Experimental Colitis Is Exacerbated by Concomitant Infection with Mycobacterium avium ssp. paratuberculosis

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Background: Crohn’s disease (CD) is a chronic inflammatory disorder of the human gastrointestinal tract. Although genetic, immunological, environmental, and bacterial factors have been implicated, the pathogenesis is incompletely understood. The histopathological appearance of CD strikingly resembles Johne’s disease, a ruminant inflammatory bowel disease, caused by Mycobacterium avium ssp. paratuberculosis (MAP), but a causative role of MAP in CD has not been established. In this work, we hypothesized that MAP might exacerbate an already existing intestinal disease.

Methods: We combined dextran sulfate sodium (DSS)-induced colitis with MAP infection in mice and monitored the immune response and bacterial count in different organs.

Results: An increased size of liver and spleen was observed in DSS-treated and MAP-infected animals (DSS + MAP) as compared with DSS-treated and certain pathogenic bacteria, and autoimmunity, an inappropriate immune response

Conclusions: Taken together, we present an in vivo model to study the role of MAP infection in CD. Our results confirm the hypothesis that MAP is able to exacerbate existing intestinal inflammation.

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Key Words: Johne’s disease, Crohn’s disease, DSS, MAP

Crohn’s disease (CD) is a human inflammatory bowel disease that has clinical symptoms, such as abdominal pain, loss of body weight, and bloody diarrhea. The transmural inflammation mainly affects the terminal ileum and colon but may extend to any part of the gastrointestinal tract from mouth to rectum. Genetic disposition,2,3 autoimmunity,4 an inappropriate immune response to the intestinal microbiota5 and certain pathogenic bacteria, and combinations of such factors have been proposed as the cause of CD.6–8 Johne’s disease (JD) is a chronic transmural inflammation mainly of the distal parts of the small intestine in ruminants. It is caused by Mycobacterium avium ssp. paratuberculosis (MAP), a very slowly growing acid-fast bacterium. The major infection route for JD is fecal oral, although intrauterine infection may occur.9 The clinical symptoms include chronic diarrhea and body weight loss, usually associated with a fatal outcome. An association of MAP with CD has been discussed for a long time. Clinical symptoms and the histopathological appearance between CD in man and JD in cattle are very similar.10,11 Environmental resistance allows MAP to survive the pasteurization process,12,13 and thus milk and dairy products may represent routes of transmission of MAP to humans. In accordance, MAP was described to cross species barriers and infect many other nonruminant animals and humans.11,14

Studies to reveal the presence of MAP in the intestine of patients with CD have given contradictory results although meta-analyses showed a positive correlation between detection of MAP in intestinal tissue and CD.10,11,15 Difficulties in cultivating MAP from clinical samples might be one explanation for the controversial reports because many MAP strains need months or even years...
to form colonies on solid media. Alternatively, MAP might be situated in anatomical locations like the mesentry that are usually not examined by standard techniques, as suggested recently. Hence, the question whether MAP plays a causative or disease-promoting role in CD remains unanswered.

Because MAP is found in the environment as well as in food, milk, and formulations for infants, humans are probably constantly exposed to MAP. Isolation of MAP from human samples might not be indicative of an infection per se or an involvement of the bacteria in disease. The higher rate of MAP detection in samples from patients with CD compared with healthy controls could suggest that either patients with IBD are more exposed or prone to MAP colonization. However, MAP might indeed actively contribute to disease initiation or progression.

In general, little is known about the infection process of MAP, its persistence in the host, and the immune responses elicited. To a large extent, this is due to the absence of an appropriate small animal model. Mice represent the most commonly used animals for infection models because of the availability of distinct inbred strains and the access to gene-deficient and transgenic animals that allow the investigation of individual host mechanisms. Although rodents and in particular mice are not natural hosts of MAP, mouse models for MAP infection have previously been developed using normal and immunocompromised. Nevertheless, improved experimental murine models should help to establish parameters of MAP infection and to clarify the involvement of MAP in CD.

Prompted by the controversy on the presence of MAP in intestinal tissues of patients with CD, we hypothesized that MAP might not cause CD but rather exacerbate a preexisting intestinal inflammation. Therefore, we combined the model of dextran sulfate sodium (DSS)–induced colitis with subsequent intraperitoneal MAP infection.

Our results show that MAP is able to infect the inflamed colonic mucosal tissue and significantly increases both mucosal and systemic signs of inflammation. We also observed large numbers of MAP in the mesenteric fat tissue causing chronic granuloma formation. Thus, the mouse model presented could be used to learn in more detail about MAP biology, immune response against MAP, correlation between MAP infection and inflammation, and might help to understand the association between MAP and CD in humans.

MATERIALS AND METHODS

Mice

Female 8-week-old C57BL/6J mice were purchased from Janvier (Le Genest-saint-Isle, France) and bred at the animal facility of the Helmholtz Centre for Infection Research (HZI) under specific pathogen-free conditions. Animal studies were carried out under good animal practice conditions in strict accordance with the German law for animal protection (Tierschutzgesetz, §7-9) from Okt.1. 2006, LAVES number: 33.14.42502-04/090/08.

Induction of DSS-induced Colitis and MAP Infection

Four percent DSS (35–50,000 kDa; MP Biomedical, Santa Ana, CA) was administered by means of the drinking water for 5 days. The bovine MAP strain DSM 44135 was grown on Watson Reid medium supplemented with mycobactin J (1 mg/L) at 37°C. For infection, mice were intraperitoneally injected with 10^8 MAP in PBS (PAA Laboratories, Pasching, Austria) 2 days after DSS administration. Body weight was monitored 2 to 3 times weekly. Mice were euthanized at day 1, 1 week, and 3 weeks after infection (1d, 1 wk, 3 wk p.i.).

Organ Plating

The liver, spleen, and mesenteric tissue homogenates were plated on Middlebrook 7H10 Agar (Difco, Heidelberg, Germany) containing Mycobactin J (IDVet Innovative Technology, Montpellier, France); colon and small intestine homogenates were also plated in the same medium supplemented with antibiotics (Vancomycin [Roth, Karlsruhe, Germany]; Amphotericin B [Roth]; and Nalidixin Acid [Sigma, Munich, Germany]). The plates were incubated at 37°C for up to 8 weeks.

Pathology and Histology

Histology was performed in the mouse pathology platform at HZI Braunschweig. Paraffin sections (3 μm) were stained with hematoxylin–eosin (H&E) and Zielh–Neelsen (ZN) staining according to standard laboratory procedures. Scoring of histopathological changes of both colon and mesenteric tissue was performed by a pathologist in a blinded fashion as described in Data, Supplemental Digital Content 1, http://links.lww.com/IBD/A539. Granuloma number and size in liver sections were analyzed in 0.2 cm² fields per section and quantified using AxioVision software (Carl Zeiss, Jena, Germany). The following antibodies were used for immunostaining of mesenteric tissue: antimyeloperoxidase (Medac, Wedel, Germany), anti-Mac-2 (Biozole) and anti-CD3 (Thermo Fischer Scientific, Schwerte, Germany).

Transmission Electron Microscopy

Mesenteric tissues were examined in a TEM 910 transmission electron microscope (Carl Zeiss, Jena, Germany) at an acceleration voltage of 80 kV. Images were recorded digitally at calibrated magnifications with a Slow-Scan CCD-Camera (ProScan, Graben, Germany) with ITEM-Software (Olympus Soft Imaging Solutions, Münster, Germany).

Enzyme-linked Immunosorbent Assay

Anti-mouse immunoglobulin A (IgA, Sigma), goat anti-mouse IgG (SIGMA) or rat anti-mouse IgM (BD Pharmingen, Heidelberg, Germany) were used for coating microtiter plates. Biotinylated rat anti-mouse IgA (Sigma), peroxidased goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), or biotinylated rat anti-mouse IgM (AbD Serotec, Kidlington, UK) antibodies were used for detection. Tumor necrosis factor (TNF–α) serum levels were detected using a mouse TNF-α enzyme-linked
imunosorbent assay kit (Biolegend, München, Germany) according to manufacturer’s protocol. Mouse cytokines and chemokines were analyzed using Bio-Plex Pro Mouse Cytokines 23-plex Assay (Bio-Rad, Munich, Germany) according to manufacturer’s protocol.

**Flow Cytometry**

Leukocytes in spleen tissue and mesenteric lymph nodes (mLN) were isolated and stained for flow cytometry analysis as described in Data, Supplemental Digital Content 2, http://links.lww.com/IBD/A540. To quantify MAP-binding IgG and IgM antibodies, equivalent volumes containing 10⁶ CFU/mL MAP and serum were mixed and incubated for 30 minutes on ice. Bacteria were washed in PBS/5 mM EDTA/2% fetal calf serum and resuspended in PBS/5 mM EDTA/2% fetal calf serum-containing anti-IgM PE (BD Pharmingen) or anti-IgG fluorescein isothiocyanate (Caltag laboratory, Burlingame, CA). Flow cytometry was performed using a LSR II analyzer (BD Bioscience, San Diego, CA). The data were analyzed using FACSDiva software (BD Bioscience) and FlowJo (TreeStar, San Carlos, CA). To quantify MAP-binding IgG and IgM antibodies, results are expressed as mean fluorescence intensity.

**Quantitative Real-time Polymerase Chain Reaction**

RNA from colon tissues was isolated with the RNeasy Mini kit (Qiagen, Limburg, Germany) according to manufacturer’s protocol. Complementary DNA synthesis was performed using the Revert Aid First Strand complementary DNA Synthesis Kit (Fermentas, Sankt Leon-Rot, Germany) according to the manufacturer’s protocol. Power SYBR Green PCR Master Mix (Applied Biosystems, Darmstadt, Germany) was used according to the manufacturer’s instructions. The primers are described in Data, Supplemental Digital Content 3, http://links.lww.com/IBD/A541.

**Statistics**

All data were analyzed with GraphPad Prism (San Diego, CA) software. In some figures, statistical difference between groups was determined by one-way analysis of variance followed by Tukey’s multiple comparison tests. In some figures, statistical difference between groups was determined by the Student’s t test. The results were considered statistically significant when P values were less than 0.05. *P values <0.05, **P ≤ 0.01, ***P < 0.001.

**RESULTS**

**MAP Infection After Colitis Induction Leads to Increased Weight Loss and Delayed Recovery**

Under the premises that MAP infection might exacerbate a preexisting colitis, we infected DSS-treated mice as described in Figure A, Supplemental Digital Content 4, http://links.lww.com/IBD/A543 and monitored the change in body weight (see Fig. B, Supplemental Digital Content 4, http://links.lww.com/IBD/A543). Untreated MAP-infected mice (H₂O + MAP) showed a reduction of body weight at 1 day p.i. and started to regain weight 2 days p.i. DSS-treated MAP-infected (DSS + MAP) and DSS-treated uninfected mice (DSS + PBS) started to lose weight after DSS administration and showed bloody diarrhea. Two days after DSS-free treatment, DSS + MAP mice were infected and showed a tendency to lose more body weight in comparison with DSS + PBS, although it did not reach statistical significance. However, all groups recovered the body weight at the end of experiment.

Dramatically elevated levels of cytokines and chemokines were observed in the sera of mice after MAP administration (Fig. 1A and see Table, Supplemental Digital Content 5, http://links.lww.com/IBD/A542). The kinetics of cytokines and chemokines levels differed significantly between both H₂O + MAP and DSS + MAP groups. H₂O + MAP mice showed higher TNF-α serum levels at 2 hours (P < 0.05) and 6 hours p.i. (P < 0.001) than DSS + MAP mice but decreased to normal at 24 hours p.i. Conversely, levels of TNF-α in DSS + MAP mice remained constitutively increased until 24 hours p.i. (Fig. 1A, P < 0.05) but had decreased to normal by 5 weeks p.i. (data not shown). The DSS + PBS group showed low TNF-α serum levels, and untreated uninfected group (H₂O + PBS) showed TNF-α serum levels below the detection limit. These results suggest that a differential response to MAP infection takes place in untreated and DSS-treated mice.

**Enlargement of Spleen and Liver in DSS-treated MAP-infected Mice**

At 1 week p.i., DSS + PBS (P < 0.01), H₂O + MAP (P < 0.05), and DSS + MAP (P < 0.001) mice showed higher total spleen weights compared with H₂O + PBS mice. DSS + MAP mice had significantly higher spleen weights at 1 (P < 0.01) and at 3 weeks p.i. compared with H₂O + MAP (P < 0.001, Fig. 1B). Similarly, a significant enlargement of the liver was observed in DSS + MAP mice at 3 weeks p.i. (see Fig. C, Supplemental Digital Content 3, http://links.lww.com/IBD/A543). Independent of MAP infection, DSS-treated mice showed a significant shortening (P < 0.001) of the colon at 1 day p.i. but recovered after 1 week p.i. (Fig. 1D).

**DSS-treated Mice Exhibit an Increased Splenic Polymorphonuclear and Monocyte Population**

At 3 weeks p.i., DSS + MAP and H₂O + MAP did not show a significantly different MAP counts in spleen tissue (see Fig. A, Supplemental Digital Content 5, http://links.lww.com/IBD/A544). However, the numbers of CD11b+Gr1bLy6Cint polymorphonuclear cells (PMN; P < 0.001) and CD11b+Ly6GbxGr1int monocytes (P < 0.01) were higher in DSS + MAP compared with H₂O + PBS and H₂O + MAP but similar to DSS-only treated animals (see Fig. B and C, Supplemental Digital Content 5, http://links.lww.com/IBD/A544). The elevation of the myeloid compartment in spleen tissue was therefore most likely induced by the DSS-induced damage of the colon that led to the dissemination of commensal bacteria. We did not observe any significant difference in the lymphocyte populations between the groups examined (data not shown).

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Increased Granuloma Formation in the Liver of DSS + MAP Mice

Severe granuloma formation was observed in both infected groups by histological examination, which was never seen in uninfected control animals (Fig. 2A, B). Granuloma formation represents the histopathological hallmark of mycobacterial infection in general and takes part in both, protective immunity and inflammatory tissue destruction.\(^2\) Number and size of granulomas (Fig. 2C, D) in DSS + MAP mice were significantly greater compared with \(H_2O+MAP\) mice \((P \leq 0.05)\). More PMNs and macrophages were detected in these areas in the DSS + MAP group, which might be the reason for the increased liver weight (Fig. 2B). In contrast, no significant differences in the MAP CFU count/g liver between \(H_2O + MAP\) and DSS + MAP mice were observed (Fig. 2E). Despite granuloma formation, MAP could be hardly visualized in liver tissue using Ziehl–Neelsen staining (data not shown). Thus, the enhanced frequency and extent of granuloma formation in DSS + MAP mice did not correlate with increased numbers of MAP. It rather might be caused by an altered immune reactivity due to the colonic inflammation or barrier break down.

Elevated Antibody Levels in DSS + MAP Mice

Immunoglobulin levels in serum and intestinal lavage were measured at 1 day, 1 week, and 3 weeks p.i. Total serum IgM and IgG increased over time in all groups most probably as a consequence of the increasing age of the animals (Fig. 3A and see Fig. A, Supplemental Digital Content 6, http://links.lww.com/IBD/A545). No significant difference was found for serum IgM levels among the groups examined (see Fig. A, Supplemental Digital Content 6, http://links.lww.com/IBD/A545). Also the total intestinal IgA and total serum IgA were not significantly altered by DSS administration and/or MAP infection (see Fig. C and D, Supplemental Digital Content 6, http://links.lww.com/IBD/A545). In contrast, total IgG levels was increased in animals of the DSS + MAP and the \(H_2O + MAP\) group as compared with \(H_2O + PBS\) and DSS + PBS animals but only the values of the DSS + MAP group became statistically significant compared with the control groups at 3 weeks p.i. (Fig. 3A, \(P \leq 0.05\)).

Similarly, flow cytometry-based quantification of MAP-binding antibodies revealed that MAP-binding IgG levels in the DSS + MAP group were significantly elevated \((P \leq 0.01)\) as compared with uninfected animals (Fig. 3B). MAP-binding IgM antibodies, however, showed no significant difference between the groups examined (see Fig. B, Supplemental Digital Content 6, http://links.lww.com/IBD/A545). Thus, the inflammation associated with DSS treatment led to increased total serum IgG levels and a significant increase of MAP-binding IgG.
Colonization of Mesenteric Tissue and Granuloma Formation by MAP

Colonization of the mesenteric tissue has been suggested to explain inconsistencies of MAP detection in biopsies of mucosal tissue from patients with CD. Therefore, we investigated the possibility of colonization of mesenteric tissue by MAP under our experimental conditions. High numbers of viable MAP could be detected in mesenteric tissue from MAP-infected mice at 3 weeks p.i. (Fig. 4A). Evidently, the presence of MAP induced accumulation of extremely high numbers of inflammatory cells, severe granuloma formation, tissue destruction, and necrosis (Fig. 4B and see Fig. B, Supplemental Digital Content 7, http://links.lww.com/IBD/A546). This was confirmed by the elevated histopathological score (Fig. 4A) and accumulation of neutrophils, macrophages, and T cells surrounding granulomas (see Fig. A, Supplemental Digital Content 7, http://links.lww.com/IBD/A546) in both MAP-infected groups. Additionally, transmission electron microscopy demonstrated MAP bacteria inside macrophages (Fig. 4C and see Fig. C, Supplemental Digital Content 7, http://links.lww.com/IBD/A546). No tissue alterations were found in DSS + PBS mice (data not shown). Unexpectedly, viable MAP counts and

FIGURE 2. Increased granuloma formation in liver of DSS + MAP mice. A and B, H&E staining of liver tissue sections at 3 weeks p.i. A, Normal liver tissue (×20 magnification) was observed in H₂O + PBS (left) and DSS + PBS (right). Scale bars, 50 μm. B, Granuloma formation (black arrow) was observed in infected mice H₂O + MAP (upper left) and DSS + MAP (upper right) in ×20 magnifications. Scale bars, 50 μm. Larger granulomas were observed in DSS + MAP (bottom right) in comparison with H₂O + MAP (bottom left) demonstrated in the ×40 magnifications. Scale bars, 100 μm. C and D, Number (C) and size (D) of granuloma in liver at 3 weeks p.i. Histological determination of granuloma numbers and size were performed in an area 0.2 cm² (n = 4–5 mice per group). C, Number of granuloma in an area 0.2 cm². D, Size of granuloma, expressed in square micrometer. E, Bacterial loads in liver were analyzed at 3 weeks p.i. by plating tissue homogenates on Middlebrook Agar (n = 3–11 mice per group). Data are representative of at least 2 independent experiments. *P < 0.05, ND = not detectable; NS, not significant.
histological score of the mesenteric tissue (Fig. 4A) were not significantly different in the DSS-treated and untreated group. Although infection of the mesenteric tissue by MAP attracted immune cells and led to severe granuloma formation, DSS treatment apparently did not influence the bacterial load and local tissue destruction.

FIGURE 3. Elevated antibody levels in DSS + MAP mice. A. Total serum IgG at day 1, 1 week, and 3 weeks p.i. were determined by enzyme-linked immunosorbent assay (n = 3–5 mice per time point per group). B. Mean fluorescence intensity of IgG bound to MAP was determined by flow cytometry. Serum was obtained 3 weeks p.i. (n = 3–5 mice per group). Data are representative of at least 2 independent experiments. *P < 0.05, **P < 0.01, NS = not significant.

FIGURE 4. MAP in mesenteric tissue generates granuloma. A. Bacterial loads in mesentery (black bars, scale in left y-axis) were analyzed at 3 weeks p.i. by plating tissue on Middlebrook Agar (n = 3–8 mice per group). Quantitative histological changes in mesentery (white bars, scale in right y-axis) were analyzed at 3 weeks p.i. (n = 3–5 mice per group). Data are representative for at least 2 independent experiments. B. H&E staining and ZN staining of mesentery at 3 weeks p.i. MAP-infected mice showed granuloma and huge numbers of inflammatory cells as seen in ×5 magnification of H&E staining (upper). ZN staining of mesentery at 3 weeks p.i. showed positive ZN staining in MAP-infected group in ×5 magnification (bottom). Scale bars, 200 μm. C. Transmission electron microscopic visualized mesentery of MAP-infected mice at 3 weeks p.i. MAP (white arrowheads) was residing inside of macrophage. Collagen fibers (black arrow) were also observed. Scale bars, 2 μm (upper) and 1 μm (bottom).
Cellularity of Mesenteric Lymph Nodes

The mLN are located in the mesenteric tissue and drain the intestine. Therefore, we analyzed the immune cell composition in mLN by flow cytometry at 3 weeks p.i. DSS treatment alone led to accumulation of CD4+ and CD8+ T lymphocytes, CD19+ B lymphocytes, CD11c+ dendritic cells, CD11b+Gr1+Ly6Cint PMNs, CD11b+Ly6C+Gr1+ monocytes, and CD11b+Gr1+Ly6Clo macrophages in the mLN (Fig. 5 and see Fig., Supplemental Digital Content 8, http://links.lww.com/IBD/A547). Concomitant MAP infection significantly (P < 0.05) reduced the numbers of CD4+ and CD8+ T cells and CD11b+Gr1+Ly6Clo macrophages (Fig. 5). Conversely, monocytes (P < 0.05) and PMNs (P < 0.05) were found in higher numbers in mLNs of DSS + MAP mice compared with DSS + PBS animals (see Fig., Supplemental Digital Content 8, http://links.lww.com/IBD/A547). Together, MAP significantly altered the immune cell composition in the mLNs with reduced lymphocytes and macrophages and increased monocytes and PMNs.

Tropism of MAP to the DSS-treated Colon and Delayed Recovery from Inflammation

MAP is known to elicit overt clinical disease in ruminants only after a prolonged period of latency. It then mainly affects the intestinal tract, but the underlying molecular mechanisms of this tissue tropism have not been resolved. Hence, we investigated the colonization of the inflamed colon by MAP under our experimental conditions. The viable count of MAP in total colon tissue was significantly higher after DSS treatment (Fig. 6A, P < 0.05). This effect was less pronounced and did not reach statistical significance in the small intestine (Fig. 6A). The tissue difference may be explained by the fact that DSS primarily damages the colonic mucosa and suggests preferential persistence of MAP in inflamed tissue.

DSS treatment leads to a severe mucosal damage and intestinal pathology in the colon. Both DSS + PBS and DSS + MAP groups showed high histopathological scores at day 1 p.i. The score was reduced after 1 week and was even less after 3 weeks p.i. The histopathological colon score of DSS + MAP mice was significantly higher (P < 0.05) than the score found in DSS + PBS mice at the 3 weeks p.i. This could be explained by a MAP-induced immune reaction and/or delay in the recovery of the colon mucosa (Fig. 6B). At that time point, H&E staining of tissue obtained from DSS + MAP animals revealed significant infiltration of inflammatory cells and epithelial hyperplasia, whereas tissue of DSS + PBS animals only showed low infiltration of inflammatory cells (Fig. 6C).

In line with the results from the histopathological score, colon tissue of DSS + MAP animals in comparison with H2O + PBS, DSS + PBS, and H2O + MAP animals showed significantly higher messenger RNA levels for ifn-γ (P < 0.01) and il-10 (Fig. 6D, P < 0.05). In contrast, messenger RNA expression of il-1β was increased to a similar degree after DSS administration, MAP infection, or both (P < 0.05). Expression of inf-α and il-6 showed no significant difference between the groups (data not shown). Taken together, the damaging effect of DSS treatment on the colonic mucosa resulted in higher MAP colonization and delayed tissue recovery possibly due to a higher inflammatory response.
DISCUSSION

DSS-induced colitis is a commonly used murine model to study inflammatory bowel disease of humans. The chemical DSS exerts a direct cytotoxic effect on enterocytes, and the mucus layer resulting in barrier damage and translocation of commensal bacteria associated with an inflammatory response. An intact innate immune system, i.e., microbial recognition by toll-like receptors and the inflammasome is required for protection from colitis and subsequent tissue repair. In addition, intestinal monocytic phagocytes, such as macrophages and dendritic cells, were shown to harbor a suppressive influence on DSS-induced colitis thus supporting the critical role of the innate immune system in this model. Therefore, we used this model to test the hypothesis that MAP infection exacerbates a preexisting colitis. In line with our hypothesis, MAP infection led to a more pronounced reduction in body weight, altered cytokine profile, and delayed recovery from DSS-induced mucosal damage.

The reduction of body weight after MAP infection might be due to a strong immune response against MAP. MAP contains several potent immunostimulatory pathogen-associated molecular patterns that elicit a strong innate immune stimulation. Concomitant colitis significantly altered the kinetic of MAP-induced TNF-α expression leading to sustained elevated TNF-α, as one of the proinflammatory cytokines.

FIGURE 6. Differential colonization of DSS-inflamed colon by MAP and delayed the recovery. A, Bacterial loads in colon (black bars, scale in left y-axis) and small intestine (white bars, scale in right y-axis) were analyzed at 3 weeks p.i. by plating tissue on Middlebrook Agar with appropriate antibiotics (n = 3–11 mice per group). Data are representative of at least 2 independent experiments. B, Quantitative histological changes in the colon were analyzed at day 1, 1 week, and 3 weeks p.i. (n = 3–5 mice per time point per group). Data are representative of 2 independent experiments. C, H&E staining of colon tissue sections at 3 weeks p.i. Scale bars, 25 μm. H2O + PBS (upper left) and H2O + MAP (bottom left) mice showed normal tissue. DSS + MAP mice (bottom right) showed mild invasion of inflammatory cells (black arrow) and mild epithelial hyperplasia. DSS + PBS mice (upper right) showed slight invasion of inflammatory cells (black arrow). D, Messenger RNA expression of ifn-γ (upper left), il-10 (upper right), tnf-α (bottom left), and il-1β (bottom right) relative to rps9 from colon at 3 weeks p.i. (n = 4–5 mice per group). *P < 0.05, **P < 0.01, NS = not significant, ND = not detectable.
Spleen enlargement in DSS + MAP mice was most likely due to an increased influx, activation, or even expansion of myeloid cells, although the MAP bacterial burden was comparable with that of the H$_2$O + MAP mice. Similarly, liver tissues were enlarged in DSS + MAP animals but harbored a similar bacterial burden. Although granuloma formation was observed in livers of both DSS- and non-DSS-treated MAP-infected animals, more frequent and larger granulomas were found in the DSS + MAP group. Again, increased numbers of myeloid cells were detected by histology in tissue from DSS + MAP animals. Granuloma formation contributes to the restriction of mycobacteria. However, intracellular infection of macrophages within granulomas supports mycobacterial survival and persistence in the host.27 Cytokines and immune cells, particularly T cells are required for the granuloma formation.26,29 DSS treatment allows translocation of commensal bacteria from the intestinal lumen and promotes immune cell activation, which might support the formation of larger granulomas in liver.

In a recent review, it was suggested that large numbers of MAP might be present in the mesenteric tissue of patients with CD.17 MAP infect endothelial cells and proliferate within them. They may thereby remain undetected when mucosal biopsies are examined. Infection of the mesenteric fat tissue might cause focal obstruction within vessels leading to neoangiogenesis and "creeping fat" of mesenteric tissue as observed in individuals with CD and JD.17 Our study confirmed the presence of large numbers of MAP in the mesenteric tissue associated with granuloma formation and local tissue destruction as well as thickening of the mesentery. However, a more detailed analysis revealed that MAP resided predominantly in tissue macrophages in contrast to the postulated residence in endothelial cells.17

As discussed above, DSS treatment and MAP infection act as "double hit." This could exacerbate the disease due to the simultaneous confrontation of the immune system by 2 challenges. However, we found that macrophages and both CD4$^+$ and CD8$^+$ T cells in the mLN of DSS + MAP-treated mice were reduced compared with DSS + PBS-treated animals. This might be due to cell death induced by the presence of MAP in mesentery or other means of bacterial immune evasion.30

A very important finding of this study was that animals of the DSS + MAP group showed an enhanced colonization of the colon by MAP. The bacteria might be attracted directly by signals of the damaged cells or by the inflammation. Most likely, MAP uses myeloid cells like monocytes/macrophages as Trojan horses to reach the inflamed tissue. This would also explain why the colon as primary target organ of DSS-induced tissue damage is most heavily colonized by MAP.

The mucosal damage itself may help MAP to better survive and persist within colonic tissue. The presence of MAP or MAP-infected macrophages in the colon, however, might impair the healing process and might allow commensal intestinal bacteria to invade the colon tissue. This would explain the observed delay in tissue recovery after MAP infection. Alternatively, MAP could directly stimulate the immune system and enhance tissue destruction. This is supported by the high expression of IFN-γ, an important cytokine of the antimycobacterial host defense. Apparently, the presence of enhanced numbers of MAP increased the IFN-γ expression. However, high expression of the anti-inflammatory cytokine IL-10 might be needed to ameliorate and protect from excessive tissue damage due to an overshooting immune response.31 One study showed that complete removal of IL-10 leads to uncontrolled inflammatory responses and disease progression in M. tuberculosis infection.32

Our data are consistent with a previous study where persistent MAP infection enhanced the effect to acute mucosal injury.33 In contrast to our work, Johnson et al hypothesized that MAP exposure would sensitize the intestine to exogenous assaults by altering the mucosal response to injury for instance by influenced repair mechanisms. Thus in both systems, chronic MAP infection together with a perturbation of the mucosal integrity resulted in enhanced inflammation in the colon. We showed MAP infection causes a prolonged and more extensive response to acute mucosal injury. Independent of the particular experimental schedule, MAP has to be considered a "disease-promoting" factor. In both systems, MAP infection in combination with DSS or another assault might lead eventually to a chronic inflammation in colon. We believe that the 2 experimental system might complement each other to decipher the molecular mechanism underlying MAP-associated IBD.

In conclusion, we provide evidence that MAP infection is able to exacerbate and prolong an existing inflammatory intestinal disease. Several aspects described for JD and CD, such as the presence of MAP in macrophages of the mesenteric tissue or strong inflammation of the colonic mucosa were also observed in our model. The presented model might thus facilitate a more in-depth analysis of the association of MAP with intestinal mucosal inflammation including the underlying molecular mechanisms in the future.

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