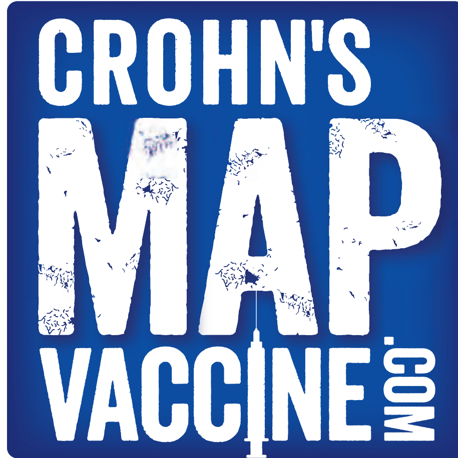


Information for Patients, General Practionioners and Specialists

Summary of Contents

This document contains the most important recently published scientific papers in this field which may be of interest to you. Please feel free to send this to your specialist as it will be more helpful for them to have PDFs than paper copies of the attachments:

1. This 2011 study reviews all the trials to date of anti-MAP antibiotic therapy (AMAT) for the treatment of CD in adults. Remission rates achieved with this treatment range from 44% - 88.5% as compared to 39% with Infliximab in the ACCENT I trial. To see the progress of the large multi-centre trial of AMAT currently in progress in the USA, visit: https://www.clinicaltrials.gov/ct2/show/NCT01951326
2. This small study from 2013 is the only published study of AMAT in children. But the remission rate of 80% - in line with the findings in adults - would certainly support the extrapolation of data from the larger studies in adults.
3. Global Warming to MAP 2014 is an excellent review paper looking at the evidence to date for MAP as the cause of CD and at therapies targeting MAP; this includes currently available AMAT and promising future therapies including the therapeutic anti-MAP vaccine.
4. Gitlin 2012 - another excellent review which addresses in particular the contentious issues re MAP in the causality of CD, looking at the latest evidence to help 'piece the puzzle together'
5. Nature 2012. This very important paper reports a meta-analysis of Genome Wide Association Scans in IBD involving over 75,000 people. It showed that there is 'considerable overlap between susceptibility loci [=genes] for IBD and mycobacterial disease'. Hence, of the two dominant hypotheses of CD causation - (1) Aberrant response of the immune system to the normal gut flora and (2) MAP infection in individuals possessing a specific immune-deficiency to mycobacterial disease - the genetic data is pointing us towards the latter. Crucially, it also indicates that this immune deficiency is primary (no-one is born with Crohn's but we are born with our genes) and not secondary to the chronic disease state resulting from Crohn's.
6. 2012 study demonstrating the anti-MAP activity of Infliximab. Many have asked 'If MAP causes CD then why do biologics, which would make TB disseminate, make CD better? Answer: (1) as Gitlin et al 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 biologics or immunosuppressants and (2) Biologics have direct anti-MAP action.
7. Olsen 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.
8. The trial of the anti-MAP Vaccine in cattle (Bull 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!
9. A patient information leaflet we have written about the treatment. Whilst this is primarily for patients, Specialists may find it helpful as a good plain-English summary.

If you want to know more about other aspects of MAP in CD, visit our website www.crohnsmapvaccine.com where you will find a comprehensive FAQ section and many informative, downloadable documents to help you.

I hope this is helpful. If your specialists would like to know more detail about the prescribing/monitoring of anti-MAP antibiotic therapy, I would suggest they contact either:

If UK based: Dr Jeremy Sanderson (St Thomas's Hospital, London, UK) or
Rest of the world: Prof Tom Borody (Centre for Digestive Diseases, Sydney Australia)
for the treatment protocol.

Best wishes and good luck!

Amy
Dr Amy Hermon-Taylor MBBS MRCGP
General Practitioner
Fundraiser and Medical lead for Crohn's MAP Vaccine

For reprint orders, please contact reprints@expert-reviews.com

Expert Reviews

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

Expert Rev. Clin. Immunol. 7(6), 751–760 (2011)

William Chamberlin[1], Thomas J Borody[2] and Jordana Campbell[†2]

[1]Texas Tech University, El Paso, TX 9905, USA
[2]Centre for Digestive Diseases, Five Dock, NSW 2046, Australia
[†]Author for correspondence:
Tel.: +61 297 134 011
Fax: +61 297 131 026
jordana.campbell@cdd.com.au

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

 10.1586/ECI.11.43 © 2011 Expert Reviews Ltd ISSN 1744-666X

(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 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 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: 68% in the ciprofloxacin/metronidazole group 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 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 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 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 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 7 patients were unable to tolerate the therapy (19.4%) 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 Histologically, 15 out of 39 (38.5%) patients experienced a marked reduction in inflammation with 6 of these (40%) displayed restoration of normal mucosa Improved histology coincided with longitudinal scarring in 12 out of 15 patients (80%) 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) Behr and Kapur (2008)	213 randomized Active (n = 102) Placebo (n = 111) 91 were withdrawn	Rifabutin, clarithromycin and clofazimine (AMAT)	16–104	ITT analysis: 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 Effects of AMAT after 16 weeks: At 52 weeks: remission sustained in 41 out of 102 (40%) patients vs 24 out of 111 (21.6%), $p < 0.005$ At 104 weeks: remission sustained in 31 out of 102 (30%) patients vs 16 out of 111(14%), $p < 0.008$ 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.

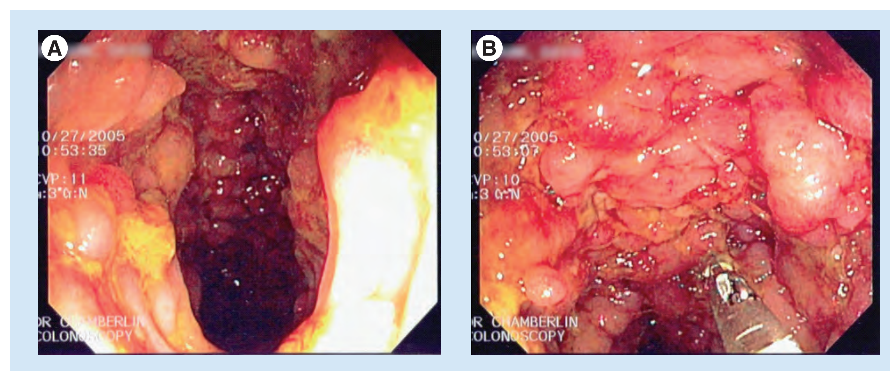


Figure 1. Before anti-*Mycobacterium avium paratuberculosis* therapy. (A) Inflamed, edematous, erythematous mucosa. **(B)** Severe mucosal ulceration, bleeding and exudates. Reproduced with permission from [59].

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.

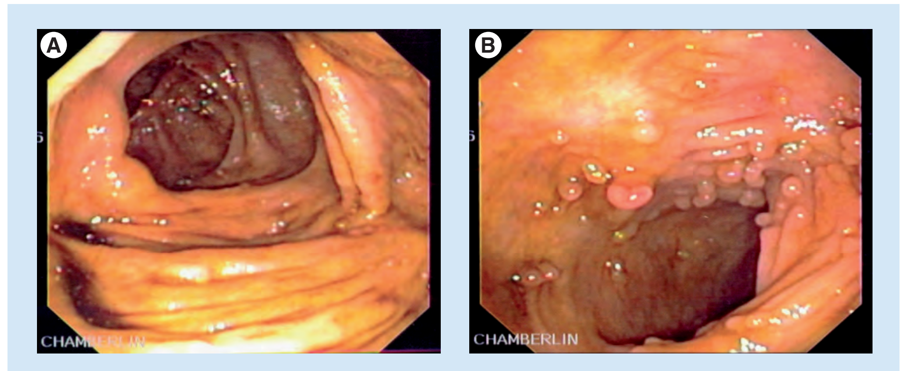


Figure 2. During anti-*Mycobacterium avium paratuberculosis* therapy. **(A)** Complete normalization of mucosa. **(B)** Residual pseudopolyps in areas of previous severe Crohn's disease.
Reproduced with permission from [59].

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.

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

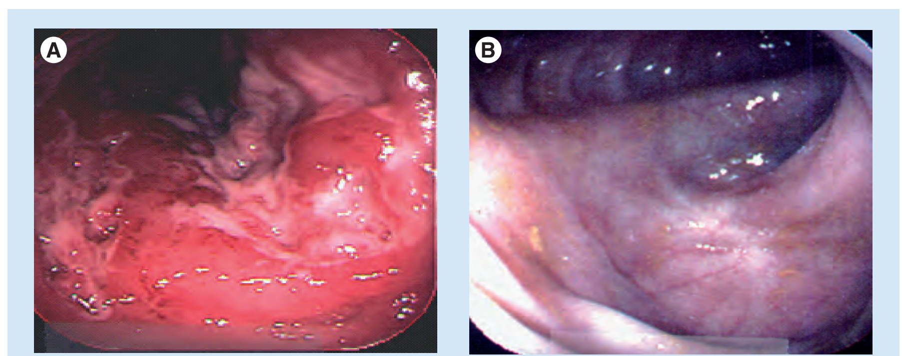


Figure 3. The transverse colon pre- and post-treatment. (A) Pretreatment: severe inflammation. **(B)** Post-treatment: no inflammation visible.
Reproduced with permission from [53].

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.

References

Papers of special note have been highlighted as:
• of interest

1 Perencevich M, Burakoff R. Use of antibiotics in the treatment of inflammatory bowel disease. *Inflamm. Bowel Dis.* 12(7), 651–664 (2006).

2 Podolsky DK. Inflammatory bowel disease. *N. Engl. J. Med.* 347, 417–429 (2002).

3 Akbulut S, Yavuz B, Koseoglu T *et al.* Crohn's disease with isolated esophagus and gastric involvement. *Turk. J. Gastroenterol.* 15(3), 196–200 (2004).

4 Malleson P. Isolated gastroduodenal Crohn's disease in a ten-year-old girl. *Postgrad. Med. J.* 56, 294–296 (1980).

5 Kotanagi H, Sone S, Fukuoka T *et al.* Liver abscess as the initial manifestation of colonic Crohn's disease: report of a case. *Surgery Today* 21(3), 348–351 (2007).

6 Ulnick KM, Perkins J. Extraintestinal Crohn's disease: case report and review of the literature. *Ear Nose Throat J.* 80(2), 97–100 (2001).

7 Sartor BR. Pathogenesis of Crohn's disease and ulcerative colitis: specific theories. *Nat. Clin. Prac. Gastroenterol. Hepatol.* 3(7), 390–407 (2006).

8 Blichfeldt P, Blonhoff JP, Myhre E *et al.* Metronidazole in Crohn's disease. A double-blind cross-over clinical trial. *Scand. J. Gastroenterol.* 13, 123–127 (1978).

9 Ursing B, Alm T, Barany F *et al.* A comparative study of metronidazole and

sulfasalazine for active Crohn's disease: the cooperative Crohn's disease study in Sweden. II Result. *Gastroenterology* 83, 550–562 (1982).

10 Ambrose NS, Allan RN, Keighley MR *et al.* Antibiotic therapy for treatment in relapse of intestinal Crohn's disease. *Dis. Colon Rectum* 28, 81–85 (1985).

11 Sutherland L, Singleton J, Sessions J *et al.* Double-blind, placebo controlled trial of metronidazole in Crohn's disease. *Gut* 32, 1071–1075 (1991).

12 De'Haens GR, Vermeire S, Van Assche G *et al.* Therapy of metronidazole with azathioprine to prevent postoperative recurrence of Crohn's disease: a controlled randomized trial. *Gastroenterology* 135(4), 1123–1129 (2008).

13 Feller M, Huwiler K, Schoepfer A *et al.* Long-term antibiotic treatment for Crohn's disease: systematic review and meta-analysis of placebo-controlled trials. *Clin. Infect Dis*. 50(4), 473–480 (2010).

- **Systematic review and meta-analysis of randomized controlled trials (RCTs) to determine the effectiveness of long-term antibiotic therapy. The authors concluded that long-term treatment with nitroimidazoles or clofazimine appear to be effective in patients with Crohn's disease (CD).**

14 Peppercorn MA. Is there a role for antibiotics as primary therapy in Crohn's disease? *J. Clin. Gatroenterol.* 17, 235–237 (1993).

15 Arnold GL, Beaves MR, Pyrjdun VO *et al.* Preliminary study of ciprofloxacin in active Crohn's disease. *Inflamm. Bowel Dis.* 8, 10–15 (2002).

16 Prantera C, Zannoni F, Scribano ML *et al.* An antibiotic treatment regimen for the treatment of active Crohn's disease. A randomized controlled clinical trial of metronidazole plus ciprofloxacin. *Am. J. Gastroenterol.* 91, 328–332 (1996).

17 Colombel JF, Lehman M, Cassagnou M *et al.* A controlled trial comparing ciprofloxacin with mesalazine for the treatment of active Crohn's disease. Groupe d'Etudes Therapeutiques des Affections Inflammatoires Digestives (GETARD). *Am. J. Gastroenterol.* 94, 674–678 (1999).

18 Steinhart AH, Feagan BG, Wong CJ *et al.* Combined budesonide and antibiotic therapy for active Crohn's disease. A randomized controlled trial. *Gastroenterology* 123, 33–40 (2002).

19 Prantera C, Berto E, Scribano ML *et al.* Use of antibiotics in the treatment of active Crohn's disease. Experience with metronidazole and ciprofloxacin. *Ital. J. Gastroenterol. Hepatol.* 30, 602–606 (1998).

20 Greenbloom SL, Steinhart AH, Greenberg GR. Combination ciprofloxacin and metronidazole for active Crohn's disease. *Can. J. Gastroenterol.* 12, 53–56 (1998).

21 Khan K, Ullman T, Ford A *et al.* Antibiotic therapy in inflammatory bowel disease: a systematic review and meta-analysis. *Am. J. Gastroenterol.* 106(4), 661–673 (2011).

- **Comprehensive systematic review of RCTs evaluating the use of all antibacterial therapy in inflammatory bowel disease. A diverse number of antibiotics were tested including anti-tuberculosis therapy, macrolides, fluroquinolones, 5-nitroimidazoles and rifaximin either alone or in combination and found that there was a statistically significant effect of antibiotics in inducing remission in active CD compared with placebo (relative risk of active CD not in remission: 0.85; 95% CI: 0.73–70.99; p = 0.03).**

22 Dignass A, Van Assche G, Lindsay JO *et al.* The second European evidence-based consensus on the diagnosis and management of Crohn's Disease: current management. *J. Crohn's Colitis* 4, 28–62 (2010).

23 Shafran I. Burgeunder P. Rifaximin for the treatment of newly diagnosed Crohn's disease: a case series. *Am. J. Gasteroenterol.* 103, 2158–2160 (2008).

24 Shafran I, Johnson LK. An open-label evaluation of rifaximin in the treatment of active Crohn's disease. *Curr. Med. Res. Opin.* 21, 1165–1169 (2005).

25 Shafran I, Burgunder P. Adjunctive antibiotic therapy with rifaximin may help reduce Crohn's disease activity. *Dig. Dis. Sci.* 55, 1079–1084 (2010).

26 Warren J, Rees H, Cox T. Remission of Crohn's disease with tuberculosis chemotherapy. *N. Engl. J. Med.* 314, 182 (1986).

27 Schultz M, Rieder H, Hersh T *et al.* Remission of Crohn's disease with antibacterial chemotherapy. *Lancet* 330, 1391–1392 (1987).

28 Ward M, McManus JP. Dapsone in Crohn's disease. *Lancet* 1, 1236–1237 (1975).

29 Prantera C, Argentieri R, Mangiarotti R *et al.* Dapsone and remission of Crohn's disease. *Lancet* 1(8584), 536 (1988).

30 Prantera C, Bothamley G, Levenstein S *et al.* Crohn's disease and mycobacteria: two cases of Crohn's disease with high anti-mycobacterial antibody levels cured by dapsone therapy. *Biomed. Pharmacother.* 43(4), 295–299 (1989).

31 Shaffer JL, Hughes S, Linaker BD *et al.* Controlled trial of rifampicin and ethambutol in Crohn's disease. *Gut* 25, 203–205 (1984).

32 Jarnerot G, Rolny P, Wickbom G *et al.* Antimycobacterial therapy ineffective in Crohn's disease after a year. *Lancet* 1, 164–165 (1989).

33 Swift GL, Srivastava ED, Stone R *et al.* Controlled trial of anti-tuberculosis chemotherapy for two years in Crohn's disease. *Gut.* 35, 363–368 (1994).

34 Rutgeerts P, Geboes K, Vantrappen G *et al.* Rifabutin and ethambutol do not help recurrent Crohn's disease in the neoterminal ileum. *J. Clin. Gastroenterol.* 15, 24–28 (1992).

35 Hampson S, Parker M, Saverymutter S *et al.* Quadruple antimycobacterial chemotherapy in Crohn's disease: results at 9 months of a pilot study in 20 patients. *Aliment. Pharmacol. Ther.* 3(4), 343–352 (1989).

36 Prantera C, Kohn A, Mangiarotti R *et al.* Antimycobacterial therapy in Crohn's disease: results of a controlled double-blind trial with a multiple antibiotic regimen. *Am. J. Gastroenterol.* 89(4), 513–518 (1994).

37 Afdhal NH, Long A, Lennon J *et al.* Controlled trial of antimycobacterial therapy in Crohn's disease: clofazimine versus placebo. *Dig. Dis. Sci.* 36, 449–453 (1991).

38 Graham DY, Al-Assi MT, Robinson M. Prolonged remission in Crohn's disease following therapy for *Mycobacterium paratuberculosis* infection. *Gastroenterology* 108, A826 (1995).

39 Leiper M. Open label trial of oral clarithromycin in active Crohn's disease. *Aliment. Pharmacol. Ther.* 14(6), 801–806 (2000).

40 Inoue S, Nakase H, Matsuura M *et al.* Open label trial of clarithromycin therapy in Japanese patients with Crohn's disease. *J. Gastro. Hepatol.* 22, 984–988 (2007).

41 Mitchell JD, Bishop A, Cafaro A *et al.* Anatomic lung resection for nontuberculous mycobacterial disease. *Ann. Thorac. Surg.* 85, 1887–1893 (2008).

42 Gangadharam, PR, Perumal, VK, Jairam, BT *et al.* Activity of rifabutin alone or in combination with clofazimine or ethambutol or both against acute and chronic experimental *Mycobacterium intracellulare* infections. *Am. Rev. Respir. Dis.* 136, 329–333 (1987).

43 Hornick DB, Dayton CS, Bedell GN *et al.* Nontuberculous mycobacterial lung disease. Substantiation of a less aggressive approach. *Chest* 93(3), 550–555 (1988).

44 Chiodini RJ. Antimicrobial activity of rifabutin in combination with two and three other antimicrobial agents against strains *Mycobacterium avium paratuberculosis*. *J. Antimicrob. Chemother.* 27, 171–176 (1991).

45 Blasquez J, Oliver A, Gomez-Gomez JM. Mutation and evolution of antibiotic resistance: antibiotics as promoters of antibiotic resistance? *Curr. Drug Targets* 3(4), 345–349 (2002).

46 Ghebremichael S, Svenson SB, Kallenius G *et al.* Antimycobacterial synergism of clarithromycin and rifabutin. *Scan. J. Infect. Dis.* 28, 387–390 (1996).

47 Rastogi N, Goh KS, Labrousse V. Activity of clarithromycin compared with those of other drugs against *Mycobacterium paratuberculosis* and further enhancement its extracellular and intracellular activities by ethambutol. *Antimicrob. Agents Chemother.* 36, 2843–2846 (1992).

48 Gui GPH, Thomas PRS, Tizard MLV *et al.* Two-year outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics. *J. Antimicrob. Chemother.* 39(3), 393–400 (1997).

49 Douglas A, Bramble MG, Silcock JC. Antimycobacterial treatment for Crohn's disease: does it prevent surgery and how fast does it act? *Gut* 48, A89 (2000).

50 Borody TJ, Leis S, Warren EF *et al.* Treatment of severe Crohn's disease using antimycobacterial triple therapy – approaching a cure? *Dig. Liver Dis.* 34(1), 29–38 (2002).

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

52 Borody TJ, Bilkey S, Wettstein AR *et al.* *Australian Gastroenterology Week Meeting*. Brisbane, Queensland, Australia, 19–22 October 2005.

53 Borody TJ, Bilkey S, Wettstein AR *et al.* Anti-mycobacterial therapy in Crohn's disease heals mucosa with longitudinal scars. *Dig. Liver Dis.* 39(5), 438–444 (2007).

- **Report on the prolonged use of anti-*Mycobacterium avium paratuberculosis* therapy in CD. 22 patients (56.4%; 22 out of 39) experiencing profound healing with unusual scarring. Scars receded in two out of six patients (33.3%) who had >3 years of treatment as full healing occurred, implying a more complete healing not seen with standard anti-inflammatory and immunosuppressant drugs.**

54 Selby W, Pavli P, Crotty B *et al.* Two-year combination antibiotic therapy with clarithromycin, rifabutin and clofazimine for Crohn's disease. *Gastroenterology* 132(7), 2313–2319 (2007).

- **RCT of CD patients treated with antimycobacterial therapy. A remission rate of 66% was achieved in CD patients treated with antimycobacterial therapy at 16 weeks (p = 0.02) despite suboptimal dosing of two of the three antibiotics and incomplete dissolution of clofazimine.**

55 Behr M, Kapur V. The evidence for *Mycobacterium paratuberculosis* in Crohn's disease. *Curr. Opin. Gastroenterol.* 24(1), 17–21 (2008).

56 Biroulet LP, Neut C, Colombel JF. Antimycobacterial therapy in Crohn's disease: the end of a long controversy? *Prac. Gastro.* 1, 11–17 (2008).

57 Yunzhao RR, Pan F, Parvez S *et al.* Clofazamine inhibits human Kv1.3 potassium channel by perturbing calcium oscillation in T lymphocytes. *PLoS One* 3(12), e4009 (2008).

58 Hanauer SB, Feagan BG, Lichtenstein GR *et al.* Maintenance infliximab for Crohn's disease: the ACCENT 1 randomised trial. *Lancet* 359, 1541–1549 (2002).

59 Keunstner J. Australian Crohn's Antibiotic Study opens new horizons. *Gastroenterology* 133(5), 1743–1744 (2007).

60 Colombel JF, Sandborn WJ, Rutgeerts P *et al.* Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 132(1), 52–65 (2007).

61 Moore TJ, Cohen MR, Furberg CD *et al.* Serious adverse drug events reported to the Food and Drug Administration, 1998–2005. *Arch. Intern. Med.* 167(16), 1752–1759 (2007).

62 Lees C, Ali A, Thompson AI, Ho GT *et al.* The safety profile of anti-TNF therapy in inflammatory bowel disease in clinical practice: analysis of 620 patient-years follow-up. *Aliment. Pharmacol. Ther.* 29(3), 286–297 (2009).

63 Chamberlin W, Ghobrial G, Chehtane M *et al.* Successful treatment of a Crohn's disease patient infected with bacteremic *Mycobacterium paratuberculosis*. *Am. J. Gastroenterol.* 102, 689–691 (2007).

- **Case report of a patient with longstanding active CD who achieved total clinical remission following treatment with clarithromycin, rifabutin and levofloxacin that resulted in resolution of abdominal pain, diarrhea and fatigue; suppression of *Mycobacterium avium paratuberculosis* markers; and complete mucosal normalization on colonoscopy. This case report is the first of its kind to correlate CD resolution with suppression of *Mycobacterium avium paratuberculosis* markers.**

64 Rahimi R, Shekoufeh N, Rezaie A *et al.* A meta-analysis of broad-spectrum antibiotic therapy in patients with active Crohn's disease. *Clin. Ther.* 28(12), 1983–1988 (2006).

65 Netea MG, Joosten LA. A NOD for autophagy. *Nature Med.* 16, 28–30 (2010).

66 Hayee B, Rahman F, Sewell G *et al.* Crohn's disease as an immunodeficiency. *Expert Rev. Clin. Immunol.* 6(4), 585–596 (2010).

67 Korzenik JR, Dieckgraefe BK, Valentine JF *et al.* Sargramostim for active Crohn's disease. *N. Engl. J. Med.* 352, 2193–2201 (2005).

68 Nicoletti F, Conrad D, Wang A *et al.* 16α-bromoepiandrosterone (HE2000) limits non-productive inflammation and stimulates immunity in lungs. *Clin. Exp. Immunol.* 158(3), 308–316 (2009).

69 Levin BR, Bull JJ. Population and evolutionary dynamics of phage therapy. *Nat. Rev. Micro.* 2, 166–173 (2004).

[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

Thomas Borody, BSc, MBBS, MD, PhD, FACG, Sarah Finlayson, BAdvSc, Anna Nowak, BMedSci, Enmoore Lin, PhD, BA/BSc. Department of Research and Innovation, Centre for Digestive Diseases, Sydney, NSW, Australia.

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

Ali Abbas, MD, MPH,[1] Rawaa Almukhtar, MD,[2] Nabeel Khan, MD[3]. 1. Department of Internal Medicine, University of Florida, Gainesville, FL; 2. Department of Epidemiology,Louisiana State University Health Science Center, New Orleans, LA; 3. Southeast Louisiana Veterans Health Care System, Section of Gastroenterology, Department of Internal Medicine, New Orleans, LA.

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.

Future
MICROBIOLOGY

EDITORIAL

For reprint orders, please contact: reprints@futuremedicine.com

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

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

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.

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 and microorganisms, especially for causality."

[1]Centre for Digestive Diseases, Level 1, Five Dock, NSW 2046, Australia
[2]Montana State University, Billings, Saint Vincent Healthcare, Billings, MT 59101, USA
*Author for correspondence: Tel.: +61 2 9713 4011; Fax: +61 2 9713 1026; drgagrawal@gmail.com

Future Medicine part of fsg

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.

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].

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

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.

References

1. Campbell J, Borody TJ, Leis S. The many faces of Crohn's disease: latest concepts in etiology. *Open J. Int. Med.* 2(2), 107 (2012).
2. Podolsky DK. Inflammatory bowel disease. *N. Engl. J. Med.* 347(6), 417–429 (2002).
3. Deretic V. Autophagy in immunity and cell-autonomous defense against intracellular microbes *Immunol. Rev.* 240(1), 92–104 (2011).
4. Greenstein RJ. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infect. Dis.* 3(8) 507–514 (2003).
5. Davis W, Madsen-Bouterse S. Crohn's disease and *Mycobacterium avium* subsp. *paratuberculosis*: the need for a study is long overdue. *Vet. Immunol. Immunopathol.* 145(1–2), 1–6 (2012).
6. Nakase H, Tamaki HH, Matsuura M, Chiba T, Okazaki K. Involvement of *Mycobacterium avium* subspecies *paratuberculosis* in TNF-α production from macrophage: possible link between MAP and immune response in Crohn's disease. *Inflamm. Bowel Dis.* 17(2), 140–142 (2011).
7. Bach H, Rosenfeld HG, Bressler B. 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. *J. Crohns Colitis* 6(5), 628–629 (2012).
8. Selby W, Pavli WP, Crotty B *et al*. Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease. *Gastroenterology* 132(7), 2313–2319 (2007).
9. Behr M, Kapur MV. The evidence for *Mycobacterium paratuberculosis* in Crohn's disease. *Curr. Opin. Gastroenterol.* 24(1), 17–21 (2008).
10. Barash JE, Lipton JE, Barash DP. Flawed Australian CD study does not end MAP controversy. *Gastroenterology* 133(5), 1742; author reply 1745–1746 (2007).
11. Gitlin L, Biesacker J. Australian Crohn's antibiotic study opens new horizons. *Gastroenterology* 133(5), 1743–1744; author reply 1745–1746 (2007).
12. Chamberlin W, Borody WT, Naser S. MAP-associated Crohn's disease: MAP, Koch's postulates, causality and Crohn's disease. *Digest. Liver Dis.* 39(8), 792–794 (2007).
13. Gui GP, Thomas PR, Tizard ML, Lake J, Sanderson JD, Hermon-Taylor J. Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics. *J. Antimicrob. Chemother.* 39(3), 393–400 (1997).
14. Efficacy and Safety of Anti-MAP Therapy in Adult Crohn's Disease (MAPUS). www.clinicaltrials.gov/ct2/show/NCT01951326
15. Click RE. A 60-day probiotic protocol with Dietzia subsp C79793-74 prevents development of Johne's disease parameters after *in utero* and/or neonatal MAP infection. *Virulence* 2(4), 337–347 (2011).
16. Click RE. A potential 'curative' modality for Crohn's disease – modeled after prophylaxis of bovine Johne's disease. *J. Mycobact. Dis.* 2, 117 (2012).
17. Zhang F, WangFH, Wang M, Cui B, Fan Z, Ji G. Fecal microbiota transplantation for severe enterocolonic fistulizing Crohn's disease. *World J. Gastroenterol.* 19(41), 7213–7216 (2013).
18. Gordon H, HarbordHM. A patient with severe Crohn's colitis responds to faecal microbiota transplantation. *J. Crohns Colitis* 8(3), 256–257 (2014).
19. Anderson JL, Edney RJ, Whelan K. Systematic review: faecal microbiota transplantation in the management of inflammatory bowel disease. *Aliment. Pharmacol. Ther.* 36(6), 503–516 (2012).
20. Bull TJ, Gilbert SC, Sridhar S *et al*. A novel multi-antigen virally vectored vaccine against *Mycobacterium avium* subspecies *paratuberculosis*. *PLoS ONE* 2(11), e1229 (2007).

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

Laura Gitlin, MD, FACG, Thomas Julius Borody, MD, PhD,† 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

From the *Gastroenterology Institute, Las Vegas, NV; †Centre for Digestive Diseases, Five Dock, NSW, Australia; and ‡Texas Tech University, El Paso, TX.

L.G. owns shares in Giaconda Ltd., the licensor of MyocondaTM, an anti-MAP therapy. T.J.B. has a pecuniary interest in the Centre for Digestive Diseases and Giaconda Ltd., the licensor of MyocondaTM, an anti-MAP therapy. W.C. owns shares in Harbor Biosciences, the bio-tech company with patent rights to the immune enhancing hormone HE2000. J.C. has no financial interest or affiliation with any institution, organization, or company relating to the manuscript.

Reprints: Jordana Campbell, BSc, Centre for Digestive Diseases, Level 1/229 Great North Road, Five Dock, NSW 2046, Australia (e-mail: jordana.campbell@cdd.com.au).

Copyright © 2012 by Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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.

 © 2012 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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 *Salmonella*, *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

© 2012 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

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."

REFERENCES

1. Sartor RB. Does *Mycobacterium avium subspecies paratuberculosis* cause Crohn's Disease? *Gut*. 2005;54:896–898.
2. Prantera C. *Mycobacteria* and Crohn's Disease: the endless story. *Digest Liver Dis*. 2007;39:452–454.
3. Quirke P. Antagonist: *Mycobacterium avium subspecies paratuberculosis* is a cause of Crohn's Disease. *Gut*. 2001;49:757–760.
4. Behr M, Kapur V. The evidence for *Mycobacterium paratuberculosis* in Crohn's Disease. *Curr Opin Gastroenterol*. 2008;24:17–21.
5. Sartor RB. Bacteria in Crohn's Disease: mechanisms of inflammation and therapeutic implications. *J Clin Gastroenterol*. 2007;41(suppl 1):S37–S43.
6. Joshi YK. *Helicobacter pylori* infection: current status. *J Indian Acad Clin Med*. 2000;5:148–155.
7. Mendoza JL, San-Pedro A, Culebras E, et al. High prevalence of viable *Mycobacterium avium subspecies paratuberculosis* in Crohn's disease. *World J Gastroenterol*. 2010;16:4558–4563.
8. Hermon-Taylor J, Barnes N, Clarke C, et al. *Mycobacterium paratuberculosis* cervical lymphadenitis, followed five years later by terminal ileitis similar to Crohn's disease. *BMJ*. 1998;316:449–453.
9. Scanu AM, Bull TJ, Cannas S, et al. *Mycobacterium avium subspecies paratuberculosis* infection in cases of irritable bowel syndrome and comparison with Crohn's Disease and Johne Disease: common neural and immune pathogenicities. *J Clin Microbiol*. 2007;45:3883–3890.
10. Burke Cm, Healy TM. Sarcoidosis and tuberculosis: variable immunologically determined reactions to a common cause? *Irish J Med Sci*. 1988;157:39–44.
11. Campbell J, Borody TJ, Leis S. The many faces of Crohn's Disease: latest concepts in etiology. *Open J Int Med*. 2012. (In press).
12. Schwartz K. Ueber penetriende Magen-imf Jejunalgeschwuer. *Bitrage Klin Chir*. 1910;68:96–111.
13. Podolsky DK. Inflammatory bowel disease. *N Engl J Med*. 2002;347:417–429.
14. Al-Orainey IO. Diagnosis of latent tuberculosis: can we do better? *Ann Thorac Med*. 2009;4:5–9.
15. Gale G. Tuberculosis of the superficial lymph nodes. *Can Med Assoc J*. 1953;69:303–309.
16. Basgoz N. Clinical manifestations; diagnosis; and treatment of miliary tuberculosis. *UpToDate*. 2010.
17. Leung A. Pulmonary tuberculosis: the essentials. *Radiology*. 1999;210:307–322.
18. Rock RB, Olin M, Baker CA, et al. Central nervous system tuberculosis: pathogenesis and clinical aspects. *Clin Microbiol Rev*. 2008;21:243–261.
19. Lamba H, Byrne M, Goldin R, et al. Tuberculosis of the cervix: case presentation and a review of the literature. *Sex Transm Infect*. 2002;78:62–63.
20. Patnaik MM, Deshpande AK. Diagnosis—Addison's Disease secondary to tuberculosis of the adrenal glands. *Clin Med Res*. 2008;6:29.
21. Edington JW, Guest D. Study of tubercle bacilli isolated from cases of surgical tuberculosis (chiefly bone and joint) in the Sheffield area. *J Hyg*. 1926;25:109–118.
22. Borody TJ, George LL, Brandl S, et al. *Helicobacter pylori*-negative duodenal ulcer. *Am J Gastroenterol*. 1991;86:1154–1157.
23. Koch R. Die Aetiologie der tuberkulose. *Mittheilungen aus dem Kaiserlichen Gesundheitsamte*. 1884;2:1–88.
24. Van Kruiningen HJ, Chiodini RJ, Thayer WR, et al. Experimental disease in infant goats induced by a *Mycobacterium* isolated from a patient with Crohn's disease. A preliminary report. *Dig Dis Sci*. 1986;31:1351–1360.
25. Fredericks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's Postulates. *Clin Microbiol Rev*. 1996;9:18–33.
26. Chamberlin W, Borody T, Naser S. MAP-associated Crohn's disease MAP, Koch's postulates, causality and Crohn's disease. *Dig Liver Dis*. 2007;39:792–794.
27. Hanauer SB. More likely than not. *Nat Clin Prac Gastroenterol Hepatol*. 2007;4:469.
28. Selby W, Pavli P, Crotty B. Author reply. *Gastroenterol*. 2007;133:1745–1746.
29. Koch R. Die aetiologie de tuberculose. *Berliner Klinische Wochenschrift*. 1882;19:221–230.
30. Marshall BJ, Armstrong JA, McGechie DB, et al. Attempt to fulfill Koch's postulates for pyloric *Campylobacter*. *Med J Aust*. 1985;142:436–439.
31. St Jean G. Treatment of clinical paratuberculosis in cattle. *Vet Clin N Am Food A Prac*. 1996;12:417–430.
32. Oki NO, Motsinger-Reif AA, Antas PRZ, et al. Novel human genetic variants associated with extrapulmonary tuberculosis: a pilot genome wide association study. *BMC Res Notes*. 2011;4:28–38.
33. Russell RK, Wilson DC, Satsangi J. Unravelling the complex genetics of inflammatory bowel disease. *Arch Dis Child*. 2004;89:598–603.
34. Shoda R, Matsueda K, Yamato S, et al. Epidemiologic analysis of Crohn disease in Japan: increased dietary intake of n-6 polyunsaturated fatty acids and animal protein relates to the increased incidence of Crohn disease in Japan. *Am J Clin Nutr*. 1996;63:741–745.
35. Nugent FW, Richmond M, Park S. Crohn's Disease of the duodenum. *Gut*. 1977;18:115–120.
36. Malleson P. Isolated gastroduodenal Crohn's Disease in a ten-year-old girl. *Postgrad Med J*. 1980;56:294–296.
37. Johne HA, Forthingham L. Ein eigenthumlicher Fall von Tuberculose beim Rind [A peculiar case of tuberculosis in a cow]. *Deutsche Zeitschrift fur Tiermedizin und vergleichende Pathologie*. 1895;21:438–455.
38. Dalziel TK. Chronic intestinal enteritis. *BMJ*. 1913;2: 1068–1070.
39. Feller M, Huwiler K, Schoepfer A, et al. Long-term antibiotic treatment for Crohn's Disease: systematic review and meta-analysis of placebo-controlled trials. *Clin Infect Dis*. 2010; 50:473–480.
40. Chiodini RJ. Crohn's Disease and the *Mycobacterioses*. *Clin Microbiol Rev*. 1989;2:90–117.
41. Borody TJ, Bilkey S, Wettstein AR, et al. Anti-mycobacterial therapy in Crohn's Disease heals mucosa with longitudinal scars. *Dig Liver Dis*. 2007;39:438–444.
42. Chamberlin W, Ghobrial G, Chehtane M, et al. Successful treatment of a Crohn's Disease patient infected with bacteremic *Mycobacterium paratuberculosis*. *Am J Gastroenterol*. 2007;102:689–691.
43. Bull TJ, McMinn EJ, Sidi-Boumedine K, et al. Detection and verification of *Mycobacterium avium subsp. paratuberculosis* in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease. *J Clin Microbiol*. 2003;41:2915–2923.
44. Autschbach F, Eisold S, Hinz U, et al. High prevalence of *Mycobacterium avium subspecies paratuberculosis* IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut*. 2005;54:944–949.
45. Schwartz D, Shafran I, Romero C, et al. Use of short-term culture for identification of *Mycobacterium avium subsp. paratuberculosis* in tissue from Crohn's disease patients. *Clin Microbiol Infect*. 2000;6:303–307.
46. Naser S, Ghobrial G, Romero C, et al. Culture of *Mycobacterium avium subspecies paratuberculosis* from the blood of patients with Crohn's Disease. *Lancet*. 2004;364: 1039–1044.
47. Nunić N, Mićić R, Popović M, et al. Generalized miliary tuberculosis during immunosuppressive therapy. *Srp Arh Celok Lek*. 1981;109:527–538.

© 2012 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

48. Fortune S, Rubin E. The complex relationship between *Mycobacteria* and macrophages: it's not all bliss. *Cell Host Microbe*. 2007;2:5–6.
49. Athreya SPK. Azathioprine in controlling type 2 reactions in leprosy: a case report. *Leprosy Rev*. 2007;78:290–292.
50. Beran V, Havelkova M, Kaustova J, et al. Cell wall deficient forms of mycobacteria: a review. *Vet Med*. 2006;7:365–389.
51. Greenstein R, Su L, Haroutunian V, et al. On the action of methotrexate and 6-mercaptopurine on *M. avium subspecies paratuberculosis*. *Plos One*. 2007;2:e161.
52. Shin S, Collins M. Thiopurine drugs azathioprine and 6-mercaptopurine inhibit *Mycobacterium paratuberculosis* growth in vitro. *Antimicrob Agents Chemother*. 2008;52:418–426.
53. Van den Brande J, Braat H, Van den Brink G, et al. Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's Disease. *Gastroenterology*. 2003;124:1774–1785.
54. Catrina AL, Trollmo C, Klint E, et al. Evidence that anti-tumor necrosis factor therapy with both etanercept and infliximab induces apoptosis in macrophages but not lymphocytes in rheumatoid arthritis joints: extended report. *Arthritis Rheum*. 2005;52:61–72.
55. Bach H, Rosenfeld G, Bressler B. 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. *J Crohn's Colitis*. 2012;6: 628–629.
56. Olsen I, Tollefsen S, Aagaard C, et al. Isolation of *Mycobacterium avium subspecies paratuberculosis* reactive CD4 T cells from intestinal biopsies of Crohn's Disease patients. *PloS One*. 2009;4:e5641.
57. Ebert E, Bhatt B, Liu S, et al. Induction of suppressor cells by *Mycobacterium paratuberculosis* antigen in inflammatory bowel disease. *Clin Exp Immunol*. 1991;83:320–325.
58. Juste R, Elguezabel N, Pavon A, et al. Association between *Mycobacterium avium subsp. paratuberculosis* DNA in blood and cellular and humoral immune response in inflammatory bowel disease patients and controls. *Int J Infect Dis*. 2009;13:247–254.
59. Ren Z, Turton J, Borody T, et al. Selective Th2 pattern of cytokine secretion in *Mycobacterium avium subsp. Paratuberculosis* infected Crohn's Disease. *J Gastroenterol Hepatol*. 2008;23: 310–314.
60. Sibarue S, Keohane J, Scully P, et al. Disturbed cytokine response to *Mycobacterium avium subspecies paratuberculosis* is dysregulated in Crohn's Disease. *Gastroenterol*. 2006;130: A-240.
61. De Almeida D, Colvin C, Coussens P. Antigen-specific regulatory T cells in bovine paratuberculosis. *Vet Immunol Immunopathol*. 2008;125:234–245.
62. Lybeck KR, Storset AK, Olsen I. Neutralisation of IL-10 from CD14 + monocytes enhances gamma interferon production in peripheral blood mononuclear cells from *Mycobacterium avium subsp. paratuberculosis* infected goats. *Clin Vaccine Immunol*. 2009;16:1071–1412.
63. Cucino C, Sonnenberg A. Occupational mortality from inflammatory bowel disease in the United States 1991–1996. *Am J Gastroenterol*. 2001;96:1101–1105.
64. Radon K, Windstetter D, Poluda AL, et al. Contact with farm animals in early life and juvenile inflammatory bowel disease: a case-control study. *Pediatrics*. 2007;120: 354–361.
65. Chiodini RJ, Thayer WR, Couta JA. Presence of *Mycobacterium paratuberculosis* antibodies in animal healthcare workers. *P Fifth Internat Colloq Para*. 1996;324–328.
66. Hermon-Taylor J. *Mycobacterium avium subspecies paratuberculosis*, Crohn's Disease and the doomsday scenario. *Gut Pathoq*. 2009;1:15–21.
67. Cirillo JD, Falkow S, Tompkins LS, et al. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infect Immun*. 1997;65:3759–3767.
68. Patel D, Danelishvilli L, Yamazaki Y, et al. The ability of *Mycobacterium avium subsp. paratuberculosis* to enter bovine epithelial cells is influenced by preexposure to a hyperosmolar environment and intracellular passage in bovine mammary epithelial cells. *Infect Immun*. 2006;74:2849–2855.
69. Reed WD, Eddleston WF, Mitchell CG, et al. Exposure and immunity to hepatitis-B virus in a liver unit. *Lancet*. 1974;303:581–583.
70. Radcliff FJ, Ferrero RL. Effect of low-dose antigen exposure on development of immunity to *Helicobacter pylori* infection in mice. *Infect Immun*. 2001;69:5186–5188.
71. Fridriksdottir V, Gunnarsson E, Sigurdarson S, et al. Paratuberculosis in Iceland: epidemiology and control measures, past and present. *Vet Microbiol*. 2000;77:263–267.
72. Bjornsson S, Johannsson JH, Oddsson E. Inflammatory bowel disease in Iceland, 1980–89. A retrospective nationwide epidemiologic study. *Scand J Gastroenterol*. 1998;33:71–77.
73. Bjornsson S, Johannsson JH. Inflammatory bowel disease in Iceland, 1990–1994: a prospective, nationwide, epidemiological study. *Eur J Gastroenterol Hepatol*. 2000;12:31–38.
74. Sechi LA, Scanu AM, Molicotti PM, et al. Detection and Isolation of *Mycobacterium avium subspecies paratuberculosis* from intestinal mucosal biopsies of patients with and without Crohn's Disease in Sardinia. *Am J Gastroenterol*. 2005;100:1529–1536.
75. Singh A, Singh S, Makharia G, et al. Presence and characterisation of *Mycobacterium avium subsp. paratuberculosis* from clinical and suspected cases of Crohn's Disease and in the healthy human population in India. *Int J Infect Dis*. 2008;12:190–197.
76. Kumar S, Singh S, Singh A, et al. Wildlife (Boselaphus tragocamelus)—small ruminant (goat and sheep) interface in the transmission of "Bison type" genotype of *Mycobacterium avium subsp. paratuberculosis* in India. *Comp Immunol Microbiol Infect Dis*. 2010;33:145–159.
77. Van Kruiningen HJ, Joossens M, Vermeire S, et al. Familial Crohn's disease in Belgium: pedigrees, temporal relationships among cases, and family histories. *J Clin Gastroenterol*. 2007;41:583–590.
78. Achord JL, Gunn CH, Jackson JF. Regional enteritis and HLA concordance in multiple siblings. *Dig Dis Sci*. 1982;37: 330–332.
79. Gelfand MD, Krone Cl. Inflammatory bowel disease in a family: observations related to pathogenesis. *Ann Intern Med*. 1970;72:903–907.
80. Bennett RA, Rubin PH, Present DH. Frequency of inflammatory bowel disease in offspring of couples both presenting with inflammatory bowel disease. *Gastroenterology*. 1991;100:1638–1643.
81. Allan RN, Pease P, Ibbotson JP. Clustering of Crohn's Disease in a Cotswold Village. *Q J Med*. 1986;59:473–478.
82. Van Kruiningen HJ, Freda BA. Clustering of Crohn's Disease in Mankato, Minnesota. *Inflamm Bowel Dis*. 2001;7:27–33.
83. Pierce ES, Borowitz SM, Naser SA. The Broad Street pump revisited: dairy farms and an ongoing outbreak of inflammatory bowel disease in Forest, Virginia. *Gut Pathoq*. 2011;3:20–24.
84. Van Kruiningen HJ, Colombel JF, Cartun RW, et al. An in-depth study of Crohn's Disease in two French families. *Gastroenterology*. 1993;104:351–360.
85. Aisenberg J, Janowitz HD. Cluster of inflammatory bowel disease in three close college friends. *J Clin Gastroenterol*. 1993;17:18–20.
86. Hornick DB, Dayton CS, Bedell GN, et al. Nontuberculous mycobacterial lung disease. Substantiation of a less aggressive approach. *Chest*. 1988;93:550–555.
87. Gui GP, Thomas PR, Tizard ML, et al. Two-year outcomes analysis of Crohn's Disease treated with rifabutin and macrolide antibiotics. *J Antimicrob Chemother*. 1997;39: 393–400.
88. Lipton J, Barach D. Flawed Australian CD study does not end MAP controversy. *Gastroenterology*. 2007;133:1745–1746.
89. Keunstner J. Australian Crohn's antibiotic study opens new horizons. *Gastroenterology*. 2007;133:1743–1744.

 © 2012 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

90. Chamberlin W. Importance of the Australian Crohn's Disease antibiotic study. *Gastroenterology*. 2007;133:1744–1745.
91. Behr M, Hanley J. Antimycobacterial therapy for Crohn's Disease: a reanalysis. *Lancet Infect Dis*. 2008;8:344.
92. Shafran I, Kugler L, El-Zataari FA, et al. Open clinical trial of rifabutin and clarithromycin therapy in Crohn's Disease. *Dig Liver Dis*. 2002;34:22–28.
93. Borody TJ, Leis S, Warren EF, et al. Treatment of severe Crohn's disease using antimycobacterial triple therapy–approaching a cure? *Dig Liver Dis*. 2002;34:29–38.
94. Borody TJ, Clancy R, Wettstein AR, et al. Anti-MAP therapy in the treatment of active Crohn's Disease. *J Gastroenterol Hepatol*. 2005;20:A2.
95. Chamberlin W, Borody TJ, Campbell J. Primary treatment of Crohn's Disease: combined antibiotics taking centre-stage. *Expert Rev Clin Immunol*. 2011;7:751–760.
96. Hanauer SB, Feagan BG, Lichtenstein GR, et al. Maintenance infliximab for Crohn's Disease: the accent 1 randomised trial. *Lancet*. 2002;359:1541–1549.
97. Borody TJ, Leis S, Torres M. Five years continuous anti-MAP therapy for Crohn's—observation of side effects. *Am J Gastroenterol*. 2008;103:S454–S455.
98. Moore TJ, Cohen MR, Furberg CD, et al. Serious adverse drug events reports to the Food and Drug Administration, 1988–2005. *Arch Intern Med*. 2007;167:1752–1759.
99. D' Haens G, Geboes K, Peeters M, et al. Early lesions of recurrent Crohn's Disease caused by infusion of the intestinal contents in excluded ileum. *Gastroenterology*. 1998;114:262–267.
100. Rutgeerts P, Geboes K, Peelers M, et al. Effect of fecal stream diversion on recurrence of Crohn's disease in the neoterminal ileum. *Lancet*. 1991;338:771–774.
101. Harper P, Lee E, Kettlewell M, et al. Role of fecal stream in the maintenance of Crohn's colitis. *Gut*. 1985;26:279–284.
102. Tvede M, Rask-Madsen J. Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. *Lancet*. 1989;1:1156–1160.
103. Eiseman B, Silen W, Bascom GS, et al. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. *Surgery*. 1958;44:854.
104. Aas J, Gessert CE, Bakken JS. Recurrent *Clostridium difficile* colitis: case series involving 18 patients treated with donor stool administered via a nasogastric tube. *Clin Infect Dis*. 2002;34:346–353.
105. Persky SE, Brandt LJ. Treatment of recurrent *Clostridium difficile*-associated diarrhea by administration of donated stool directly through a colonoscope. *Am J Gastroenterol*. 2000;95:3283.
106. Yoon S, Brandt L. Treatment of refractory/recurrent *C. difficile*-associated disease by donated stool transplanted via colonoscopy: a case series of 12 patients. *J Clin Gastroenterol*. 2010;44:562–566.
107. Silverman M, Davis I, Pillai D. Success of self-administered home fecal transplantation for chronic *Clostridium difficile* infection. *Clin Gastroenterol Hepatol*. 2010;8:471–473.
108. Rohlke F, Surawicz C, Stollman N. Fecal flora reconstitution for recurrent *Clostridium difficile* infection: results and methodology. *J Clin Gastroenterol*. 2010;44:567–570.
109. You DM, Franzos A, Holman RP. Successful treatment of fulminant *Clostridium difficile* infection with fecal bacteriotherapy. *Ann Int Med*. 2008;148:632–633.
110. Borody TJ, George L, Andrews P, et al. Bowel-flora alteration: a potential cure for inflammatory bowel disease and irritable bowel syndrome? *Med J Aust*. 1989;150:604.
111. Bennet JD, Brinkman M. Treatment of ulcerative colitis by implantation of normal colonic flora. *Lancet*. 1989;1:164.
112. Borody TJ, Warren EF, Leis S, et al. Treatment of ulcerative colitis using fecal bacteriotherapy. *J Clin Gastroenterol*. 2003;37:42–47.
113. Ramakrishnan K, Mold JW. Urinary catheters: a review. *Int J Fam Prac*. 2005;3. Available at: http://www.ispub.com/journal/the-internet-journal-of-family-practice/volume-3-number-2/urinary-catheters-a-review.html. Accessed January 19, 2012.

© 2012 Lippincott Williams & Wilkins

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

LETTER

doi:10.1038/nature11582

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

A list of authors and their affiliations appears at the end of the paper.

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 (λ_{GC}) = 2.00 to λ_{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

©2012 Macmillan Publishers Limited. All rights reserved

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)
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	rs11612508§	*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	rs194749§	*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	rs7240004§	*SMAD7* (2)
18	67.53	rs727088	***CD226*** (2)
19	10.49*	**rs11879191**	***TYK2*** (27)

©2012 Macmillan Publishers Limited. All rights reserved

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	rs1042058§	***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 1×10^{-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

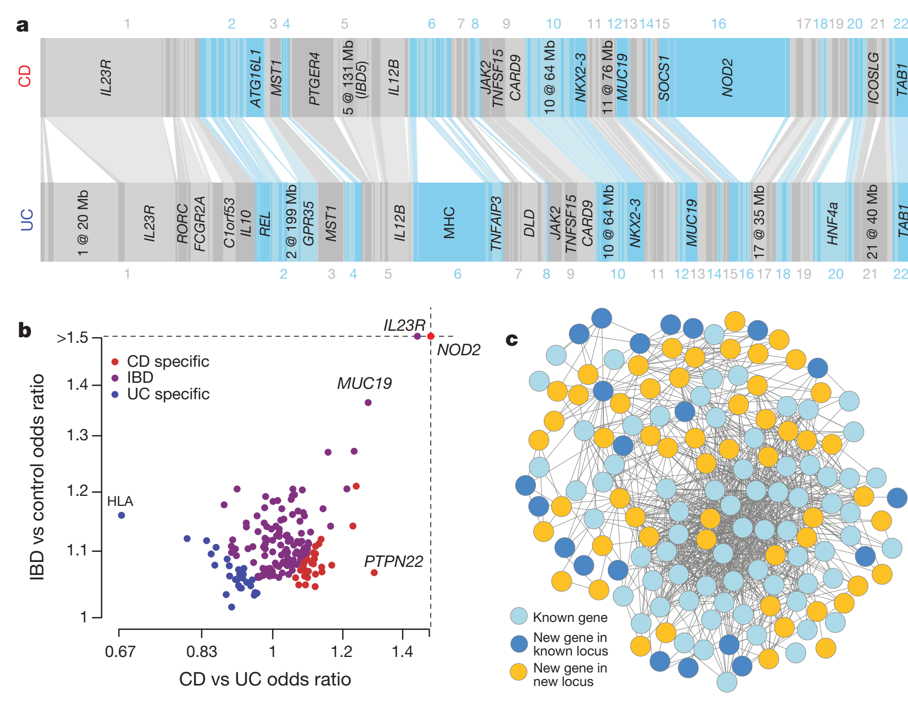


Figure 1 | The IBD genome. a, Variance explained by the 163 IBD loci. Each bar, ordered by genomic position, represents an independent locus. The width of the bar is proportional to the variance explained by that locus in Crohn's disease (CD) and ulcerative colitis (UC). Bars are connected together if 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.

©2012 Macmillan Publishers Limited. All rights reserved

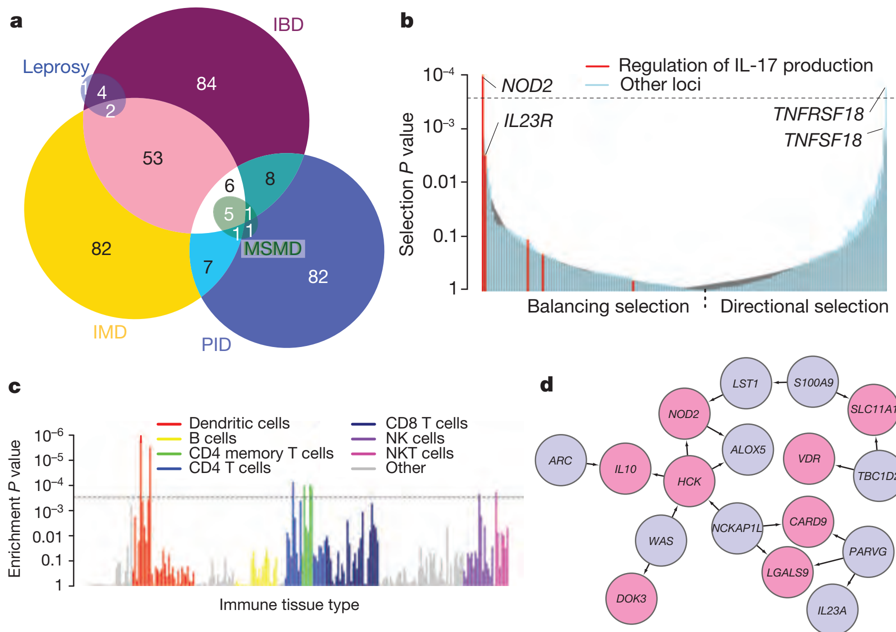


Figure 2 | Dissecting the biology of IBD. **a**, Number of overlapping IBD loci with other immune-mediated diseases (IMD), leprosy and Mendelian PIDs. Within PID, we highlight MSMD. **b**, Signals of selection at IBD SNPs, from strongest balancing on the left to strongest directional on the right. The 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 $P_{stimulated} < 1 \times 10^{-6}$ compared with $P_{unstimulated} = 0.0015$, 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

©2012 Macmillan Publishers Limited. All rights reserved

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.

Received 17 May; accepted 12 September 2012.

1. Molodecky, N. A. *et al.* Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. *Gastroenterology* **142,** 46–54 (2012).
2. Anderson, C. A. *et al.* Meta-analysis identifies 29 additional ulcerative colitis risk loci, increasing the number of confirmed associations to 47. *Nature Genet.* **43,** 246–252 (2011).
3. Franke, A. *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nature Genet.* **42,** 1118–1125 (2010).
4. Khor, B., Gardet, A. & Xavier, R. J. Genetics and pathogenesis of inflammatory bowel disease. *Nature* **474,** 307–317 (2011).
5. Cho, J. H. & Gregersen, P. K. Genomics and the multifactorial nature of human autoimmune disease. *N. Engl. J. Med.* **365,** 1612–1623 (2011).
6. Cortes, A. & Brown, M. A. Promise and pitfalls of the Immunochip. *Arthritis Res. Ther.* **13,** 101 (2011).
7. Zuk, O., Hechter, E., Sunyaev, S. R. & Lander, E. S. The mystery of missing heritability: genetic interactions create phantom heritability. *Proc. Natl Acad. Sci. USA* **109,** 1193–1198 (2012).
8. Raychaudhuri, S. *et al.* Identifying relationships among genomic disease regions: predicting genes at pathogenic SNP associations and rare deletions. *PLoS Genet.* **5,** e1000534 (2009).
9. Hindorff, L. A. *et al.* Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proc. Natl Acad. Sci. USA* **106,** 9362–9367 (2009).
10. Notarangelo, L. D. *et al.* Primary immunodeficiencies: 2009 update. *J. Allergy Clin. Immunol.* **124,** 1161–1178 (2009).
11. Bustamante, J., Picard, C., Boisson-Dupuis, S., Abel, L. & Casanova, J. L. Genetic lessons learned from X-linked Mendelian susceptibility to mycobacterial diseases. *Ann. NY Acad. Sci.* **1246,** 92–101 (2011).
12. Patel, S. Y., Doffinger, R., Barcenas-Morales, G. & Kumararatne, D. S. Genetically determined susceptibility to mycobacterial infection. *J. Clin. Pathol.* **61,** 1006–1012 (2008).
13. Zhang, F. *et al.* Identification of two new loci at *IL23R* and *RAB32* that influence susceptibility to leprosy. *Nature Genet.* **43,** 1247–1251 (2011).
14. Holland, S. M. *et al.* *STAT3* mutations in the hyper-IgE syndrome. *N. Engl. J. Med.* **357,** 1608–1619 (2007).
15. Minegishi, Y. *et al.* Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature* **448,** 1058–1062 (2007).
16. Glocker, E. O. *et al.* A homozygous *CARD9* mutation in a family with susceptibility to fungal infections. *N. Engl. J. Med.* **361,** 1727–1735 (2009).
17. Hu, X. *et al.* Integrating autoimmune risk loci with gene-expression data identifies specific pathogenic immune cell subsets. *Am. J. Hum. Genet.* **89,** 496–506 (2011).
18. Zhang, B. & Horvath, S. A general framework for weighted gene co-expression network analysis. *Stat. Appl. Genet. Mol. Biol.* **4,** Article 17 (2005).
19. Greenawalt, D. M. *et al.* A survey of the genetics of stomach, liver, and adipose gene expression from a morbidly obese cohort. *Genome Res.* **21,** 1008–1016 (2011).
20. Emilsson, V. *et al.* Genetics of gene expression and its effect on disease. *Nature* **452,** 423–428 (2008).
21. Schadt, E. E. *et al.* Mapping the genetic architecture of gene expression in human liver. *PLoS Biol.* **6,** e107 (2008).
22. Chen, Y. *et al.* Variations in DNA elucidate molecular networks that cause disease. *Nature* **452,** 429–435 (2008).
23. Zhong, H. *et al.* Liver and adipose expression associated SNPs are enriched for association to type 2 diabetes. *PLoS Genet.* **6,** e1000932 (2010).
24. Zhu, J. *et al.* Increasing the power to detect causal associations by combining genotypic and expression data in segregating populations. *PLOS Comput. Biol.* **3,** e69 (2007).
25. Lewis, S. J., Baker, I. & Davey Smith, G. Meta-analysis of vitamin D receptor polymorphisms and pulmonary tuberculosis risk. *Int. J. Tuberc. Lung Dis.* **9,** 1174–1177 (2005).
26. Li, X. *et al.* *SLC11A1* (NRAMP1) polymorphisms and tuberculosis susceptibility: updated systematic review and meta-analysis. *PLoS ONE* **6,** e15831 (2011).
27. Kumar, D. *et al.* Genome-wide analysis of the host intracellular network that regulates survival of Mycobacterium tuberculosis. *Cell* **140,** 731–743 (2010).
28. Glocker, E. O. *et al.* Infant colitis–it's in the genes. *Lancet* **376,** 1272 (2010).
29. Franke, A. *et al.* Sequence variants in *IL10*, *ARPC2* and multiple other loci contribute to ulcerative colitis susceptibility. *Nature Genet.* **40,** 1319–1323 (2008).
30. Bhattacharjee, A., Pal, S., Feldman, G. M. & Cathcart, M. K. Hck is a key regulator of gene expression in alternatively activated human monocytes. *J. Biol. Chem.* **286,** 36709–36723 (2011).

Supplementary Information is available in the online version of the paper.

Acknowledgements We thank all the subjects who contributed samples and the physicians and nursing staff who helped with recruitment globally. UK case collections were supported by the National Association for Colitis and Crohn's disease; Wellcome Trust grant 098051 (L.J., C.A.A., J.C.B.); Medical Research Council UK; the Catherine McEwan Foundation; an NHS Research Scotland career fellowship (R.K.R.); Peninsula College of Medicine and Dentistry, Exeter; the National Institute for Health Research, through the Comprehensive Local Research Network, and through Biomedical Research Centre awards to Guy's & Saint Thomas' National Health Service Trust, King's College London, Addenbrooke's Hospital, University of Cambridge School of Clinical Medicine and to the University of Manchester and Central Manchester Foundation Trust. The British 1958 Birth Cohort DNA collection was funded by Medical Research Council grant G0000934 and Wellcome Trust grant 068545/Z/02, and the UK National Blood Service controls by the Wellcome Trust. The Wellcome Trust Case Control Consortium projects were supported by Wellcome Trust grants 083948/Z/07/Z, 085475/B/08/Z and 085475/Z/08/Z. North American collections and data processing were supported by funds to the National Institute of Diabetes, Digestive and Kidney diseases (NIDDK) IBD Genetics Consortium, which is funded by the following grants: DK062431 (S.R.B.), DK062422 (J.H.C.), DK062420 (R.H.D.), DK062432 (J.D.R.), DK062423 (M.S.S.), DK062413 (D.P.M.), DK076984 (M.J.D.), DK084554 (M.J.D. and D.P.M.) and DK062429 (J.H.C.). Additional funds were provided by funding to J.H.C. (DK062429-S1 and Crohn's & Colitis Foundation of America, Senior Investigator Award (5-2229)) and R.H.D. (CA141743). K.Y.H. is supported by the National Institutes of Health (NIH) MSTP TG T32GM07205 training award. Cedars-Sinai is supported by USPHS grant PO1DK046763 and the Cedars-Sinai F. Widjaja Inflammatory Bowel and Immunobiology Research Institute Research Funds, National Center for Research Resources (NCRR) grant M01-RR00425, UCLA/Cedars-Sinai/Harbor/Drew Clinical and Translational Science Institute (CTSI) Grant (UL1 TR000124-01), the Southern California Diabetes and Endocrinology Research Grant (DERC) (DK063491), The Helmsley Foundation (D.P.M.) and the Crohn's and Colitis Foundation of America (D.P.M.). R.J.X. and A.N.A. are funded by DK83756, AI062773, DK043351 and the Helmsley Foundation. The Netherlands Organization for Scientific Research supported R.K.W. with a clinical fellowship grant (90.700.281) and C.W. (VICI grant 918.66.620). C.W. is also supported by the Celiac Disease Consortium (BSIK03009). This study was also supported by the German Ministry of Education and Research through the National Genome Research Network, the Popgen biobank, through the Deutsche Forschungsgemeinschaft (DFG) cluster of excellence 'Inflammation at Interfaces' and DFG grant no. FR 2821/2-1. S.B. was supported by DFG BR 1912/6-1 and the Else Kröner-Fresenius-Stiftung (Else Kröner-Exzellenzstipendium 2010_EKES.32). Italian case collections were supported by the Italian Group for IBD and the Italian Society for Paediatric Gastroenterology, Hepatology and Nutrition and funded by the Italian Ministry of Health GR-2008-1144485. Activities in Sweden were supported by the Swedish Society of Medicine, Ihre Foundation, Örebro University Hospital Research Foundation, Karolinska Institutet, the Swedish National Program for IBD Genetics, the Swedish Organization for IBD, and the Swedish Medical Research Council. D.F. and S.V. are senior clinical investigators for the Funds for Scientific Research (FWO/FNRS) Belgium. We acknowledge a grant from Viborg Regional Hospital, Denmark. V. Andersen was supported by SHS Aabenraa, Denmark. We acknowledge funding provided by the Royal Brisbane and Women's Hospital Foundation, National Health and Medical Research Council, Australia and by the European Community (5th PCRDT). We acknowledge the following groups that provided biological samples or data for this study: the Inflammatory Bowel in South Eastern Norway (IBSEN) study group, the Norwegian Bone Marrow Donor Registry (NMBDR), the Avon Longitudinal Study of Parents and Children, the Human Biological Data Interchange and Diabetes UK, and Banco Nacional de ADN, Salamanca. This research also uses resources provided by the Type 1 Diabetes Genetics Consortium, a collaborative clinical study sponsored by the NIDDK, National Institute of Allergy and Infectious Diseases (NIAID), National Human Genome Research Institute (NHGRI), National Institute of Child Health and Human Development (NICHD), and Juvenile Diabetes Research Foundation (JDRF) and supported by U01 DK062418. The KORA study was initiated and financed by the Helmholtz Zentrum München – German Research Center for Environmental Health, which is funded by the German Federal Ministry of Education and Research (BMBF) and by the State of Bavaria. KORA research was supported within the Munich Center of

©2012 Macmillan Publishers Limited. All rights reserved

Health Sciences (MC Health), Ludwig-Maximilians-Universität, as part of LMUinnovativ.

Author Contributions R.K.W., R.H.D., D.P.M., C.G.M., J.D.R., E.E.S., M.J.D., A.F., M.P. and S.V. contributed equally to the manuscript. J.H.C., J.C.B., R.K.W., R.H.D., D.P.M., A.F., M.P., C.G.M., J.D.R., S.V., M.D.A. and V. Annese conceived, designed and managed the study and managed the funding. J.H.C., J.C.B., L.J., S. Ripke, R.K.W., R.H.D., D.P.M., M.J.D., M.P. and C.G.M. were involved in manuscript preparation. J.H.C., J.C.B., L.J., S. Ripke, R.K.W., K.Y.H., C.A.A., J.E., K.N., S.L.S., S. Raychaudhuri, Z.W., C.A., A.C., G.B., M.H., X.H., B.Z., C.K.Z., H.Z., J.D.R., E.E.S. and M.J.D. performed or supervised statistical and computational analyses. R.K.W., R.H.D., D.P.M., J.C.L., L.P.S., Y.S., P.G., J.-P.A., T.A., L.A., A.N.A., V. Andersen, J.M.A., L.B., P.A.B., A.B., S.B., C.B., S.C., M.D.A., D.D.J., K.L.D., M.D., C.E., L.R.F., D.F., M.G., C.G., R.G., J.G., A.H., C.H., T.H.K., L.K., S.K., A.L., D.L., E.L., I.C.L., C.W.L., A.R.M., C.M., G.M., J.M., W.N., O.P., C.Y.P., U.P., N.J.P., M.R., J.I.R., R.K.R., J.D.S., M.S., J. Satsangi, S.S., L.A.S., J. Sventoraityte, S.R.T., M.T., H.W.V., M.D.V., C.W., D.C.W., J.W., R.J.X., S.Z., M.S.S., V. Annese, H.H., IIBDGC, S.R.B., J.D.R., G.R.S., C.G.M., A.F., M.P., S.V. and J.H.C. were involved in study subject recruitment and assembling phenotypic data. R.K.W., R.H.D., D.P.M., L.P.S., Y.S., M.M., I.C., E.T., T.B., D.E., K.F., T.H., K.D.T., C.G.M., A.F., M.P. and J.H.C. established DNA collections, genotyping and data management. All authors read and approved the final manuscript before submission.

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).

Luke Jostins[1]*, Stephan Ripke[2,3]*, Rinse K. Weersma[4], Richard H. Duerr[5,6], Dermot P. McGovern[7,8], Ken Y. Hui[9], James C. Lee[10], L. Philip Schumm[11], Yashoda Sharma[12], Carl A. Anderson[1], Jonah Essers[13], Mitja Mitrovic[14,15], Kaida Ning[12], Isabelle Cleynen[16], Emilie Theatre[17,18], Sarah L. Spain[19], Soumya Raychaudhuri[20,21,22], Philippe Goyette[23], Zhi Wei[24], Clara Abraham[12], Jean-Paul Achkar[25,26], Tariq Ahmad[27], Leila Amininejad[28], Ashwin N. Ananthakrishnan[29], Vibeke Andersen[30], Jane M. Andrews[31], Leonard Baidoo[5], Tobias Balschun[32], Peter A. Bampton[33], Alain Bitton[34], Gabrielle Boucher[23], Stephan Brand[35], Carsten Büning[36], Ariella Cohain[37], Sven Cichon[38], Mauro D'Amato[39], Dirk De Jong[4], Kathy L. Devaney[29], Marla Dubinsky[40], Cathryn Edwards[41], David Ellinghaus[32], Lynnette R. Ferguson[42], Denis Franchimont[28], Karin Fransen[5,43], Richard Gearry[44,45], Michel Georges[17], Christian Gieger[46], Jürgen Glas[34], Talin Haritunians[8], Ailsa Hart[47], Chris Hawkey[48], Matija Hedl[12], Xinli Hu[20], Tom H. Karlsen[49], Limas Kupcinskas[50], Subra Kugathasan[51], Anna Latiano[52], Debby Laukens[53], Ian C. Lawrance[54], Charlie W. Lees[55], Edouard Louis[18], Gillian Mahy[56], John Mansfield[57], Angharad R. Morgan[42], Craig Mowat[58], William Newman[59], Orazio Palmieri[52], Cyriel Y. Ponsioen[60], Uros Potocnik[14,61], Natalie J. Prescott[19], Miguel Regueiro[5], Jerome I. Rotter[8], Richard K. Russell[62], Jeremy D. Sanderson[63], Miquel Sans[64,65], Jack Satsangi[55], Stefan Schreiber[32,66], Lisa A. Simms[67], Jurgita Sventoraityte[50], Stephan R. Targan[7], Kent D. Taylor[7,8], Mark Tremelling[68], Hein W. Verspaget[69], Martine De Vos[53], Cisca Wijmenga[43], David C. Wilson[62,70], Juliane Winkelmann[71], Ramnik J. Xavier[29,72], Sebastian Zeissig[66], Bin Zhang[37], Clarence K. Zhang[73], Hongyu Zhao[73], The International IBD Genetics Consortium (IIBDGC)†, Mark S. Silverberg[74], Vito Annese[52,75], Hakon Hakonarson[76,77], Steven R. Brant[78], Graham Radford-Smith[67,79], Christopher G. Mathew[19], John D. Rioux[23], Eric E. Schadt[37], Mark J. Daly[2,3], Andre Franke[32], Miles Parkes[10], Severine Vermeire[16,80], Jeffrey C. Barrett[1]* & Judy H Cho[9,12]*

[1]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge CB10 1HH, UK. [2]Analytic and Translational Genetics Unit, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, USA. [3]Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, USA. [4]Department of Gastroenterology and Hepatology, University of Groningen and University Medical Center Groningen, Groningen 9700 RB, The Netherlands. [5]Division of Gastroenterology, Hepatology and Nutrition, Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA. [6]Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania 15261, USA. [7]F. Widjaja Foundation Inflammatory Bowel and Immunobiology Research Institute, Los Angeles, California 90048, USA. [8]Medical Genetics Institute, Cedars-Sinai Medical Center, Los Angeles, California 90048, USA. [9]Department of Genetics, Yale School of Medicine, New Haven, Connecticut 06520, USA. [10]Inflammatory Bowel Disease Research Group, Addenbrooke's Hospital, University of Cambridge, Cambridge CB2 0QQ, UK. [11]Department of Health Studies, University of Chicago, Chicago, Illinois 60637, USA. [12]Department of Internal Medicine, Section of Digestive Diseases, Yale School of Medicine, New Haven, Connecticut 06520, USA. [13]Center for Human Genetic Research, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, USA. [14]University of Maribor, Faculty of Medicine, Center for Human Molecular Genetics and Pharmacogenomics, Maribor 2000, Slovenia. [15]University Medical Center Groningen, Department of Genetics, Groningen 9700 RB, The Netherlands. [16]Department of Clinical and Experimental Medicine, Gastroenterology section, KU Leuven, Leuven 3000, Belgium. [17]Unit of Animal Genomics, Groupe Interdisciplinaire de Génoprotéomique Appliquée (GIGA-R) and Faculty of Veterinary Medicine, University of Liège, Liège 4000, Belgium. [18]Division of Gastroenterology, Centre Hospitalier Universitaire, Université de Liège, Liège 4000, Belgium. [19]Department of Medical and Molecular Genetics, Division of Genetics and Molecular Medicine, King's College London School of Medicine, Guy's Hospital, London SE1 9RT, UK. [20]Division of Rheumatology Immunology and Allergy, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA. [21]Program in Medical and Population Genetics, Broad Institute, Cambridge, Massachusetts 02142, USA. [22]Division of Genetics, Brigham and Women's Hospital, Boston, Massachusetts 02115, USA. [23]Université de Montréal and the Montreal Heart Institute, Research Center, Montréal, Québec H1T 1C8, Canada. [24]Department of Computer Science, New Jersey Institute of Technology, Newark, New Jersey 07102, USA. [25]Department of Gastroenterology & Hepatology, Digestive Disease Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA. [26]Department of Pathobiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio 44195, USA. [27]Peninsula College of Medicine and Dentistry, Exeter EX1 2LU, UK. [28]Erasmus Hospital, Free University of Brussels, Department of Gastroenterology, Brussels, 1070 Belgium. [29]Massachusetts General Hospital, Harvard Medical School, Gastroenterology Unit, Boston, Massachusetts 02114, USA. [30]Viborg Regional Hospital, Medical Department, Viborg 8800, Denmark. [31]Inflammatory Bowel Disease Service, Department of Gastroenterology and Hepatology, Royal Adelaide Hospital, and School of Medicine, University of Adelaide, Adelaide 5000, Australia. [32]Institute of Clinical Chemistry, Christian-Albrechts-University, Kiel 24105, Germany. [33]Department of Gastroenterology and Hepatology, Flinders Medical Centre and School of Medicine, Flinders University, Adelaide 5000, Australia. [34]Division of Gastroenterology, McGill University Health Centre, Royal Victoria Hospital, Montréal, Québec H3A 1A1, Canada. [35]Department of Medicine II, University Hospital Munich-Grosshadern, Ludwig-Maximilians-University, Munich 80336, Germany. [36]Department of Gastroenterology, Charité, Campus Mitte, Universitätsmedizin Berlin, Berlin 10117, Germany. [37]Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York City, New York 10029, USA. [38]Department of Genomics, Life & Brain Center, University Hospital Bonn, Bonn 53012, Germany. [39]Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm 14 183, Sweden. [40]Department of Pediatrics, Cedars Sinai Medical Center, Los Angeles, California 90048, USA. [41]Torbay Hospital, Department of Gastroenterology, Torbay, Devon TQ2 7AA, UK. [42]School of Medical Sciences, Faculty of Medical & Health Sciences, The University of Auckland, Auckland 1142, New Zealand. [43]University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen T9700 RB, The Netherlands. [44]Department of Medicine, University of Otago, Christchurch 8140, New Zealand. [45]Department of Gastroenterology, Christchurch Hospital, Christchurch 8011, New Zealand. [46]Institute of Genetic Epidemiology, Helmholtz Zentrum München - German Research Center for Environmental Health, Neuherberg 85764, Germany. [47]St Mark's Hospital, Watford Road, Harrow, Middlesex HA1 3UJ, UK. [48]Nottingham Digestive Diseases Centre, Queens Medical Centre, Nottingham NG7 1AW, UK. [49]Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo 0424, Norway. [50]Kaunas University of Medicine, Department of Gastroenterology, Kaunas 44307, Lithuania. [51]Department of Pediatrics, Emory University School of Medicine, Atlanta, Georgia 30322, USA. [52]Unit of Gastroenterology, Istituto di Ricovero e Cura a Carattere Scientifico-Casa Sollievo della Sofferenza (IRCCS-CSS) Hospital, San Giovanni Rotondo 71013, Italy. [53]Ghent University Hospital, Department of Gastroenterology and Hepatology, Ghent 9000, Belgium. [54]School of Medicine and Pharmacology, The University of Western Australia, Fremantle, Western Australia 6009, Australia. [55]Gastrointestinal Unit, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK. [56]Department of Gastroenterology, The Townsville Hospital, Townsville, Queensland 4810, Australia. [57]Institute of Human Genetics, Newcastle University, Newcastle upon Tyne NE1 7RU, UK. [58]Department of Medicine, Ninewells Hospital and Medical School, Dundee DD1 9SY, UK. [59]Genetic Medicine, MAHSC, University of Manchester, Manchester M13 9PL, UK. [60]Academic Medical Center, Department of Gastroenterology, Amsterdam 1105 AZ, The Netherlands. [61]University of Maribor, Faculty for Chemistry and Chemical Engineering, Maribor 2000, Slovenia. [62]Royal Hospital for Sick Children, Paediatric Gastroenterology and Nutrition, Glasgow G3 8SJ, UK. [63]Guy's & St Thomas' NHS Foundation Trust, St Thomas' Hospital, Department of Gastroenterology, London SE1 7EH, UK. [64]Department of Gastroenterology, Hospital Clinic/Institut d'Investigacions Biomédiques August Pi i Sunyer (IDIBAPS), Barcelona, Spain. [65]Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBER-EHD), Barcelona 08036, Spain. [66]Department for General Internal Medicine, Christian-Albrechts-University, Kiel, Kiel 24118, Germany. [67]Inflammatory Bowel Diseases, Genetics and Computational Biology, Queensland Institute of Medical Research, Brisbane 4029, Australia. [68]Norfolk and Norwich University Hospital, Norwich NR4 7UY, UK. [69]Department of Gastroenterology, Leiden University Medical Center, Leiden 2333 ZA, The Netherlands. [70]Child Life and Health, University of Edinburgh, Edinburgh, Scotland EH9 1UW, UK. [71]Institute of Human Genetics and Department of Neurology, Technische Universität München, Munich 80336, Germany. [72]Center for Computational and Integrative Biology, Massachusetts General Hospital, Boston, Massachusetts 02114, USA. [73]Department of Biostatistics, School of Public Health, Yale University, New Haven, Connecticut 06520, USA. [74]Mount Sinai Hospital Inflammatory Bowel Disease Centre, University of Toronto, Toronto, Ontario M5G 1X5, Canada. [75]Azienda Ospedaliero Universitaria (AOU) Careggi, Unit of Gastroenterology SOD2, Florence 50134, Italy. [76]Center for Applied Genomics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. [77]Department of Pediatrics, Center for Pediatric Inflammatory Bowel Disease, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. [78]Meyerhoff Inflammatory Bowel Disease Center, Department of Medicine, School of Medicine, and Department of Epidemiology, Bloomberg School of Public Health, Johns Hopkins University, Baltimore, Maryland 21205, USA. [79]Department of Gastroenterology, Royal Brisbane and Women's Hospital, and School of Medicine, University of Queensland, Brisbane 4029, Australia. [80]Department of Gastroenterology, University Hospital Leuven, Leuven 3000, Belgium.

*These authors contributed equally to this work.
†Lists of participants and their affiliations appear in the Supplementary Information.

©2012 Macmillan Publishers Limited. All rights reserved

Journal of Crohn's and Colitis (2012) 6, 628–629

Available online at www.sciencedirect.com

SciVerse ScienceDirect

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

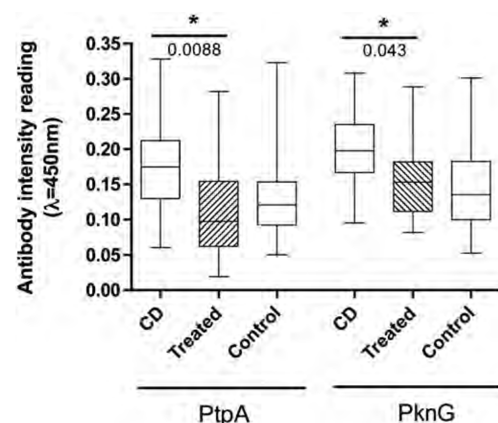


Figure 1 Distribution of the antibody intensity readings. A boxplot analysis shows the distribution of the antibody intensity readings obtained by ELISA in CD patients, CD patients under treatment with infliximab, and healthy controls (n = 20 individuals in each category). *P-value obtained using Wilconox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

1873-9946/$ - see front matter © 2012 European Crohn's and Colitis Organisation. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.crohns.2012.01.011

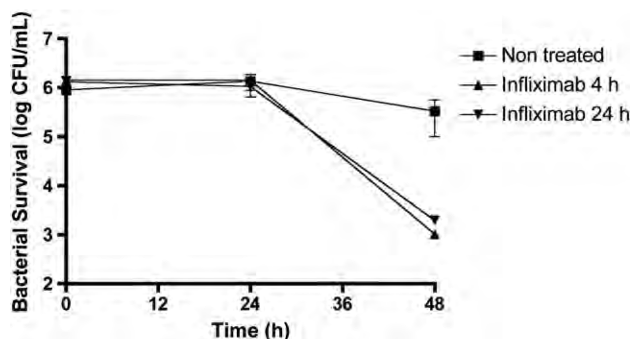


Figure 2 Survival of MAP in infected human macrophages THP-1 cell lines treated with infliximab (5 μg/mL) for 4 h (triangle) and 24 h (inverted triangle). Non-treated cells (square) were used as control. Shown are the mean values (±SD) of three independent experiments. CFU = Colony Forming Unit.

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.

References

1. Abubakar I, Myhill D, Aliyu SH, Hunter PR. Detection of *Mycobacterium avium* subspecies *paratuberculosis* from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis. *Inflamm Bowel Dis* 2008;**14**(3):401–10.
2. Autschbach F, Eisold S, Hinz U, Zinser S, Linnebacher M, Giese T, et al. High prevalence of *Mycobacterium avium* subspecies *paratuberculosis* IS900 DNA in gut tissues from individuals with Crohn's disease. *Gut* 2005;**54**(7):944–9.
3. Naser SA, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium* subspecies *paratuberculosis* from the blood of patients with Crohn's disease. *Lancet* 2004;**364**(9439):1039–44.
4. Naser SA, Schwartz D, Shafran I. Isolation of *Mycobacterium avium* subsp *paratuberculosis* from breast milk of Crohn's disease patients. *Am J Gastroenterol* 2000;**95**(4):1094–5.
5. Clancy R, Ren Z, Turton J, Pang G, Wettstein A. Molecular evidence for *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in Crohn's disease correlates with enhanced TNFalpha secretion. *Dig Liver Dis* 2007;**39**(5):445–51.
6. Nakase H, Tamaki H, Matsuura M, Chiba T, Okazaki K. Involvement of *Mycobacterium avium* subspecies *paratuberculosis* in TNF-α production from macrophage: possible link between MAP and immune response in Crohn's disease. *Inflamm Bowel Dis* 2011;**17**(2):E140–2.
7. Bach H, Sun J, Hmama Z, Av-Gay Y. Mycobacterium avium subsp. paratuberculosis PtpA is an endogenous tyrosine phosphatase secreted during infection. *Infect Immun* 2006;**74**(12):6540–6.
8. Bach H, Ko HH, Raizman EA, Attarian R, Cho B, Biet F, et al. Immunogenicity of *Mycobacterium avium* subsp. *paratuberculosis* proteins in Crohn's disease patients. *Scand J Gastroenterol* 2011;**46**(1):30–9.
9. Bach H, Papavinasasundaram KG, Wong D, Hmama Z, Av-Gay Y. *Mycobacterium tuberculosis* virulence is mediated by PtpA dephosphorylation of human vacuolar protein sorting 33B. *Cell Host Microbe* 2008;**3**(5):316–22.
10. Walburger A, Koul A, Ferrari G, Nguyen L, Prescianotto-Baschong C, Huygen K, et al. Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* 2004;**304**(5678):1800–4.

Horacio Bach
Division of Infectious Diseases, Department of Medicine, University of British Columbia, Vancouver, BC, Canada
*Corresponding author.
E-mail address: horacio.bach@gmail.com.

Greg Rosenfeld
Brian Bressler
Division of Gastroenterology, Department of Medicine, University of British Columbia, Vancouver, BC, Canada

12 January 2012

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

Ingrid Olsen[1,2]*, Stig Tollefsen[2], Claus Aagaard[3], Liv J. Reitan[1], John P. Bannantine[4], Peter Andersen[3], Ludvig M. Sollid[2], Knut E. A. Lundin[2,5]

1 Department of Animal Health, National Veterinary Institute, Oslo, Norway, 2 Centre for Immune Regulation, Institute of Immunology, University of Oslo and Rikshospitalet University Hospital, Oslo, Norway, 3 Department of Infectious Disease Immunology, Statens Serum Institut, Copenhagen, Denmark, 4 National Animal Disease Center, USDA-Agricultural Research Service, Ames, Iowa, United States of America, 5 Department of Medicine, Rikshospitalet University Hospital, Oslo, Norway

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.

Citation: Olsen I, Tollefsen S, Aagaard C, Reitan LJ, Bannantine JP, et al. (2009) Isolation of *Mycobacterium avium* Subspecies *paratuberculosis* Reactive CD4 T Cells from Intestinal Biopsies of Crohn's Disease Patients. PLoS ONE 4(5): e5641. doi:10.1371/journal.pone.0005641

Editor: Derya Unutmaz, New York University School of Medicine, United States of America

Received March 23, 2009; **Accepted** April 27, 2009; **Published** May 22, 2009

This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Funding: EU FP6 ParaTBTools Contract No 023106 (to I.O. and C.A.), the Broad Medical Research Program of The Broad Foundation (to I.O.), The Research Council of Norway (to L.M.S.), USDA-ARS and JDIP (to J.P.B.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ingrid.olsen@vetinst.no

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 $\alpha\beta$ FITC (IgM, clone T10B9.1A-31), anti-CCR6 PE (IgG1κ, clone 11A9), anti-TCR$\gamma\delta$ 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^{TM} Medicon, 35 μm Sterile). The single cells from each biopsy were centrifuged, dissolved in 1 ml complete medium containing 2×10^6 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 1×10^6 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

doi:10.1371/journal.pone.0005641.t001

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 10th and 11th 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
doi:10.1371/journal.pone.0005641.t002

(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*
doi:10.1371/journal.pone.0005641.t003

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.

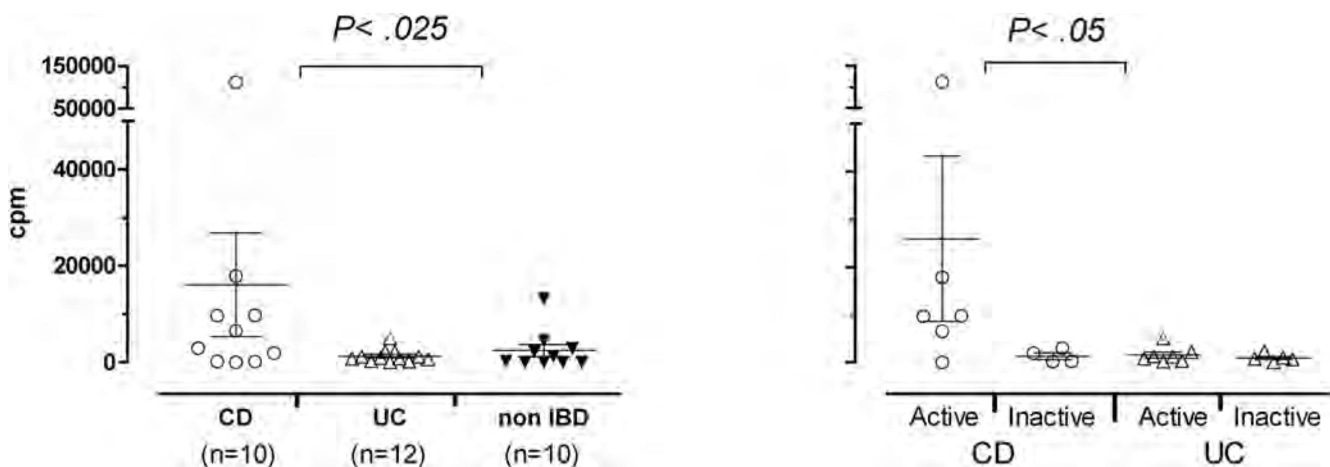


Figure 1. T-cell responses to MAP. Autologous adherent cells were incubated with antigen overnight and T cells were added followed by incubation for three days. ^{3}H thymidine was added for the last 20 hours, and proliferation was assessed by scintillation counting after harvesting of the cultures. 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.
doi:10.1371/journal.pone.0005641.g001

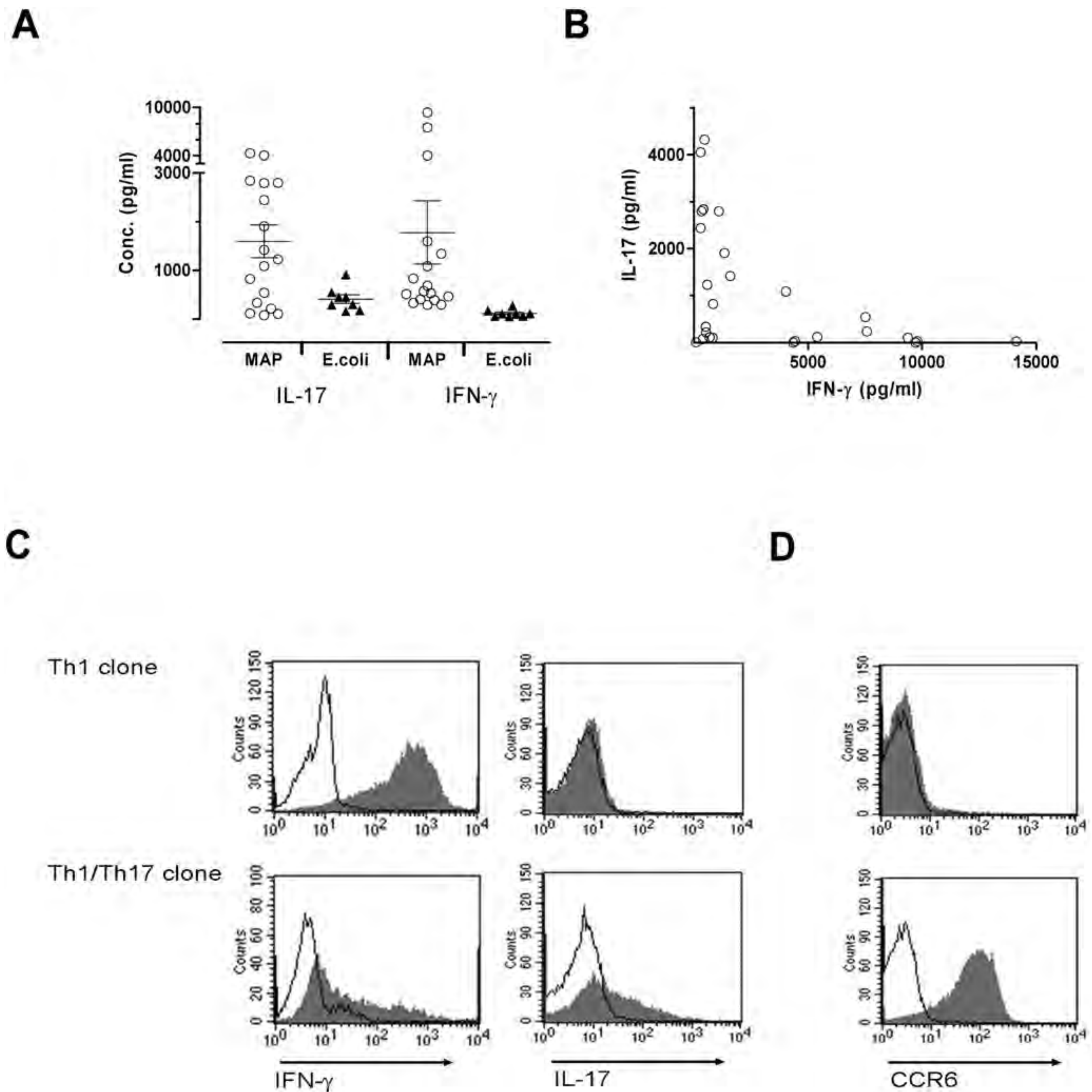


Figure 2. Cytokine responses in T-cell clones. A) Comparison of cytokine response in MAP reactive (n = 17) and *E. coli* reactive (n = 8) T-cell clones from the same CD patient (CD-46). T-cell clones were stimulated with MAP or *E. coli* antigens (10 µg/ml) for 48 hours using HLA class II matched irradiated 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.
doi:10.1371/journal.pone.0005641.g002

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*

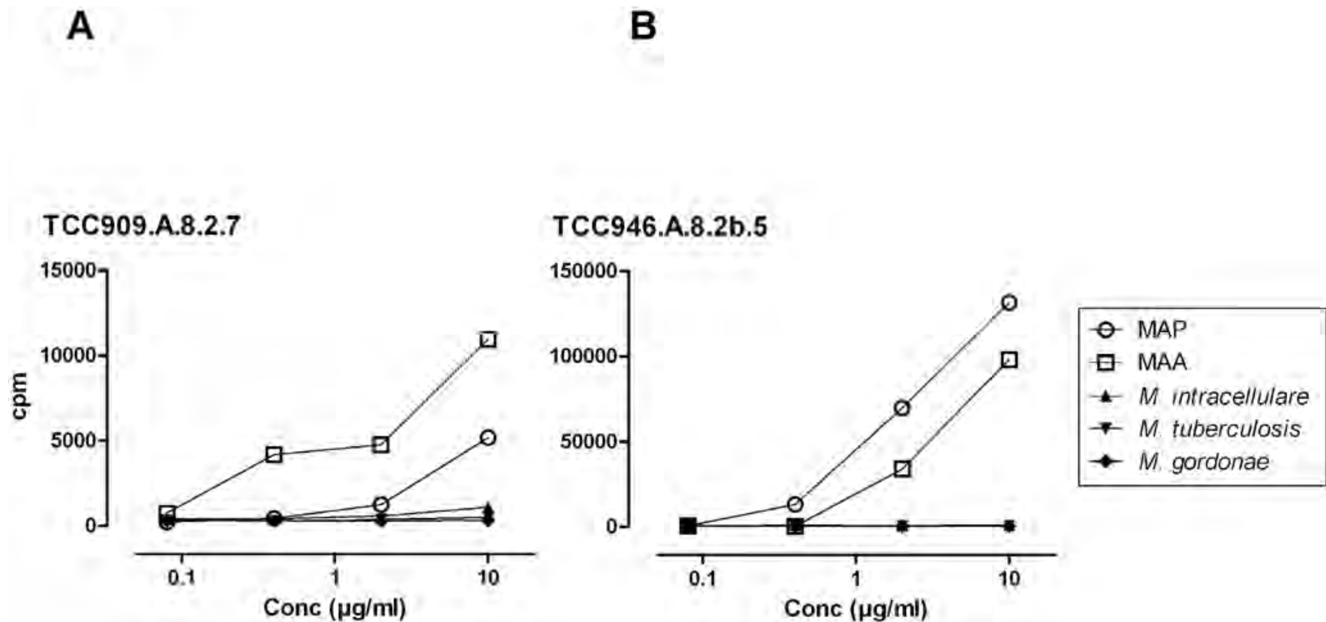


Figure 3. Responses of T-cell clones to various mycobacteria. Proliferation of T-cell clones from two different CD patients (CD-9 and CD-46) after stimulation with crude antigen preparations from various mycobacteria. HLA-II matched adherent cells were used as APC and were incubated with antigen overnight. 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.
doi:10.1371/journal.pone.0005641.g003

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

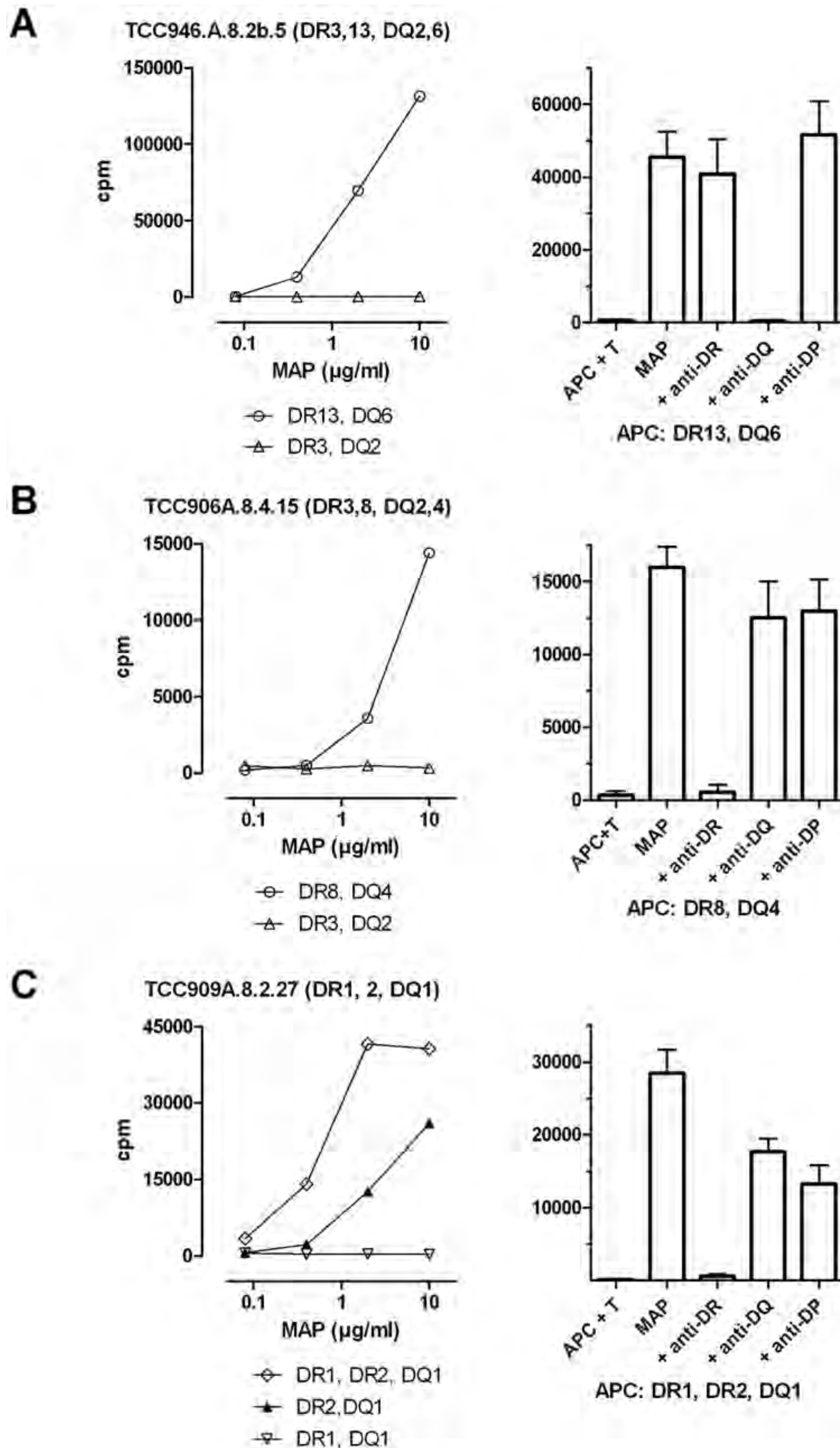
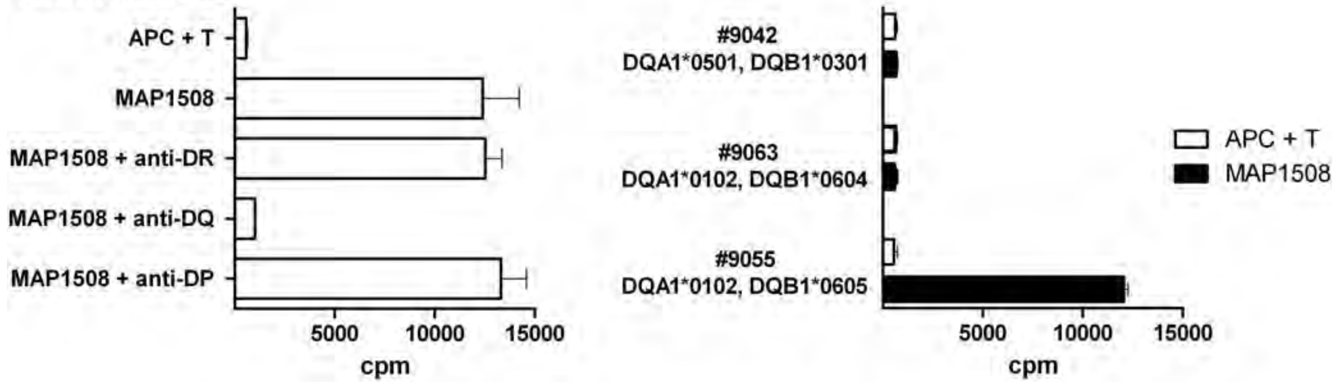


Figure 4. HLA restriction of T-cell clones. A) Patient CD-46, B) Patient CD-6 and C) Patient CD-9. The individual patients HLA DR/DQ serotype of the clone donor is given in brackets after the clone identification tag. Adherent cells from HLAII DR/DQ haplotype matched donors were used as APC and incubated 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



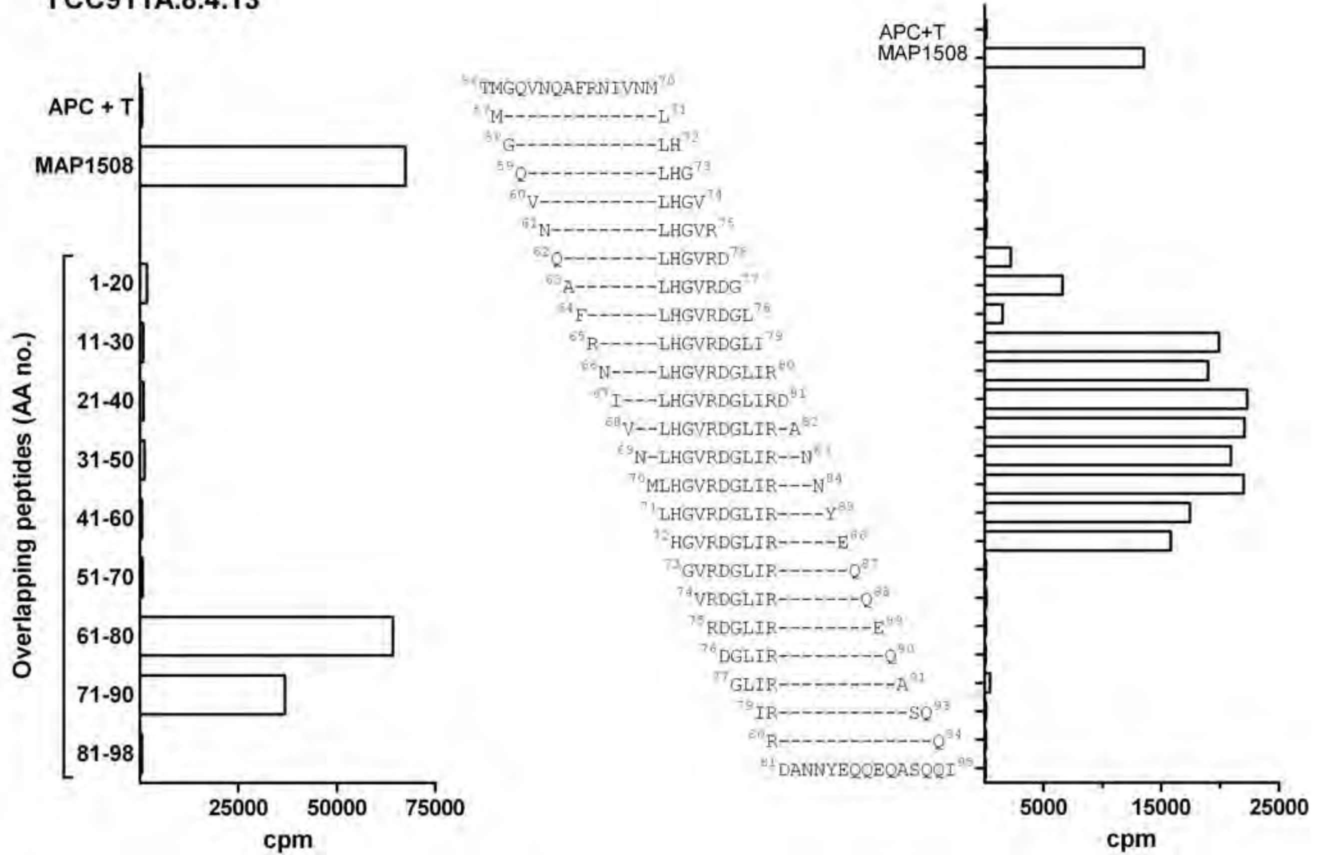


Figure 5. Epitope mapping of a MAP1508 specific T-cell clone. A) HLA-II restriction of TCC911.A.8.4.13. Left; blocking of responses by HLA class II specific monoclonal antibodies. Right; HLA-DQ matched EBV cells were used as APC to identify HLA restriction. The patient was DQB1*0609 and DQB1*0301. Error 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.

doi:10.1371/journal.pone.0005641.g005

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)

Acknowledgments

We thank the subjects donating biological material, and Tore Jensen and Anne Kari Tveter for the HLA-typing.

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.

References

1. Baumgart DC, Carding SR (2007) Inflammatory bowel disease: cause and immunobiology. Lancet 369: 1627–1640.
2. Ogura Y, Bonen DK, Inohara N, Nicolae DL, Chen FF, et al. (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. Nature 411: 603–606.
3. Hugot JP, Chamaillard M, Zouali H, Lesage S, Cezard JP, et al. (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. Nature 411: 599–603.
4. Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, et al. (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. Nat Genet 39: 207–211.
5. Parkes M, Barrett JC, Prescott NJ, Tremelling M, Anderson CA, et al. (2007) Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. Nat Genet 39: 830–832.
6. Eckmann L, Karin M (2005) NOD2 and Crohn's disease: loss or gain of function? Immunity 22: 661–667.
7. Strober W, Fuss I, Mannon P (2007) The fundamental basis of inflammatory bowel disease. J Clin Invest 117: 514–521.
8. Cadwell K, Liu JY, Brown SL, Miyoshi H, Loh J, et al. (2008) A key role for autophagy and the autophagy gene Atg16l1 in mouse and human intestinal Paneth cells. Nature 456: 259–263.
9. Rich KA, Burkett C, Webster P (2003) Cytoplasmic bacteria can be targets for autophagy. Cell Microbiol 5: 455–468.
10. Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, et al. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. Nat Genet 39: 596–604.
11. Singh SB, Davis AS, Taylor GA, Deretic V (2006) Human IRGM induces autophagy to eliminate intracellular mycobacteria. Science 313: 1438–1441.
12. Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, et al. (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. Nat Genet 40: 955–962.
13. Boudeau J, Glasser AL, Masseret E, Joly B, Darfeuille-Michaud A (1999) Invasive ability of an *Escherichia coli* strain isolated from the ileal mucosa of a patient with Crohn's disease. Infect Immun 67: 4499–4509.
14. Feller M, Huwiler K, Stephan R, Altpeter E, Shang A, et al. (2007) *Mycobacterium avium* subspecies *paratuberculosis* and Crohn's disease: a systematic review and meta-analysis. Lancet Infect Dis 7: 607–613.
15. Abubakar I, Myhill D, Aliyu SH, Hunter PR (2008) Detection of *Mycobacterium avium* subspecies *paratuberculosis* from patients with Crohn's disease using nucleic acid-based techniques: a systematic review and meta-analysis. Inflamm Bowel Dis 14: 401–410.
16. Annunziato F, Cosmi L, Santarlasci V, Maggi L, Liotta F, et al. (2007) Phenotypic and functional features of human Th17 cells. J Exp Med 204: 1849–1861.
17. Duchmann R, Marker-Hermann E, Meyer zum Buschenfelde KH (1996) Bacteria-specific T-cell clones are selective in their reactivity towards different enterobacteria or *H. pylori* and increased in inflammatory bowel disease. Scand J Immunol 44: 71–79.
18. Duchmann R, May E, Heike M, Knolle P, Neurath M, et al. (1999) T cell specificity and cross reactivity towards enterobacteria, *Bacteroides*, *Bifidobacterium*, and antigens from resident intestinal flora in humans. Gut 44: 812–818.
19. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.
20. Olsen I, Reitan LJ, Holstad G, Wiker HG (2000) Alkyl hydroperoxide reductases C and D are major antigens constitutively expressed by *Mycobacterium avium* subsp. *paratuberculosis*. Infect Immun 68: 801–808.
21. Molberg O, Solheim FN, Jensen T, Lundin KE, rentz-Hansen H, et al. (2003) Intestinal T-cell responses to high-molecular-weight glutenins in celiac disease. Gastroenterology 125: 337–344.
22. Molberg O, McAdam S, Lundin KE, Sollid LM (2000) Studies of Gliadin-Specific T-cells in Celiac Disease. In: Marsh MN, ed. Celiac Disease Humana Press. pp 105–124.
23. Li L, Bannantine JP, Zhang Q, Amonsin A, May BJ, et al. (2005) The complete genome sequence of *Mycobacterium avium* subspecies *paratuberculosis*. Proc Natl Acad Sci U S A 102: 12344–12349.
24. Seldenrijk CA, Drexhage HA, Meuwissen SG, Meijer CJ (1990) T-cellular immune reactions (in macrophage inhibition factor assay) against *Mycobacterium paratuberculosis*, *Mycobacterium kansasii*, *Mycobacterium tuberculosis*, *Mycobacterium avium* in patients with chronic inflammatory bowel disease. Gut 31: 529–535.
25. Dalton HR, Hoang P, Jewell DP (1992) Antigen induced suppression in peripheral blood and lamina propria mononuclear cells in inflammatory bowel disease. Gut 33: 324–330.
26. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL (2003) Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 278: 1910–1914.
27. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, Muller FJ, Hommes DW, et al. (2007) Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. Immunity 27: 660–669.
28. Azuma I, Thomas DW, Adam A, Ghuysen JM, Bonaly R, et al. (1970) Occurrence of N-glycolylmuramic acid in bacterial cell walls. A preliminary survey. Biochim Biophys Acta 208: 444–451.
29. Raymond JB, Mahapatra S, Crick DC, Pavelka MS Jr (2005) Identification of the namH gene, encoding the hydroxylase responsible for the N-glycolylation of the mycobacterial peptidoglycan. J Biol Chem 280: 326–333.
30. Pinedo PJ, Buergelt CD, Donovan GA, Melendez P, Morel L, et al. (2009) Association between CARD15/NOD2 gene polymorphisms and paratuberculosis infection in cattle. Vet Microbiol. pp 346–352.
31. Behr MA, Wilson MA, Gill WP, Salamon H, Schoolnik GK, et al. (1999) Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science 284: 1520–1523.
32. Garnier T, Eiglmeier K, Camus JC, Medina N, Mansoor H, et al. (2003) The complete genome sequence of *Mycobacterium bovis*. Proc Natl Acad Sci U S A 100: 7877–7882.
33. Hemmer B, Gran B, Zhao Y, Marques A, Pascal J, et al. (1999) Identification of candidate T-cell epitopes and molecular mimics in chronic Lyme disease. Nat Med 5: 1375–1382.
34. Hiemstra HS, Duinkerken G, Benckhuijsen WE, Amons R, De Vries RR, et al. (1997) The identification of CD4+ T cell epitopes with dedicated synthetic peptide libraries. Proc Natl Acad Sci U S A 94: 10313–10318.
35. Coler RN, Dillon DC, Skeiky YA, Kahn M, Orme IM, et al. (2009) Identification of *Mycobacterium tuberculosis* vaccine candidates using human CD4(+) T-cells expression cloning. Vaccine 27: 223–233.
36. van Pinxteren LA, Ravn P, Agger EM, Pollock J, Andersen P (2000) Diagnosis of tuberculosis based on the two specific antigens ESAT-6 and CFP10. Clin Diagn Lab Immunol 7: 155–160.

Bull et al. Veterinary Research 2014, 45:112
http://www.veterinaryresearch.org/content/45/1/112

VETERINARY RESEARCH

RESEARCH Open Access

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

Tim J Bull[1*], Christina Vrettou[2], Richard Linedale[1], Catherine McGuinnes[2], Sam Strain[3], Jim McNair[4], Sarah C Gilbert[5] and Jayne C Hope[2]

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

* Correspondence: tim.bull@sgul.ac.uk
[1]Institute of Infection and Immunity, St. George's University of London, Cranmer Terrace, London SW17 0RE, UK
Full list of author information is available at the end of the article

BioMed Central

© 2014 Bull et al.; licensee BioMed Central Ltd. This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly credited. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

[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 5×10^8 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 5×10^8 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, 5×10^{-5} M β-mercaptoethanol, 50 μg/mL gentamicin). Cell concentrations were estimated using a haemocytometer, adjusted to 1×10^7 cells/mL then aliquoted (5×10^6 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 1×10^2-10^8 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), $5 \times$ 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:

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

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 (2×10^6 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 1×10^7/mL in RPMI medium (RPMI1640, 10% FCS, 50 μg/mL

Hygromycin B) then plated into 96 well flat bottom tissue culture plates at 4×10^5 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 8×10^5 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-γ^+ and $CD8^+$IFN-γ^+ 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.

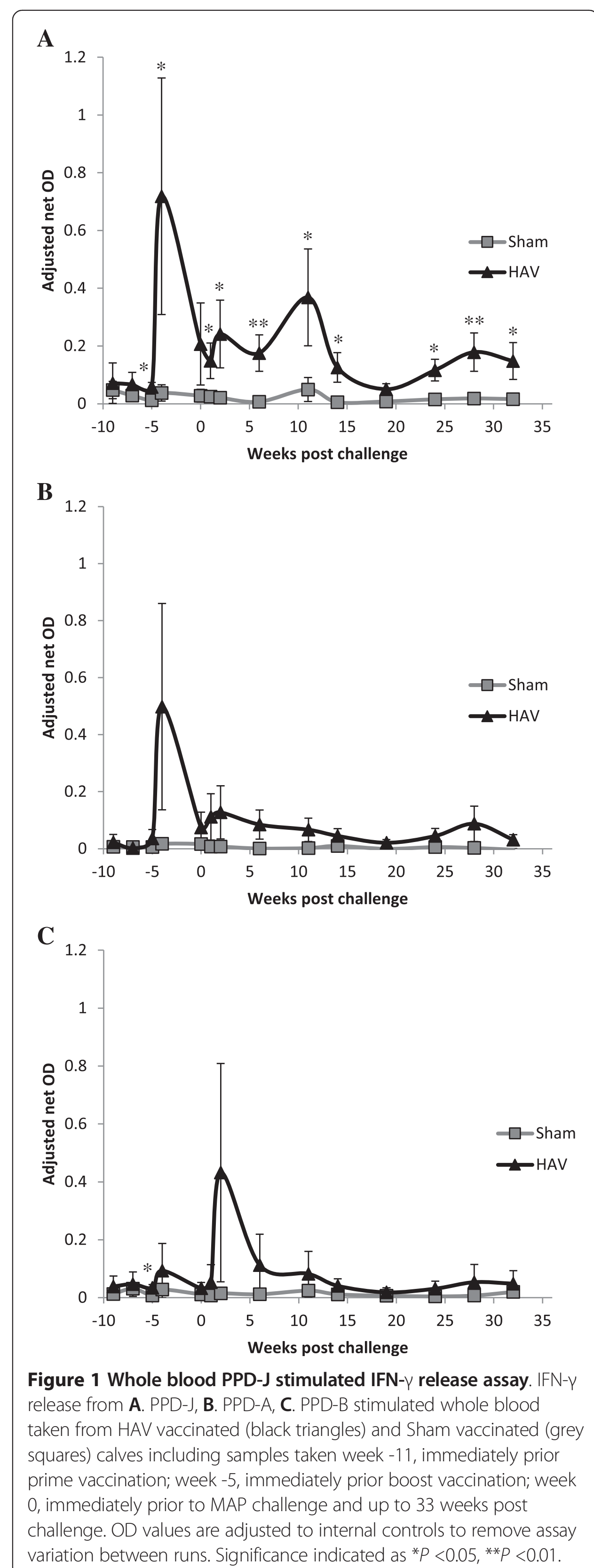


Figure 1 Whole blood PPD-J stimulated IFN-γ release assay. IFN-γ release from **A**. PPD-J, **B**. PPD-A, **C**. PPD-B stimulated whole blood taken from HAV vaccinated (black triangles) and Sham vaccinated (grey squares) calves including samples taken week -11, immediately prior prime vaccination; week -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.

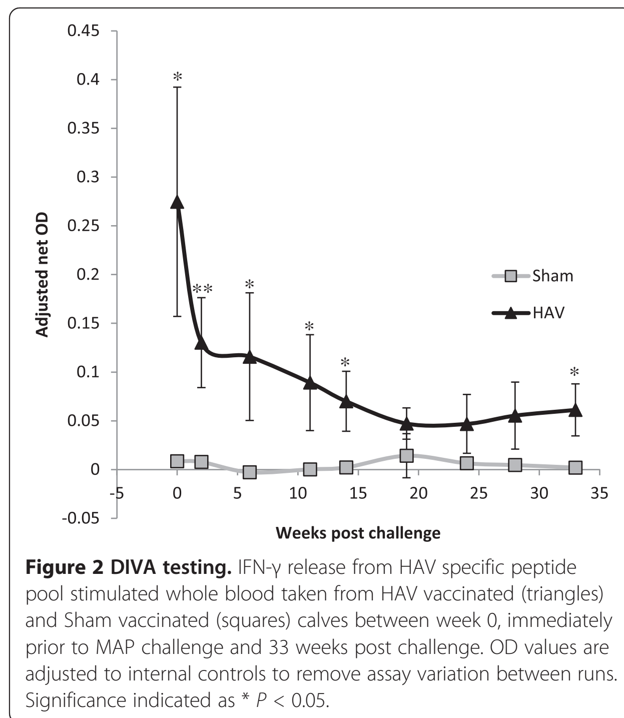


Figure 2 DIVA testing. IFN-γ release from HAV specific peptide pool stimulated whole blood taken from HAV vaccinated (triangles) and Sham vaccinated (squares) calves between week 0, immediately prior to MAP challenge and 33 weeks post challenge. OD values are adjusted to internal controls to remove assay 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-γ^+ 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

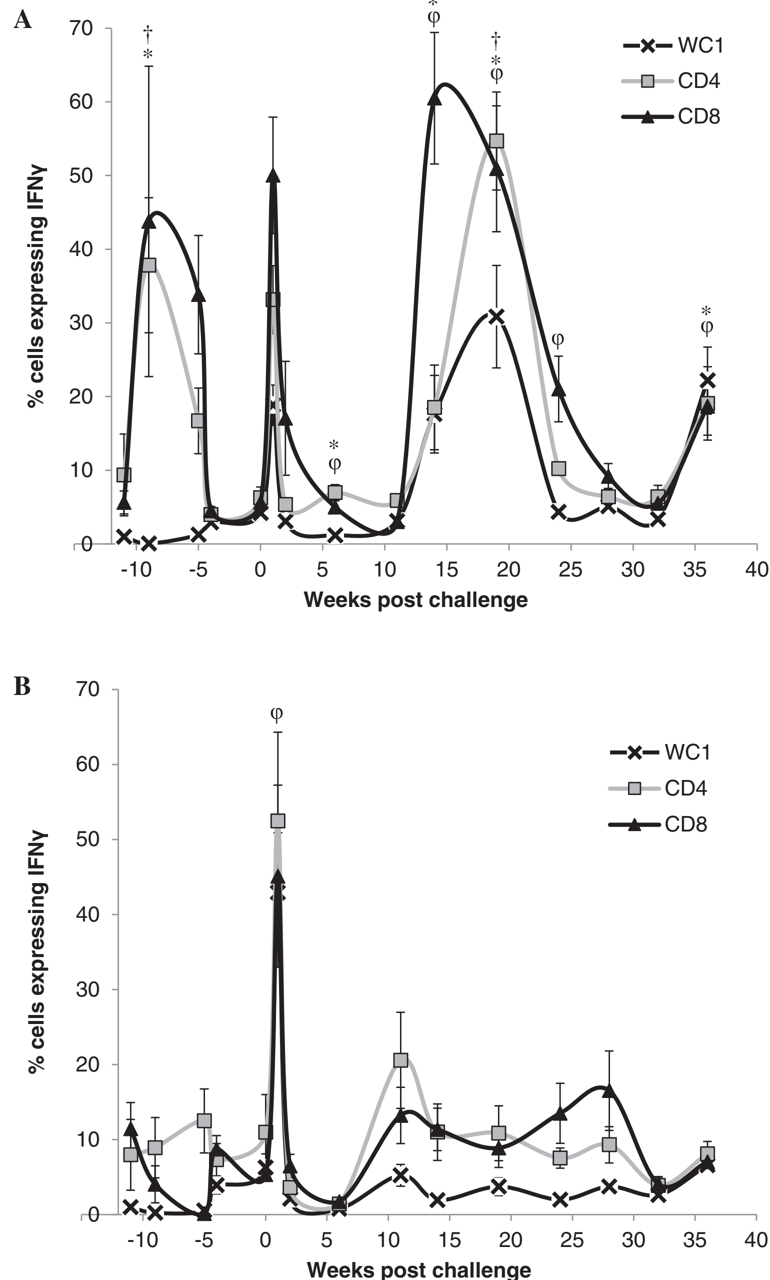


Figure 3 Intracytoplasmic expression of IFN-γ by sub-populations of peripheral T cells in HAV and Sham-vaccinated calves. Percentage of live CD4^{+} (squares), CD8 $^{+}$ (triangles) WC1 $^{+}$ (crosses) expressing IFN-γ after 24 h stimulation with PPD-J from A. HAV vaccinated calves B. Sham vaccinated 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

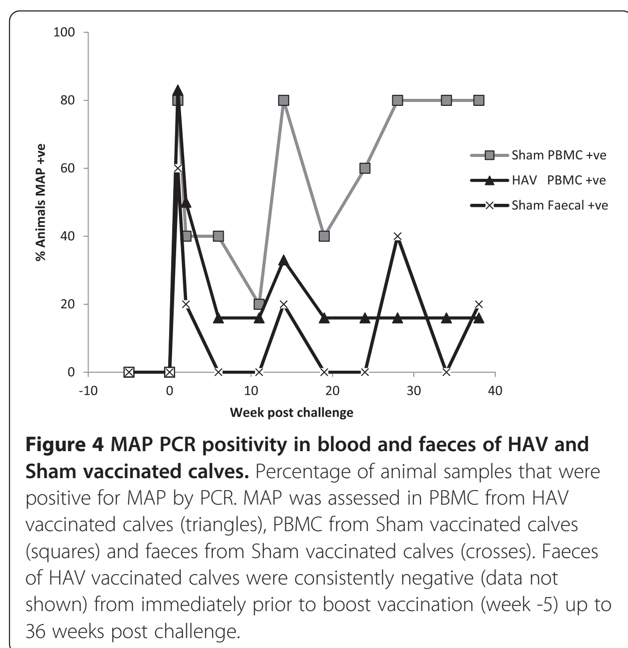


Figure 4 MAP PCR positivity in blood and faeces of HAV and Sham vaccinated calves. Percentage of animal samples that were positive for MAP by PCR. MAP was assessed in PBMC from HAV vaccinated calves (triangles), PBMC from Sham vaccinated calves (squares) and faeces from Sham vaccinated calves (crosses). 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

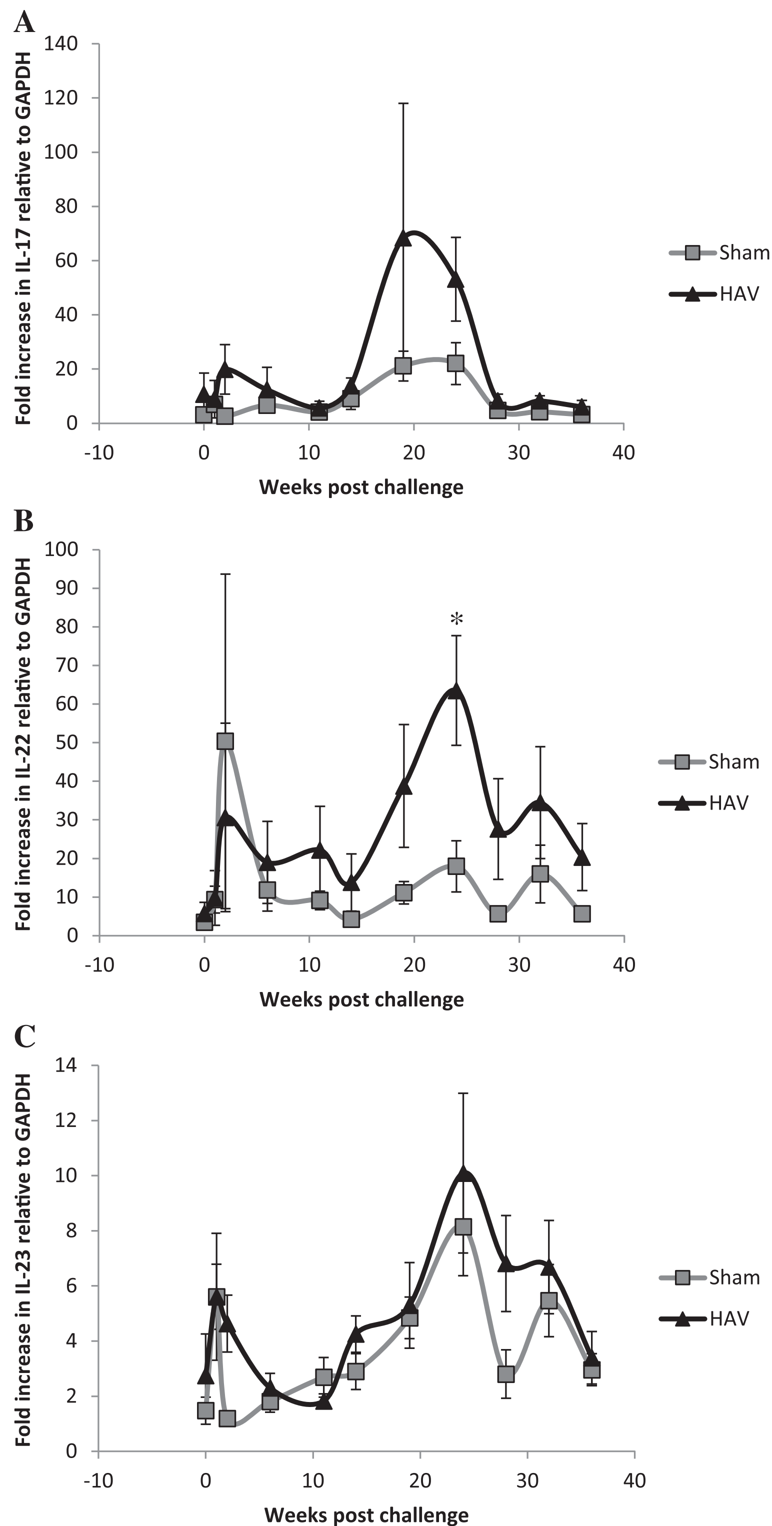


Figure 5 Expression of antigen specific IL-17, IL-22 and IL-23 by PBMC from HAV- and Sham-vaccinated calves. Fold increases, relative to GAPDH. in expression of cytokines **A**. IL-17, **B**. IL-22 and **C**. IL-23 measured by qPCR from RNA extracted from 24 h PPD-J stimulated PBMC isolated from HAV 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 * $P < 0.05$.

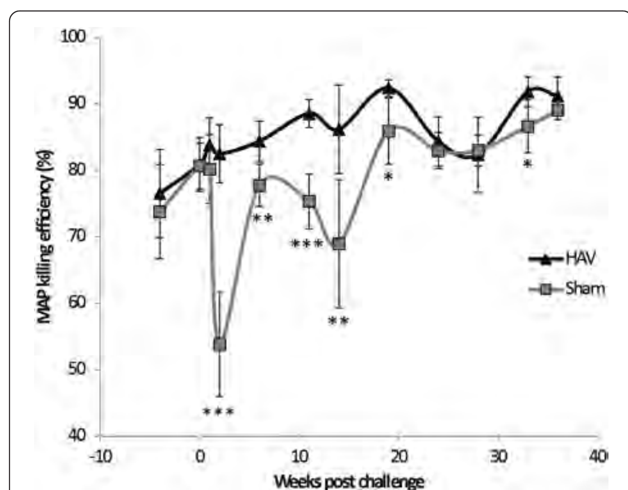


Figure 6 MAP killing efficiency of macrophage fractions. Percentage of an initial MAP inoculum killed after infection for 5 days in macrophages isolated from HAV vaccinated (diamonds) or Sham vaccinated (squares) calves taken immediately prior to HAV boost (week -5) up to 36 weeks post challenge. Values 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

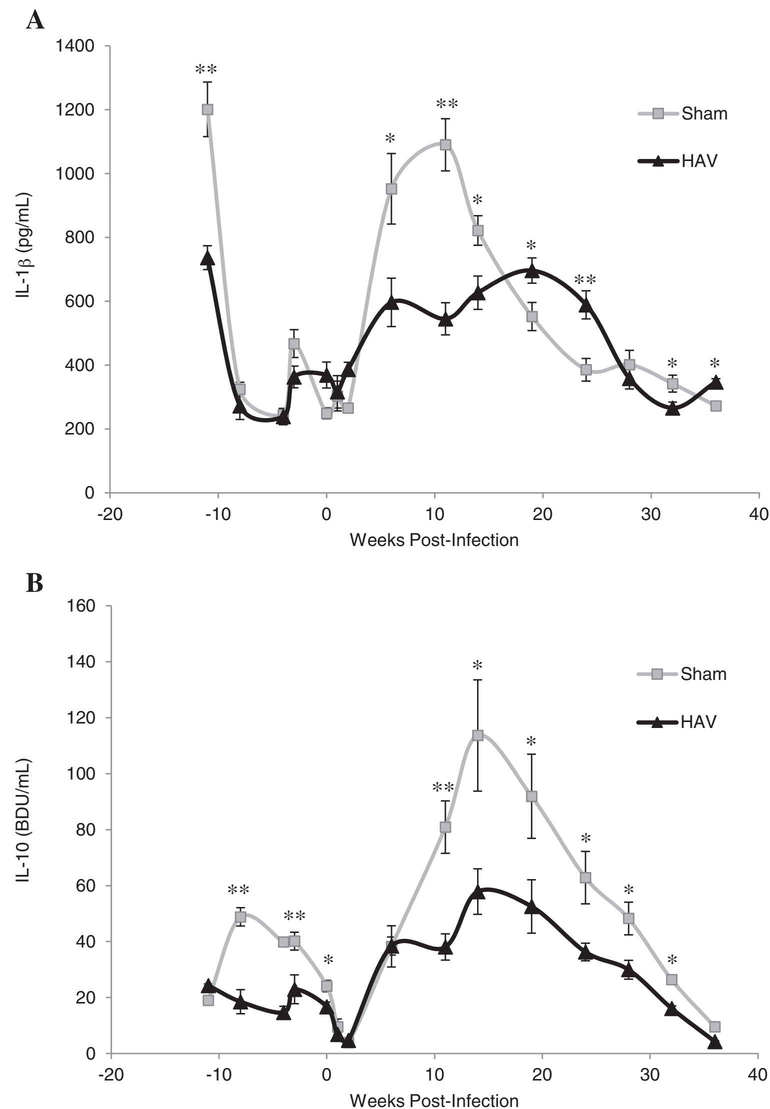


Figure 7 Secretion of IL-1β and IL-10 by PBMC from HAV- and Sham-vaccinated animals. At the indicated time points post-MAP challenge PBMC were isolated from HAV-vaccinated (triangle) or Sham-vaccinated (grey square) calves and stimulated for 24 h with PPD-J or left unstimulated (control). Supernatants 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

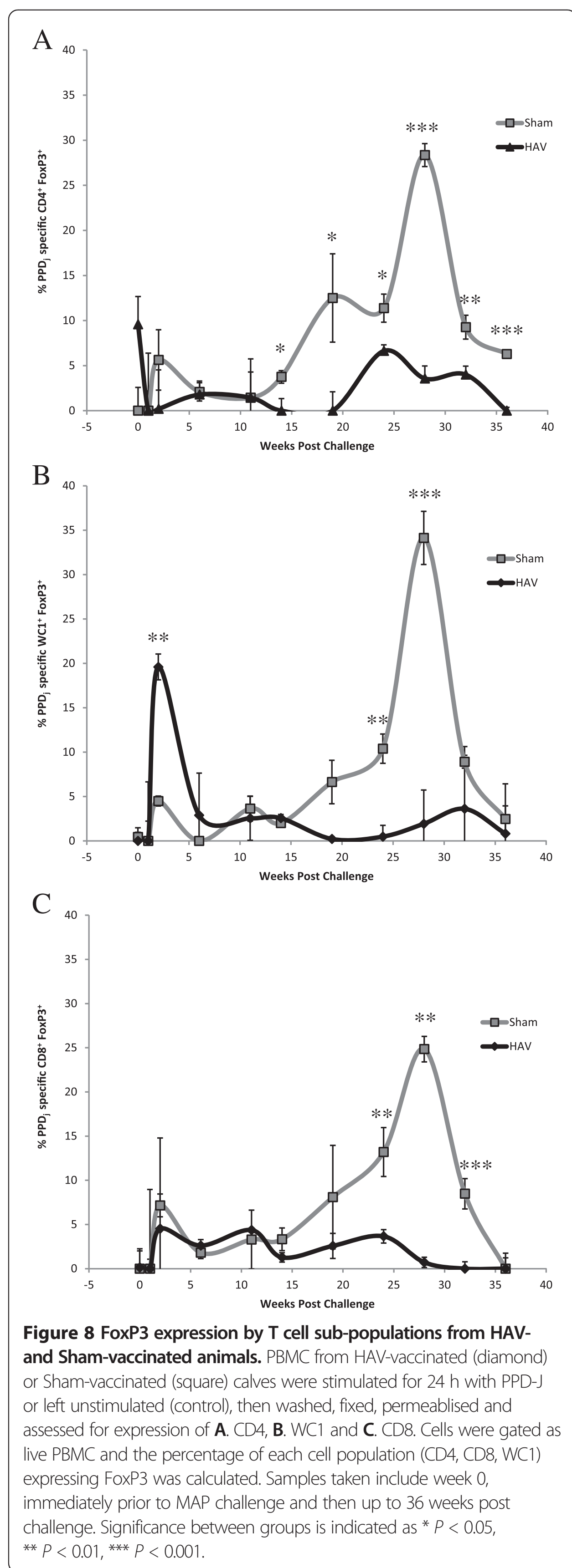


Figure 8 FoxP3 expression by T cell sub-populations from HAV- and Sham-vaccinated animals. PBMC from HAV-vaccinated (diamond) or Sham-vaccinated (square) calves were stimulated for 24 h with PPD-J or left unstimulated (control), then washed, fixed, permeablised and assessed for expression of **A**. CD4, **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

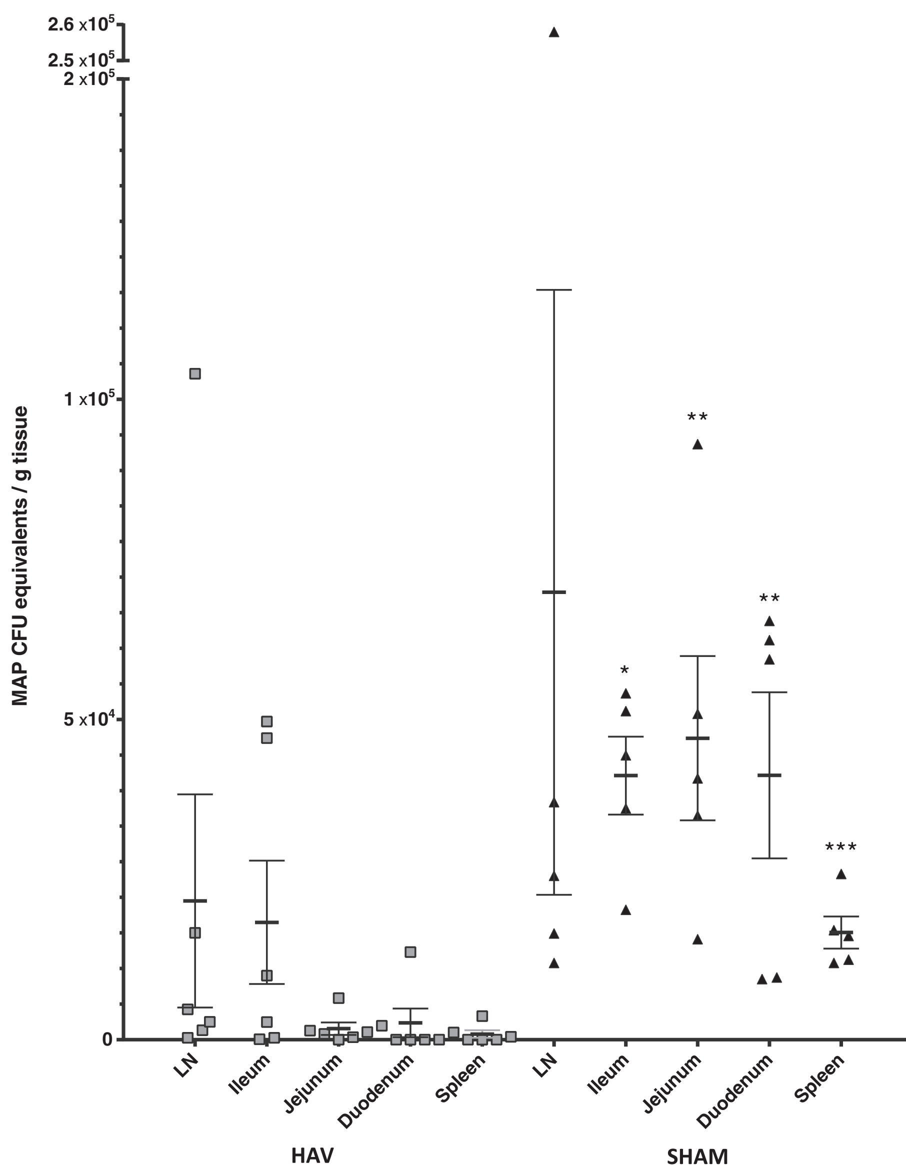


Figure 9 MAP load in tissue 38 weeks post challenge. Mean and SEM of genome equivalents determined by MAP specific IS900 qPCR (assuming 17 copies per organism) in weighed samples of various tissue sites (4 lymph node, 3 ileum, 6 jejunum, 2 duodenum, 1 spleen, per animal) from HAV vaccinated (triangles) 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

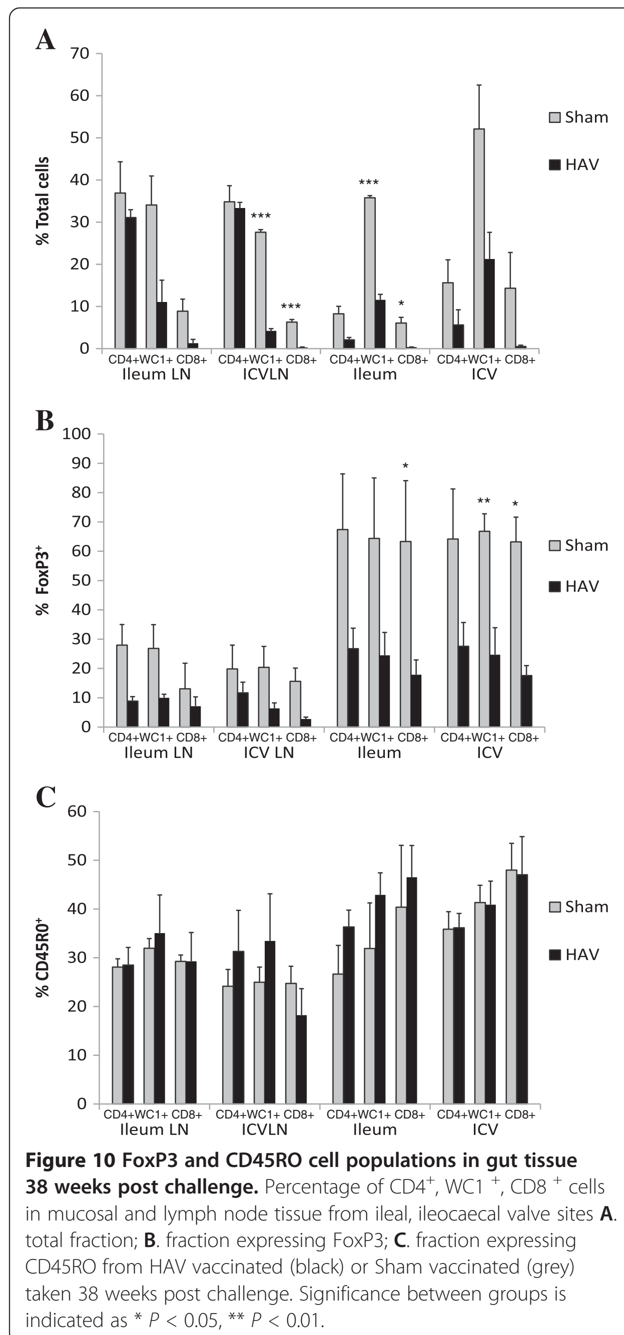


Figure 10 FoxP3 and CD45RO cell populations in gut tissue 38 weeks post challenge. Percentage of CD4+, WC1 +, CD8 + cells in mucosal and lymph node tissue from ileal, ileocaecal valve sites **A**. total fraction; **B**. fraction expressing FoxP3; **C**. fraction expressing CD45RO from HAV vaccinated (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.

Acknowledgements
This work was supported by Biotechnology and Biological Sciences Research Council (BBSRC) grants BB/H010556/1 and BB/H010718/1. Jayne Hope and Irene McGuinnes were supported by Institute Strategic Grant funding from the BBSRC. We would also like to thank Professor WC Davis, Washington State University, USA for kindly providing anti-bovine FoxP3 mAb; Professor D Bakker, CVL, Lelystad, The Netherlands for provision of PPD-J and Professor Gary Entrican, Moredun Research Institute, UK for bovine IL-10. The authors also would like to acknowledge the expertise of Drs Lyanne McCallan and David Corbett for their contributions to the animal infection model and Clare Barry for assistance with IFNγ measurements. We acknowledge the statistical expertise and advice of Dr Helen Brown, The Roslin Institute.

Author details
[1]Institute of Infection and Immunity, St. George's University of London, Cranmer Terrace, London SW17 0RE, UK. [2]The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian EH25 9RG, UK. [3]Animal Health and Welfare Northern Ireland, 97 Moy Road, Dungannon, Co. Tyrone BT71 7DXNorthern Ireland, UK. [4]Agri-Food and Biosciences Institute, Stoney Road, Stormont, Belfast BT4 3SDNorthern Ireland, UK. [5]The Jenner Institute, University of Oxford, Old Road Campus Research Building, Oxford OX3 7DQ, UK.

Received: 10 June 2014 Accepted: 10 October 2014
Published online: 29 October 2014

References
1. Harris NB, Barletta RG: **Mycobacterium avium subsp. paratuberculosis in Veterinary Medicine.** *Clin Microbiol Rev* 2001, **14:**489–512.
2. Olsen I, Lundin KE, Sollid LM: **Increased frequency of intestinal CD4+ T cells reactive with mycobacteria in patients with Crohn's disease.** *Scand J Gastroenterol* 2013, **48:**1278–1285.
3. Hines ME 2nd, Turnquist SE, Ilha MR, Rajeev S, Jones AL, Whittington L, Bannantine JP, Barletta RG, Grohn YT, Katani R, Talaat AM, Li L, Kapur V: **Evaluation of novel oral vaccine candidates and validation of a caprine model of Johne's disease.** *Front Cell Infect Microbiol* 2014, **4:**26.
4. Juste RA, Alonso-Hearn M, Molina E, Geijo M, Vazquez P, Sevilla IA, Garrido JM: **Significant reduction in bacterial shedding and improvement in milk production in dairy farms after the use of a new inactivated paratuberculosis vaccine in a field trial.** *BMC Res Notes* 2009, **2:**233.
5. Reddacliff L, Eppleston J, Windsor P, Whittington R, Jones S: **Efficacy of a killed vaccine for the control of paratuberculosis in Australian sheep flocks.** *Vet Microbiol* 2006, **115:**77–90.
6. Sweeney RW, Whitlock RH, Bowersock TL, Cleary DL, Meinert TR, Habecker PL, Pruitt GW: **Effect of subcutaneous administration of a killed Mycobacterium avium subsp paratuberculosis vaccine on colonization of tissues following oral exposure to the organism in calves.** *Am J Vet Res* 2009, **70:**493–497.
7. Kalis CH, Hesselink JW, Barkema HW, Collins MT: **Use of long-term vaccination with a killed vaccine to prevent fecal shedding of Mycobacterium avium subsp paratuberculosis in dairy herds.** *Am J Vet Res* 2001, **62:**270–274.
8. Faisal SM, Chen J-W, Yan F, Chen T-T, Useh NM, Yan W, Guo S, Wang S-J, Glaser AL, McDonough SP, Singh B, Davis WC, Akey BL, Chang YF: **Evaluation of a Mycobacterium avium subsp. paratuberculosis leuD mutant as a vaccine candidate against challenge in a caprine model.** *Clin Vaccine Immunol* 2013, **20:**572–581.
9. Lu Z, Schukken YH, Smith RL, Mitchell RM, Grohn YT: **Impact of imperfect Mycobacterium avium subsp. paratuberculosis vaccines in dairy herds: a mathematical modeling approach.** *Prev Vet Med* 2013, **108:**148–158.
10. Cho J, Tauer LW, Schukken YH, Gomez MI, Smith RL, Lu Z, Grohn YT: **Economic analysis of Mycobacterium avium subspecies paratuberculosis vaccines in dairy herds.** *J Dairy Sci* 2012, **95:**1855–1872.
11. Borsuk S, Newcombe J, Mendum TA, Dellagostin OA, McFadden J: **Identification of proteins from tuberculin purified protein derivative (PPD) by LC-MS/MS.** *Tuberculosis (Edinb)* 2009, **89:**423–430.
12. Thomsen VT, Nielsen SS, Thakur A, Jungersen G: **Characterization of the long-term immune response to vaccination against Mycobacterium avium subsp. paratuberculosis in Danish dairy cows.** *Vet Immunol Immunopathol* 2012, **145:**316–322.
13. Benedictus G, Verhoeff J, Schukken YH, Hesselink JW: **Dutch paratuberculosis programme history, principles and development.** *Vet Microbiol* 2000, **77:**399–413.
14. Kaufmann SH: **Tuberculosis vaccine development at a divide.** *Curr Opin Pulm Med* 2014, **20:**294–300.
15. Dean G, Whelan A, Clifford D, Salguero FJ, Xing Z, Gilbert S, McShane H, Hewinson RG, Vordermeier M, Villarreal-Ramos B: **Comparison of the immunogenicity and protection against bovine tuberculosis following immunization by BCG-priming and boosting with adenovirus or protein based vaccines.** *Vaccine* 2014, **32:**1304–1310.
16. Perez de Val B, Villarreal-Ramos B, Nofrarias M, Lopez-Soria S, Romera N, Singh M, Abad FX, Xing Z, Vordermeier HM, Domingo M: **Goats primed with Mycobacterium bovis BCG and boosted with a recombinant adenovirus expressing Ag85A show enhanced protection against tuberculosis.** *Clin Vaccine Immunol* 2012, **19:**1339–1347.
17. Vordermeier HM, Villarreal-Ramos B, Cockle PJ, McAulay M, Rhodes SG, Thacker T, Gilbert SC, McShane H, Hill AV, Xing Z, Hewinson RG: **Viral booster vaccines improve Mycobacterium bovis BCG-induced protection against bovine tuberculosis.** *Infect Immun* 2009, **77:**3364–3373.
18. Guzman E, Cubillos-Zapata C, Cottingham MG, Gilbert SC, Prentice H, Charleston B, Hope JC: **Modified vaccinia virus Ankara-based vaccine vectors induce apoptosis in dendritic cells draining from the skin via both the extrinsic and intrinsic caspase pathways, preventing efficient antigen presentation.** *J Virol* 2012, **86:**5452–5466.
19. Norbury CC, Malide D, Gibbs JS, Bennink JR, Yewdell JW: **Visualizing priming of virus-specific CD8+ T cells by infected dendritic cells in vivo.** *Nat Immunol* 2002, **3:**265–271.
20. Reyes-Sandoval A, Rollier CS, Milicic A, Bauza K, Cottingham MG, Tang CK, Dicks MD, Wang D, Longley RJ, Wyllie DH, Hill AV: **Mixed vector immunization with recombinant adenovirus and MVA can improve vaccine efficacy while decreasing antivector immunity.** *Mol Ther* 2012, **20:**1633–1647.
21. McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, Fletcher HA, Hill AV: **Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans.** *Nat Med* 2004, **10:**1240–1244.
22. de Cassan SC, Pathan AA, Sander CR, Minassian A, Rowland R, Hill AV, McShane H, Fletcher HA: **Investigating the induction of vaccine-induced Th17 and regulatory T cells in healthy, Mycobacterium bovis BCG-immunized adults vaccinated with a new tuberculosis vaccine, MVA85A.** *Clin Vaccine Immunol* 2010, **17:**1066–1073.
23. Bull TJ, Gilbert SC, Sridhar S, Linedale R, Dierkes N, Sidi-Boumedine K, Hermon-Taylor J: **A novel multi-antigen virally vectored vaccine against Mycobacterium avium subspecies paratuberculosis.** *PLoS One* 2007, **2:**e1229.
24. Pozzato N, Gwozdz J, Gastaldelli M, Capello K, Dal Ben C, Stefani E: **Evaluation of a rapid and inexpensive liquid culture system for the**

detection of Mycobacterium avium subsp. paratuberculosis in bovine faeces. *J Microbiol Methods* 2011, **84**:413–417.
25. Bull TJ, McMinn EJ, Sidi-Boumedine K, Skull A, Durkin D, Neild P, Rhodes G, Pickup R, Hermon-Taylor J: **Detection and verification of Mycobacterium avium subsp. paratuberculosis in fresh ileocolonic mucosal biopsy specimens from individuals with and without Crohn's disease.** *J Clin Microbiol* 2003, **41**:2915–2923.
26. Bull TJ, Schock A, Sharp JM, Greene M, McKendrick IJ, Sales J: **Linedale R.** *Stevenson K: Genomic variations associated with attenuation in Mycobacterium avium subsp. paratuberculosis vaccine strains. BMC Microbiol* 2013, **13**:11.
27. Jeulin H, Salmon A, Bordigoni P, Venard V: **Comparison of in-house real-time quantitative PCR to the Adenovirus R-Gene kit for determination of adenovirus load in clinical samples.** *J Clin Microbiol* 2010, **48**:3132–3137.
28. Pfaffl MW: **From Quantification strategies in real-time PCR.** In *A-Z of Quantitative PCR.* Edited by Bustin SA. La Jolla, CA: IUL Biotechnology Series, International University Line; 2004:87–120.
29. Hines ME 2nd, Stabel JR, Sweeney RW, Griffin F, Talaat AM, Bakker D, Benedictus G, Davis WC, de Lisle GW, Gardner IA, Juste RA, Kapur V, Koets A, McNair J, Pruitt G, Whitlock RH: **Experimental challenge models for Johne's disease: a review and proposed international guidelines.** *Vet Microbiol* 2007, **122**:197–222.
30. Rosseels V, Roupie V, Zinniel D, Barletta RG, Huygen K: **Development of luminescent Mycobacterium avium subsp. paratuberculosis for rapid screening of vaccine candidates in mice.** *Infect Immun* 2006, **74**:3684–3686.
31. Barry C, Corbett D, Bakker D, Andersen P, McNair J, Strain S: **The effect of Mycobacterium avium complex infections on routine Mycobacterium bovis diagnostic tests.** *Vet Med Int* 2011, **2011**:145092.
32. Kwong LS, Hope JC, Thom ML, Sopp P, Duggan S, Bembridge GP, Howard CJ: **Development of an ELISA for bovine IL-10.** *Vet Immunol Immunopathol* 2002, **85**:213–223.
33. Seo KS, Davis WC, Hamilton MJ, Park YH, Bohach GA: **Development of monoclonal antibodies to detect bovine FOXP3 in PBMCs exposed to a staphylococcal superantigen.** *Vet Immunol Immunopathol* 2009, **128**:30–36.
34. Coussens PM: **Model for immune responses to Mycobacterium avium subspecies paratuberculosis in cattle.** *Infect Immun* 2004, **72**:3089–3096.
35. Bower KL, Begg DJ, Whittington RJ: **Tissue localisation of Mycobacterium avium subspecies paratuberculosis following artificially induced intracellular and naked bacteraemia.** *Vet Microbiol* 2013, **162**:112–118.
36. Bower KL, Begg DJ, Whittington RJ: **Culture of Mycobacterium avium subspecies paratuberculosis (MAP) from blood and extra-intestinal tissues in experimentally infected sheep.** *Vet Microbiol* 2011, **147**:127–132.
37. Rosseels V, Huygen K: **Vaccination against paratuberculosis.** *Exp Rev Vacc* 2008, **7**:817–832.
38. Khare S, Lawhon SD, Drake KL, Nunes JES, Figueiredo JF, Rossetti CA, Gull T, Everts RE, Lewin HA, Galindo CL, Garner HR, Adams LG: **Systems biology analysis of gene expression during in vivo Mycobacterium avium paratuberculosis enteric colonization reveals role for immune tolerance.** *PLoS One* 2012, **7**:e42127.
39. Stabel JR, Robbe-Austerman S: **Early immune markers associated with Mycobacterium avium subsp. paratuberculosis infection in a neonatal calf model.** *Clin Vaccine Immunology* 2011, **18**:393–405.
40. Smeed JA, Watkins CA, Rhind SM, Hopkins J: **Differential cytokine gene expression profiles in the three pathological forms of sheep paratuberculosis.** *BMC Vet Res* 2007, **3**:18.
41. Balcewicz-Sablinska MK, Gan H, Remold HG: **Interleukin 10 produced by macrophages inoculated with Mycobacterium avium attenuates mycobacteria-induced apoptosis by reduction of TNF-alpha activity.** *J Infect Dis* 1999, **180**:1230–1237.
42. Subharat S, Shu D, Wedlock DN, Price-Carter M, de Lisle GW, Luo D, Collins DM, Buddle BM: **Immune responses associated with progression and control of infection in calves experimentally challenged with Mycobacterium avium subsp. paratuberculosis.** *Vet Immunol Immunopathol* 2012, **149**:225–236.
43. Lamont EA, O'Grady SM, Davis WC, Eckstein T, Sreevatsan S: **Infection with Mycobacterium avium subsp. paratuberculosis results in rapid interleukin-1beta release and macrophage transepithelial migration.** *Infect Immun* 2012, **80**:3225–3235.
44. Koets A, Rutten V, Hoek A, Van MF, Muller K, Bakker D, Gruys E, Van EW: **Progressive bovine paratuberculosis is associated with local loss of CD4 (+) T cells, increased frequency of gamma delta T cells, and related changes in T-cell function.** *Infect Immun* 2002, **70**:3856–3864.
45. Coussens PM, Sipkovsky S, Murphy B, Roussey J, Colvin CJ: **Regulatory T cells in cattle and their potential role in bovine paratuberculosis.** *Comp Immunol Microbiol Infect Dis* 2012, **35**:233–239.
46. Hoek A, Rutten VPMG, Kool J, Arkesteijn GJA, Bouwstra RJ, Van Rhijn I, Koets AP: **Subpopulations of bovine WC1(+) gammadelta T cells rather than CD4(+)CD25(high) Foxp3(+) T cells act as immune regulatory cells ex vivo.** *Vet Res* 2009, **40**:6.
47. Guzman E, Hope J, Taylor J, Smith AL, Cubillos-Zapata C, Charleston B: **Bovine γδ T cells are a major regulatory T cell subset.** *J Immunol* 2014, **193**:208–222.
48. Purdie AC, Plain KM, Begg DJ, de Silva K, Whittington RJ: **Expression of genes associated with the antigen presentation and processing pathway are consistently regulated in early Mycobacterium avium subsp. paratuberculosis infection.** *Comp Immunol Microbiol Infect Dis* 2012, **35**:151–162.
49. Morrison WI, Bourne FJ, Cox DR, Donnelly CA, Gettinby G, McInerney JP, Woodroffe R: **Pathogenesis and diagnosis of infections with Mycobacterium bovis in cattle. Independent Scientific Group on Cattle TB.** *Vet Rec* 2000, **146**:236–242.

doi:10.1186/s13567-014-0112-9
Cite this article as: Bull *et al.*: Immunity, safety and protection of an Adenovirus 5 prime - Modified Vaccinia virus Ankara boost subunit vaccine against *Mycobacterium avium* subspecies *paratuberculosis* infection in calves. *Veterinary Research* 2014 **45**:112.

Submit your next manuscript to BioMed Central and take full advantage of:

- **Convenient online submission**
- **Thorough peer review**
- **No space constraints or color figure charges**
- **Immediate publication on acceptance**
- **Inclusion in PubMed, CAS, Scopus and Google Scholar**
- **Research which is freely available for redistribution**

Submit your manuscript at
www.biomedcentral.com/submit

Dr Hermon-Taylor A, Dr Agrawal G, Prof J Hermon-Taylor, Prof Borody TJ
26.01.2015

Patient Information Leaflet: Anti-MAP antibiotic therapy for the treatment of Crohn's Disease

Important note: This leaflet is for information purposes only and not to be construed as medical advice. Treatment should only be undertaken with close medical supervision from a specialist.

Anti-MAP antibiotic therapy (AMAT) is one option for the treatment of Crohn's Disease. It has been used by some specialists since the 1990's. The rationale for this treatment is based on growing evidence to support the hypothesis that Crohn's Disease is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (MAP) [1]. The protocol for this combination therapy includes Rifabutin, Clarithromycin and usually a 3rd agent, Clofazimine. These are DIFFERENT antibiotics to those such as ciprofloxacin or metronidazole which mainly kill the overgrowth of other gut bacteria rather than having significant action against MAP itself. Side effects of AMAT can limit its use in some people. But in those who can take it, the majority will benefit from treatment and profound remissions can occur, even in those with severe disease. In some cases, major surgery has been avoided.

What is the success rate of AMAT?

In ordinary terms, of 5 people who can take it, 2 will get a complete remission, 2 will get a partial remission and one will not respond, probably because the MAP in that person is already resistant.

A review of the published scientific literature reveals 7 clinical trials which have investigated the effects of AMAT in Crohn's Disease, with clinical remission rates ranging from 44-89% [2]. Of these, the 'landmark study' is considered to be that conducted by Selby *et al.*[2] in Australia in 2007 –it is the only large randomized controlled trial and hence the most well-known. The failure of this study to show a long-term benefit of anti-MAP therapy in Crohn's disease is regarded by many as the 'final nail in the coffin' for the MAP/Crohn's hypothesis and is a major reason for the reluctance of some Gastroenterologists to recommend this treatment to their patients. However, the trial has since been widely criticized, both in terms of the protocol used and the subsequent analysis [2,4,5], such that it cannot be relied upon as having any clinical significance.

Major flaws include the following:

- Patients were not tested for MAP before entry into the trial
- Sub-therapeutic doses of all 3 antibiotics were used
- The Clofazimine capsules used failed to dissolve, thereby not releasing the active ingredient within
- The analysis of the data was flawed; the results were not based on an intention-to-treat analysis and thus underestimated the beneficial effect of treatment [5].

Despite these flaws, the trial still demonstrated a remission rate of 66% (of 102 patients with Crohn's Disease) at 16 weeks ($p<0.02$), as compared with a remission rate of 39% for Infliximab (in a separate study) over a comparable time frame.

A fresh start has been badly needed for a long time - but there is new hope on the horizon that the true potential of this treatment may finally be revealed. In the USA, a large multi-centered randomized-controlled trial is currently underway entitled 'Efficacy and Safety of Anti-MAP Therapy in Adult Crohn's Disease (MAPUS)'. The AMAT being used in this trial is a fixed-dose combination of Rifabutin, Clarithromycin and Clofazimine combined into a new 3-in-1 pill called RHB-104:
http://clinicaltrials.gov/ct2/show/NCT01951326?term=crohn%27s+disease+redhill&rank=1

What are the possible side effects or complications of AMAT?

Overall, AMAT is well tolerated by the majority of patients, but as with most medicines, there are potential side effects which may be experienced by some people. These include:

- *Common:* a 'tanned' appearance (use of sunscreens may help to minimise this), aching joints (anecdotally N-acetyl glucosamine supplements may help), pinkish discolouration of body fluids (urine, saliva, tears), increased frequency of urination
- *Less commonly:* rash, fevers (usually transient and subside with a step-up dose approach) Healing of the bowel (i.e. successful treatment) can sometimes result in scarring –if the scar tissue causes irreversible narrowing (in some, bowel narrowing may soften over time) surgery to remove the small area that is narrowed may be required
- *Rarely:* uveitis (characterised by eye pain, reversible on stopping treatment)

Are there any contra-indications to AMAT?

- Known allergy to any of the antibiotics included in the AMAT protocol
- Concomitant use of the medication Colchicine
- Drug interactions would need to be assessed on an individual basis. Other commonly prescribed treatments for Crohn's (e.g. biological agents, immunosuppressants, steroids) do not prohibit the use of AMAT; indeed they may sometimes be co-prescribed.

Why do some people with Crohn's not improve with AMAT?

- A small proportion of people won't be able to tolerate treatment.
- Mycobacterial resistance to antibiotics is well-described in the literature and becoming more common; MAP may already be resistant to AMAT due to previous antibiotic exposure. It is not possible to do sensitivity studies prior to starting therapy because of the difficulty in culturing MAP.
- Prescribing problems: The preferred regime is triple therapy with Rifabutin, Clarithromycin and Clofazimine although in some people Rifabutin and Clarithromycin alone work well. Antibiotic monotherapy is well-known to lead to resistance. Correct dosing is important as opposed to that used in the trial by Selby *et al*.
- Inadequate duration of treatment: As with other mycobacterial infections, treatment needs to be continued for a long time (24-36 months) because of MAP's ultraslow growth and ability to become 'latent' –a state of hibernation in which it is very difficult to kill with drugs.

How soon will I see a response to treatment?

In some people, particularly those who are newly diagnosed with Crohn's and not previously treated with antibiotics, improvement can occur within 4-6 weeks. In others with longstanding disease, it can take 6 to 12 months before a real improvement occurs. In these people there is a lot of damage to the gut wall, the immune system and the delicate gut nervous system which has to be repaired. At the end of the treatment phase, lower dose 'maintenance therapy' may be advisable.

Will my Crohn's symptoms return after stopping AMAT?

Most people who relapse have stopped their therapy themselves as they feel 'cured' with AMAT. Invariably the high majority relapse within the year and it is often difficult to achieve the same treatment effect upon recommencement. Hence it is important to continue treatment even if you feel well and stop only on the advice of your doctor. Unfortunately there are some patients whose symptoms have returned despite completing the full course of AMAT as prescribed.

Will minimising exposure to MAP help to maintain remission following treatment?

Environmental exposure to MAP cannot currently be controlled and hence it is impossible to avoid MAP altogether. However dietary modifications can be made to help minimise exposure to MAP in the food chain. Live MAP has been detected in pasteurised milk and dairy products as well as meat.

Although studies have been done looking ways of eliminating MAP from milk, there is no consensus in the literature on how high a temperature and for how long milk has to be heated to, to guarantee 100% eradication of MAP. Whilst there is no scientific data to support a benefit from avoiding MAP in the diet, some patients wish to take a precautionary approach in their lifestyle choices.

Dietary modifications to reduce MAP exposure include:

- Only consume dairy products if they have been extensively boiled or cooked to a high temperature for a prolonged period e.g. rice pudding
- AND/OR change to UHT milk (although this is less likely to contain MAP than standard HTST pasteurised milk, occasional MAP-positive UHT milk samples have been reported)
- OR cut out dairy products altogether
- Only eat meat that is well-cooked. This is especially important with mince meat products e.g. beef burgers

Whilst antibiotic treatments can be a real help, they are not always a long-term solution, due to the ever-present threat of developing resistance and MAP's ability to avoid destruction by becoming latent. The prospect of the therapeutic anti-MAP Vaccine (currently pending trial), which would avoid all of these problems, is therefore a very exciting one.

(1) Agrawal G, Borody TJ, Chamberlin W. Global warming to *Mycobacterium avium* subspecies *paratuberculosis. Future Microbiol*. 2014; 9(7): 829-832
(2) Chamberlin W, Borody TJ, Campbell J. Primary Treatment of Crohn's Disease: Combined antibiotics taking centre stage. *Expert Rev Clin Immunol* 2011; 7(6): 751-760
(3) Selby W, Pavli P, Crotty B *et al.* Two-year combination antibiotic therapy with clarithromycin, rifabutin and clofazimine for Crohn's disease. *Gastroenterology* 2007; 132(7): 2313–2319
(4) Lipton JE, Barash DP. Flawed Australian DC trial does not end MAP controversy. *Gastroenterology* 2007; 133:1742
(5) Feller M, Huwiler K, Schoepfer A *et al.* Long-term antibiotic treatment for Crohn's disease: systematic review and meta-analysis of placebo-controlled trials. *Clin. Infect Dis.* 2010; 50(4): 473–480