



## On deaf ears, mycobacterium avium paratuberculosis in pathogenesis Crohn's and other diseases

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### Abstract

The historic suggestion that *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) might be a zoonotic pathogen was based on the apparent similarity of lesions in the intestine of patients with Crohn's disease (CD) with those present in cattle infected with *Map*, the etiological agent of Johne's disease. Reluctance to fully explore this possibility has been attributed to the difficulty in demonstrating the presence of *Map* in tissues from patients with CD. Advances in technology have resolved this problem and revealed the presence of *Map* in a significant proportion of patients with CD and other diseases. The seminal finding from recent investigations, however, is the detection of *Map* in healthy individuals with no clinical signs of disease. The latter observation indicates all humans are susceptible to infection with *Map* and lends support to the thesis that *Map* is zoonotic, with a latent stage of infection similar to tuberculosis, where infection leads to the development of an immune response that controls but does not eliminate the pathogen. This clarifies one of the reasons why it has been so difficult to document that *Map* is zoonotic and associated with the pathogenesis of CD and other diseases. As discussed in the present review, a better understanding of the immune response to *Map* is needed to determine how infection is usually kept under immune control during the latent stage of infection and elucidate the triggering events that lead to disease progression in the natural host and pathogenesis of CD and immune related diseases in humans.

**Key words:** Crohn's disease; Johne's disease; *Mycobacterium avium* subsp. *paratuberculosis*; Animal model; Monoclonal antibodies; Flow cytometry; Cytokines

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**Core tip:** The seminal finding from recent investigations is the detection of *Map* in healthy individuals with no clinical signs of disease. The latter observation indicates all humans are susceptible to infection with *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) and lends support to the thesis that *Map* is zoonotic, with a latent stage of infection similar to *M. tuberculosis*, where infection leads to the development of an immune response that controls but does not eliminate the pathogen. This clarifies one of the reasons why it has been so difficult to document that *Map* is zoonotic and associated with the pathogenesis of Crohn's disease and other diseases.

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## INTRODUCTION

A controversy has persisted since the initial suggestion that *Map* may be a zoonotic pathogen and the causative agent of Crohn's disease (CD). *Map* is a mycobacterial pathogen with a broad host range that can infect many species<sup>[1]</sup>. The primary hosts are ruminants. It was initially diagnosed as the causative agent of Johne's disease (JD), a chronic enteritis in cattle<sup>[2]</sup>. The similarities in the clinical manifestations of JD with CD led Dalziel *et al*<sup>[3]</sup> and later Crohn *et al*<sup>[4]</sup> to suggest *Map* may be the causative agent of CD<sup>[1]</sup>. As reviewed comprehensively by Naser *et al*<sup>[5]</sup> and Gitlin *et al*<sup>[6]</sup>, the debate continues. The reluctance to fully explore the issue of whether *Map* is associated with the pathogenesis of CD has been the difficulty in isolating *Map* from all patients with CD. Chiodini *et al*<sup>[7-9]</sup> were the first investigators to successfully isolate *Map* from 3 patients. Initial attempts by others, however, proved unsuccessful raising questions as to the role of *Map* in the pathogenesis of CD. Even with improvements in technology, success in detection of *Map* has varied with some investigators more successful than others in isolating *Map* from patients with CD. Sufficient evidence, however, has been obtained over recent years to clearly show that many patients with CD are infected with *Map*. Investigations have revealed the presence of *Map* or *Map* DNA in blood or lesions from adults and children with CD<sup>[10-16]</sup>. Of special interest, *Map* has also been found in patients with other diseases as well as healthy subjects using several methods of detection<sup>[17-20]</sup>. A study long overdue, has now been conducted by Singh *et al*<sup>[21,22]</sup>. The study was conducted in India. It was designed to examine the level of infection in humans where *Map* is endemic in livestock and the general

environment. They obtained specimens of blood, serum and stool from 42400 patients, submitted to pathology laboratories and hospitals in different areas of India, where *Map* is endemic, for evaluation over a span of two plus years. The samples were from patients presenting with a spectrum of infectious and non-infectious diseases including typhoid, TB, malaria, skin disorders, type 1 diabetes, anemia, thyroid, ion imbalance, abdominal disorders, lipid profile disorders, and intestinal inflammatory illness of different origins. They also collected samples from healthy individuals. *Map* was detected in all categories of patients at different frequencies, including healthy individuals, reflecting the consequence of large scale exposure of the resident populations to *Map* in the environment, infected animals and the food supply. The predominant biotype of *Map* detected in the samples was the bison type prevalent in livestock. The results clearly show, humans like other species, are equally susceptible to infection with *Map* regardless of health status. It can be predicted that any further large scale screening will provide similar results with the frequency of infected individuals dependent on the prevalence of *Map* in the environment and presence in food supplies<sup>[23]</sup>. The question remaining and reason for the unending debate is, "is *Map* a zoonotic pathogen and if so, what role does it play in the pathogenesis of CD and other diseases?" The answer is yes for at least a subset of patients presenting with the clinical signs of CD<sup>[5,24]</sup>. Koch's postulates were actually fulfilled in subsequent studies by Chiodini *et al*<sup>[25]</sup> where he and his associates demonstrated that passage of one of the first human isolates of *Map* in kid goats led to the development of enteritis similar to the enteritis in CD and JD. A later investigation of an enteritis in a colony of stump-tail macaque (*Macaca arctoides*) revealed *Map* was the causative agent, the first report showing the susceptibility of non-human primates to infection with *Map*<sup>[26]</sup>. The study was the first to show that *Map* could be isolated from feces as an acid fast bacillus, extending what was known about the host range of species susceptible to infection with *Map*. The ensuing studies have shown *Map* isolated from patients with CD may be present in affected tissues with or without the mycobacterial cell wall<sup>[9]</sup>, a characteristic of other mycobacterial pathogens<sup>[27]</sup>. Studies of healthy individuals and patients with clinical signs of CD and other diseases have shown *Map*, with an intact cell wall, can be detected in blood, tissues, and feces<sup>[22]</sup>. Cell wall deficient forms have also been found in tissues from animals with JD<sup>[28]</sup>.

## ROLE OF MAP IN CROHN'S AND OTHER DISEASES

The difficulty in elucidating the role of *Map* in CD and other diseases has been the lack of understanding of the mechanisms of pathogenesis of JD. *Map*, like

other pathogenic mycobacteria doesn't cause epidemic disease, where all infected animals develop clinical disease. This is similar to tuberculosis in humans where infection leads to the development of an immune response that controls but does not clear infection. Only an estimated 10% of individuals infected with *Mtb* develop clinical disease. Disease progression is associated with a breakdown in protective immunity. Intensive ongoing investigations have not yet identified the triggering events associated with disruption of protective immunity during the latent stage of infection. No comprehensive studies have been conducted on the immune response to *Map* in humans. However, the presence of an immune response to *Map* in patients with clinical manifestations of CD and the presence of antibody to *Map* in healthy individuals and patients with various diseases show exposure leads to development of an immune response to *Map*<sup>[22,29,30]</sup>. The detection of *Map* in blood and affected tissues shows that infection persists providing a constant stimulus to the immune system.

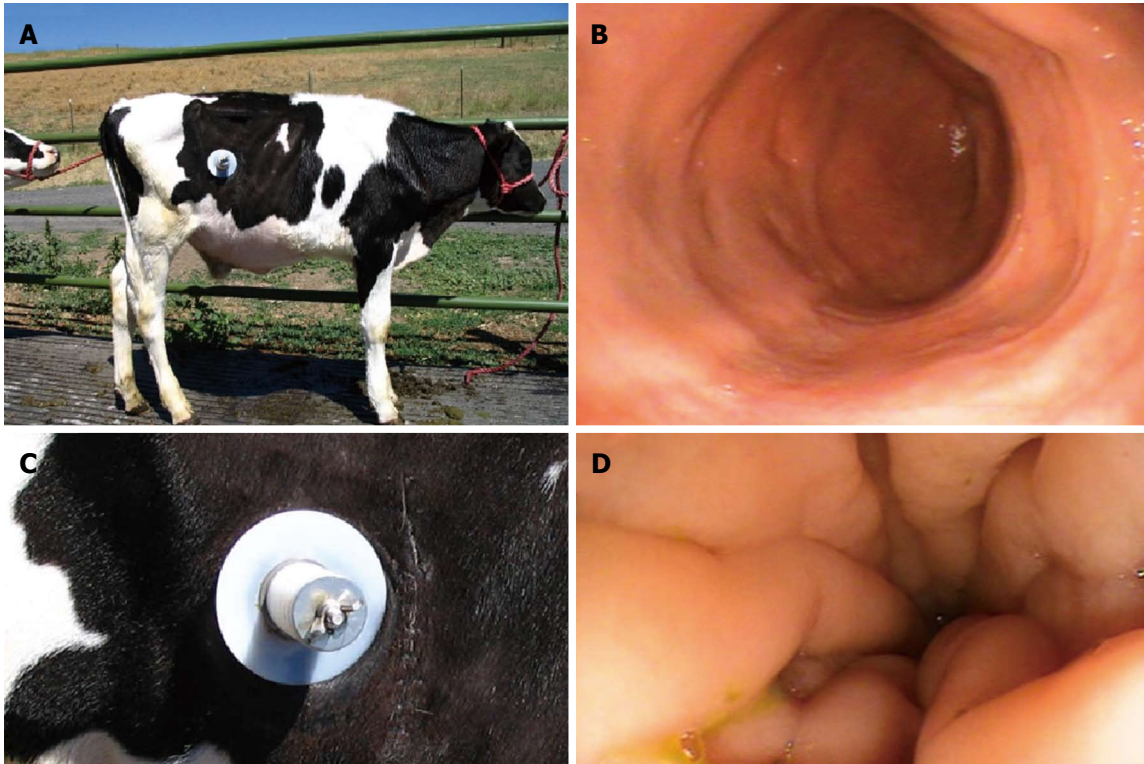
#### ***Map as a model pathogen to study the mechanism of immunopathogenesis of mycobacterial pathogens***

There are two ways *Map* could contribute to the pathogenesis of diseases in humans. The first is as the etiologic agent of CD, where there is a breakdown of immunity present during the latent stage of infection, similar to the breakdown that occurs with tuberculosis<sup>[5,24,31]</sup>. The second is through mimicry where mimics of proteins present in humans are produced by *Map* that interfere with immune function and or elicit development of cross-reactive antibodies associated with autoimmune diseases<sup>[32,33]</sup>.

For ethical reasons much of the information needed to elucidate the role of *Map* in pathogenesis of CD and other diseases can't be obtained through investigations conducted with human subjects. The alternative is the use of cattle, the natural host of *Map*, as a model species to study the role of *Map* in the immunopathogenesis of CD and other diseases. The added advantage is that it affords an opportunity address the broader problem, the need to elucidate the mechanisms of immunopathogenesis of mycobacterial pathogens in general. The similarities in latency suggest the pathogens use the same signaling pathways to dysregulate the immune response and establish a persistent infection that eventually, over time, leads to a complete breakdown of protective immunity and development of clinical disease. The unique advantage of using *Map* to explore this possibility is that it can be used to study the interaction of the pathogen with the host at all stages of infection. Our studies have been based on the premise that *Map* is a zoonotic pathogen that is the etiologic agent of a subset of patients with CD<sup>[5,24,31]</sup> and that it plays a role in pathogenesis of other disease through mimicry<sup>[22,32]</sup>.

To provide some background and a review of the

current status of knowledge on the mechanisms of immunopathogenesis of JD in cattle, we became interested in the potential role of *Map* in CD when limited information was available on the immune response to *Map* in cattle. Progress was limited by the lack of immune reagents needed to characterize the immune system in cattle and study the cell mediated immune response to *Map*. Studies in dairy herds had shown the disease is characterized by a long latency period, where infected animals were difficult to detect by available diagnostic techniques during the initial stages of infection. Some animals could be detected by development of cell mediated immunity using the tuberculin skin test (reviewed in<sup>[1]</sup>). At later stages of infection, infected animals could be detected by intermittent shedding of *Map* in feces. Persistent shedding was associated with development of clinical disease where the absorptive mucosa of the intestine is destroyed, leading to death by starvation. The long latency suggested that *Map* could be passed through the gastrointestinal system without infecting all animals and constant exposure was needed for infection to occur. The latency also suggested that there was an age related difference in susceptibility to infection, with older animals more resistant to infection than young animals. Development of immune reagents to characterize the immune system of cattle and a flow cytometric assay provided an opportunity to begin analyzing the immune response to *Map* ex vivo and determine whether there was an age difference in susceptibility to infection and whether it was possible for "pass through" of *Map* without infection<sup>[34-36]</sup>. Studies in neonatal calves revealed exposure through application of *Map* to tonsillar crypts<sup>[36]</sup> or by ingestion of *Map* in milk supplement, leads to uniform infection in all animals and development of a lymphoproliferative response ex vivo to *Map* purified protein derivative (PPD) and soluble *Map* antigens (Ag) dominated by memory CD45R0<sup>+</sup> CD4 T cells 5 mo post infection (PI)<sup>[34]</sup>. Peripheral colonization of tissue was detected in some tissues at necropsy, with some bacteria detectable in feces in the initial study<sup>[36]</sup>. In the second study, no gross or microscopic lesions were detectable in intestinal tissue at necropsy one year PI, with *Map* detectable in some tissues by PCR<sup>[34]</sup>. A surgical intervention model used to directly inoculate the ileum showed *Map* is taken up rapidly and disseminated to other tissues within an hour PI. A proliferative response was detected by 9 mo but no humoral response<sup>[37]</sup>. Development of a cannulated ileum model allowed us to continuously monitor the early stages of infection in experimentally infected calves for up to 18 mo PI (Figure 1)<sup>[35]</sup>. Use of the model showed bacteria directly introduced into the ileum, once or multiple times over several days, were rapidly cleared with detection of bacteria in feces only during the first weeks PI. No gross or microscopic lesions were found in biopsies during the 18 mo of



**Figure 1** Early stages of infection in experimentally infected calves for up to 18 mo post infection. Pictures showing the cannula used in the studies (A, B); Endoscopic field showing the condition of ileal mucosa 8 mo following infection with *Map* (C); Endoscopic field taken before necropsy of a naturally infected animal showing the characteristic swelling and corrugation of the ileal mucosa that occurs at the clinical stage of infection (D). Modified from<sup>[35,38]</sup>.

the studies. However, *Map* was detected by PCR with IS900 and culture of some tissues. An immune response was detected using a flow cytometric assay at 3 mo<sup>[35]</sup>. A subsequent study, with the cannulation model, was focused on determining whether passage of *Map* in humans altered their capacity to infect the natural host and establish a persistent infection. Two of the original isolates obtained from humans by Chiodini *et al*<sup>[7]</sup> (Linda and Ben) were used to compare the capacity of isolates from humans and cattle to infect calves. The comparison revealed no difference in infectivity or the immune response to the bovine or human isolates of *Map*<sup>[38]</sup>. Sequence analysis of the human isolate obtained from tissue had identical single sequence repeat as the original isolate, 7g-4ggt, a sequence common in isolates from cattle<sup>[39]</sup>. This observation indicates it will not be necessary to use human isolates of *Map* to model mechanisms of persistence and pathogenesis in cattle.

## MECHANISMS OF IMMUNE EVASION AND IMMUNOPATHOGENESIS

The early events of infection by pathogenic mycobacteria that lead to the development of an immune response that controls but fails to eliminate the pathogen remain to be elucidated. Also, the triggering events that disrupt protective immunity have not been identified. Efforts to breakdown the immune response

to *Map* in cattle and accelerate progression to clinical disease have revealed the immune response to *Map* is resilient. Exposure of calves to massive doses of *Map* by different routes or administration of dexamethasone to alter the immune response to *Map* did not modulate the immune response to *Map*<sup>[40]</sup>. In addition, transient depletion of CD4 T cells in calves with a monoclonal antibody to CD4 did not impair the development of an immune response to *Map* or accelerate progression to clinical disease<sup>[41]</sup>. Under all conditions tested, including exposure at birth, an immune response develops that controls but does not clear infection. These observations suggest a similar resilient response also occurs in the majority of humans exposed to *Map* that do not develop clinical disease. The challenge that remains is identifying the factors that trigger a breakdown in protective immunity.

Thus far, analysis of the immune response to *Map* has shown the cellular response, under experimental conditions, is characterized by development of an immune response dominated by CD45R0<sup>+</sup> memory CD4 T cells using live *Map*, *Map* PPD, and soluble *Map* Ags<sup>[34,35,38]</sup>. Although a CD8 T cell response also develops, the proliferative response to *Map* *ex vivo* is less than the proliferative response of CD4 T cells. The response increases over time, however. In a comparative study, the CD8 T cell proliferative response was equivalent to the response observed with naturally infected animals at the clinical stage of infection<sup>[38]</sup>. qRT-PCR showed IFN- $\gamma$ , IL-17, and IL-22

gene expression was up-regulated to equivalent extent in experimentally and naturally infected animals<sup>[38]</sup>.

Ongoing efforts to develop a vaccine have provided additional information on the immune response to *Map* and an approach for elucidating how *Map* and mycobacterial pathogens dysregulate the immune response to establish a persistent infection. We used site directed mutation to develop 3 mutants to genes associated with virulence (*relA*, *lsr2*, and *pknG*)<sup>[42,43]</sup> as part of a multi-institutional endeavor to identify deletion mutants as vaccine candidates<sup>[44]</sup>. Mutants, made by other investigators, selected for challenge studies in mice and goats based on reduced capacity to survive in macrophages were unsuccessful. All the mutants were able to establish an infection<sup>[44]</sup>. In contrast, one of the mutants we selected for a challenge study in goats and calves,  $\Delta$ *Map-relA*, was unable to establish a persistent infection in goats and calves<sup>[45]</sup>. Further analysis in calves showed no clear difference in the proliferative response to *Map* antigens. The increases in expression IFN- $\gamma$ , IL-17, and IL22 were comparable indicating more in depth studies are needed to determine how deletion of *relA* abrogates the capacity of *Map* to establish a persistent infection.

The last observation of interest is on the effect of therapeutic vaccination with BCG on the immune response to *Mtb* in guinea pigs and humans at the clinical stage of infection made by Koch<sup>[38,46]</sup>. Vaccination led to a reversal of pathology, showing the triggering events associated with a breakdown in protective immunity, could be reversed by vaccination. Although subsequent studies revealed vaccination only led to a transient reversal, the observation is of interest because studies of vaccination at the clinical stage of infection could reveal which components of the immune response are restored that account for a reversal of pathology. Similar observations have been made more recently by Singh *et al*<sup>[47,48]</sup> with *Map* in goats and cattle. Although the results looked very promising, reversal of pathology was transient with no improvement even with repeated vaccination (personal communication). The important observation made by Koch *et al*<sup>[47,48]</sup> and Singh *et al*<sup>[47,48]</sup> is that reversal of pathology was associated with an increase in the inflammatory response following vaccination that resolved lesions containing infected cells. *Map* affords an opportunity to explore the cellular and molecular basis for this phenomenon. A preliminary test with a cow at a late clinical stage of infection, with an anergic response to a skin test with *Map* Ag and a diminished proliferative response to Ag *ex vivo*, showed vaccination with  $\Delta$ *MaprelA* restored the proliferative response to *Map*. With the cannulation model it will be possible to isolate affected tissue before and after vaccination for analysis and characterize the effector cells restored by vaccination and characterize their effector activity.

## CONCLUSION

Cumulative studies clearly show humans, like other species, are equally susceptible to infection with *Map*<sup>[22]</sup>. This places the whole populations at risk of infection, depending on the prevalence of *Map* in the environment and food supply<sup>[22,23]</sup>. Ongoing studies have shown CD may contribute to the pathogenesis of disease in humans as the etiologic agent of a subset of patients with CD<sup>[5]</sup> and through antigenic mimicry<sup>[32]</sup>. Use of the natural host as a model, to understand the immunopathogenesis of CD mediated by *Map*, has provided opportunity to gain insight into the immune response to *Map* in humans during the early, latent, and clinical stages of infection that cannot be obtained through study of *Map* in humans, except at the clinical stage of infection<sup>[29]</sup>. Analysis has shown the immune response during the latent stage of infection is resilient and not readily disrupted by methods of immunosuppression<sup>[40,41]</sup>. The components of the immune response to *Map* that control infection during latency remain to be elucidated. The development of a mutant that cannot establish a persistent infection offers 2 opportunities to identify these components, one through analysis of the early stages of infection and the second through analysis of the immune response following restoration by vaccination at the clinical stage of infection.

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