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Experimental infection of a Bovine Model with Human Isolates of *Mycobacterium avium* subsp. *paratuberculosis*

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Abstract

Mycobacterium avium subsp. *paratuberculosis (Map)*, the etiologic agent of Johne's disease (JD) in ruminants, has been implicated in the pathogenesis of Crohn's disease (CD) in humans. We developed a bovine ileal cannulation model to facilitate comparison of the immune response to *Map* and the mechanisms of pathogenesis in cattle and humans. Initial studies showed a T cannula could be maintained for up to a year in calves without inducing inflammation or adversely affecting intestinal function. *Map* introduced through the cannula established a persistent low level of infection without inflammation. Infection elicited an immune response to *Map* antigens detectable by flow cytometry. Further studies now show the cannulation model can be used with cows during the later stage of infection, affording access to the target tissue at all stages of infection. The studies also revealed no difference in infectivity or immunogenicity of isolates of *Map* obtained from cattle or humans with CD. Comparison of the immune response to *Map* during the early and late stages of infection using PCR, flow cytometry and QRT-PCR, showed the immune response early in the disease process is dominated by CD4 T cells. A CD8 response is

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delayed but comparable at later stages of infection. Genes for pro-inflammatory cytokines IFN-γ and the recently identified genes encoding IL-17 and IL-22 are up regulated in infected animals. These findings reveal that both human and bovine isolates of *Map* can establish infection and induce similar immune responses in a bovine model. They also reveal the cytokine responses elicited in cattle are similar to those implicated in CD pathogenesis.

Keywords

Crohn's disease; Johne's disease; *Mycobacterium avium* subsp. *paratuberculosis*; animal model; flow cytometry; cytokines

Introduction

Over the past few years, extensive studies have been conducted to determine whether *Mycobacterium avium* subsp. *paratuberculosis* (*Map*) is involved in the pathogenesis of Crohn's disease (CD), a chronic inflammatory bowel disease that occurs in humans. The initial suggestion that *Map* may be involved was based on the apparent similarity in the clinical manifestations of CD with Johne's disease (JD) a chronic enteritis in cattle caused by *Map* (Dalziel, 1913; Crohn et al., 1932). Until recently, however, the difficulty in detecting *Map* in affected tissues left open the question on the role *Map* might play in the pathogenesis of CD. Improvements in methods of isolation and detection have now revealed that *Map* can be detected in peripheral blood as well as in affected tissues from patients with CD (Naser et al., 2009). The improved ability to detect *Map* has also shown the presence of *Map* in patients with other types of disease (Autschbach et al., 2005; Bull et al., 2003; Kirkwood et al., 2009) as well as healthy subjects (Juste et al., 2009). The importance of the latter observation is that it appears that exposure and infection with *Map* is similar to other domestic species (Juste et al., 2009; Singh et al., 2008). Exposure to *Map* leads to the development of a protective immune response that controls but does not eliminate the pathogen (Juste et al., 2009). This is similar to what is observed following exposure to *M. tuberculosis* (*Mtb*); the majority of individuals infected with *Mtb* develop a latent infection with only about 10% developing disease. Thus the question is whether *Map* is present in all patients with CD or only a subset of patients. An answer to this question would provide support for the contention that *Map* is the etiological agent of CD (Mendoza et al., 2010) or that it is a modifying factor that plays a role in pathogenesis (Sibartie et al., 2010). Although these questions remain to be answered, studies of CD show pathogenesis is associated with an alteration in the functional interaction of regulatory T (Tr) cells and T cell subsets secreting the newly identified proinflammatory cytokines IL-17 and IL-22 (Brand, 2009; Hölttä et al., 2008; Schmidt et al., 2005; Fuss et al., 2006). Alteration in the function of these components of the immune system are also involved in the pathogenesis of tuberculosis, providing support for the hypothesis that a mycobacterial pathogen could be involved in the pathogenesis of CD (Dheda et al., 2008; Robinson and Nau, 2008; Kursar et al., 2007; Sharma et al., 2009).

The recent observation that *Map* DNA can be detected more frequently in the blood of healthy humans and not just CD patients indicates a possible controlled infection (Juste et al., 2009). Information on the immune response to *Map* is mainly from patients with CD. The immune response to *Map* in healthy subjects as well as patients with CD has yet to be fully characterized. Additionally, similarities in the immune response to *Map* in humans and the natural host (ruminants) during the early and late stages of infection need to be examined. Comparative studies would reveal the similarities and differences between the mechanisms of pathogenesis in CD and JD. To facilitate such studies we developed a bovine ileal cannulation model to study the mechanisms of pathogenesis of JD (Allen et al., 2009;

Hines et al., 2007). The model provides an opportunity to conduct studies not possible in humans. The initial observations with the model have shown an indwelling T cannula can be maintained for over a year with minimal problems (Allen et al., 2009). Endoscopic examinations have shown that the ileum remains patent and functional with minimal signs of inflammation caused by the cannula. Bacteria are rapidly cleared in experimental infection through the cannula, with *Map* becoming difficult to detect in biopsies within days post inoculation (PI) even after multiple inoculations; no lesions or swelling of the mucosa are detectable during the first year PI. A proliferative response to PPD and soluble antigen extract that is dominated by CD4 memory T cells becomes consistently detectable by flow cytometry 3 months PI. A CD8 T cell response to antigens is present but less pronounced during the first months PI. The specific aims of our study were to examine whether the model can be used at all stages of infection and to gain additional information on the immune response to *Map*. We were specifically interested in whether it would be necessary to conduct comparative studies using isolates of *Map* obtained from patients with CD versus bovine isolates. To our knowledge studies have never been conducted to determine if passage through humans alters infectivity or pathogenicity when re-introduced into the natural host. We were also interested in whether the immune response to *Map* involves newly identified T cell subsets producing IL-17 and IL-22.

Material and methods

Animals

Four Holstein bull calves were obtained from the Washington State University (WSU) research dairy (127, 129, 131, 132). The dairy is a closed herd considered free of paratuberculosis since it has had no cases diagnosed for over 35 years and because the dairy performs periodic, whole-herd, Johne's enzyme linked immunosorbent assays (ELISA) (IDEXX, Westbrooke, ME). All calves were removed from their dams at birth and were fed 4 liters of maternally derived colostrum. They were maintained in a Biosafety Level 2 (BSL-2) isolation facility for the duration of the study. At 8 weeks of age the calves were cannulated as described (Allen et al., 2009). Three mixed breed adult cows were obtained from regional dairies in Washington State with endemic Johne's disease (1181, 3696, 1111). All three cows had clinical signs of JD and were positive via Johne's ELISA (PARACHECK, Prionics, Switzerland). Cows were housed in an isolation facility, fed alfalfa hay and provided with water ad libitum. All cows were fecal culture positive for *Map* at necropsy.

Samples (mesenteric lymph nodes, ileocecal lymph nodes, and ileum) were collected from the experimentally and clinically infected animals at necropsy. Samples were also obtained from three 17-18 month old, mixed breed beef steers (from the WSU Meats laboratory) for use as negative controls following necropsy of the animals used in the study. The steers were considered negative for *Map* based on results showing intestinal mucosal tissues were negative for *Map* by culture and PCR and also negative by fecal culture.

All protocols and procedures were approved by the Washington State University Institutional Animal Care and use Committee.

Cannulation surgery

Cannulation surgeries involving the 4 calves were performed as previously described (Allen et al., 2009). Two of the three *Map* infected, adult dairy cows were cannulated as described with a modifications (Allen et al., 2009). Briefly; surgery of adult cattle was performed while standing under regional anesthesia utilizing a right flank approach. Regional anesthesia was provided via an inverted L block using approximately 200 ml of 2%

lidocaine HCl (Turner et al. 1989). The remainder of the procedure including placement of the canula was performed as described (Allen et al., 2009).

Bacteria and culture

Map isolates used in this study included Linda (ATCC 43015) and Ben (ATCC 43544), isolates originally obtained from humans with CD (Chiodini et al., 1984). Both isolates were fully characterized and shown to contain multilocus short sequence repeats (SSRs) of *Map* genotypes identical to those obtained from cattle (Ghadiali et al., 2004). The isolates have also been shown to carry typical bovine *Map* genotypes using whole genome scans with comparative microarray hybridizations (Paustian et al., 2008).

Map isolates were prepared as a 1:1 mixture with 1×10^8 CFU diluted in 20ml of phosphate buffered saline (PBS). A mixture of the two isolates was used due to the limited number of calves available for the study. In addition, it was not clear whether either isolate would establish and infection following passage through a human. Calves 127, 129, and 131 were inoculated daily for a total of 3 days as described (Allen et al., 2009). Briefly, a size 20 French Foley catheter was inserted into the cannula, directed orally, and the cuff inflated with air to obstruct ingesta flow. The inoculum was injected into the isolated section of ileum via the Foley catheter and the catheter was left in place for 1 hour to facilitate adequate bacterial-mucosal contact.

Endoscopic evaluation and Biopsies

Endoscopic evaluation was performed as described (Allen et al., 2009). Biopsies were performed by inserting the gastroscope through the cannula into the ileum and directed orally or aborally. Air was used to insufflate the intestinal lumen to facilitate advancement of the scope. Following visual examination, a 2.8 mm biopsy instrument was inserted via the endoscope port to retrieve 15 to 20 mucosal and submucosal biopsies from a range of areas located within 10 cm of the cannula. Biopsies samples were obtained the day prior to inoculation, weekly for 4 weeks, and then monthly for up to 10 months.

Polymerase chain reaction (PCR) for *Map* DNA detection

Biopsy and necropsy samples for PCR detection of *Map* DNA were stored in micro-centrifuge tubes at -20° C until processed. The tissue DNA was extracted using a commercial kit (DNeasy® tissue kits; QIAGEN Valencia, CA) as described by Quist et. al. (Quist et al., 2002).

Oligonucleotide primers were derived from the DNA insertion sequence IS*900*. Primers are shown in (Table I). The first step PCR reaction mixture consisted of 25 μl of Go Taq® Green Master Mix (Promega, Madison, WI), 1 μl of (25 pmole/μl) upstream primer, 1 μl of (25 pmole/μl) downstream primer, 5 μl of the DNA sample, 18 μl of nuclease free water. The PCR cycling conditions included: 35 cycles at 95° C for 1 minute, 58° C for 1.5 minute, and a final extension at 72° C for 1.5 minute in a Thermal cycler PCR System 9700 (Gene Amp, Foster City, CA). The second step (nested) PCR reaction mixture consisted of 25 μl of Go Taq® Green Master Mix, 1 μl of (25 pmole/μl) upstream primer, 1 μl of (25 pmole/μl) downstream primer, 1 μl of the DNA sample, 22 μl of nuclease free water. The PCR cycling conditions included: 25 cycles at 95° C for 1 minute, 58° C for 1.5 minute, and a final extension at 72° C for 1.5 minute. Negative controls consisted of sterile water and DNA extracted from intestinal mucosa samples taken from a JD negative animal. *Map* DNA extracted from intestinal mucosa from a clinical cow was used as positive control. The amplification product was analyzed using gel electrophoresis on 2% agarose gel.

RNA extraction and quantitative real time reverse transcriptase PCR (QRT-PCR)

Ileocecal and mesenteric lymph node tissues (50 to 100 mg) were collected from experimentally infected and control calves at necropsy for analysis of cytokine profiles. Tissues were snap-frozen in liquid nitrogen and stored at -80° C until processed. Similar tissues were collected from three uninfected control steers and three naturally infected cows (1181, 3696, 1111) for comparison of cytokine profiles. Tissue RNA was stabilized before processing by adding 10 volumes of RN*Alater*®-ICE (Applied Biosystems, TX) and stored at -20° C overnight. Tissue was transferred into a 2 ml screw cap tube containing 200 μl of 1 mm glass beads (BioSpec Products, Inc., OK) and 1 ml of TRIzol Reagent (Invitrogen, CA). The tube was processed two times in a FastPrep®-24 instrument (MP biomedicals, CA) for 45 sec at a setting of 6.5 with a 5 min interval on ice. The tube was centrifuged at 12,000 × g at 4° C. The supernatant was transferred into a new micro-centrifuge tube, and RNA extracted. Genomic DNA contamination was removed with a Turbo DNA-free kit (Applied Biosystems, TX), and 2μg of RNA were reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA).

The RT-PCR reaction mixture was prepared as a 20 μl volume by mixing Power SYBR Green PCR Master Mix (Applied Biosystems, CA), template cDNA, and a gene specific primer set for each gene listed in (Table I). QRT-PCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, CA). After 40 cycles of amplification, dissociation curves for each primer set were generated to confirm a single peak. The QRT-PCR data were analyzed with StepOne software v2.1. Two housekeeping genes (β-actin and GAPDH) were used as controls.

Culture of Tissues and Feces

Map was isolated from tissues and fecal samples as previously described (Allen et al., 2009).

Blood and tissue processing for flow cytometry

Blood was obtained from the control and experimentally calves and the clinically infected cows to evaluate the immune response to *Map* antigens ex vivo using a flow cytometric assay (Allen et al., 2009). Blood was processed for tissue culture and flow cytometry as described (Koo et al., 2004; Allen et al., 2009). Johnin purified protein derivative (jPPD) and a preparation of soluble antigen (SAg) were used to stimulate cultures of PBMC from the control and infected animals (Allen et al., 2009).

Flow Cytometric Analysis

Flow cytometric analysis was performed as described (Allen et al., 2009). Freshly isolated cells and preparations of cells cultured for 6 days were labeled with the following monoclonal antibodies (mAb): CD335 (AKS1, IgG1)(a gift from A. Storset), CD2 (MUC2A, IgG2a), CD4 (ILA11A, IgG2a), CD8 (7C2B, IgG2a), CD25 (CACT116A, IgG1 and LCTB2A, IgG3), CD45R0 (ILA116A, IgG3), γδ TCR δ chain specific (GB21A, IgG2b) (all other mAbs were obtained from the Washington State University Monoclonal Antibody Center). Combinations of 3 or 4 mAbs were used to distinguish NK cells, CD4 and CD8 T cell subsets, and $CD2^+$ and $CD2^-$ γδ T cell subsets. $CD2^+$ and $CD2^-$ γδ T lymphocytes correspond to the two major $WC1^-$ and $WC1^+$ subsets of γδ T lymphocytes respectively in ruminants (Davis et al., 1996). The mAbs specific for CD4 and CD8 were combined with CD45R0 to distinguish naïve versus memory T lymphocyte subsets. Anti-CD25 was included in each combination to determine the proportion of activated proliferating cells in each cell subset. CD335 was a gift from Anne Storset (Storset et al., 2004). The remaining mAbs were obtained from the WSU Monoclonal Antibody Center.

A FACSort flow cytometer equipped with argon and red lasers, a Macintosh computer, and Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA) was used to collect data. FCS Express software (De Novo software, Thornton, Ontario, Ca) was used to analyze data. The gating strategy used to obtain the data is illustrated in Figure 1.

Histopathology

Biopsy specimens and ileal tissue samples obtained at necropsy were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (HE) for morphologic evaluation (Rosadio et al., 1988). Replicate sections were stained with Fite's acid fast stain and examined at 400 × magnification.

Statistics analysis

One way ANOVA with repeated measures and Newman-Keuls multiple comparison tests were used to compare pre-inoculation flow cytometric data to 4, 5, and 11 month post inoculation data (PI). CD4 and CD8 CD25 RPMI (non- antigen stimulated) control samples were subtracted from antigen stimulation samples to show the difference post stimulation. Data were transformed using arcsin to normalize data from percentages. An unpaired t-test was run on GraphPad Prism 5 (GraphPad Software, INC. La Jolla, CA 92037 USA) to compare flow cytometric data from clinically infected cows to data from calves 11 months PI. One way ANOVA and Newman-Keuls multiple comparison tests were used to compare cytokine levels between negative control animals and experimentally and clinically infected animals.

Results

Animal health

All 4 cannulated calves recovered without complication from surgery. Ileal cannulas were largely well tolerated, producing no adverse systemic effects, only minor, local, superficial skin irritation that was readily treated. While all calves remained healthy throughout the duration of the study, at 5 months post inoculation, calf 131 developed more marked irritation around the cannula necessitating its removal. The infection resolved following removal of the canula. The calf remained in the study for 4 more months without access to the ileum for biopsies. Calf 129 was euthanized 11 months post inoculation due to leg fracture. Calf 127 was maintained for 15 months PI. Clinically affected cows were maintained for one week post cannulation surgery to demonstrate the technical feasibility of cannulation in older infected animals and to obtain FC data on the proliferative response following antigenic stimulation. The cows were then necropsied to obtain tissue and cells for FC and QRT-PCR analysis.

Endoscopic evaluation of ileum over time

Calves 129, 131 and 132 possessed grossly normal appearing ileal mucosa on endoscopic evaluation throughout the trial (Fig. 2). At 10 months post inoculation, the ileal mucosa of calf 127 appeared mildly thickened and corrugated. Thickening of the ileal mucosa was clearly evident in clinically affected cows (Fig. 2).

Detection of *Map* in tissues and Feces

Two of three experimentally inoculated calves (127 and 129) were culture positive for *Map* on biopsy tissues obtained endoscopically at 10 months post inoculation. All experimentally inoculated calves possessed culture positive tissues obtained at necropsy. Tissue and feces obtained from the uninfected control calf (132) were *Map* culture negative during the entire study.

Map DNA was obtained from biopsy samples from all 3 infected calves including: 8 of 11 samples for calf 127, 3 of 11 for calf 129, and 1 of 6 samples for calf 131 (Table II). Culture and PCR positive preparations of tissue were also obtained from the clinical cows at necropsy.

Fecal samples from infected calves and the negative control calf were culture negative during the duration of the study.

Flow cytometric (FC) analysis of PBMC

The FC results with PBMC from the calves infected with the human isolates of *Map* were very similar to the results obtained from calves infected with bovine *Map* isolates (Allen et al., 2009; Koo et al., 2004) and naturally infected cows. CD4 lymphocytes comprised an average of 18.65% (14.02% - 27.16%) in calves and 17.08% (8.31% - 26.66%) in clinical cows, of total lymphocyte population at the initiation of 6 day cultures. At 4 months post inoculation, CD25 was consistently up regulated on proliferating CD4 memory T cells from experimentally infected calves compared to the uninfected control (Fig 3). Because data for SAg and jPPD were essentially identical, only data for jPPD are shown. CD8 lymphocytes comprised an average of 10.96% (8.12% - 14.72%) in calves and 9.78% (4.48% - 13.85%) in clinically infected cows at the initiation of culture. The CD8 response was less vigorous than CD4 responses during the first months PI in experimentally inoculated calves. In contrast, there was a strong CD4 and CD8 proliferative response to SAg and PPD with PBMC obtained from cows at the late clinical stage of infection (Fig. 3).

Pre inoculation γδ T cells comprised an average of 24.48% (22.38% – 29.41%) of the total lymphocyte population at the beginning of culture for calves and 8.07% (3.88% - 17.41%) for clinically infected cows. WC1+ CD2- cells averaged 19.27% (15.38% - 25.96%) in experimentally inoculated calves and 5.89% (3.24% - 11.56%) in clinically infected cows. WC1- CD2+ cells averaged 5.21% (3.45% - 8.20%) in calves and 2.17% (.22% - 8.71%) in clinically infected cows. The γδ T cells did show an increase in expression of CD25, however, the increase was inconsistent in cells cultured with and without antigen, consistent with previous observations (Koo et al., 2004).

NK cells comprised an average of 6.26% (2.81% - 11.09%) of the total lymphocyte population at the beginning of 6 day cultures in calves and 3.77% (1.02% - 9.91%) in clinically infected cows. $CD2^+$ NK cells averaged 5.48% (2.23% - 10.43%) in calves and 2.9% (.87% -7.52%) in clinically infected cows while $CD2^-$ NK cells averaged .78% (.58% - 1.24%) and 1.04% (.15% - 2.39%) respectively. As previously noted, the NK cells proliferated in the presence and absence of antigen in some cultures (Allen et al., 2009). NK cells from the control negative animal did not proliferate in culture.

QRT-PCR

Gene profiles of cells present in samples of the ileocecal (ILN) and mesenteric lymph nodes (MLN) were obtained for analysis from 3 experimentally inoculated calves and three clinically infected cows. Data are only shown for the ILN since results were similar to the MLN (Fig. 4). Analysis revealed up regulation of multiple cytokine genes in both the experimentally infected calves and clinical infected cows in comparison to control animals. Of particular interest, expression of genes encoding IFN-γ, IL-22, and IL-17 were up regulated in experimentally and clinically infected animals in comparison with controls. (Fig. 4).

Histopathology

There were very few histopathological changes seen in tissues from the experimentally infected calves except for a transient infiltration of eosinophils during the first month PI. Very few multinucleated giant cells were visualized in any of the biopsy and tissue samples examined at necropsy. No acid fast bacteria were observed in tissues from the control and experimentally infected calves. In contrast, multinucleated giant cells containing acid fast bacteria were abundant in ileal tissues from clinically infected cows.

Discussion

The present study extends our initial observations that demonstrated an indwelling ileal cannula can be maintained in calves for up to 15 months, affording continuous access to study the interaction of *Map* with mucosal tissues. As demonstrated here, it is also possible to introduce and maintain cannulas in adult cows during the later clinical stage of infection. This finding indicates that this model can be used at all stages of infection to compare the immune response to *Map* in cattle with the response in humans. Initial use of the model has provided information on infectivity and the early immune response to *Map* isolates from bovine and human origin compared to the response in naturally infected cows at the late stage of infection. These findings reveal similarities to the immune response to *Mtb* and that pathogenesis involves the regulatory networks implicated in pathogenesis of CD.

Sequence analysis of bovine-origin and human-origin isolates used in this study (the first isolates obtained from patients with CD (Chiodini et al., 1984)) revealed that the SSR genotypes and genome contents are indistinguishable (Ghadiali et al., 2004; Motiwala et al., 2004; Paustian et al., 2008). Moreover, 52% of one of the bovine isolates analyzed in this survey showed they had the same single SSR genotype (7g-4ggt) as the one present in the isolate from humans. In addition, comparative transcriptional analysis of the human and bovine isolate responses following phagocytosis by primary cultures of bovine macrophages showed no appreciable difference in response, indicating that there is little or no functional difference in human isolates versus bovine isolates in in-vitro macrophage studies (Zhu et al., 2008). Similarly, no appreciable differences were observed in the cytokine responses to the bovine and human isolates following phagocytosis by a human macrophage cell line (THP-1), indicating that human isolates did not differ in their capacity to infect macrophages (Motiwala et al., 2006). As shown in the present study, no differences were observed in the capacity of the human isolates to infect, persist, and elicit an immune response compared to isolates from cattle. The studies extend our previous findings on the early and late stages of infection and offer initial evidence that the same cell subsets are involved in the immune responses of JD and CD. Exposure to *Map* through ingestion, direct inoculation into the peritoneum, or introduction directly into the ileum leads to a persistent infection (Allen et al., 2009; Koo et al., 2004; Stabel et al., 2009; Sweeney et al., 2006) and development of CMI that controls infection during the early stages of disease. The response is characterized by the development of a vigorous CD4 and less vigorous CD8 T cell response to *Map* antigens during the first year PI. The CD8 response increases over time and is more evident in experimentally infected animals maintained for over a year (Koo et al., 2004) and naturally infected clinically diseased animals as seen in this study.

We observed that γδT cells comprised a much higher proportion of PBMC in calves; both experimentally infected and in negative controls than in adult cows. This is attributable to a normal physiological change that occurs as calves age. Wyatt et al. have shown that the $WC1^+$ γδT cell frequency decrease as animals age (Wyatt et al., 1994). As noted previously, activation and proliferation of γδT and NK cells was inconsistent. They proliferated in some cultures and not others. No clear difference was observed between cells cultured with and without antigen (Koo et al., 2004).

As noted in the present study, there was an increase in expression of IFN-γ, IL-22, and IL-17 message in ileocecal lymph nodes obtained from experimentally and naturally infected animals compared to ileocecal lymph nodes obtained from negative control animals. Of interest, T cell clones derived from intra-lesional biopsies from CD patients also exhibited high expression of IL-17 and IFN-γ message. The IL-17/IL-23 axis of the immune response appears to be involved in the pathogenesis of CD, with IL-23 playing a pivotal role in regulating induction of Th17 cells and Tregs. An additional proinflammatory cytokine, IL-22, may also be involved (Brand, 2009). CCL20, a chemo-attractant chemokine secreted by activated mucosal epithelial cells appears to play a role in attracting $CCR6^+$ Th17 cells to areas of inflammation where they secrete IL-17 and IL-22 and contribute to maintenance of the inflammatory response (Brand, 2009).

Conclusion

The recent advances in elucidating the mechanisms of pathogenesis of CD and TB and our findings that the same cell subsets appear to be involved in the pathogenesis of JD provide insight into how *Map* could be involved in the pathogenesis of CD. Disease progression is associated with dysregulation of the IL-23/IL-17 axis and regulatory activity of $Foxp3^+$ Tr cells in TB (Kursar et al., 2007; Sharma et al., 2009; Khader and Cooper, 2008; Dheda et al., 2008; Scriba et al., 2008) and CD (Fuss et al., 2006; Hölttä et al., 2008; Saruta et al., 2007). As noted in our studies, increased levels of IFN-γ, IL-17 and IL-22 have also been observed in cells from the mesenteric lymph nodes of patients with CD (Sakuraba et al., 2009; Brand et al., 2006). Further studies are clearly needed for comparison of the immune response to *Map* in cattle and humans. The bovine ileal cannulation model affords an opportunity to investigate the mechanism of pathogenesis in the natural host and show how closely pathogenesis compares with CD at the cellular and molecular level.

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References

Allen AJ, Park KT, Barrington GM, Hamilton MJ, Davis WC. Development of a bovine lleal cannulation model to study the immune response and mechanisms of pathogenesis paratuberculosis. Clin Vaccine Immunol. 2009; 16:453–463. [PubMed: 19225077]

Autschbach F, Eisold S, Hinz U, Zinser S, Linnebacher M, Giese T, Löffler T, Büchler MW, Schmidt J. High prevalence of Mycobacterium avium subspecies paratuberculosis IS900 DNA in gut tissues from individuals with Crohn's disease. Gut. 2005; 54:944–949. [PubMed: 15951539]

Brand S. Crohn's disease: Th1, TH17 or both? The change of a paradigm: new immunological and genetic insights implicate Th17 cells in the pathogenesis of Crohn's disease. Gut. 2009; 58:1152–1167. [PubMed: 19592695]

Brand S, Beigel F, Olszak T, Zitzmann K, Eichhorst ST, Otte JM, Marquardt A, Jaga W, Popp A, Leclair S, Herrmann K, Seiderer J, Ochsenkühn T, Göke B, Auernhammer CJ, Dambacher J. IL-22 is increased in active Crohn's disease and promotes proinflammatory gene expression and intestinal epithelial cell migration. Am J Physiol Gastrointest Liver Physiol. 2006; 290:G827–G838. [PubMed: 16537974]

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. [PubMed: 12843021]

Chiodini RJ, Van Kruiningen HJ, Merkal RS, Thayer WR Jr, Coutu JA. Characteristics of an unclassified Mycobacterium species isolated from patients with Crohn's disease. J Clin Microbiol. 1984; 20:966–971. [PubMed: 6511878]

Crohn BB, Ginzburg L, Oppenheimer G. Regional ileitis: a pathologic and clinical entity. JAMA. 1932; 99:1323–1329.

Dalziel TK. Chronic interstitial enteritis. Br Med J. 1913; ii:1068–1070.

Davis WC, Brown WC, Hamilton MJ, Wyatt CR, Orden JA, Khalid AM, Naessens J. Analysis of monoclonal antibodies specific for the γδ TcR. Vet Immunol Immunopathol. 1996; 52:275–283. [PubMed: 8896216]

Dheda K, Chang JS, Lala S, Huggett JF, Zumia A, Rook GAW. Gene expression of IL-17 and IL-23 in the lungs of patients with active tuberculosis. Thorax. 2008; 63:566–568. [PubMed: 18511642]

Fuss IJ, Becker C, Yang Z, Groden C, Hornung RL, Heller F, Neurath MF, Strober W, Mannon PJ. Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. Inflamm Bowel Dis. 2006; 12:9–15. [PubMed: 16374252]

Ghadiali AH, Strother M, Naser SA, Manning EJB, Sreevatsan S. Mycobacterium avium subsp paratuberculosis strains isolated from Crohn's disease patients and animal species exhibit similar polymorphic locus patterns. J Clin Microbiol. 2004; 42:5345–5348. [PubMed: 15528739]

Hines ME II, Stabel JR, Sweeney RW, 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; 122:197–122. [PubMed: 17467201]

Hölttä V, Klemetti P, Sipponen T, Kociubinski G, Westerholm-Ormio M, Salo H, Räsänen L, Kolho KL, Färkkilä M, Savilahti E, Vaarala O. IL-23/IL-17 immunity as a hallmark of Crohn's disease. Inflamm Bowel Dis. 2008; 14:1175–1184. [PubMed: 18512248]

Juste RA, Elguezabal N, Pavón A, Garrido JM, Geijo M, Sevilla I, Cabriada JL, Tejada A, García-Campos F, Casado R, Ochotorena I, Izeta A. Asssociation 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. [PubMed: 18922720]

Khader SA, Cooper AM. IL-23 and IL-17 in tuberculosis. Cytokine. 2008; 41:79–83. [PubMed: 18218322]

Kirkwood CD, Wagner J, Boniface K, Vaughn J, Michalski WP, Catto-Smith A, Cameron DJS, Bishop RF. Mycobacterium avium subspecies paratuberculosis in children with early-onset Crohn's disease. Inflamm Bowel Dis. 2009; 15:1643–1655. [PubMed: 19462429]

Koo HC, Park YH, Hamilton MJ, Barrington GM, Davies CJ, Kim JB, Dahl JL, Waters WR, Davis WC. Analysis of the immune response to Mycobacterium avium subsp paratuberculosis in experimentally infected calves. Infect Immun. 2004; 72:6870–6883. [PubMed: 15557608]

Kursar M, Koch M, Mittrücker HW, Nouailles G, Bonhagen K, Kamradt T, Kaufmann SHE. Cutting edge: regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. J Immunol. 2007; 178:2661–2665. [PubMed: 17312107]

Mendoza JL, San-Pedro A, Culebras E, Cies R, Taxonera C, Lana R, Urcelay E, de la Torre F, Picazo JJ, Diaz-Rubio M. High prevalence of viable Mycobacterium avium subspecies paratuberculosis in Crohn's disease. World J Gastroenterol. 2010; 16:4558–4563. [PubMed: 20857526]

Motiwala AS, Amonsin A, Strother M, Manning EJB, Kapur V, Sreevatsan S. Molecular epidemiology of Mycobacterium avium subsp. paratuberculosis isolates recovered from wild animal species. J Clin Microbiol. 2004; 42:1703–1712. [PubMed: 15071028]

Motiwala AS, Janagama HK, Paustian ML, Zhu X, Bannantine JP, Kapur V, Sreevatsan S. Comparative transcriptional analysis of human macrophages exposed to animal and human isolated of Mycobacterium avium subspecies paratuberculosis with diverse genotypes. Infect Immun. 2006; 74:6046–6056. [PubMed: 17057086]

Naser SA, Collins MT, Crawford JT, Valentine JF. Culture of Mycobacterium avium subspecies paratuberculosis (MAP) from the blood of patients with Crohn's disease: a follow-up blind multi center investigation. The Open Inflamm J. 2009; 2:22–23.

Paustian ML, Zhu X, Sreevatsan S, Robbe-Austerman S, Kapur V, Bannantine JP. Comparative genomic analysis of Mycobacterium avium subspecies obtained from multiple host species. BMC Genomics. 2008; 9:135. [PubMed: 18366709]

Quist CF, Nettles VF, Manning EJ, Hall DG, Gaydos JK, Wilmers TJ, Lopez RR. Paratuberculosis in key deer (Odocoileus virginianus clavium). J Wildl Dis. 2002; 38:729–737. [PubMed: 12528439]

Robinson CM, Nau GJ. Interleukin-12 and interleukin-27 regulate macrophage control of Mycobacterium tuberculosis. J Infect Dis. 2008; 198:359–366. [PubMed: 18557702]

Rosadio RH, Lairmore MD, Russell HI, DeMartini JC. Retrovirus-associated ovine pulmonary carcinoma (sheep pulmonary adenomatosis) and lymphoid interstital pneuomina. I. Lesion development and age susceptibility. Vet Pathol. 1988; 25:475–483. [PubMed: 3212891]

Sakuraba A, Sato T, Kamada N, Kitazume M, Sugita A, Hibi T. Th1/Th17 immune response is induced by mesenteric lymph node dendritic cells in Crohn's disease. Gastroenterology. 2009; 137:1736–1745. [PubMed: 19632232]

Saruta M, Yu QT, Fleshner PR, Mantel PY, Schmidt-Weber CB, Banham AH, Papadakis KA. Characterization of FOXP3+CD4+ regulatory T cells in Crohn's disease. Clin Immunol. 2007; 125:281–290. [PubMed: 17897887]

Schmidt C, Giese T, Ludwig B, Mueller-Molaian I, Marth T, Zeuzem S, Meuer SC, Stallmach A. Expression of interleukin-12-related cytokine transcripts in inflammatory bowel disease: elevated interleukin-23p19 and interleukin-27p28 in Crohn's disease but not in ulcerative colitis. Inflamm Bowel Dis. 2005; 11:16–23. [PubMed: 15674109]

Scriba TJ, Kalsdorf B, Abrahams DA, Isaacs F, Hofmeister J, Black G, Hassan HY, Wilkinson RJ, Walzl G, Gelderbloem SJ, Mahomed H, Hussey GD, Hanekom WA. Distinct, specific IL-17 and IL-22 producing CD4+ T cell subsets contribute to the human anti-mycobacterial immune response. J Immunol. 2008; 180:1962–1970. [PubMed: 18209095]

Sharma PK, Saha PK, Singh A, Sharma SK, Ghosh B, Mitra DK. FoxP3+ regulatory T cells suppress effector T cell function at pathologic sites in miliary tuberculosis. Am J Respir Crit Care Med. 2009; 179:1061–1070. [PubMed: 19246720]

Sibartie S, Scully P, Keohane J, O'Neill S, O'Mahony J, O'Hanlon D, Kirwan WO, O'Mahony L, Shanahan F. Mycobacterium avium subsp paratuberculosis (MAP) as a modifying factor in Crohn's disease. Inflamm Bowel Dis. 2010; 16:296–304. [PubMed: 19824071]

Singh AV, Singh SV, Makharia GK, Singh PK, Schal JS. Presence and characterization of Mycobacterium avium subspecies paratuberculosis from clinical and suspected cases of Crohn's disease and in healthy human population in India. Int J Infect Dis. 2008; 12:190–197. [PubMed: 17913536]

Stabel JR, Palmer MV, Harris B, Plattner B, Hostetter J, Robbe-Austerman S. Pathogenesis of Mycobacterium avium subsp paratuberculosis in neonatal calves after oral or intraperitoneal experimental infection. Vet Microbiol. 2009; 136:306–313. [PubMed: 19135813]

Storset AK, Kulberg S, Berg I, Boysen P, Hope JC, Dissen E. NKp46 defines a subset of bovine leukocytes with natural killer cell characteristics. Eur J Immunol. 2004; 34:669–676. [PubMed: 14991596]

Sweeney RW, Uzonna J, Whitlock RH, Habecker PL, Chilton P, Scott P. Tissue predilection sites and effect of dose on Mycobacterium avium subs. paratuberculosis organism recovery in a short-term bovine experimental oral infection model. Res Vet Sci. 2006; 80:253–259. [PubMed: 16165171]

Turner, AS. Anesthesia. In: Turner, AS.; McIlwraith, CW., editors. Techniques in Large Animal Surgery. A Lea & Febiger Book, Williams & Wilkins; Baltimore: 1989. p. 10-11.

Wyatt CR, Madruga C, Cluff C, Parish S, Hamilton MJ, Goff W, Davis WC. Differential distribution of γδ T cell receptor positive lymphocyte subpopulations in blood and spleen of young and adult cattle. Vet Immunol Immunopathol. 1994; 40:187–199. [PubMed: 8160359]

Zhu X, Tu ZJ, Coussens PM, Kapur V, Janagama H, Naser S, Sreevatsan S. Transcriptional analysis of diverse strains Mycobacterium avium subspecies paratuberculosis in primary bovine monocyte derived macrophages. Microbes Infect. 2008; 10:1274–1282. [PubMed: 18692151]

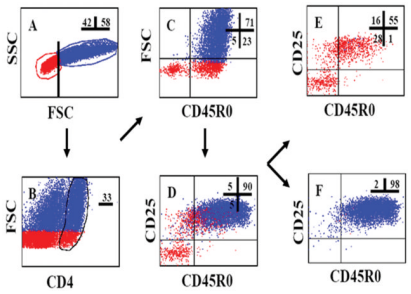

Figure 1.

Representative dot plots of CD4 T cells labeled with anti-CD4, -CD45R0, and -CD25, showing the gating strategy used to electronically isolate activated proliferating cells for analysis, (SSC vs FSC). Panel A, electronic gates were used to identify and color code resting (red) and activated proliferating (blue) cells. Panel B, an additional gate was used to isolate cell subsets for analysis (FSC vs CD4). Panel C, gated CD4 cells showing relative proportion of naïve and $CD25^-$ and $CD25^+$ memory CD4 T cells. Panel D, gated CD4 cells showing the proportion of naïve and memory T cells expressing CD25. Panels E and F, additional gates were placed on resting and activated cells to show the relative proportion of resting and activated naïve and memory CD4 cells expressing CD25. Note that all activated CD4 cells expressed CD25. For presentation of the data, the proportion of activated cells present in preparations of PBMC cultured in RPMI alone (as shown in panel C) were subtracted from the proportion of activated cells present in preparations of PBMC cultured in jPPD or SAg.

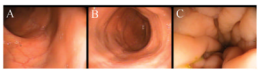

Figure 2.
Three endoscopic pictures of cannulated ileums. Picture A is of a normal ileum from the control negative calf 132. Picture B is from calf 129 (experimentally inoculated) with little to no changes in the ileal mucosa, and picture C shows the corrugated thickened appearance of the ileal mucosa from a naturally infected cow 1181 with clinical Johne's disease.

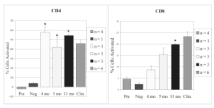

Figure 3.
Comparison of the expression of CD25 on activated CD4 and CD8 memory cells. The first bar shows pre-inoculation samples that were taken from all of the calves. The second bar shows the mean of the control negative calf sampled over 3 time points and bars 3-5 show the means of the inoculated calves at 4, 5 and 11 months PI. Bar 6 shows expression of CD25 on cells from clinical cows was similar to the expression on cells from experimentally inoculated calves. Asterisks indicate statistical significance difference $P < .05$ compared to pre inoculation results. Results from clinical cows were not significantly different from results from calves 11 months PI.

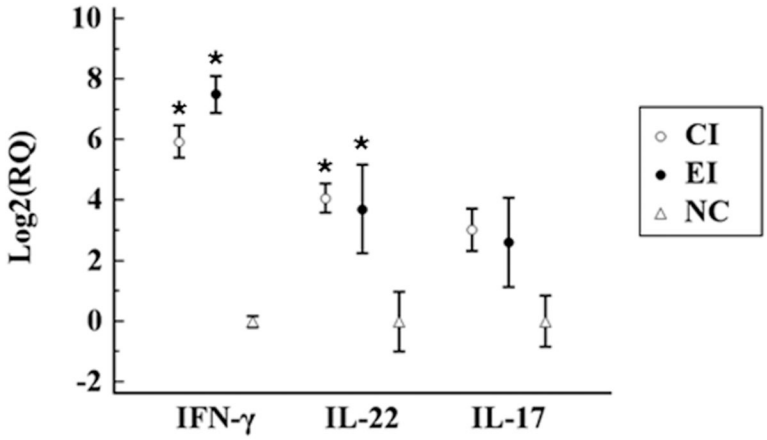

Figure 4.

Cytokine gene expression from ileocecal lymph nodes from clinically (naturally) infected cows (CI) n = 3 and experimentally inoculated calves (EI) n = 2 compared to negative controls (NC) n = 3. Control negative values were set at 0. IFN-γ and IL-22 are statistically significant with a P > .05.

Table I

Nucleotide sequences of Nested PCR and RT-PCR primers.

Target gene	Forward (5′ – 3′)	Reverse (5′-3′)	Source
	Nested PCR Primers		
IS900(1st set)	GTTCGGGGCCGTCGCTTAGG	GAGGTCGATCGCCCACGTGA	(Chamberlin et al., 2001)
IS900(2nd set)	ATGTGGTTGCTGTGTTGGATGG	CCGCCGCAATCAACTCCAG	
	RT-PCR Primers		
β-actin	GGAATCCTGCGGCATTCAC	GGATGTCGACGTCACACTTCA	This study
GAPDH	GCATCGTGGAGGGACTTATGA	GGGCCATCCACAGTCTTCTG	(Stabel et al., 2009)
IFN-γ	CTTGAATGGCAGCTCTGAGAAA	TGCAGATCATCCACCGGAAT	(Sweeney et al., 2006)
IL17	GGACTCTCCACCGCAATGAG	TGGCCTCCCAGATCACAGA	This study
IL22	GAGGTGCTGTTCCCCCAAT	GAAGGGCACCACCTTTTCC	This study

Table II

PCR results for calves 127 – 132.

Calf #	Day 0	1 Wk	2 Wk	3 Wk	1 Mo	2 Mo	3 Mo	4 Mo	5 Mo	6 Mo	8 Mo	10 Mo
127	Neg	Pos	Pos	Pos	Pos	NA	Neg	Pos	Neg	Pos	Pos	Pos
129	Neg	Pos	Pos	NA	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg
131	Neg	Neg	NA	NA	Pos	Neg	Neg	Neg	NA	NA	NA	NA
132	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg