-γ in response to MAP, were stimulated with PMA/ionomycin. The results were comparable to what was seen after antigen stimulation of the same clones. No IL-17 was detected in the IFN-γ secreting clones while the IL-17 producing clones made both cytokines (Figure 2C). This suggested that MAP reactive clones with a Th1 and a Th1/Th17 mixed phenotype were present in CD patients. A typical marker of IL-17 producing cells is the chemokine receptor CCR6, and all but one T-cell clone expressed CCR6 (Figure 2D). The CCR6 negative clone, TCC906A.8.4.15, had a Th1 phenotype with no detectable IL-17 in response to MAP or PMA/ionomycin.

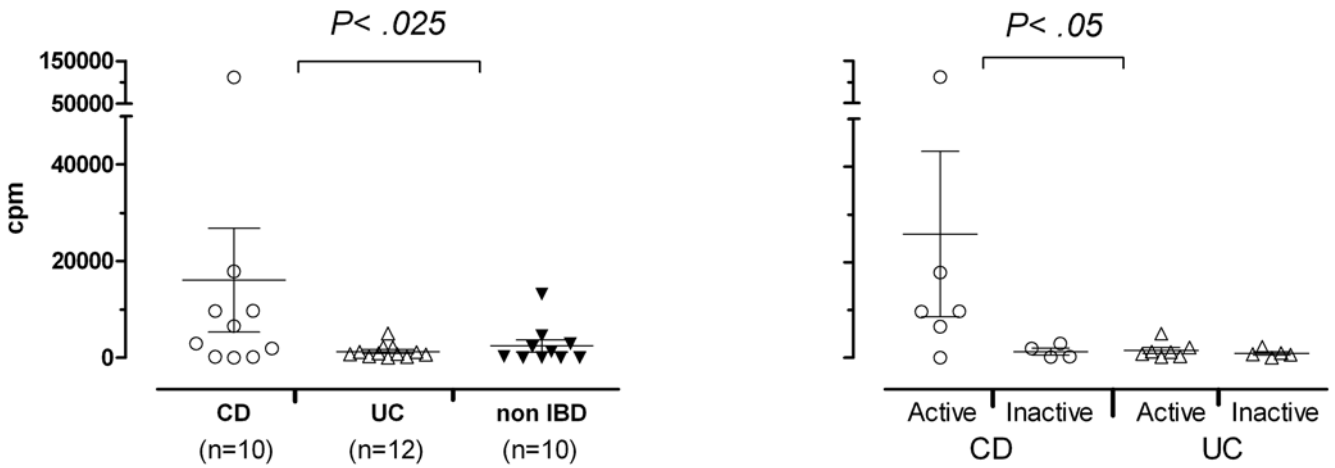


**Figure 1. T-cell responses to MAP.** Autologous adherent cells were incubated with antigen overnight and T cells were added followed by incubation for three days. $^{3}$H thymidine was added for the last 20 hours, and proliferation was assessed by scintillation counting after harvesting of the cultures. A) Depiction of results from all patients. B) Depiction of the same results, but where the material is divided into subgroups with active and inactive disease; active CD; n = 6, inactive CD; n = 4, active UC; n = 7, inactive UC; n = 5. Each symbol represents the mean response in four T-cell lines made from one patient. The lines were screened in triplicates and the results are given as CPM [ (T+ APC + MAP)−(T+APC)]. There was a significant difference between CD and UC patients ($p<0.025$) and between patients with active and inactive CD ($p<0.05$) using the non-parametric Wilcoxon Mann-Whitney test. Error bars indicate the mean response in the group±SEM.
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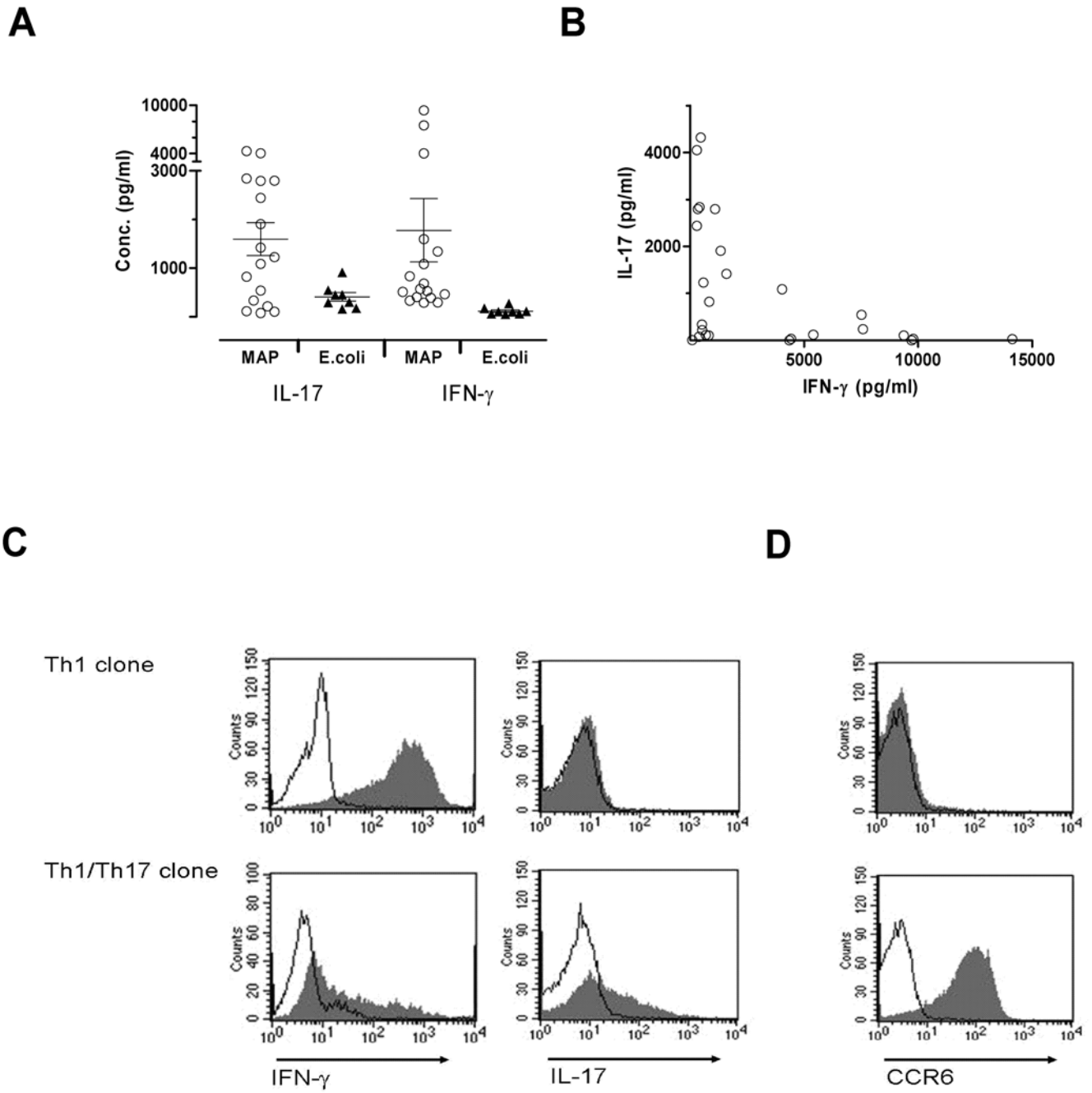


**Figure 2. Cytokine responses in T-cell clones**. A) Comparison of cytokine response in MAP reactive (n = 17) and *E. coli* reactive (n = 8) T-cell clones from the same CD patient (CD-46). T-cell clones were stimulated with MAP or *E. coli* antigens (10 μg/ml) for 48 hours using HLA class II matched irradiated adherent cells as APC. Supernatants from duplicate wells were sampled and tested for cytokine production. Cytokine production in control wells was subtracted. One symbol represents one clone. Error bars indicate mean ± SEM. B) IL-17 and IFN-γ production in MAP reactive T-cell clones (n = 26) from four different CD patients in response to MAP antigen. C) Intracellular staining of IFN-γ and IL-17 after stimulation with PMA/ionomycin in a Th1 (TCC906.A.8.4.15) clone and a Th1/Th17 clone (TCC946.A.8.2b.17). Filled histogram represent PMA/ionomycin samples and open histograms represent unstimulated samples. D) CCR6 expression in a Th1 clone and (top) and a Th1/Th17 clone (bottom). Filled histograms represent CCR6 expression and open histograms represent isotype control.
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## MAP reactive T-cell clones from CD patients showed a dominant response to the M. avium-intracellulare complex

MAP share several highly cross-reactive antigens with other mycobacteria, and exposure to environmental mycobacteria could lead to detectable T-cell responses. The T-cell clones were subsequently screened for reactivity to various crude antigen preparations from different mycobacterial species. Most of the T-cell clones showed some degree of cross-reactivity, however one clone (TCC946.A.8.2b.5 from CD-46) responded only to MAP and the very closely related *Mycobacterium avium* subspecies *avium*

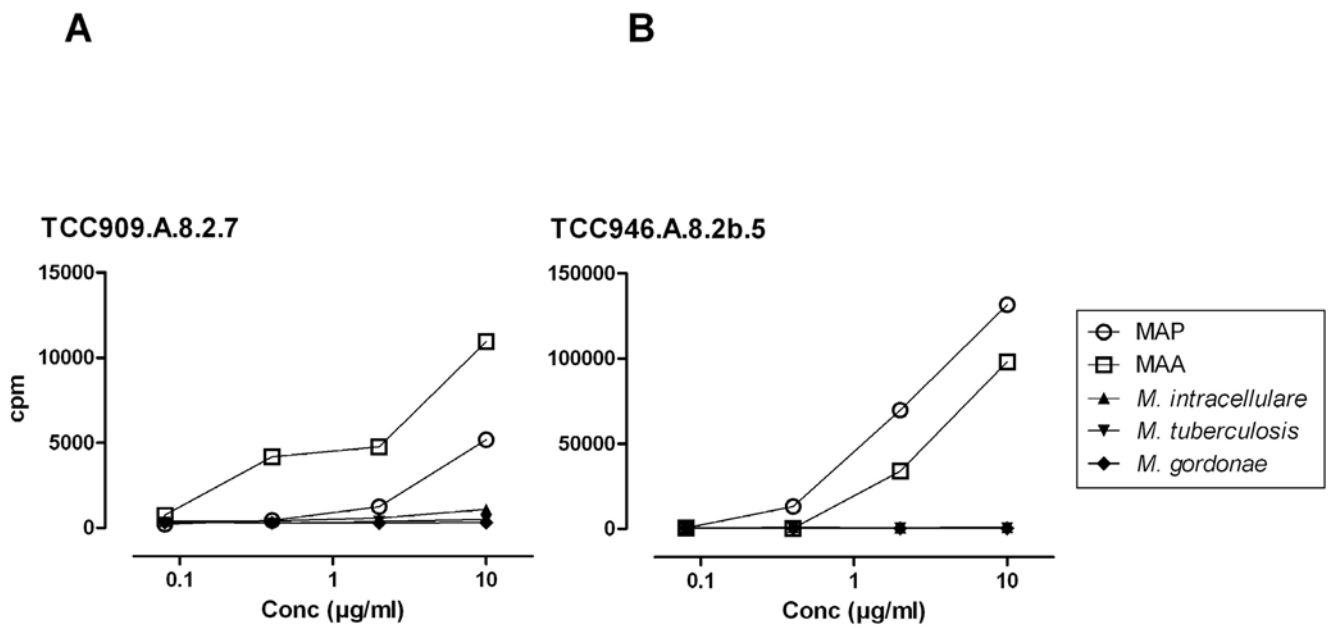


**Figure 3. Responses of T-cell clones to various mycobacteria.** Proliferation of T-cell clones from two different CD patients (CD-9 and CD-46) after stimulation with crude antigen preparations from various mycobacteria. HLA-II matched adherent cells were used as APC and were incubated with antigen overnight. The T cells were added followed by incubation for three days. $^{3}$H thymidine was added for the last 20 hours. Each concentration of antigen was tested in duplicates. Most T-cell clones showed some degree of cross-reactivity to mycobacterial antigens. A) Depiction of results of one clone (TCC909.A.8.2.7) which responded to MAP, MAA and *M. intracellulare* B) Depiction of results of one clone (TCC946.A8.2b.5) which responded only to MAP and MAA. The data are representative for three independent experiments.
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MAA (Figure 3). Another clone (TCC909A.8.2.7) from a different patient (CD-9) responded to MAP, MAA and had a low, but detectable response to *Mycobacterium intracellulare*. The percentages of MAP reactive T-cell clones responding to the other mycobacteria were, MAA 100%, *M. intracellulare* 92%, *M. gordonae* 65% and *M. tuberculosis* 31%.

### HLA restriction

The T-cell restriction was determined using APCs from DR/DQ haplotype matched donors together with blocking of the response in a T-cell assay by adding specific anti-HLA-DP, anti-HLA-DQ and anti-HLA-DR antibodies (Figure 4). The MAP reactive clones from two of the patients (CD-6 and CD-9) were DR restricted (n = 7) while DQ restricted clones (n = 18) were obtained from the two others (CD-11 and CD-46). The response could be blocked in all clones except three. These clones were CD4+, TCRαβ+, CD56- and appeared to be conventional T cells. One could speculate that they might recognize antigen in the context of CD1 which is seen in other mycobacterial infections. However, this was not pursued in the present study.

### Characterization of a T-cell clone responding to MAP1508

Finally we aimed to identify which antigen in the crude MAP preparation the T cells responded to. T-cell lines and a selection of T-cell clones were thus tested for responses against a range of available recombinant purified MAP antigens or pools of overlapping peptides (Table S1). One T-cell line had a strong response to pooled peptides from MAP1508 [23] which is 87% identical to esxP from *M. tuberculosis*. By cloning of this line we isolated a T-cell clone (TCC911.A.8.4.13) responding to this antigen. The T-cell clone recognized the peptides in the context of HLA-DQ as showed by adding anti-HLA-DP, anti-HLA-DQ or anti-HLA-DR antibodies (Figure 5A). The patient was DQB1*0609 and DQB1*0301. HLA-DQ matched EBV cells were used as APC, and the results indicated that this clone recognized the peptide in the context of DQ6 (i.e. DQA1*0102/DQB1*0605). The clone did not recognize the peptide in the context of DQA1*0102/DQB1*0604, which might be due to a histidine at position 30 in DQB1*0604 compared to a tyrosine in DQB1*0605 and DQB1*0609. Epitope mapping demonstrated that the epitope was located at aa position 71-80 of MAP1508 (Figure 5B). Protein Blast using these 10 aa confirmed that the epitope is conserved in several of the pathogenic mycobacteria including the *M. avium* complex and the *M. tuberculosis* complex, while it was not found in the non-pathogenic *Mycobacterium smegmatis* mc$^{2}$ 155. Staining for TCR Vβ showed that the clone expressed the TCR Vβ8 chain. Furthermore, the clone was CD4+, Tαβ+ CCR6+, and it produced IFN-γ and not IL-17 in response to PMA/ionomycin (data not shown). The T cells from the other patients did not recognize any of the available purified antigens.

## Discussion

This study demonstrated that T cells reacting to various bacteria were present in the intestine of patients with CD, however a majority of the patients had a dominant response to MAP. The T cells secreted IFN-γ and IL-17, and a role for mycobacteria in the excessive inflammation seen in CD cannot be excluded. The isolation of T cells together with identification of their specificity is a useful approach to get answers about the relative importance of various bacteria in CD.

Although the CD lesions have increased number of CD4 T cells producing inflammatory cytokines, very few studies have focused

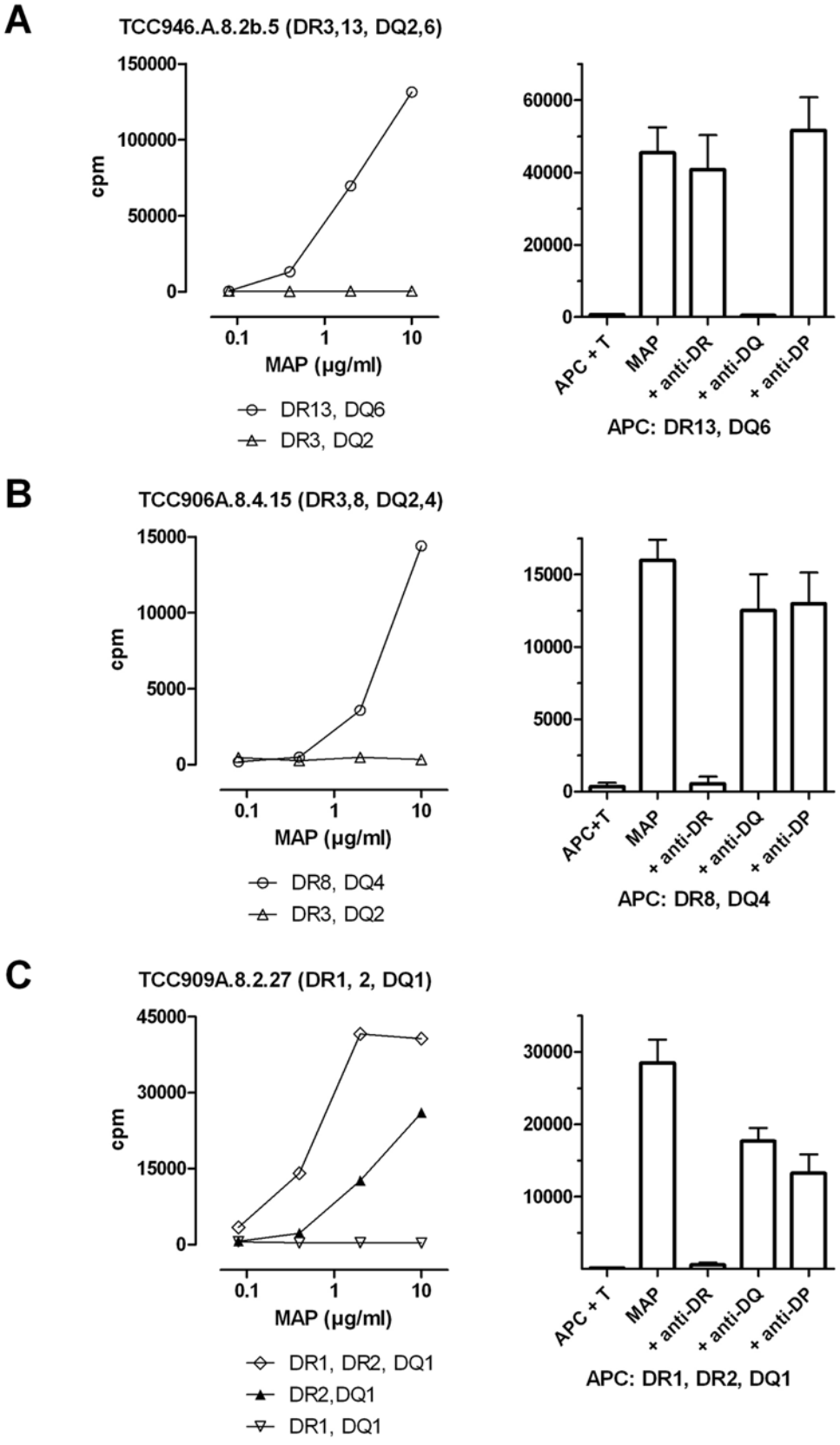


**Figure 4. HLA restriction of T-cell clones**. A) Patient CD-46, B) Patient CD-6 and C) Patient CD-9. The individual patients HLA DR/DQ serotype of the clone donor is given in brackets after the clone identification tag. Adherent cells from HLAII DR/DQ haplotype matched donors were used as APC and incubated with antigen overnight. T cells were added followed by further incubation for three days with addition of $^{3}H$ thymidine for the last 20 hours. Left panel depicts responses using different APC. Right panel depicts blocking of the responses by addition of monoclonal antibodies specific for either HLA-DR, HLA-DQ or HLA-DP two hours prior to addition of the T cells. The blocking assay was done in triplicates and repeated three times. Error bars indicate mean±SD.

doi:10.1371/journal.pone.0005641.g004

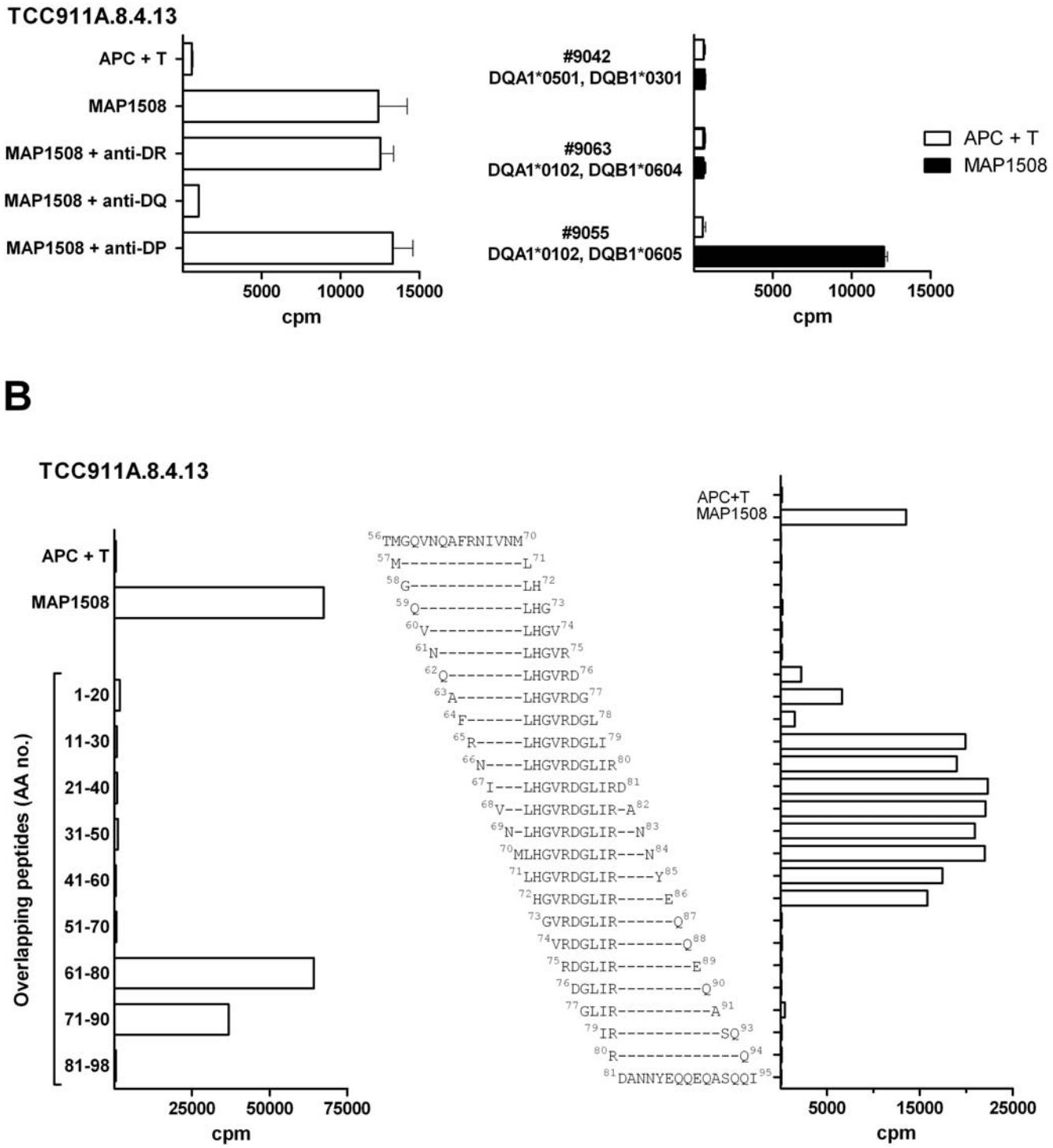


**Figure 5. Epitope mapping of a MAP1508 specific T-cell clone**. A) HLA-II restriction of TCC911.A.8.4.13. Left; blocking of responses by HLA class II specific monoclonal antibodies. Right; HLA-DQ matched EBV cells were used as APC to identify HLA restriction. The patient was DQB1*0609 and DQB1*0301. Error bars indicate mean±SD. B) Proliferation of the T-cell clone in response to overlapping peptides (10 μM) of the MAP1508 protein. Left; peptides of 20 aa with10 aa overlap. Right; peptides of 15 aa overlapping with one aa ranging from position 56 to 95. The sequences are shown.

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on the specificity of these intestinal T cells. Duchmann et al published two studies that showed the presence of T cells responding to commensal bacteria in equal frequencies in UC and CD patients with active disease [17,18], which is in agreement with our findings. Interestingly, we found that intestinal T cells from CD patients responded more vigorously to MAP antigens compared with T cells from UC patients. There was also an apparently higher frequency of MAP reactive T cells compared with T cells responding to commensal bacteria in the CD patients while no such difference was found in UC patients. MAP causes a disease in ruminants with similarities to CD and the bacterium has been found in higher frequencies in CD patients compared to controls [14,15]. However, convincing data showing a cellular immune response to MAP are lacking. Some studies have looked into T cell responses against mycobacteria in CD patients, however the methodology in the current work is vastly different. Previous studies have largely used PBMC and/or looked at antigen induced suppression [24,25]. To the best of our knowledge this is the first study where isolated intestinal T cells were used to investigate cellular immune responses to MAP in CD patients.

All the T-cell clones in the present study produced IL-17 and/or IFN-γ. T cells that produced the inflammatory cytokines IFN-γ and IL-17 have been shown to be increased in the intestine of CD patients [16], but the specificity of any of these Th1/Th17 clones has not previously been identified. The genetic associations of *NOD2* and *IL23R* with CD suggest that IL-17 is relevant for disease development. It has been shown that IL-23 induced IL-17 production from memory T cells [26] and that stimulation of NOD2 promoted IL-17 production through a synergistic effect of IL-23 and IL-1 [27] . The ligand for NOD2 is known to be muramyl dipeptide (MDP) which is present in the cell wall of bacteria. However, it is recognized that most bacterial species produce only N-acetyl-MDP, in contrast to mycobacteria which also produce N-glycolyl MDP [28,29]. Studies comparing these forms of MDP have shown that N-glycolyl MDP is more potent than N-acetyl MDP at inducing NOD2-dependent pro-inflammatory responses (Behr, personal communication). Furthermore an association between NOD2 polymorphism and resistance to MAP in cattle has been described [30]. Altogether these recent studies suggest a link between mycobacteria, NOD2 and the IL-23/IL-17 pathway and are in agreement with our findings. It cannot be excluded that the method used to isolate the T-cell clones influenced their cytokine profile, and a future challenge is to confirm that the MAP reactive T cells also produce IL-17 *in vivo*.

In the present study there was a strong T-cell response to MAP in the CD group, but some of the control patients also showed some reactivity. This was not surprising considering that a crude mycobacterial antigen preparation was used. Mycobacteria contain several antigens with high degree of homology, and humans can be exposed to a range of environmental mycobacteria that might trigger an immune response. In addition, the Norwegian population is vaccinated with Bacille Calmette Guerin (BCG) and one could speculate that BCG reactive T cells can be found at the site of inflammation in IBD patients. Although this cannot be totally excluded, it is not likely to be a major confounding factor. The majority (69%) of the T-cell clones did not respond to *M. tuberculosis*. BCG is attenuated from *Mycobacterium bovis* by deletion of several genetic regions, and all of the genes in this vaccine strain are also present in *M. tuberculosis* [31,32]. Consequently, BCG reactive T cells are likely to cross-react with antigens from *M. tuberculosis*. To find conclusive evidence that the responses were caused by MAP is difficult since proteins from MAP and MAA have a extremely high degree of identity [23]. However, we isolated one T-cell clone that responded only to MAP and MAA, while another clone from another patient responded to MAA, MAP and *M. intracellulare*. MAP and MAA are both subspecies of *M. avium* while *M. intracellulare* is the mycobacterial species that is phylogenetically closest to *M. avium*. Of these bacteria, MAP is the only organism that has a predilection for the intestinal mucosa while MAA and *M. intracellulare* usually causes cervical lymphadenitis in children or also disseminated or pulmonary disease particularly in immunocompromised individuals. These findings suggest that at least in two of the patients the responses were triggered by MAP or a closely related bacterium belonging to the *M. avium* complex.

A future challenge is to identify how many, and which patients have a MAP or an *M. avium* complex specific T-cell response. T-cell cloning is tedious and not an option for screening of large number of patients. An alternative is to use MAP specific epitopes and test for recognition of these in polyclonal T cell lines derived from intestinal biopsies of patients carrying the relevant HLA class II restriction element. Identification of such epitopes is challenging, but may be achieved using a panel of MAP specific T-cell clones to screen peptide libraries or MAP expression libraries. These methods have previously been used successfully to identify the epitopes of T cells of unknown specificity [33–35]. To date we have identified the specificity of one T-cell clone. The epitope was located on an esx protein which belongs to the highly immunogenic ESAT family [36]. The epitope was conserved in several pathogenic mycobacterial species, but not found in the genome of the saprophytic *M. smegmatis*. Further studies will focus on identification of MAP specific epitopes that can be used to screen T-cell lines from a larger number of CD patients and controls

CD presents with a variety of clinical manifestation and genes associated with CD differs between populations. It is thus possible that certain bacteria can be of importance in a subgroup of patients.We believe that the isolation of tissue derived T cell clones followed by characterization of their specificity can give novel answers about the bacteria involved in the inappropriate inflammatory response seen in CD. This study demonstrated the presence of MAP reactive intestinal T-cell clones producing IFN-γ and IL-17 suggesting that they may contribute to the intestinal inflammation.

## Supporting Information

**Table S1** Recombinant antigens and pooled peptides used in the present study

Found at: doi:10.1371/journal.pone.0005641.s001 (0.06 MB DOC)

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## Author Contributions

Conceived and designed the experiments: IO ST LJR LS KEAL. Performed the experiments: IO ST. Analyzed the data: IO ST. Contributed reagents/materials/analysis tools: IO CA LJR JB PA LS KEAL. Wrote the paper: IO ST LS KEAL. Contributed to writing of the manuscript: CA LJR JB PA.

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VETERINARY RESEARCH

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# Immunity, safety and protection of an Adenovirus 5 prime - Modified Vaccinia virus Ankara boost subunit vaccine against *Mycobacterium avium* subspecies *paratuberculosis* infection in calves

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## Abstract

Vaccination is the most cost effective control measure for Johne's disease caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP) but currently available whole cell killed formulations have limited efficacy and are incompatible with the diagnosis of bovine tuberculosis by tuberculin skin test. We have evaluated the utility of a viral delivery regimen of non-replicative human Adenovirus 5 and Modified Vaccinia virus Ankara recombinant for early entry MAP specific antigens (HAV) to show protection against challenge in a calf model and extensively screened for differential immunological markers associated with protection. We have shown that HAV vaccination was well tolerated, could be detected using a differentiation of infected and vaccinated animals (DIVA) test, showed no cross-reactivity with tuberculin and provided a degree of protection against challenge evidenced by a lack of faecal shedding in vaccinated animals that persisted throughout the 7 month infection period. Calves given HAV vaccination had significant priming and boosting of MAP derived antigen (PPD-J) specific $CD4^+$, $CD8^+$ IFN-γ producing T-cell populations and, upon challenge, developed early specific Th17 related immune responses, enhanced IFN-γ responses and retained a high MAP killing capacity in blood. During later phases post MAP challenge, PPD-J antigen specific IFN-γ and Th17 responses in HAV vaccinated animals corresponded with improvements in peripheral bacteraemia. By contrast a lack of IFN-γ, induction of FoxP3+ T cells and increased IL-1β and IL-10 secretion were indicative of progressive infection in Sham vaccinated animals. We conclude that HAV vaccination shows excellent promise as a new tool for improving control of MAP infection in cattle.

## Introduction

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) is the causative agent of Johne's disease (JD), a chronic granulomatous inflammation of the intestines primarily in ruminants [1] and which has been linked to Crohn's disease in humans [2]. The increasing prevalence of MAP infection in cattle, the associated economic losses and zoonotic potential indicate the need for an effective MAP vaccine as a sustainable and economically viable solution for disease control [3]. Whole cell killed MAP vaccines can improve milk productivity [4] reduce the incidence of clinical JD [5] and faecal abundance of MAP [6], however the proportion of animals harbouring and excreting MAP remain similar [7]. Attempts to provide live attenuated whole cell vaccines have mostly been ineffective [3]. Although some have shown promise in goats [8], none have been able to eliminate faecal excretion. This is an important pre-requisite, however recent modelling studies indicate that to achieve effective MAP control in cattle in the shortest time period, priority should be given to vaccines that are able to reduce susceptibility to MAP infection [9]. In contrast, vaccines targeted at only reducing clinical cases and decreasing but not eliminating MAP shedding would not be economically beneficial to dairy producers when compared with an alternative non-vaccine control, particularly when herds were highly infected with MAP

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[10]. In the study reported here we have assessed protective capacity as an ability of vaccination to reduce bacterial numbers in peripheral blood, gut tissues and in faeces as this is likely to impact significantly on disease progression and transmission.

Importantly whole cell MAP vaccines contain generic mycobacterial cell wall antigens cross-reactive with tuberculin [11] used in diagnostic testing for bovine tuberculosis caused by *Mycobacterium bovis*. In addition animals vaccinated with whole cell MAP vaccines cannot be differentiated, using current diagnostic tests, from MAP infected animals and there is therefore a need for any new MAP vaccine to have a Differentiation of Infected from Vaccinated Animals (DIVA) test capability [12]. Eradication programs are currently impossible whilst inter-animal spread and seeding into environmental or wildlife reservoirs remains high. Therefore whole cell vaccines have limited value in cattle control programmes given their limited efficacy and their interference with current bovine TB diagnostics, particularly in areas with ongoing endemic bovine tuberculosis [13].

Of increasing importance is the understanding that MAP disease involves an insidious onset of dysregulatory immune mechanisms that need to be normalised or prevented from accumulating if protection is to be achieved. Comparisons with studies in other mycobacterial diseases suggest lasting effective immunity would require combinations of humoral and mucosal immunity along with priming and maintenance of pathogen specific Th1 responses [14]. Development of an effective vaccine thus requires rational, focused design using novel delivery methods able to circumvent dysregulated antigen priming invoked during MAP persistence whilst specifically targeting and enhancing the crucial immunological processes able to arrest pathogenesis.

The ability of viral vectored vaccines to positively address these processes has already shown promise in other mycobacterial diseases including cattle [15-17]. Viral delivery provides the advantages of high antigen delivery to antigen presenting cells [18], increased antigen specific $CD4^+$ and $CD8^+$ responses [19,20] and maintenance of IFN-γ release driving increased macrophage activation and killing capacity [21]. Prime-boost vaccination with MVA-Ag85A induced altered Th1/Th17 related responses [22] that were shown to correlate with the induction of protective immunity [17].

We have previously demonstrated that a priming vaccination with non-replicative Adenovirus 5 followed by boosting with Modified Vaccinia virus Ankara delivery vectors expressing a fusion of critical epitopes from four intracellular phase codon optimised MAP antigens (HAV) was able to confer both therapeutic and prophylactic protection against MAP challenge in mice [23]. In this study we evaluate the same prime boost strategy in cattle and investigate immunological responses associated with protection. We show that HAV vaccination was well tolerated, could be detected by DIVA testing, did not cross react with the tuberculin test and provided a high degree of protection against challenge evidenced by a lack of faecal shedding that persisted throughout the 38-week test period.

## Materials and methods

### Vaccine construction and manufacture

HAV vaccine comprised non replicative human adenovirus serotype 5 (Ad5) and Modified Vaccinia virus Ankara (MVA) expressing a 838aa polypeptide fusion of regions sourced from four non-essential, non-toxigenic, immunogenic, early intracellularly expressed MAP proteins with no significant BLASTP homologies to either mammalian or known tuberculosis PPD proteins [11]. Vaccine design and production including extensive codon optimisation and addition of expression enhancement features were as described previously [23]. Vaccine doses were derived from a single batch preparation manufactured at the Viral Vector Core Facility, Jenner Institute, Oxford, UK from seed stocks using specific pathogen free CEF and T-Rex 293A cell lines for MVA and Ad5 respectively in certified pathogen free media. Ad5 and MVA vectors expressing GFP in place of the HAV polypeptide were manufactured in parallel to a similar viral titre and used for Sham vaccination.

### Preparation of MAP challenge inoculum

A strain of MAP (R0808) isolated from a cow with JD was inoculated into modified liquid Middlebrooks 7H9 medium [24] and grown at 37 °C with gentle agitation to an $OD_{600}$ of 1.8. The culture was extensively passed through a 20G needle and any remaining large clumps were allowed to settle for 5 min. The upper suspension was then separated and adjusted to an $OD_{600}$ of 1.0 with sterile PBS then aliquoted in 5 mL doses. qPCR using MAP specific primers (see below) estimated that each dose contained $5 \times 10^8$ MAP genome equivalents of MAP organisms. Prior to dosing aliquots were centrifuged at $3500 \times g$ for 10 min and the pellet resuspended with a syringe into 20 mL of PBS.

### Vaccination, challenge and sampling regimen

Calves were selected from herds with an absence of tuberculin skin test reactivity over the previous 10 years, and without bovine tuberculosis disclosed at abattoir over the same period. These same herds were selected on the basis that increases in skin thickness following injection of PPD-A were minimal, below 4 mm, indirectly indicating an absence of immune responses to MAP. Eight week old male Holstein Friesian calves in two groups of six were held under regulated category containment at AFBINI, Stormont, Northern Ireland and vaccinated intradermally

into the neck region with 1 mL of either Ad5.HAV ($10^9$ vp/mL) at week -11 and boosted with MVA.HAV ($10^9$ pfu/mL) at week -5 (HAV) or vaccinated with Ad5 ($10^9$ vp/mL) and MVA ($10^9$ pfu / mL) vectors expressing GFP in the same regime (Sham). Five weeks after boosting (week 0) both vaccinated groups were challenged on two consecutive days with an oral dose of $5 \times 10^8$ MAP R0808 mixed into PBS.

Faecal and blood samples were taken immediately prior and 1 week post- prime and boost vaccinations and post-challenge, then monthly over the 38 week post challenge study period. Whole bloods were processed for IFN-γ release assays. PBMC fractions were aliquoted and processed separately for MAP killing assays, MAP presence using a 2 week pre-liquid culture followed by subculture for MAP colonies on solid slopes and direct differential lysis DNA extraction for MAP by nested PCR, cytokine expression or presence of HAV transgene using cDNA extractions followed by specific PCR, cytokine release assays by ELISA and cell population analysis using flow cytometry. Faecal samples were processed for MAP presence by 2 week pre-liquid culture followed by dividing for subculture on solid slopes and differential lysis DNA extraction for MAP by nested PCR. Direct DNA extraction was also performed separately on faecal samples for HAV transgene PCR testing. Animals were euthanized at 38 weeks post challenge and samples from mesenteric lymph nodes, duodenum, spleen, ileum and jejunum taken at necropsy. Tissue samples were processed for MAP load by direct differential mycobacterial DNA extraction followed by qPCR, for cytokine expression and HAV transgene presence by PCR from cDNA preparations and cell population analysis using flow cytometry.

All animal husbandry and procedures were carried out by trained and experienced animal care workers under the direction of a senior Named Veterinary Surgeon and in compliance with the UK Home Office Regulations including the Animals (Scientific Procedures) Act 1986. The study was authorized by the local Ethical Review Committee at AFBINI, in compliance with national guidelines and EU regulations for projects using animals for research purposes.

### Tissue preparation

All tissue samples were dissected at necropsy and, for MAP culture and DNA isolation, were initially stored/transported for up to two days at RT in 1 mL RPMI1640 (Sigma, Gillingham, UK) plus 10% FBS and 100 μg/mL ampicillin. For cell isolation for flow cytometry and RNA isolation tissues were processed immediately following retrieval. Fat was removed from the tissue and mucosal tissue (if present) was scraped, washed with PBS, diced using sterile scalpels and then weighed. Lymph nodes were gently dissociated using a scalpel and a single cell suspension was obtained by filtration through a 70 μm cell strainer (Becton Dickinson, Oxford, UK). For MAP culture and DNA PCR, samples were digested overnight with slow agitation at 37 °C in 1 mL Pen/Strep free digest buffer (0.15 mM NaCl, 0.3 mM $CaCl_2$, 1 mg/mL collagenase B, 1 mg/mL trypsin (Sigma)) then pelleted in a microfuge at 16 000 × *g* for 5 min. For flow cytometric analysis and host RNA extraction, ileum and ileocecal valve tissue was digested for 80 min at 37 °C in digestion medium (RPMI1640, 1% FBS, 25 μg/mL gentamicin, 100 U/mL Pen/Strep, 75 U/mL collagenase B (Sigma), 20 μg/mL Dispase I (Roche, Welwyn Garden City, UK)). The cells were then washed gently in PBS. For flow cytometric analysis tissue cells were fixed with 1% paraformaldehyde and stored at 4 °C. For RNA expression analysis preparations were resuspended in 1 mL of RLT Plus buffer (Qiagen, Manchester, UK) containing 1 μL/mL β-mercaptoethanol prior to RNA extraction.

### Isolation and stimulation of PBMC

PBMC were isolated by density gradient centrifugation (Histopaque 1083 (Sigma), resuspended in tissue culture medium ([TCM]; RPMI +10% foetal calf serum, $5 \times 10^{-5}$ M β-mercaptoethanol, 50 μg/mL gentamicin). Cell concentrations were estimated using a haemocytometer, adjusted to $1 \times 10^7$ cells/mL then aliquoted ($5 \times 10^6$ cells) for MAP and HAV transgene PCR or stimulated with either 10 μg/mL PPD-J (a kind gift from D Bakker, CVL, Lelystad, Netherlands) or an equal volume of TCM alone as control for 24 h at 37 °C in 5% $CO_2$ in air. Stimulated or unstimulated PBMC were then pelleted and supernatants stored at −70 °C prior to cytokine analysis by ELISA. Parallel stimulations were established for the subsequent analysis of cytokine expression by qPCR and multiparametric analysis of cell populations by flow cytometry.

### DNA extraction

Pelleted samples were suspended in 600 μL GTC buffer (4 M Guanidium thiocyanate (Sigma), 10 mM TrisHCl, pH 8.0, 1 mM EDTA), transferred to a 1.5 mL lysing matrix B ribolyser tube (MP Biomedicals, Leicester, UK) mixed and lysed overnight at 4 °C. DNA extraction included mechanical disruption in a FastPrep-24 ribolyser (MP Biomedicals) at 6500 rpm for 45 s followed by standard extraction using phenol/chloroform, chloroform/isoamyl alcohol and precipitation overnight at −20 °C with 7.5 M ammonium acetate in ethanol [25].

### MAP specific PCR

qPCR of DNA extracted from tissue samples was performed as previously described with minor adjustments [26]. Briefly, reactions comprised 2 μL DNA sample,

12.5 μL Power SYBR green mastermix (Applied Biosystems, Paisley, UK), 2 pMoles primer pair (AV1: ATGTGGTT GCTGTGTTGGATGG, AV2: CCGCCGCAATCAACTC CAG), made to 25 μL with RNAse free water. PCR cycling used 95 °C: 15 min (1 cycle); at 95 °C: 30 s, 58 °C: 1 min, 72 °C: 1 min (40 cycles) with data collection at 76 °C (10 s) using a CFX96 qPCR cycler (BioRad, Hemel Hempstead, UK). Sample copy numbers were estimated from an averaged value of three qPCR's on each sample using a dilution curve of a control total genomic DNA MAP K-10 stock preparation serially diluted 10 fold to contain between $1 \times 10^2$-$10^8$ genome equivalents. Nested MAP specific PCR of DNA extracted from PBMC and liquid pre-culture faecal sample preparations was performed as previously described [25].

#### HAV transgene specific PCR

Blood and faecal samples taken 1 week prior to prime vaccination then at intervals post vaccination (week -10, -4, 0, 6 and 33) and spleen samples taken post mortem (week 38) from each vaccinated animal were screened for the presence of the HAV transgene. DNA was extracted from PBMC and tissue samples as described above. Faecal samples (200 mg) were processed using a QIAamp Stool DNA extraction kit (Qiagen) using a standard protocol optimised for viral DNA extraction [27]. DNA extracted from MVA.HAV cultured in CEF cells (48 h: MOI 50:1) using either blood or faecal extraction method were used as positive reagent/lysis controls. PCR sensitivity was estimated at 20-50 copies (positive in > 80% replicates) by dilution curves of a reference DNA standard stock comprising a plasmid containing one copy of the HAV transgene construct.

#### RNA extraction and cDNA generation

Total RNA was isolated from PBMC stored in 1 mL of RLT Plus buffer (Qiagen) containing 1 μL/mL β-mercaptoethanol using the ALLPrep DNA/RNA mini kit (Qiagen) and the robotic workstation for the automated isolation of DNA and RNA, Qiacube (Qiagen), according to manufacturer's instructions. First strand cDNA was synthesised from 250 ng mRNA sample aliquots using standard reverse transcription reaction buffer (10 mM dNTPs (Bioline, London, UK), 500 μg/mL oligo$(dT)_{15}$ primers (Promega, Southampton, UK), 5 × RT Buffer, 0.1 M DTT and Superscript II Reverse Transcriptase (Invitrogen, Paisley, UK)) denatured at 65 °C for 10 min and incubated at 42 °C for 50 min.

#### cDNA qPCR for IL-17, IL-22 and IL-23 expression

IL-17, IL-22, IL-23 and the reference gene GAPDH were amplified by qPCR using the LightCycler 480 DNA SYBR Green I Master on the LightCycler 480 qPCR machine (Roche). The primers and conditions used were: IL-17A: FW: TAACCGGAGCACAAACTCCAGA ; RV: GGTGGAGAGTCCAAGGTGAGGT; 95 °C: 5 min (1 cycle), then 95 °C: 20 s, 62 °C: 20 s, 72 °C: 30 s (45 cycles): IL-22: FW: CCGCTGGCTGCCTCCTT; RV: AGGG CTCCTGGAAGTCGGA ; 95 °C: 5 min (1 cycle), then 95 °C: 20 s, 60 °C: 20 s, 72 °C: 30 s (40 cycles): IL-23: FW: ACC AATGGGACATGTGGATCTAC; RV: AGGGCTTGGAG TCTGCTCAGTT: 95 °C: 5 min (1 cycle), then 95 °C: 20 s, 60 °C: 20 s, 72 °C: 30 s (45 cycles): GAPDH: FW: GATG CTGGTGCTGAGTATGTAGTG RV: ATCCACAACAG ACACGTTGGGAG 95 °C: 5 min (1 cycle), then 95 °C: 20 s, 60 °C: 20 s, 72 °C: 45 s (40 cycles).

All reactions were run in duplicate in a final volume of 20 μL. Relative gene quantities were calculated using LightCycler480 1.5.0 software by comparing each sample with a serial dilution of standard PCR purified products in the same run. Concentrations of genes of interest were then calculated from standard curves in arbitrary units. Antigen-specific gene induction was calculated according to the method described by Pfaffl [28] from values of each target gene normalized to the reference gene (ref; GAPDH) for each sample. Briefly, values were calculated according to the following:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta \text{Cp target (control-sample)}}}{(E_{\text{ref}})^{\Delta \text{Cp ref (control-sample)}}}$$

Where E is efficiency calculated by the standard curve for each gene and ΔCp is the difference calculated by the Lightcycler of the samples treated with media alone minus the samples treated with PPD-J.

#### MAP culture

Samples were decontaminated and extracted following previously described recommended guidelines [29] then cultured on liquid Middlebrooks 7H9 medium [24] at 37 °C for 2 weeks, then plated onto the same medium (with added agar) and incubated for up to 12 weeks or until colonies appeared. PBMC samples ($2 \times 10^6$ cells) were added to 5 mL of sterile distilled water and lysed for 30 min then centrifuged for 15 min at $3500 \times g$ and the pellet resuspended in 1 mL modified liquid Middlebrooks 7H9 medium and cultured as faecal samples. Colony identity was confirmed with MAP specific PCR as above and representative isolates from the initial inoculum and a final faecal sample also subjected to MIRU typing.

#### MAP killing assay

Whole blood (15 mL) in EDTA tubes was mixed with 15 mL PBS and centrifuged at room temperature onto 15 mL Histopaque 1083 (Sigma) for $1200 \times g$ for 1 h with no brake applied. Buffy coats were pipetted off and washed once in PBS. Cells were diluted to $1 \times 10^7$/mL in RPMI medium (RPMI1640, 10% FCS, 50 μg/mL

Hygromycin B) then plated into 96 well flat bottom tissue culture plates at $4 \times 10^5$ cells per well in quadruplicate. Two duplicates were activated with 30 ng/mL bovine IFN-γ (Fisher Scientific, Loughborough, UK) then incubated at 37 °C in 5% $CO_2$ overnight to attach. Media was exchanged with 200 μL of RPMI containing $8 \times 10^5$ luminescent MAP 19698 L [30] and incubated for 5 days in 5% $CO_2$, changing media at 3 days. Cells were washed once in PBS then lysed in 200 μL 0.4% SDS final in PBS and read immediately in an injector Luminometer Glo-Max 20/20 (Promega) set at 1 s delay, using 1% v/v decanal (Sigma) as substrate. Relative killing values were calculated as the percentage of luminosity lost from an average of both IFN-γ activated and non-activated infected cell cultures relative to an RPMI only infection control set of wells.

### Whole blood IFN-γ release assay

Whole blood IFN-γ release assay was performed as previously described [31]. Briefly heparinised whole blood was stimulated within one hour of sampling with either PBS (control), avian-purified protein derivatives (PPD-A) at 4 μg/mL final concentration (Veterinary Laboratories Agency, Guildford, UK), bovine-purified protein derivative (PPD-B) at 8 μg/mL final concentration (Veterinary Laboratories Agency, UK), and Johnin-purified protein derivative (PPD-J) at 4 μg/mL final concentration (Central Veterinary Institute, Copenhagen,Denmark), pokeweed mitogen (Sigma) (positive control) and a set of peptides (Pool J) spanning MAPK_1565 (C-term) plus MAP K_2533 (N-term) region of the HAV transcript (GK RHTQAVLALARRR; QAVLALARRRLNVLW; LARRRL NVLWAMLRD; LNVLWAMLRDHAVYH; AMLRDHA VYHPATTT; HAVYHPATTTAAARL; SIVGQTYRE VEVVLD; TYREVEVVLVDGGST; EVVLVDGGSTDRT LD; DGGSTDRTLDIANSF) at a final concentration of 2 μg/mL each [23]. After 24 h, plasma was tested in duplicate by Bovigam ELISA (Prionics, Lelystad, Netherlands) for the release of bovine IFN-γ. Values are expressed as a Net OD (OD of antigen stimulated sample minus OD of negative control).

### ELISA for IL-1β and IL-10 expression

The supernatants were assessed for the presence of IL-1β using a bovine IL-1β kit (Thermofisher, Loughborough, UK) and for IL-10 as previously described [32]. The concentration of IL-1β is expressed as pg/mL and for IL-10 as biological units (BU)/mL relative to a standard curve. For IL-10 the standard preparation was CHO cell expressed IL-10 (a kind gift from G Entrican, Moredun Research Institute, Edinburgh). Each sample assayed was measured in duplicate by ELISA; the variability between samples was less than 5%.

### Multi-colour immunofluorescent labelling

PBMC stimulated for 24 h with PPD-J or TCM were harvested and subjected to multi-parametric staining protocols. Unless indicated all primary monoclonal antibodies were from AbD-Serotec (Kidlington, UK) and secondary antibodies were: goat anti-mouse IgG1-alexa-fluor 647 (Life Technologies, Paisley, UK), goat anti-mouse IgG2a-PECy7 (Abcam, Cambridge, UK), goat anti-mouse IgG2b-RPE and goat anti-mouse IgG3-FITC (Cambridge BioScience, Cambridge, UK). All antibodies were used at predetermined optimal concentrations. The fluorescence without the presence of primary mAb was used as a control for analysis. Four colour flow cytometry was utilised to define cell subsets. T lymphocyte subsets were detected using mAbs specific for bovine CD4 (CC30, IgG1 or CC8, IgG2a), CD8 (CC58, IgG1 or CC63, IgG2a), the WC1 γδ TCR (CC15, IgG2a) or pan-γδ TCR (GB21a, IgG2b; VMRD, Pullman, USA). The expression of CD25 (IL-A111, IgG1) and CD45RO (IL-A116, IgG3) was determined on subsets of T cells. Intracellular expression of FoxP3 was determined within cells that were fixed with 1% paraformaldehyde and permeabilised (BD FACSPerm) using mAb Fox5A (anti-bovine Foxp3, IgG1 [33]; a gift from Professor WC Davis, Washington State University, USA). For intracellular staining of IFN-γ, cells were pre-incubated with TCM with and without PPD-J supplemented with PMA, ionomycin and brefeldin A (Sigma), then fixed and permeabilised as described above. Cytokine expression was determined using anti-bovine IFN-γ (CC330, IgG1). Flow cytometric analysis was conducted using the FACSCalibur (for intracellular IFN-γ expression) or the LSR II Fortessa (Becton Dickinson) and a minimum of 10 000 events were collected. Flow cytometric data was analysed using FlowJo software (v.7.6.5).

### Statistical analysis

Group sizes were calculated using G*Power program (v.3.15) based upon standard deviations from a similar study [31] calculated at alpha significance of 0.05 to derive an expected 90% power probability using a two tailed t-test. Statistical analyses were calculated using a standard statistics package software (GraphPad Prism v.6.04, La Jolla, USA) or in SAS using a mixed model for repeated measures analysis.

## Results

### Vaccination and general condition of animals

Calves were vaccinated at week -11 with Ad5-HAV, boosted at week -5 with MVA-HAV (HAV vaccinated group) then challenged orally with MAP at week 0. A second group were vaccinated and challenged under the same regime but with Ad5-GFP and MVA-GFP controls (Sham vaccinated group). Vaccine preparations gave no adverse reactions at any time during the experiment. No

significant swelling or induration was observed at any of the vaccination sites. PCR for HAV transgene specific DNA, carried out on blood and faecal samples taken at intervals throughout the experiment and spleen tissue at necropsy, was uniformly negative demonstrating that no vaccine was shed from the animals (data not shown). One calf in the Sham vaccinated group developed an unrelated illness (determined by post-mortem examination as septicaemia related to a navel infection) 3 weeks post vaccination and was euthanized. All other calves appeared healthy throughout the experiment. There was no significant decrease in the final body weights of the groups (data not shown). It became evident during data analysis that there were distinct phases in several parameters that differed between groups. We therefore report these findings in relation to each of these sequential phases.

### Pre-challenge

All of the calves in the HAV vaccinated group, but not the Sham vaccinated group responded to vaccination with an increase in MAP (PPD-J) specific IFN-γ release (Figure 1A). A significant increase ($P < 0.05$) in the level of PPD-J specific IFN-γ released from stimulated whole blood was evident at one week post-MVA-HAV boosting and this remained significantly elevated throughout all but one testing month in the experimental period ($P < 0.05$). By contrast no PPD-J specific IFN-γ was detected following Sham vaccination. Increases in avium (PPD-A) and bovine (PPD-B) specific IFN-γ release were also evident in the HAV- but not the Sham vaccinated calves immediately post-MVA boost but these did not reach significance and rapidly declined to baseline prior to challenge (Figure 1B and C). In parallel we assessed IFN-γ release by whole blood stimulated with a pool of HAV specific peptides in order to determine whether these could be used to distinguish vaccinated from infected animals (DIVA). In response to HAV peptides we observed a significant increase in IFNγ responses ($P < 0.05$) from blood of HAV vaccinated but not Sham vaccinated animals. This remained elevated for the duration of the experiment (Figure 2).

In stimulated PBMC we observed differences in PPD-J specific IFN-γ expression by subsets of T lymphocytes from HAV (Figure 3A) and Sham (Figure 3B) vaccinated calves. In Sham vaccinated calves no significant differences in the percentage of cells expressing IFN-γ in response to PPD-J were observed pre-challenge. By contrast, significant differences ($P < 0.05$) in PPD-J specific $CD4^+IFN\text{-}\gamma^+$ and $CD8^+IFN\text{-}\gamma^+$ cells were observed pre-challenge in HAV vaccinated calves which peaked 2 weeks (week -9) post Ad5-HAV vaccination. No differences were observed in IFN-γ expression by $WC1^+$ γδ $TCR^+$ T cell populations.

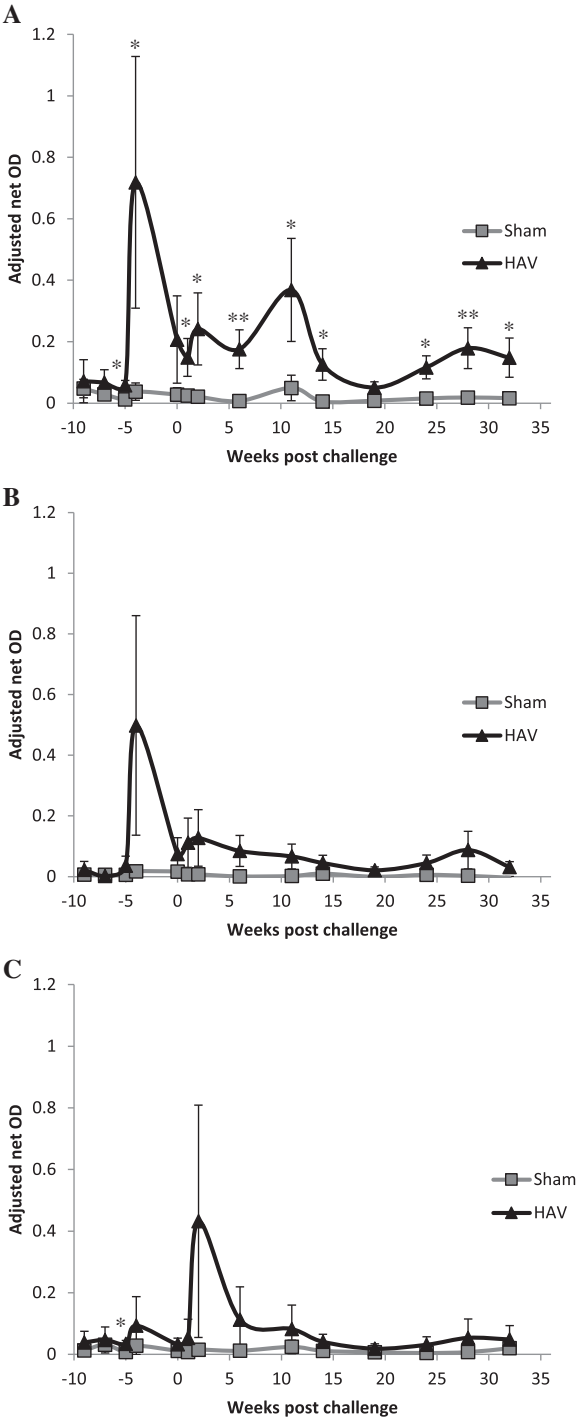


**Figure 1 Whole blood PPD-J stimulated IFN-γ release assay**. IFN-γ release from **A**. PPD-J, **B**. PPD-A, **C**. PPD-B stimulated whole blood taken from HAV vaccinated (black triangles) and Sham vaccinated (grey squares) calves including samples taken week -11, immediately prior prime vaccination; week -5, immediately prior boost vaccination; week 0, immediately prior to MAP challenge and up to 33 weeks post challenge. OD values are adjusted to internal controls to remove assay variation between runs. Significance indicated as *$P$ <0.05, **$P$ <0.01.

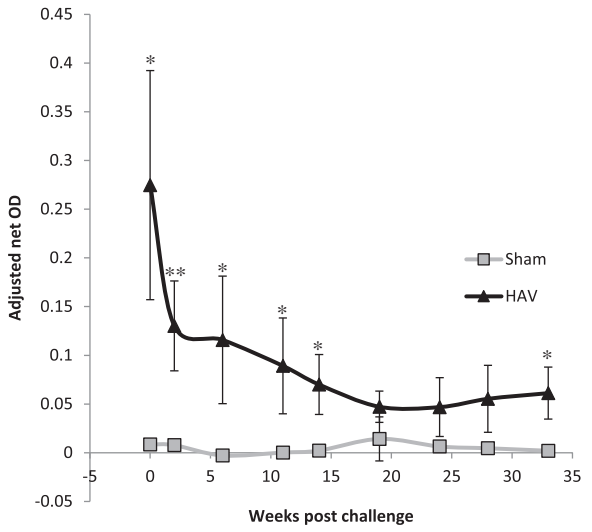


**Figure 2 DIVA testing.** IFN-γ release from HAV specific peptide pool stimulated whole blood taken from HAV vaccinated (triangles) and Sham vaccinated (squares) calves between week 0, immediately prior to MAP challenge and 33 weeks post challenge. OD values are adjusted to internal controls to remove assay variation between runs. Significance indicated as * $P < 0.05$.

### Post challenge (1-5 weeks)

Post oral MAP challenge (week 0) we systematically assessed antigen-specific immune responses in whole blood and isolated PBMC populations, the presence of MAP DNA in blood and faecal samples and macrophage killing efficacies. One week following oral challenge PBMC isolated from 5/6 (83%) HAV vaccinated and from 4/5 (80%) Sham vaccinated calves became MAP PCR positive and 3/5 (60%) Sham vaccinated calves shed MAP in faeces (Figure 4). Faecal cultures for HAV vaccinated calves were negative for MAP throughout the experiment. Two weeks following challenge PBMC from 3/6 (50%) HAV vaccinated and 2/5 (40%) Sham vaccinated calves were positive for MAP by PCR and 1 of the 5 calves (20%) in the Sham vaccinated group was still shedding MAP in the faeces (Figure 4). At week 1 post challenge a transient but significant (relative to week 0) peak in the percentage of PPD-J specific IFN-$\gamma^+$ T cells ($CD4^+$, $CD8^+$; $P < 0.001$) and $WC1^+$ subsets ($P < 0.01$) was observed in all animals (Figure 3). In whole blood significantly ($P < 0.05$) higher levels of PPD-J specific IFN-γ release were also detected in the HAV vaccinated group compared to the Sham vaccinated calves and this remained significantly elevated throughout the course of the experiment (Figure 1). Alongside alterations in T cell populations expressing IFN-γ occurring at this early time point post-MAP challenge, we observed a significant increase in IL-22 ($P < 0.05$) and a trend towards increased IL-17 expression in the HAV vaccinated but not the Sham vaccinated animals (Figure 5).

Immediately prior to challenge PBMC isolated from Sham vaccinated calves were equally capable of killing MAP compared to PBMC from HAV vaccinated calves. However, within one week of challenge the efficacy of PBMC fractions to kill MAP dropped dramatically by ~30% in the Sham group whereas the capacity for MAP killing was retained within the HAV animals (Figure 6). This large drop in killing efficacy was of relatively short duration with some recovery of killing capacity by 2 weeks post-challenge but a significant difference ($P < 0.05$) between HAV and Sham vaccinated animals remained evident between weeks 1 to 24 post-challenge.

### Post challenge (6-19 weeks)

Significant differences between the HAV and Sham vaccinated groups across a range of parameters were observed between weeks 6 and 14. The MAP killing capacity of PBMC from the Sham vaccinated group remained reduced (Figure 6) and there was an increase in the number of animals with MAP positive PBMC. PBMC from one animal were positive for MAP by PCR in each group at week 11 and this had increased to 4/5 (80%) Sham vaccinated and 2/6 (33%) HAV vaccinated calves positive at 14 weeks (Figure 4).

Within a similar timeframe altered cytokine expression profiles were detected with significant differences between the HAV and Sham vaccinated groups (Figure 7). Levels of IL-1β peaked at week 11 (Figure 7A) followed a few weeks later by IL-10 (Figure 7B) with a significantly greater increase ($P < 0.05$) in the secretion of both cytokines by PPD-J stimulated PBMC isolated from Sham vaccinated calves when compared to the HAV vaccinated group. In contrast an increase in PPD-J specific IFN-γ secretion was evident in the HAV vaccinated group at week 11 (Figure 1A) and antigen-specific expression of IFN-γ was significantly elevated in $CD4^+$, $CD8^+$ and $WC1^+$ T cells from HAV vaccinated animals between 11 and 19 weeks (Figure 3A). No significant increases in IFN-γ were detected in Sham vaccinated animals in this time period. Within PBMC, alterations in the number of PPD-J stimulated cells expressing FoxP3 were evident from week 14 post-challenge with significant increases in $CD4^+FoxP3^+$ cells evident in the Sham vaccinated group at weeks 14 and 19 ($P < 0.05$; Figure 8A).

### Post challenge (20 - 38 weeks) and post-mortem

Towards the end of the challenge period the differences between HAV vaccinated and Sham vaccinated animals became more pronounced for a number of the measured parameters. The MAP killing capacity of the PBMC fraction returned in the Sham vaccinated group to levels similar to that of the HAV vaccinated group (Figure 6). The frequency of detection of MAP within PBMC

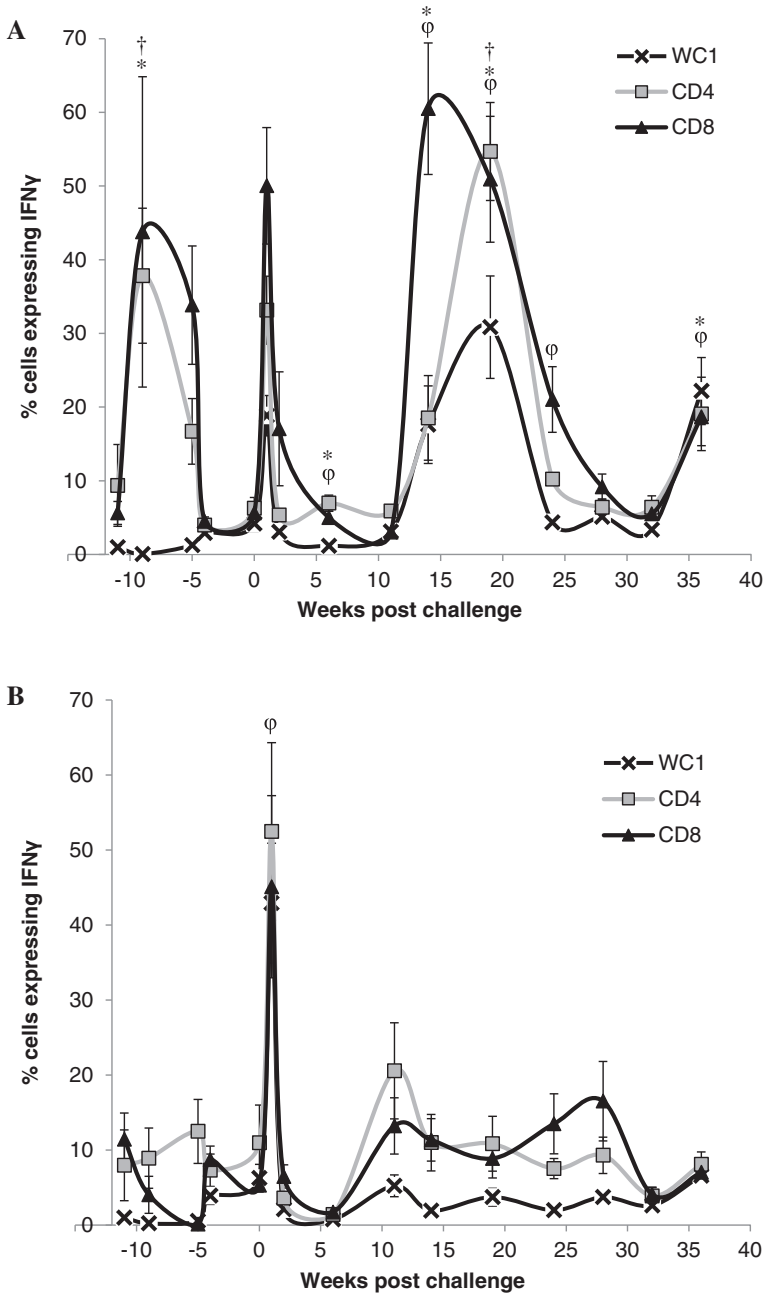


**Figure 3 Intracytoplasmic expression of IFN-γ by sub-populations of peripheral T cells in HAV and Sham-vaccinated calves.** Percentage of live $CD4^+$ (squares), $CD8^+$ (triangles) $WC1^+$ (crosses) expressing IFN-γ after 24 h stimulation with PPD-J from A. HAV vaccinated calves B. Sham vaccinated calves including samples taken week -11, immediately prior prime vaccination; week -5, immediately prior boost vaccination; week 0, immediately prior to MAP challenge and up to 36 weeks post challenge. Significant differences between groups in **A**. and **B**. of $P < 0.05$ are indicated as † ($CD4^+$), * ($CD8^+$), φ ($WC1^+$) for each time point.

increased in the Sham vaccinated group with 3/5 animals being consistently positive and all animals in this group testing positive at least once during this period (Figure 4). In contrast PBMC from 4/6 HAV vaccinated calves remained consistently negative and 2/6 only tested positive once within this period (Figure 4).

Antigen-specific cytokine levels decreased from week 20 onwards. At week 24 in the HAV vaccinated group significantly elevated levels of PPD-J specific IL-22 and raised levels of IL-17 were observed compared to the Sham vaccinated calves (Figure 5) which decreased along with antigen specific IL-1β and IL-10 over time

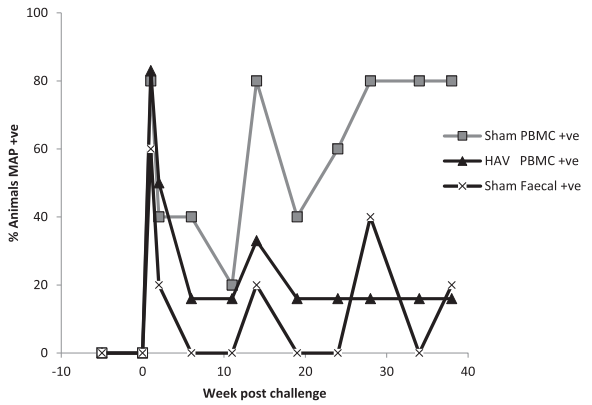


**Figure 4 MAP PCR positivity in blood and faeces of HAV and Sham vaccinated calves.** Percentage of animal samples that were positive for MAP by PCR. MAP was assessed in PBMC from HAV vaccinated calves (triangles), PBMC from Sham vaccinated calves (squares) and faeces from Sham vaccinated calves (crosses). Faeces of HAV vaccinated calves were consistently negative (data not shown) from immediately prior to boost vaccination (week -5) up to 36 weeks post challenge.

(Figure 7). IL-10 levels remained significantly higher in the Sham vaccinated compared to the HAV vaccinated calves throughout the remainder of the study period.

The percentage of PPD-J specific IFN-γ expressing cells (Figure 3) and secreted IFN-γ (Figure 1) began to decrease in the HAV vaccinated group during this final stage whilst levels in the Sham vaccinated group remained low. A highly significant increase ($P < 0.001$) in PPD-J specific FoxP3 expressing $CD4^+$ (Figure 8A), $WC1^+$ (Figure 8B) and $CD8^+$ (Figure 8C) T cells was observed in the Sham vaccinated group compared to the HAV vaccinated animals from week 24 onwards (Figure 8).

Standard tuberculin skin testing was carried out at week 36. All calves had similar skin reactivity to both PPD-A and PPD-B. The difference in PPD-B:PPD-A specific response was consistently < 1 mm indicating that none of the calves would be classified as TB reactors (see Additional file 1). At this time point DIVA testing using HAV specific antigens could still identify the HAV vaccinated from the Sham vaccinated calves (Figure 2). Examination of tissues taken post-mortem (38 weeks) revealed significant differences between the HAV vaccinated and Sham vaccinated groups. Measurement of the number of MAP present within tissues demonstrated significant reductions in load averages between HAV and Sham groups with samples obtained from duodenum ($P = 0.003$), jejunum ($P = 0.009$) and spleen ($P = 0.002$) (Figure 9). A significant decrease ($P = 0.016$) in overall total load was also observed when averages of all 5 sites were combined. All calves had at least one tissue sample positive for MAP indicating that whilst there was a significant degree of protection based on a significant reduction in bacterial load, sterilising immunity was not induced by HAV vaccination. All tissue samples from Sham vaccinated calves were positive for MAP. By contrast only 36/106 (34%) of all samples and 8/36 (17%) jejunum samples from the HAV vaccinated group were positive for MAP by qPCR. Nearly half (48%) of the total load present in HAV animals was located in mesenteric lymph node samples with 38% represented in one lymph node sample alone.

MAP cultured from two samples was shown to have the same genomic identity profile as the challenge strain (see Additional file 2). There were no obvious clinical manifestations or major macroscopic lesions at post mortem suggestive of progression towards clinical JD in any of the calves. Assessment of lymphoid cell populations within gut mucosal tissue from the ileum, ileocaecal valve region and associated lymph nodes showed larger populations of $CD4^+FoxP3^+$, $CD8^+FoxP3^+$ and $WC1^+FoxP3^+$ cells in the Sham vaccinated group compared to the HAV vaccinated group with the latter two reaching statistical significance ($P < 0.05$; Figure 10). There was also trend towards an increased presence of IL-17 and IL-22 in lymph node tissue of the HAV vaccinated group but this did not reach significance (see Additional file 3).

## Discussion

Johne's disease (JD) is a disease with economic significance in many dairy producing countries. Despite awareness of the problem and long term implementation of extensive control policies, MAP prevalence in domestic livestock worldwide has been rapidly increasing, particularly in dairy cattle. Vaccination is the most cost effective disease control measure but current whole cell killed JD vaccines have limited efficacy and are incompatible with diagnosis of MAP infection. Notably these also interfere with bovine tuberculosis tuberculin skin tests. In previous studies using a mouse model we have shown that a prime-boost viral delivery regimen of early entry MAP specific antigens (HAV vaccine) showed significant protection and efficacy in prevention of colonisation [23].

In this study we have applied this approach to cattle and shown that prime-boost HAV vaccination prior to MAP challenge offered a high degree of protection relative to a Sham vaccinated challenged group. We report protective capacity as an ability of vaccination to significantly reduce bacterial numbers in peripheral blood, gut tissues and in faeces as this is likely to impact significantly on disease progression and transmission.

Significant infection of Sham vaccinated calves with MAP was shown herein. MAP could be detected in 100% of tissue samples at 38 weeks post challenge at high numbers (up to 5 logs of MAP load per gram of

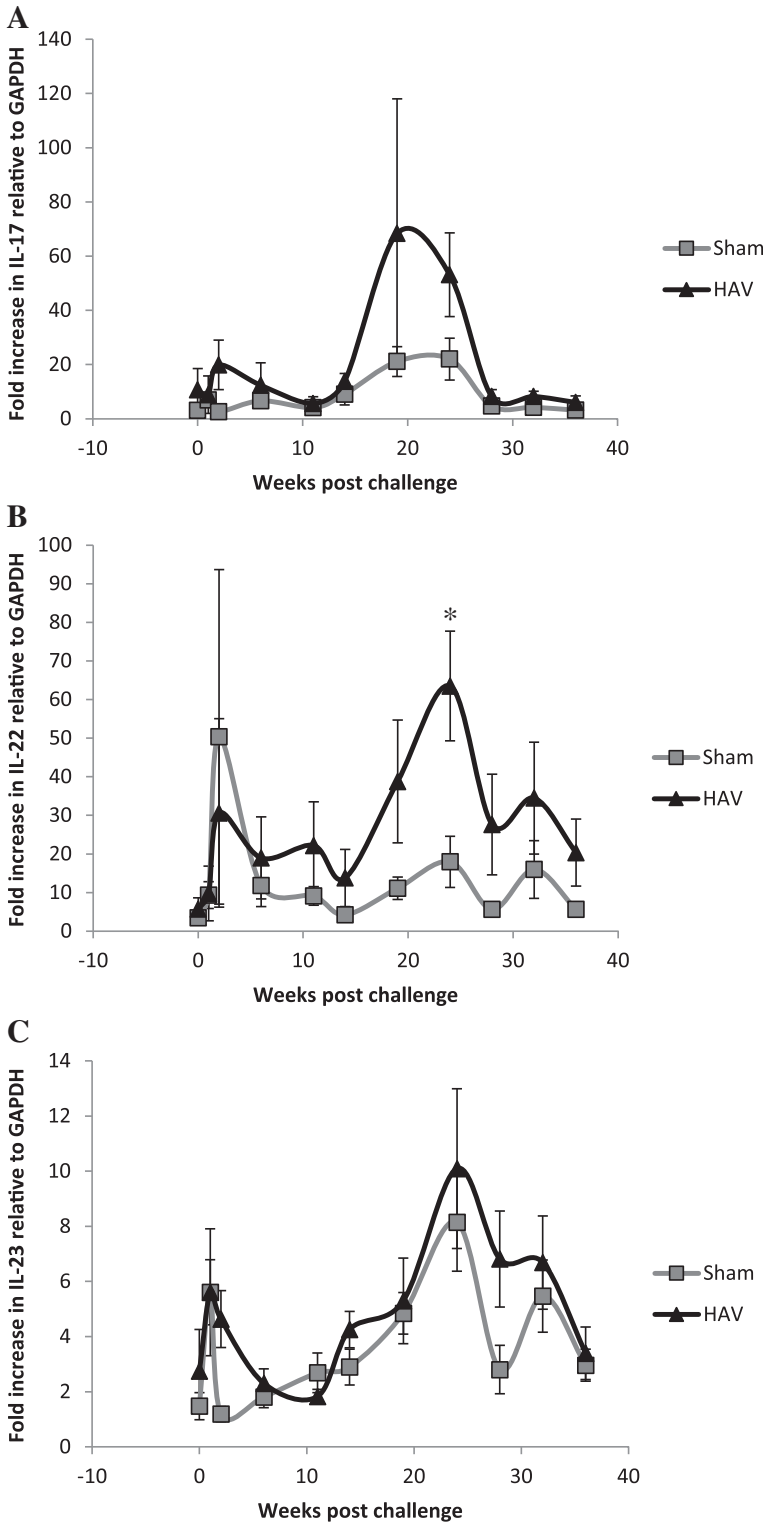


**Figure 5 Expression of antigen specific IL-17, IL-22 and IL-23 by PBMC from HAV- and Sham-vaccinated calves.** Fold increases, relative to GAPDH. in expression of cytokines **A**. IL-17, **B**. IL-22 and **C**. IL-23 measured by qPCR from RNA extracted from 24 h PPD-J stimulated PBMC isolated from HAV vaccinated (triangles) or Sham vaccinated (squares) calves, taken immediately prior to MAP challenge (week 0) up to 36 weeks post challenge. Significance between groups is indicated as * $P < 0.05$.

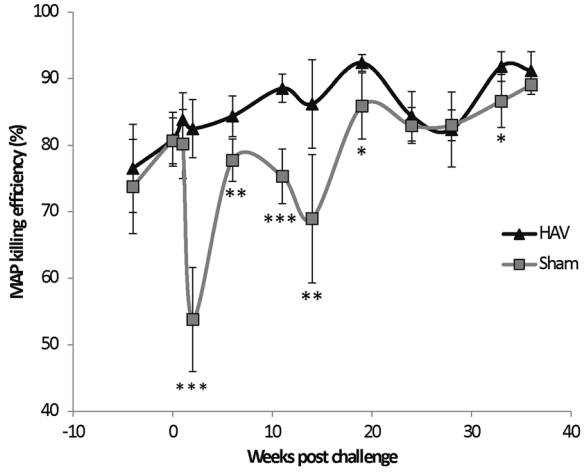


**Figure 6 MAP killing efficiency of macrophage fractions.** Percentage of an initial MAP inoculum killed after infection for 5 days in macrophages isolated from HAV vaccinated (diamonds) or Sham vaccinated (squares) calves taken immediately prior to HAV boost (week -5) up to 36 weeks post challenge. Values given are averages of bovine IFN-γ stimulated and unstimulated preparations performed in duplicate. Significance between groups is indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

tissue) and shedding was detected at least once in faecal samples from 3/5 Sham vaccinated challenged animals. Contrastingly, all faecal samples and at least one tissue sampling site from 4/6 HAV vaccinated calves tested negative (less than 100 organisms per gram) for the presence of MAP.

This reduction in faecal shedding would have a major impact on disease control strategies and would contribute to minimising animal to animal spread of MAP infection. Examination of a range of immunological parameters suggested that these occurred in distinct phases related to the infectious load of MAP and these differed between HAV and Sham vaccinated animals. During the initial phase HAV vaccination significantly primed and boosted antigen specific $CD4^+$, $CD8^+$ but not $WC1^+$ IFN-γ producing T-cell populations. No adverse events and no excretion of vaccine were detected one week and up to 43 weeks post vaccination indicating that, as in other studies, MVA and adenoviral delivery systems are well tolerated in cattle. The DIVA peptide pool used, despite being derived from MAP specific epitopes, showed reactivity only with HAV vaccinated animals and was significantly raised throughout the experiment. Reactivity was not increased after MAP challenge and importantly no response to the HAV specific peptides was observed in in Sham vaccinated animals post-MAP challenge suggesting that these epitopes are not recognised during early natural MAP infection in cattle. In addition none of the cattle in the current study tested positive in the tuberculin skin test used for diagnosis of bovine TB. This is important as it is essential not only to be able to distinguish HAV vaccinated from MAP infected cattle in DIVA tests but also to be able to identify these from cattle infected with *M. bovis*. This will be of particular importance in countries with ongoing bovine TB control programmes such as the UK. All animals were challenged orally with MAP, which resulted in rapid and efficient uptake as demonstrated by the high degree of transient MAP bacteraemia in the peripheral blood observed at week 1 post-challenge. Some early passive shedding was seen along with pro-inflammatory Th1 immunological responses and T cell proliferative responses characteristic of established MAP infection [34]. Importantly in the HAV vaccinated group we observed a more rapid and significantly higher expression of IFN-γ compared with the Sham-vaccinated cattle likely to indicate early Th1 polarisation. This enhanced secretion of IFN-γ in HAV vaccinated calves could activate macrophages for enhanced cytotoxicity. Indeed in this study we demonstrated that macrophages from HAV vaccinated cattle had, at early time points post-infection, significantly greater capacity to kill MAP compared to PBMC taken from Sham vaccinated animals. This may be a crucial early event determining the eventual outcome of infection. Interestingly, early post-challenge there was a dramatic loss in the capacity of PBMC derived macrophages to kill MAP that was only observed in the Sham vaccinated animals and not the HAV vaccinated calves. This may reflect the capacity of MAP to actively evade killing within macrophages and to alter their lytic capacity such that in infected calves the normal killing capacity is significantly reduced. An alternative, but not exclusive explanation is that HAV vaccination induced immune responses (including IFNγ release and T cell activation) activate macrophages for enhanced killing. Our studies have not defined which parameters are required for the maintenance of killing capacity observed in HAV vaccinated cattle but this will be an important aspect to dissect in future studies.

After 2 weeks the number of animals with MAP detectable within PBMC declined; this was evident in both the HAV vaccinated and Sham vaccinated animals. For the remainder of the experiment only one animal in the HAV vaccinated group continued to have persistent MAP present in the PBMC fraction. However, in the Sham vaccinated animals the reduction observed at 2 weeks was transient and persistent bacteraemia returned, consistent with the hypothesis that vaccination significantly affects the capacity of the host to control MAP. The transient reduction in the number of MAP present within the blood of Sham vaccinated animals may correspond to a translocation of MAP to tissues where they begin to divide before again populating the blood at later stages of infection. By contrast the ongoing immune response in the HAV

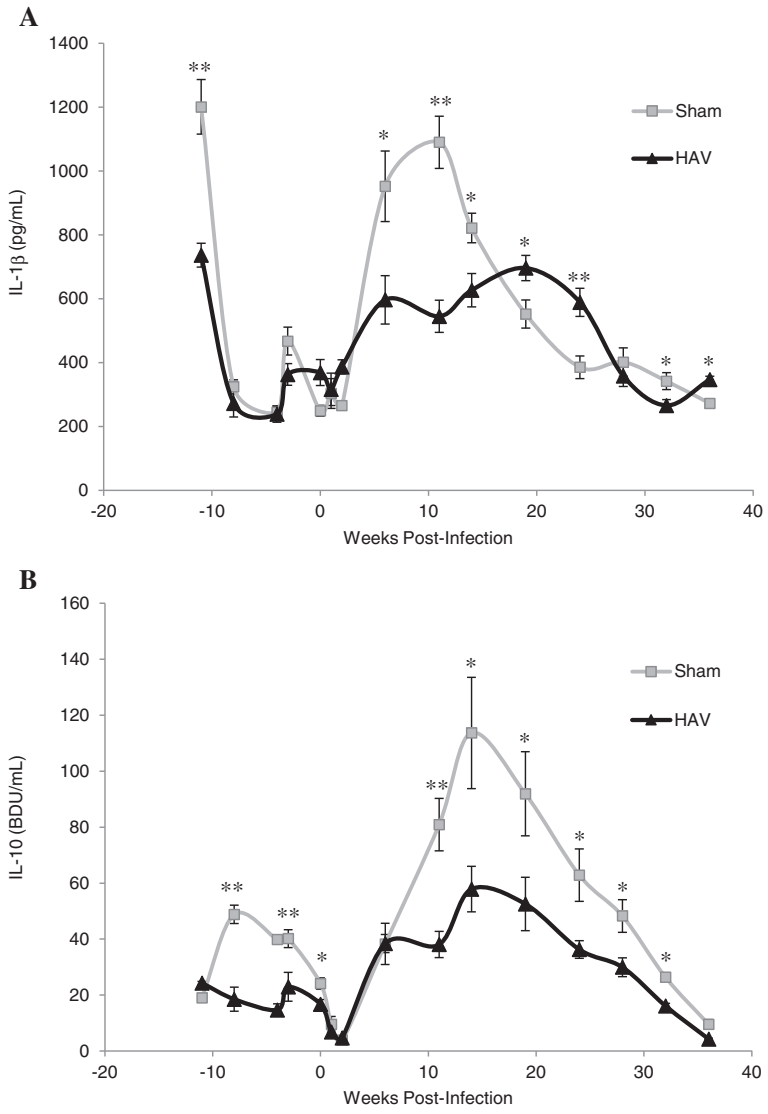


**Figure 7 Secretion of IL-1β and IL-10 by PBMC from HAV- and Sham-vaccinated animals.** At the indicated time points post-MAP challenge PBMC were isolated from HAV-vaccinated (triangle) or Sham-vaccinated (grey square) calves and stimulated for 24 h with PPD-J or left unstimulated (control). Supernatants were assessed for the presence of **A**. IL-1β and **B**. IL-10 in triplicate by ELISA and concentrations of secreted cytokine were assessed relative to standard curve. The mean +/- SD PPD-J specific (PPD-J induced cytokine concentration – unstimulated cytokine concentration) is shown for n = 6 (HAV-vaccinated) or n = 5 (Sham-vaccinated) animals including samples taken week -11, immediately prior prime vaccination; week -5, immediately prior boost vaccination; week 0, immediately prior to MAP challenge and up to 36 weeks post challenge. Significance between groups is indicated as * $P < 0.05$, ** $P < 0.01$.

vaccinated calves is likely to contribute to the level of MAP proliferation and/or survival. Rapid dissemination of MAP post infection and consistent bacteraemia has been demonstrated in several animal models for at least 72 hours post challenge [35]. Long term bacteraemia in naturally infected animals has been linked with progression towards disease, particularly that of the multibacillary type [36]. Thus, early control of peripheral bacteraemia by vaccination may be critical for long term protection from disease.

Small increases in IL-17 were also detected in HAV vaccinated cattle early post-infection, however due to a lack of antibodies for detection of intra-cytoplasmic IL-17 in cattle, we were not able to determine the cellular source of IL-17 herein. Both IFN-γ and IL-17 have been implicated as protective cytokines induced by MAP vaccination in previous studies [37]. Conversely, the cytokine response in the Sham vaccinated animals at early time points were dominated by IL-1β and IL-10 with little induction of IFN-γ. Similar profiles have previously

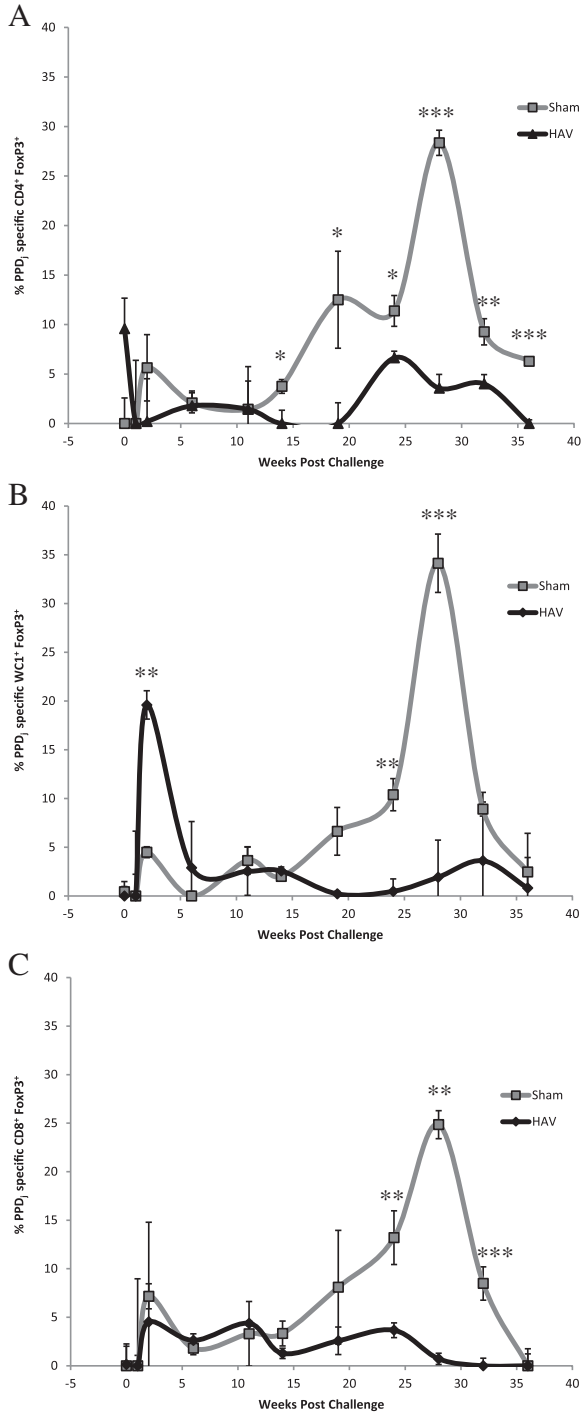


**Figure 8 FoxP3 expression by T cell sub-populations from HAV- and Sham-vaccinated animals.** PBMC from HAV-vaccinated (diamond) or Sham-vaccinated (square) calves were stimulated for 24 h with PPD-J or left unstimulated (control), then washed, fixed, permeablised and assessed for expression of **A**. CD4, **B**. WC1 and **C**. CD8. Cells were gated as live PBMC and the percentage of each cell population (CD4, CD8, WC1) expressing FoxP3 was calculated. Samples taken include week 0, immediately prior to MAP challenge and then up to 36 weeks post challenge. Significance between groups is indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

been associated with late stage intracellular processing of mycobacteria [38] and progression of MAP infection [39]. The source, timing and magnitude of IL-10 production can be a major determinant on disease outcome [40] and the induction of an IL-10 response in animals with significant MAP burden (i.e. the Sham vaccinated calves) is an indicator of immune regulatory imbalance which could facilitate intracellular mycobacterial survival [41]. A number of studies have shown the source of IL-10 from MAP infected cattle to be largely $CD4^+$ T cells, although monocytes may also be involved. In vitro up-regulation of expression of IL-10 is a major response mechanism of bovine macrophages infected with MAP and is associated with reduced IFN-γ secretion and immune evasion. In bovine intestinal tissues early post-infection, MAP induces anti-inflammatory genes such as IL-10 [42] associated with increased intracellular survival. Furthermore we have recently demonstrated that knockdown of IL-10 by siRNA significantly inhibits intracellular survival of BCG indicating a key role for IL-10 in enabling mycobacterial growth and persistence in macrophages (Professor Liz Glass, personal communication to J Hope).

Up-regulated IL-1β has been described in the tissues of animals affected by JD [40], the expression of which appeared to correlate with inflammation. In an epithelial cell line-bone marrow-derived macrophage (bMDM) co-culture model, MAP invasion of the epithelial cells induced up-regulation of IL-1β, leading to the transmigration of the bMDM [43]. This may be a mechanism whereby MAP promotes its own uptake and intracellular survival. Since IL-1β (along with IL-23/IL-17) is regulated by autophagy, interference with expression of these cytokines could also indicate that MAP is directly subverting this pathway to promote its survival within macrophages enabling growth and establishment within the host. This is in line with the decreased capacity of PBMC to kill MAP that we observed in the infected Sham vaccinated calves.

In the final phase of this study (> 19 weeks) MAP bacteraemia steadily increased and persisted in the Sham vaccinated calves but stayed low in the HAV vaccinated group. The final MAP load in tissues of HAV vaccinated animals at 38 weeks was significantly reduced in gut and lymphoid tissues with most (73%) gut mucosal tissue samples testing MAP negative and the majority (55%) of detectable MAP organisms were located in lymph nodes. Faecal shedding was not an expected outcome measure in this model due to the low challenge dose and short study duration post challenge [30]. However abrogation of faecal shedding is a major requirement for an effective MAP vaccine [9] so it was interesting to note that all HAV vaccinated animals were negative for MAP in faecal samples collected throughout the experiment whilst

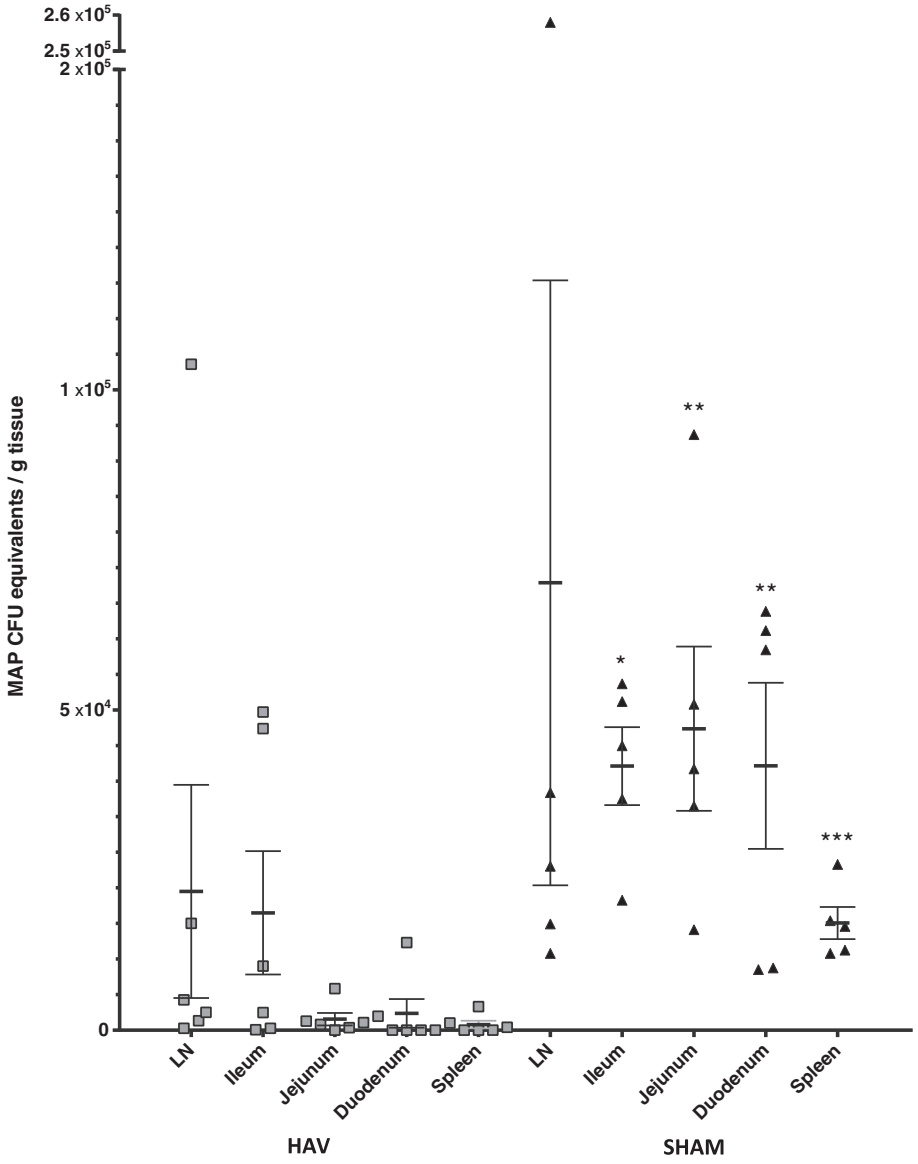


**Figure 9 MAP load in tissue 38 weeks post challenge.** Mean and SEM of genome equivalents determined by MAP specific IS900 qPCR (assuming 17 copies per organism) in weighed samples of various tissue sites (4 lymph node, 3 ileum, 6 jejunum, 2 duodenum, 1 spleen, per animal) from HAV vaccinated (triangles) and Sham vaccinated (squares) calves taken 38 weeks post MAP challenge. Each point represents an average of all samples taken from each tissue site in a single animal with individual sample values being derived from averages of duplicate qPCR performed on DNA extracted from each sample. Mann-Whitney U tests show Sham vaccinated animals had significantly greater loads than HAV vaccinated animals in duodenum ($P = 0.003$), jejunum ($P = 0.009$) and spleen ($P = 0.002$).

intermittent faecal positives were detected in the Sham vaccinated group.

Additionally in this final phase, the Sham vaccinated group developed increased proportions of PPD-J reactive FoxP3$^+$ T cell populations which were also evident in gut tissues taken at the end of the study period. This may be consistent with the development of a regulatory population of T cells, although we have not demonstrated herein that these cells display such functions. Reduced CD4+ effector T cell capacity and the development of regulatory T cell populations has been reported to correlate with disease progression in other studies of MAP infection in cattle, consistent with our observations [44,45]. Interestingly we found significant alterations in the proportion of WC1$^+$ γδ TCR$^+$ T cells present within the tissues of HAV vaccinated compared to Sham vaccinated cattle. A significant proportion of WC1$^+$ γδ TCR$^+$ T cells are regulatory, expressing high levels of IL-10 [46,47] which could contribute to the continued ability of MAP to proliferate within the tissue. A caveat to the observations and interpretations of our data

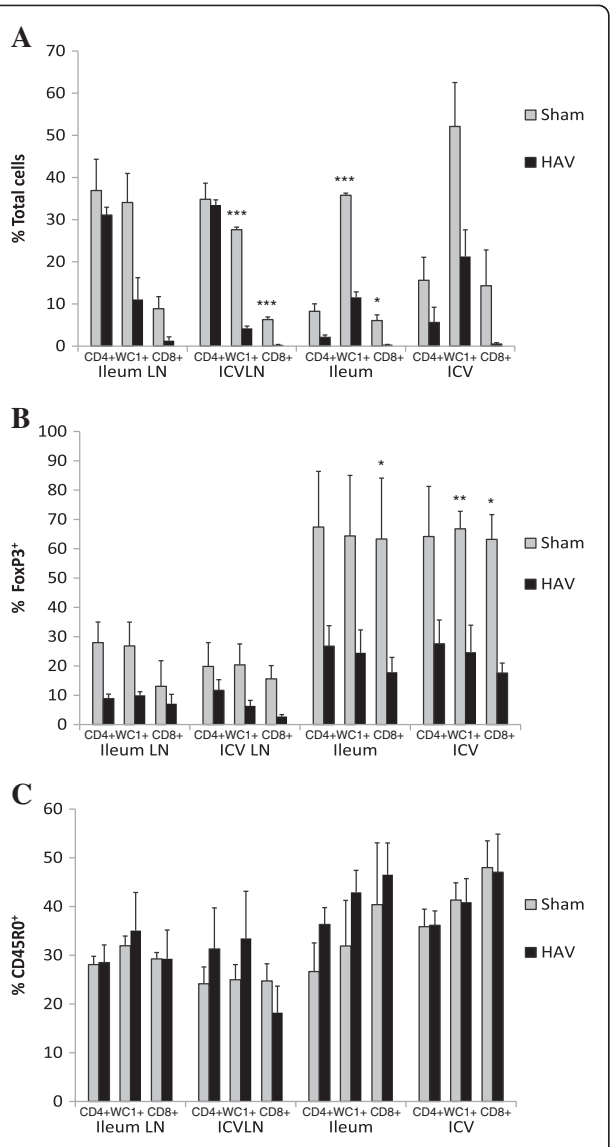


**Figure 10 FoxP3 and CD45RO cell populations in gut tissue 38 weeks post challenge.** Percentage of $CD4^+$, $WC1^+$, $CD8^+$ cells in mucosal and lymph node tissue from ileal, ileocaecal valve sites **A**. total fraction; **B**. fraction expressing FoxP3; **C**. fraction expressing CD45RO from HAV vaccinated (black) or Sham vaccinated (grey) taken 38 weeks post challenge. Significance between groups is indicated as * $P < 0.05$, ** $P < 0.01$.

is the relatively short duration of the experimental infection model used herein. More extensive studies in a long term model or in a field study where natural exposure to MAP occurs will be required to confirm whether we can reproducibly eliminate faecal shedding. This would represent a major advance in disease control as breaking the transmission cycle would have a significant impact on the incidence and spread of disease within and between herds. Such longitudinal studies in large cohorts of MAP exposed cattle would enable us to define the impact of HAV vaccination not only on transmission but also on disease progression. This is an essential next step in confirming the protective efficacy of the HAV vaccine.

The majority of recent MAP vaccination strategies have relied on MAP whole cell formulations to effect nonspecific multi-antigen delivery, thus it is difficult to make detailed comparisons between our novel specific multi-epitope viral delivery and other vaccine regimens. However, studies have shown that protective immunity is associated with high IFN-γ levels and increased Th-17 related responses [22]. There is evidence that the failure of whole cell vaccines to eliminate MAP shedding in faeces may be due partly to interference from non-specific immune regulators present in the mycobacterial cell wall that can deflect appropriate induction of Th1 responses critical for disease resolution [34] and reduce antigen presentation [48]. By contrast HAV vaccination appeared to induce appropriate immune bias, enhanced MAP-specific killing and eliminated MAP shedding.

A long term challenge will be the implementation of HAV vaccination in the field since the strategy that we have assessed involves prime-boost with viral, genetically modified, vectors rather than a single subunit vaccination. Assessment of the MAP-specific antigens contained within the HAV vaccine in combination with adjuvants or other delivery systems will be important, as will determination of the duration of immunity induced by vaccination and the long term impact of vaccination on MAP infection and Johne's disease in the face of potential high-level exposure in heavily affected herds.

In conclusion we have shown that prime-boost viral delivery of MAP antigens to young calves was well tolerated, vaccine was not excreted and vaccination was able to prime a range of cell mediated immune responses which may correlate with the induction of protective immunity. We have shown significant efficacy of HAV vaccination of young calves to reduce the tissue burden of MAP associated with abrogated faecal shedding of MAP. These features, alongside a clear capacity to differentiate vaccinated from infected animals by a novel DIVA test, lack of tuberculin cross reactivity and definition of immunological parameters associated with varied stages post-infection highlight the promise of the HAV vaccine for the improved control of MAP infection in cattle.

## Additional files

**Additional file 1: Tuberculin testing of Sham vaccinated (grey) and HAV vaccinated (black) animals 35 weeks post MAP challenge.** Table showing skin thickness measurements at separate PPA-A, PPD-B inoculum sites pre and 72 h post inoculation. A standard positive tuberculin test requires > 5 mm difference between PPD-B and PPD-A at 72 h. A standard positive tuberculin test requires > 5 mm difference. No significant difference between groups was demonstrated ($P = 0.523$) [49].

**Additional file 2: MIRU-VNTR typing of MAP isolates.** Gel files showing specific MIRU-VNTR PCR products profiles comparing Control K10 reference strain, challenge strain MAP R0808 and a MAP isolate from faeces of Sham vaccinated animal obtained at 36 weeks.

**Additional file 3: IL-17 and IL-22 in tissue from Sham vaccinated and HAV vaccinated animals 36 weeks post MAP challenge.** Bar graphs showing fold increases relative to GAPDH in expression of cytokines Graph A. IL-17 and Graph B. IL-22 in mucosal and lymph node tissue from ileal, ileocaecal valve sites obtained from HAV vaccinated (black) or Sham vaccinated (grey) calves obtained 38 weeks post challenge. There were no significance between groups ($P > 0.05$).

#### Competing interests
TJB is a minor shareholder in HAV Vaccines Ltd.

#### Authors' contributions
TJB and JH wrote the manuscript. All authors read and approved the manuscript. Microbiological investigations were carried out by TJB and RL. Immunological investigations were carried out by CV, IMcG, CB and JH. HAV vaccine preparation was supervised by SCG. Animal vaccination, challenge and sampling were performed and supervised by JMcN and SS. The project was conceived and co-ordinated by TJB and JH.

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