

Lactobacillus reuteri promotes *Helicobacter hepaticus*-associated typhlocolitis in gnotobiotic B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice

Mark T. Whary,¹ Nancy S. Taylor,¹
Yan Feng,¹ Zhongming Ge,¹ Suresh
Muthupalani,¹ James Versalovic²
and James G. Fox¹

¹Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA, and ²Department of Pathology, Baylor College of Medicine and Texas Children's Hospital, Houston, TX, USA

Summary

To model inflammatory bowel disease, we assessed infection with *Helicobacter hepaticus* 3B1 (ATCC 51449) and a potential probiotic *Lactobacillus reuteri* (ATCC PTA-6475) in gnotobiotic B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice. No typhlocolitis developed in germ-free controls ($n = 21$) or in *L. reuteri* ($n = 8$) or *H. hepaticus* ($n = 18$) mono-associated mice for 20 weeks post-infection. As positive controls, three specific pathogen-free IL-10^{-/-} mice dosed with *H. hepaticus* developed severe typhlocolitis within 11 weeks. Because *L. reuteri* PTA-6475 has anti-inflammatory properties *in vitro*, it was unexpected to observe significant typhlocolitis ($P < 0.0001$) in mice that had been infected with *L. reuteri* followed in 1 week by *H. hepaticus* ($n = 16$). The *H. hepaticus* colonization was not affected through 20 weeks post-infection but *L. reuteri* colonization was lower in co-infected compared with *L. reuteri* mono-associated mice at 8–11 weeks post-infection ($P < 0.05$). Typhlocolitis was associated with an increased T helper type 1 serum IgG2c response to *H. hepaticus* in co-infected mice compared with *H. hepaticus* mono-associated mice ($P < 0.005$) and similarly, mRNA expression in caecal-colonic tissue was elevated at least twofold for chemokine ligands and pro-inflammatory interleukin-1 α (IL-1 α), IL-1 β , IL-12 receptor, tumour necrosis factor- α and inducible nitric oxide synthase. Anti-inflammatory transforming growth factor- β , lactotransferrin, peptidoglycan recognition proteins, Toll-like receptors 4, 6, 8 and particularly 9 gene expression, were also elevated only in co-infected mice ($P < 0.05$). These data support that the development of typhlocolitis in *H. hepaticus*-infected IL-10^{-/-} mice required co-colonization with other microbiota and in this study, required only *L. reuteri*. Although the effects other microbiota may have on *H. hepaticus* virulence properties remain speculative, further investigations using this gnotobiotic model are now possible.

Keywords: bacteria/bacterial immunity; gut immunology/disease; inflammatory bowel disease; innate immunity; mucosal immunity

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Correspondence: Dr M. T. Whary, 77 Mass Ave 16-825A, Cambridge, MA 02139, USA.

Email: mwhary@mit.edu

Senior author: Mark T. Whary

Introduction

Select human inflammatory bowel diseases such as Crohn's disease, are characterized by a genetically predisposed inflammatory response to commensal bacteria.¹ The idiopathic nature of human inflammatory bowel diseases has promoted the use of interleukin-10-deficient (IL-10^{-/-}) mice to fulfill Koch's postulates using potential bacterial pathogens,^{2,3} to examine the role of commensal organ-

isms⁴ and to investigate the interplay between otherwise non-pathogenic bacterial species in causing or ameliorating disease.^{5,6} *Helicobacter hepaticus* infection of IL-10^{-/-} mice has been used to model mechanisms of human inflammatory bowel diseases ever since the association between enterohepatic helicobacter infections and chronic typhlocolitis in immunocompromised mice was first suspected and later experimentally established.^{7–10} Similar features between Crohn's enterocolitis and the

H. hepaticus-infected IL-10^{-/-} mouse model include a pro-inflammatory T helper type 1 (Th1) immune response to enteric microbiota.¹¹ IL-10^{-/-} mice develop a Th1-biased inflammatory response to *H. hepaticus* driven by macrophages, CD4⁺ T cells, IL-12 and interferon- γ (IFN- γ) in the absence of IL-10-secreting T regulatory cells.^{12,13} Comparable to Crohn's lesions, typhlocolitis in *H. hepaticus*-infected IL-10^{-/-} mice is characterized by transmural inflammation, epithelial hyperplasia and dysplasia, primarily at the caecal-colonic junction with varying degrees of colitis and proctitis.¹⁴

Despite controlling for host background genetics, variable severity of disease in the *H. hepaticus* IL-10^{-/-} mouse model has been reported.^{13,15} Genetic variation in a 70-kb genomic island (*HHGI1*), considered a putative pathogenicity island in the *H. hepaticus* strain 3B1 (ATCC 51449), was identified and shown to be important for the A/J mouse model of hepatitis and the IL-10^{-/-} mouse model of colitis.^{2,16} Similarly, cytolethal distending toxin (CDT) present in *H. hepaticus*^{17,18} promoted typhlocolitis in the IL-10^{-/-} mouse model.¹⁹ One study suggested that the commensal microbiota may be the primary promoter of colitis in the IL-10^{-/-} model and that *H. hepaticus* does not induce or potentiate typhlocolitis in IL-10^{-/-} mice.¹⁵

For these reasons, to further evaluate the virulence potential of *H. hepaticus* to cause colitis in the IL-10^{-/-} mouse model under well-defined conditions, gnotobiotic IL-10^{-/-} B6.129 mice were infected with *H. hepaticus* 3B1 (ATCC 51449). The *H. hepaticus* 3B1 was selected because it is the prototypic enterohepatic helicobacter that was first shown to cause chronic active hepatitis²⁰ and hepatocellular carcinoma²¹ in male A/JCr mice and severe typhlocolitis in IL-10^{-/-} mice.¹³ We further assessed co-infection with human-derived *Lactobacillus reuteri* (ATCC PTA-6475) because it was previously shown to have probiotic immunomodulatory activity *in vitro*²² and had not previously been evaluated in a mouse model of inflammatory bowel diseases. Our objectives were to determine if *H. hepaticus* mono-association would cause typhlocolitis and if inflammation developed, could it be ameliorated by a probiotic *L. reuteri* PTA-6475, as had been shown in *H. hepaticus*-infected B6.129 IL-10^{-/-} male mice co-colonized with murine-derived *Lactobacillus paracasei* 1602 and *L. reuteri* 6798.⁶

Methods

Mice

B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice were originally obtained from Jackson Laboratories (Bar Harbor, ME) and were maintained specific pathogen-free (SPF) for murine viruses, pathogenic bacteria (including helicobacters) and parasites in an Association for Assessment and Accreditation of Laboratory Animal Care-accredited

barrier facility. These IL-10^{-/-} mice were re-derived by embryo transfer into the germ-free health status and subsequently bred to generate experimental groups. Germ-free IL-10^{-/-} mice were housed in sterile plastic film isolators confirmed to be maintained free of all aerobic and anaerobic bacteria and fungi by weekly microbiological monitoring. After experimental infections, isolator interior surfaces, drinking water, food and faeces were sampled weekly for contaminants by culture. Within isolators segregated by infection status, mice were housed in sterile open-top polycarbonate cages on autoclaved hardwood bedding and fed autoclaved water and diet (ProLab 2000; Purina Mills, St Louis, MO) *ad libitum*. Macroenvironmental conditions included a 14 : 10-hr light/dark cycle and temperature maintenance at 20 \pm 1°C. Control SPF IL-10^{-/-} mice were housed in a separate facility in standard microisolator cages under similar environmental conditions except their food and water were not autoclaved. Experiments were approved by the MIT Committee on Animal Care.

Experimental infection

Lactobacillus reuteri (ATCC PTA-6475) was originally isolated from human milk and *in vitro* inhibition of tumour necrosis factor- α (TNF- α) activity has been demonstrated.²² The *H. hepaticus* strain 3B1 (ATCC 51449) was the original isolate from an outbreak of hepatitis and hepatocellular carcinoma in control A/J mice used in carcinogenesis assays.^{20,23} The 3B1 was sequenced²⁴ and found to contain CDT and a putative pathogenicity island important for development of typhlocolitis in IL-10^{-/-} mice.² At 6–8 weeks of age, approximately equal numbers of male and female germ-free IL-10^{-/-} mice were randomly assigned to separate isolators to be either unmanipulated as controls ($n = 21$) or to be orally gavaged every other day for three doses of 2×10^8 colony-forming units (CFU) of *L. reuteri* alone ($n = 8$), 2×10^8 CFU of *H. hepaticus* alone ($n = 18$), or *L. reuteri* followed in 1 week by *H. hepaticus* infection ($n = 16$). Three helicobacter-free SPF IL-10^{-/-} mice were dosed with the same *H. hepaticus* inoculum as the gnotobiotic mice as a positive control to demonstrate its ability to cause typhlocolitis. Negative control SPF IL-10^{-/-} mice ($n = 3$) were maintained helicobacter-free²⁵ and then killed and examined post-mortem after 20 weeks.

Colonization levels of *L. reuteri* and *H. hepaticus* at the caecal-colonic junction

At 8–11 weeks post-infection (p.i.), tissue samples of the caecal-colonic junction from experimentally infected mice were obtained post-mortem and weighed using sterile technique for quantification of *L. reuteri* by limiting dilution culture and for *H. hepaticus* by real-time quantitative

PCR, as previously described.²⁶ In brief, samples for *L. reuteri* were 10-fold diluted in sterile saline and plated on blood agar under anaerobic conditions at 37°C and colony counts were performed after 24 hr of incubation. Because *H. hepaticus* does not grow in discrete colonies on agar, bacterial and host DNA were extracted from the caecum using the Boehringer-Mannheim High Pure PCR Template Preparation kit (Roche Molecular Biochemicals, Indianapolis, IN). Samples were probed with 18S rRNA-based primers for quantifying host DNA (Applied Biosystems, Foster City, CA) and with *H. hepaticus* DNA-specific primers and probe generated from the *H. hepaticus cdtB* gene as previously described²⁶ using PRIMER EXPRESS software (Applied Biosystems). At 20 weeks p.i., the caecal-colonic junction of experimentally dosed mice was cultured for *L. reuteri* to confirm colonization but *L. reuteri* was not quantified.

Clinical assessment and post-mortem examination

Mice were assessed for evidence of diarrhoea, dehydration and deteriorating body condition²⁷ with final body weights recorded at the post-mortem examination. Approximately 50% of randomly selected mice from each infection or control group were killed with carbon dioxide and examined post-mortem at 8–11 weeks p.i.; the remaining mice were killed and examined post-mortem at 20 weeks p.i. Serum and tissues were aseptically obtained for serology, microbiology, pathology and mRNA expression levels. Tissues for culture recovery of *L. reuteri* and *H. hepaticus* were stored in Brucella broth containing 30% glycerol at -70°C pending culture. Tissues for mRNA expression levels were flash frozen in liquid nitrogen and stored at -70°C pending extraction and PCR assays. Tissues for histology were fixed in 10% buffered formalin and processed routinely.

Histology

Formalin-fixed tissues were embedded in paraffin, sectioned at 4 µm, stained with haematoxylin and eosin and evaluated by a board-certified veterinary pathologist (S.M.) blinded to the sample identity. Lesion scores for inflammation, oedema, epithelial defects, hyperplasia and dysplasia of the caecal-colonic junction were evaluated on a scale of zero for normal to four for ascending severity and invasiveness of inflammatory lesions. Distinct from transmural inflammatory lesions, gut-associated lymphoid tissue (GALT) was semi-quantitatively assessed using a scale of zero (for minimal cellularity) to four for increased cellular expansion of organized lymphoid follicles, inclusive of Peyer's patches, and scattered aggregates of lymphoid cells that varied from small foci to isolated but organized follicles with germinal centres. To generate comparative scores, haematoxylin and eosin-stained longi-

tudinal section of coiled small intestine, the entire caecum with adjacent terminal ileum and proximal colon as well as multiple representative linear sections from transverse and distal colon were examined under both low-power and high-power objective fields.

Immunohistochemistry

Immunohistochemistry was performed to phenotype B cells, T cells, macrophages and proliferating cells at the caecal-colonic junction of three germ-free and three gnotobiotic IL-10^{-/-} mice infected with *H. hepaticus* alone, three infected with *L. reuteri* alone and three mice with *H. hepaticus* and *L. reuteri* infection at 20 weeks p.i. Formalin-fixed sections from the caecal-colonic junction were stained for the pan-B-cell marker CD45/B220 using monoclonal rat anti-mouse antibody (#550286; BD Pharmingen, San Diego, CA) and secondary goat anti-rat antibody (#559286; BD Pharmingen), pan-T-cell marker CD3 using rabbit anti-mouse CD3 antibody (#A0452; Dako, Carpinteria, CA), macrophage-restricted cell surface glycoprotein F4/80 using rat monoclonal antibody (#MF48015; Invitrogen, Carlsbad, CA) and secondary goat anti-rat antibody (#559286; BD Pharmingen), and nuclear cell proliferation marker Ki67 using mouse anti-human antibody (#550609; BD Pharmingen), developed using Dako Art kit (#K3954) per instructions. Methods for antigen retrieval, blocking steps and counterstaining were as previously described.²⁸

ELISA for serum IgG2c and IgG1 and mucosal IgA responses to H. hepaticus

Serum Th1-associated IgG2c²⁹ and Th2-associated IgG1 responses to outer membrane antigens of *H. hepaticus* were measured by ELISA as previously described.³⁰ Antigen was coated on Immulon II plates (Thermo Fischer Scientific, Pittsburgh, PA) at a concentration of 10 µg/ml with sera diluted 1 : 100. Biotinylated secondary antibodies included monoclonal anti-mouse antibodies produced by clones A85-1 and 5.7 (BD Biosciences, San Jose, CA) for detecting IgG1 and IgG2c, respectively. Incubation with extravidin peroxidase (Sigma, St. Louis, MO) was followed by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS[®]) substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for colour development. Absorbance (or optical density) development at 405 nm was recorded by an ELISA plate reader (Dynatech MR7000; Dynatech Laboratories, Inc., Chantilly, VA).

To measure mucosal IgA responses to *H. hepaticus*, four fresh faecal pellets were emulsified in protease inhibitor (Sigma), centrifuged briefly to pellet debris and stored at -70°C pending ELISA. Assay conditions were as for serum IgG1 and IgG2c except the faecal sample was used neat and biotinylated goat anti-mouse IgA (Southern

Biotechnology Associates, Birmingham, AL) diluted 1 : 2000 was used as the secondary antibody. Optical density values for *H. hepaticus*-specific IgA were normalized by optical densities measured for total IgA content of the faecal sample. Sheep anti-mouse IgA (Sigma M-1272) was used as a capture antibody at 5 µg/ml, followed by sample incubation and additional steps of extravidin peroxidase, ABTS[®] substrate as above.

PCR array for innate and adaptive immunity gene expression

Tissue samples of the caecal–colonic junction collected at post-mortem examination from three mice of each infection status at 8–11 weeks p.i. were snap frozen in liquid nitrogen. Total RNA was isolated using Trizol reagent following the supplier's instruction (Invitrogen) with further purification by removing DNA contamination using the RNeasy kit (Qiagen, Valencia, CA). Caecal–colonic mRNA (2 µg) was reverse-transcribed into cDNA using the High Capacity cDNA Archive kit following the supplier's instructions (Applied Biosystems). Expression of mRNA of mouse genes involved in innate and adaptive immunity was measured with an RT² Profiler PCR array (SABioscience Corporation) using real-time quantitative PCR (qPCR) performed in the 7500 Fast Real-Time PCR System (Applied Biosystems). Transcript levels of the detected genes were normalized to the endogenous control *GAPDH* transcript and expressed as fold change in reference to germ-free control mice using the Comparative C_T method (Applied Biosystems User Bulletin #2). Messenger RNA expression was considered significant if twofold or higher multiples of expression were measured compared with values obtained from the germ-free control mice.

Statistical analysis

Analysis of variance, regression and the Student's *t*-test were used to determine the influence of infection status on serum levels of antibodies, colonization levels of *H. hepaticus* and *L. reuteri* and gene expression levels in tissues collected from the caecal–colonic junction. Experimental groups were compared for severity of typhlocolitis using the Mann–Whitney non-parametric *t*-test or the Kruskal–Wallis one-way analysis of variance with the Dunns' post-test (GraphPad Software Inc., San Diego, CA). Results were considered significant at *P* < 0.05.

Results

Co-infection of IL-10^{-/-} mice with *L. reuteri* and *H. hepaticus* caused significant typhlocolitis

As positive controls for demonstrating the virulence of the *H. hepaticus* 3B1 strain, three SPF IL-10^{-/-} mice

experimentally infected with *H. hepaticus* 3B1 developed rectal prolapse with declining body condition by 11 weeks p.i. and were killed and examined post-mortem. There was gross thickening of the caecum and colon, moderately enlarged mesenteric lymph nodes and histologically, the caecal–colonic junction mucosa and submucosa had severe mononuclear inflammation, submucosal oedema, mild epithelial defects (glandular epithelial necrosis and luminal cell debris) and hyperplasia (Fig. 1c,d). Mild dysplasia was characterized by architectural distortion involving glandular elongation, gland splitting, loss of columnar orientation and mild cytological atypia. Two of three additional SPF IL-10^{-/-} mice maintained under helicobacter-free husbandry conditions through 20 weeks p.i. were free of significant gross or histological lesions at post-mortem examination (Fig. 1a). One SPF IL-10^{-/-} mouse had developed rectal prolapse and a thickened proximal colon but was PCR negative for *H. hepaticus*, as were the two clinically normal cage mates. Spontaneous colitis occurs in this helicobacter-free SPF IL-10^{-/-} mouse colony with a very low incidence.

Germ-free IL-10^{-/-} mice that were mono-associated with *L. reuteri* or *H. hepaticus* or co-infected with *L. reuteri* and *H. hepaticus* were killed and examined post-mortem at 8–11 and 20 weeks p.i. to confirm colonization with one or both bacteria and to assess potential pathology. The IL-10^{-/-} mice mono-associated with *L. reuteri* or *H. hepaticus* were clinically normal except for gross dilation of the gallbladder and caecum, characteristic of germ-free mice, and lacked gross evidence of caecal–colonic inflammation. In *L. reuteri* mono-associated mice (Fig. 1b) and in *H. hepaticus* mono-associated mice (Fig. 1g), there was histological evidence of small numbers of neutrophils, lymphocytes and macrophages scattered in the lamina propria of the small intestine, caecal–colonic junction and colon, without qualitative differences in number or distribution of these cell types compared with the germ-free control mice (Fig. 1h). Notably, there was no significant inflammation at the caecal–colonic junction (typhlocolitis) unless mice were co-infected with *L. reuteri* and *H. hepaticus* (Fig. 1e,f). At post-mortem examination 8–11 and 20 weeks p.i., IL-10^{-/-} mice co-infected with *L. reuteri* and *H. hepaticus* had gross dilation of the caecum comparable with the germ-free mice and the *L. reuteri* and *H. hepaticus* mono-associated mice but dual infection also produced gross enlargement of the mesenteric lymph nodes and thickening of the caecal–colonic junction. At both 8–11 and 20 weeks p.i., there was significant infiltration of inflammatory cells in the lamina propria of the caecal–colonic junction accompanied by distinct lymphoid aggregates in the submucosa with associated mild oedema, epithelial defects (surface epithelial tethering, decrease in goblet cells, focal crypt necrosis and abscessation), mild hyperplasia and minimal to mild dysplasia (occasional loss of

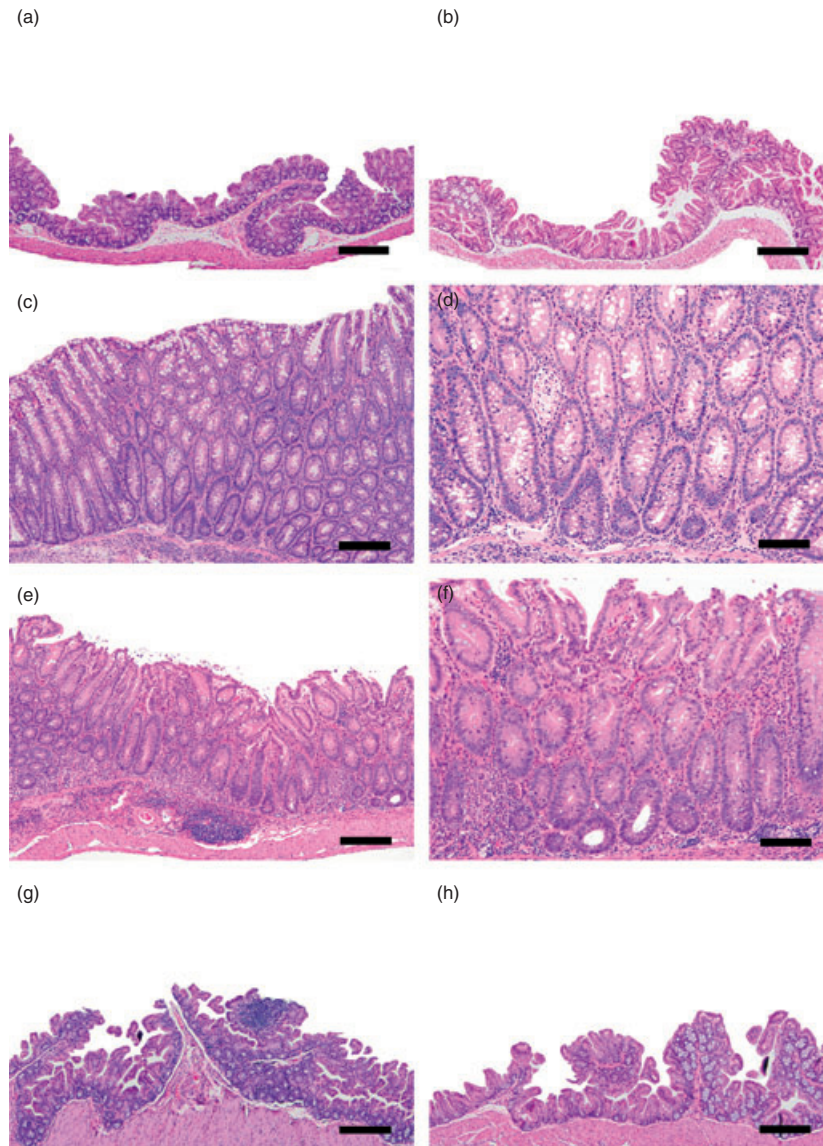


Figure 1. Germ-free B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice were colonized at 6–8 weeks of age with *Lactobacillus reuteri*, *Helicobacter hepaticus* or *L. reuteri* followed in 1 week by *H. hepaticus* and killed and examined post-mortem at 8–11 or 20 weeks post-infection (p.i.). (a) Caecal–colonic junction from helicobacter-free specific-pathogen-free (SPF) IL-10^{-/-} mouse at 28 weeks of age that lacked gross or histological lesions at necropsy. (b) Caecal–colonic junction from IL-10^{-/-} mouse mono-associated with *L. reuteri* for 20 weeks. Note absence of lymphoid development. (c, d) Representative low and high magnification images of the caecal–colonic junction from a positive control, 19-week-old SPF IL-10^{-/-} mouse infected with *H. hepaticus* for 11 weeks characterized by mucosal and submucosal mononuclear inflammation, submucosal oedema, mild epithelial defects (glandular epithelial necrosis and luminal cell debris), hyperplasia and mild dysplasia characterized by architectural distortion involving glandular elongation, gland splitting, loss of columnar orientation and mild cytological atypia. (e, f) Representative low and high magnification images of the caecal–colonic junction of a gnotobiotic IL-10^{-/-} mouse co-infected with *L. reuteri* and *H. hepaticus* for 20 weeks. Note mixed population of inflammatory cells in the lamina propria and distinct lymphoid aggregate in submucosa with associated mild oedema, epithelial defects (surface epithelial tethering, decrease in goblet cells, focal crypt necrosis and abscessation), mild hyperplasia and minimal to mild dysplasia (occasional loss of glandular columnar orientation and mild cytological atypia). (g) The *H. hepaticus* mono-association for 20 weeks did not result in significant inflammation and only mild lymphoid development in the caecal–colonic junction. (h) Caecal–colonic junction from germ-free IL-10^{-/-} mouse at the 20 weeks p.i. time-point. Note lack of lymphoid development or histological lesions. Bars: a, b, c, e, g, h = 100 μ m, d and f = 50 μ m.

glandular columnar orientation and mild cytological atypia). Compared with germ-free and mono-associated mice (Fig. 2a–c), there was significant inflammatory cell

infiltration in co-infected mice at both 8–11 and 20 weeks p.i. (both $P < 0.0001$) accompanied by submucosal oedema ($P < 0.03$, $P < 0.01$), and epithelial defects

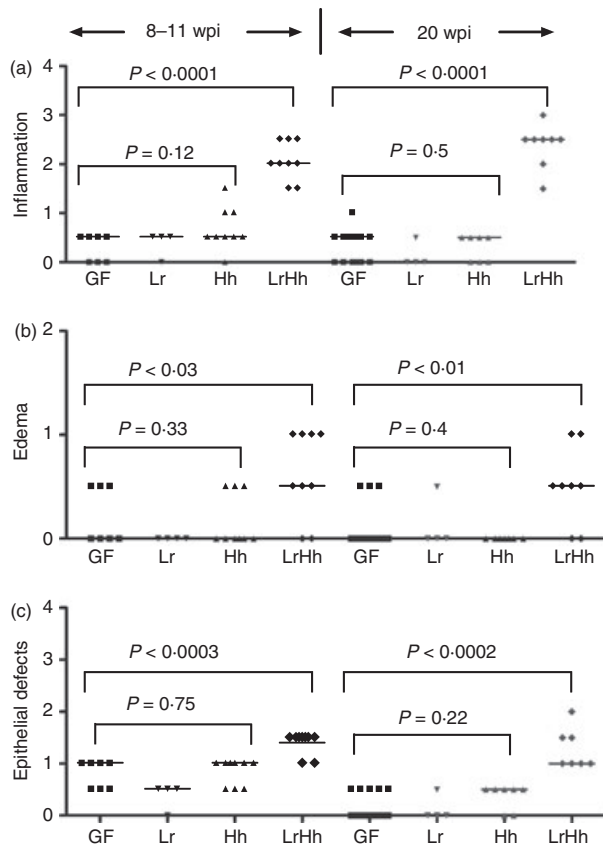


Figure 2. Germ-free B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice were colonized at 6–8 weeks of age with *Lactobacillus reuteri* (Lr) *Helicobacter hepaticus* (Hh) or *L. reuteri* followed in 1 week by *H. hepaticus* (LrHh) and killed and examined post-mortem at 8–11 or 20 weeks post-infection (p.i.). Median scores were significantly higher for (a) inflammation ($P < 0.0001$; $P < 0.0001$) (b) submucosal oedema ($P < 0.03$; $P < 0.01$) and (c) epithelial defects ($P < 0.0003$; $P < 0.0002$) at the caecal–colonic junction of IL-10^{-/-} mice co-infected with *L. reuteri* and *H. hepaticus* (LrHh) for 8–11 or 20 weeks p.i. (respectively) compared with germ-free (GF) mice or mice mono-associated with *L. reuteri* (Lr) or *H. hepaticus* (Hh). There were no significant differences in inflammation, submucosal oedema or epithelial defects in mice that were germ-free or mono-associated with either *L. reuteri* or *H. hepaticus* (analysis of variance P -values as shown).

($P < 0.0003$, $P < 0.0002$). Scores for crypt atrophy, hyperplasia and dysplasia were only significant in co-infected mice (Fig. 3a; all $P < 0.05$) and did not differ in extent by time-point. Additionally, typhlocolitis in co-infected mice was similar in severity at 8–11 and 20 weeks p.i. with no gender differences in lesion severity among co-infected mice.

Colitis distal to the caecal–colonic junction developed as a result of co-infection with *L. reuteri* and *H. hepaticus* but was not observed in mice mono-infected with *L. reuteri* or *H. hepaticus* (colitis scores not shown). In co-infected mice, colitis was less severe in the mid to distal colon

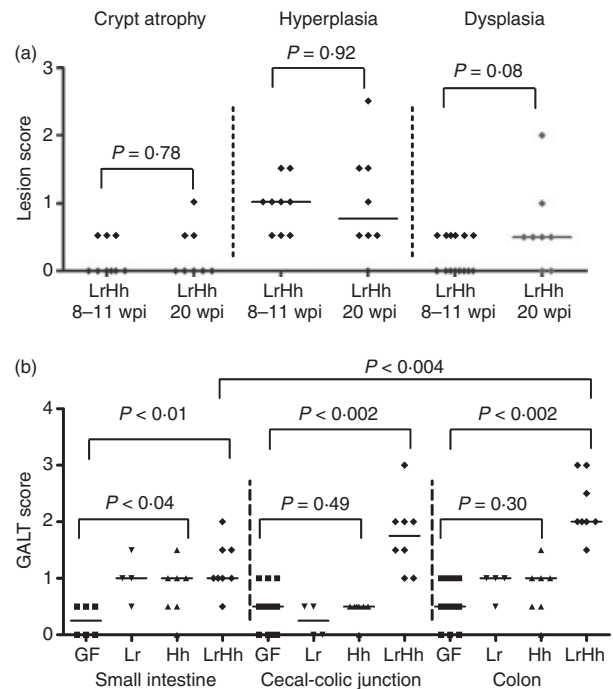


Figure 3. Germ-free B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice were colonized at 6–8 weeks of age with *Lactobacillus reuteri* (Lr) *Helicobacter hepaticus* (Hh) or *L. reuteri* followed in 1 week by *H. hepaticus* (LrHh), then killed and examined at post-mortem at 8–11 or 20 weeks post-infection (p.i.). (a) Crypt atrophy, epithelial hyperplasia and dysplasia developed only in co-infected IL-10^{-/-} mice (LrHh); these features were absent in all other experimental groups so median scores were all significant for co-infected mice only (all $P < 0.05$). Differences between time-points were not significant. (b) Median gut-associated lymphoid tissue (GALT) scores for small intestine, caecal–colonic junction and the colon at 20 weeks p.i. were significantly greater in co-infected IL-10^{-/-} mice (LrHh) compared with uninfected germ-free mice (GF) ($P < 0.01$, $P < 0.002$, $P < 0.002$, respectively). In co-infected mice the GALT scores were higher at the caecal–colonic junction and in representative sections of transverse and distal colon compared with small intestine ($P < 0.004$).

than at the more proximal caecal–colonic junction for inflammation ($P < 0.0007$), oedema ($P < 0.012$) and epithelial defects ($P < 0.0015$). Scores of the more distal colon for inflammation-driven crypt atrophy, epithelial hyperplasia and dysplasia were similar to scores observed for the caecal–colonic junction of co-infected mice.

In comparison to co-infected mice, significant inflammation did not develop at any level of the intestinal tract of mice mono-associated with *L. reuteri* or *H. hepaticus*. Compared with germ-free mice, GALT in the small intestine of mono-associated and co-infected mice had increased numbers of lymphoid mononuclear cells in Peyer’s patches and in small, scattered foci within the lamina propria (Fig. 3b; $P < 0.01$). Consistent with minimal inflammation, GALT in the caecal–colonic junction

and colon of mice mono-associated with *L. reuteri* or *H. hepaticus* were similar to those in germ-free mice with low lymphoid cell content (Figs 3b and 4). As an example, *H. hepaticus*-infected mice had a few small to medium-sized mucosal, and occasionally submucosal, foci of lymphoid aggregates and some distinct lymphoid follicles that contained mostly B cells (Fig. 4a), fewer T cells (Fig. 4c) and sparse macrophages (Fig. 4e). Proliferative (Ki67) activity in these lymphoid patches was minimal and was restricted to the basal aspect of the glandular acini and crypts (Fig. 4g). Mice co-infected with *L. reuteri* and *H. hepaticus* had similarly higher GALT scores at the

caecal-colonic junction and in representative sections of transverse and distal colon (Fig. 3b; $P < 0.002$). Co-infection with *L. reuteri* and *H. hepaticus* stimulated multifocal mucosal and submucosal expansion of lymphoid follicles at the caecal-colonic junction that were predominantly B cells (Fig. 4b), indicative of B-cell expansion, with T cells surrounding the B-cell germinal centres (Fig. 4d). Small numbers of B cells and considerable numbers of T cells were distributed throughout the lamina propria. Macrophages were also increased in the lamina propria of the mucosa and submucosa (Fig. 4f). Intraepithelial T lymphocytes and macrophages were also increased compared

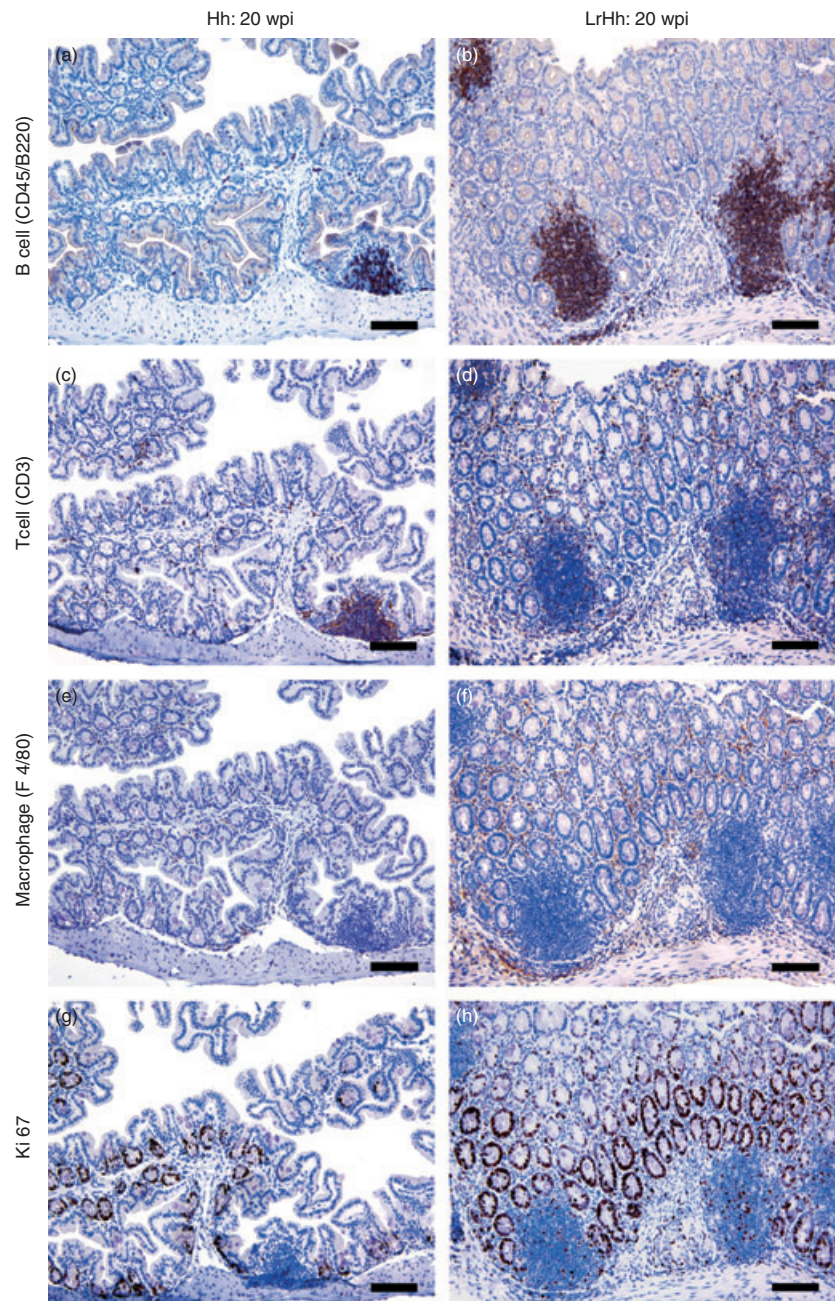


Figure 4. Immunohistochemistry to phenotype B cells (CD45/B220⁺), T cells (CD3⁺), macrophages (F4/80⁺) and proliferating cells (Ki67⁺) at the caecal-colonic junction of gnotobiotic IL-10^{-/-} mice infected with *Helicobacter hepaticus* alone (Hh) or with *H. hepaticus* and *Lactobacillus reuteri* (LrHh) for 20 weeks post-infection (20 wpi). Germ-free controls and mice mono-associated with *L. reuteri* alone were similar to *H. hepaticus* alone and are not shown. Mice with *H. hepaticus* alone had a few foci of lymphoid aggregates and some distinct lymphoid follicles that contained mostly B cells (a), fewer T cells (c) and sparse macrophages (e). Ki67 activity was minimal and was restricted to the acini and crypts (g). The co-infected mice (LrHh) developed prominent lymphoid follicles that were predominantly B cells (b), with T cells surrounding the germinal centres (d). Small numbers of B cells, considerable numbers of T cells and macrophages (f) were also increased in the lamina propria. Intraepithelial T lymphocytes and macrophages were also increased. Ki67 activity was prominent in the glands (h), within lymphoid follicles and in the inflammatory infiltrates. Magnification $\times 200$ (bar = 80 μm).

with very low numbers observed in mice colonized with *H. hepaticus* (Fig. 4c) or *L. reuteri* alone (not shown). Consistent with epithelial hyperplasia induced by co-infection (Fig. 3a), Ki67 activity was prominently increased in the glands (Fig. 4h). Mild to moderate Ki67 activity was also present within the distinct lymphoid follicles in the mucosa and submucosa of co-infected mice and scattered Ki67⁺ cells were evident as part of the inflammatory infiltrates within the lamina propria and glands.

Lactobacillus reuteri colonization was suppressed in co-infected IL-10^{-/-} mice but had no effect on *H. hepaticus* colonization dynamics

Colonization dynamics between *L. reuteri* and *H. hepaticus* were examined by assaying either CFU (*L. reuteri*) or the equivalent for *H. hepaticus* as estimated by qPCR and genome size. *Lactobacillus reuteri* was recovered by culture of caecal-colonic tissue from experimentally dosed mice at both 8–11 and 20 weeks p.i. The CFU of *L. reuteri* were determined in mice killed and examined post-mortem between 8 and 11 weeks p.i. and were significantly lower in mice co-colonized with *L. reuteri* and *H. hepaticus* compared with mice mono-associated with *L. reuteri* alone ($P < 0.05$, approximately 10^6 and 10^8 CFU per gram of tissue, respectively). We used qPCR to estimate *H. hepaticus* colonization levels because *H. hepaticus* grows in a mucoid film making colony counts impossible; there was no significant impact of *L. reuteri* on *H. hepaticus* colonization levels at the caecal-colonic junction at either 8–11 or 20 weeks p.i. ($P = 0.22$). Additionally, *H. hepaticus* colonization levels at 8–11 weeks p.i. persisted at similar levels until 20 weeks p.i. in mono-associated mice (mean \pm SE of CFU equivalent of $1.15E+06 \pm 2.59E+05$ and $1.15E+06 \pm 1.9E+05$, respectively) and in co-infected IL-10^{-/-} mice ($1.82E+06 \pm 8.68E+05$ and $4.44E+06 \pm 1.8E+06$, respectively).

Pro-inflammatory IgG2c seroconversion to *H. hepaticus* was more robust in mice co-infected with *L. reuteri*

The Th1-associated IgG2c and Th2-associated IgG1 serological responses to *H. hepaticus* were measured as an index of the pro-inflammatory and anti-inflammatory host responses. Mice co-infected with *L. reuteri* and *H. hepaticus* developed higher Th1-associated IgG2c and Th2-associated IgG1 responses to *H. hepaticus* at 8–11 weeks p.i. compared with mice mono-associated with *H. hepaticus* ($P < 0.004$, $P < 0.0005$, respectively, Fig. 5a). At 20 weeks p.i., the Th1-associated IgG2c response of co-infected mice remained significantly higher than that of *H. hepaticus* mono-associated mice ($P < 0.0005$). The *H. hepaticus* mono-associated mice still

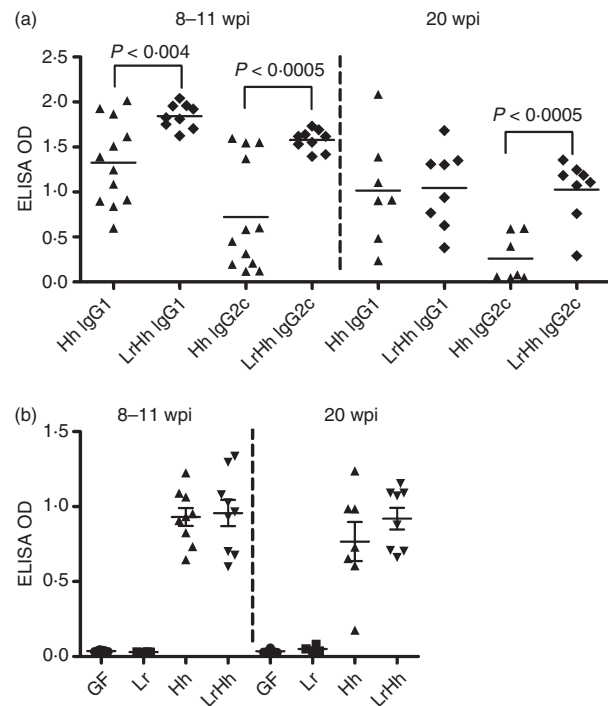


Figure 5. Germ-free B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice were colonized at 6–8 weeks of age with *Lactobacillus reuteri* (Lr), *Helicobacter hepaticus* (Hh) or *L. reuteri* followed in 1 week by *H. hepaticus* (LrHh) then killed and examined post-mortem at 8–11 or 20 weeks post-infection (p.i.). (a) The mean T helper type 2 (Th2)-associated IgG1 and Th1-associated IgG2c responses in co-infected mice were higher at 8–11 weeks p.i. compared with responses in *H. hepaticus* mono-associated mice ($P < 0.004$, < 0.0005 , respectively) and IgG2c was higher in co-infected mice at 20 weeks p.i. ($P < 0.0005$). The anti-inflammatory IgG1 response to *H. hepaticus* in mono-associated mice remained elevated through 20 weeks p.i. whereas the pro-inflammatory IgG2c response decreased over time. (b) Mucosal IgA responses to *H. hepaticus* were similarly robust in *H. hepaticus* mono-associated or co-infected mice (LrHh). Levels of IgA were similar at both time-points and as expected, germ-free and *L. reuteri*-infected mice had no detectable IgA to *H. hepaticus*.

had a robust anti-inflammatory associated IgG1 response, but a very weak pro-inflammatory IgG2c response, indicating that anti-inflammatory bias towards *H. hepaticus* was substantial. Notably the Th2-associated IgG1 to Th1-associated IgG2c ratio was higher and more variable in *H. hepaticus* mono-associated mice compared with co-infected mice, particularly at 20 weeks p.i. (mean ratio of 13 ± 15 (SD) versus 1.2 ± 0.6 , respectively).

Mucosal IgA responses were similar in mice mono-associated with *H. hepaticus* or co-infected with *L. reuteri*

Mucosal IgA responses to *H. hepaticus* were similarly robust in mice mono-associated with *H. hepaticus* or co-infected with *L. reuteri* and *H. hepaticus* (Fig. 5b).

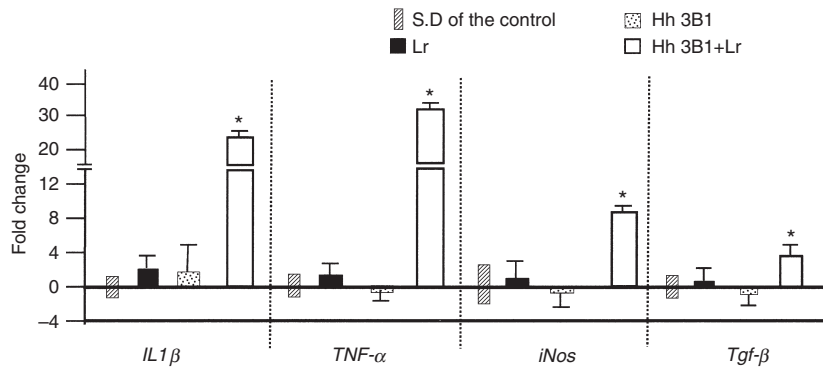


Figure 6. Germ-free B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice were colonized at 6–8 weeks of age with *Lactobacillus reuteri* (Lr) *Helicobacter hepaticus* (Hh) or *L. reuteri* followed in 1 week by *H. hepaticus* (LrHh) then killed and examined post-mortem at 8–11 or 20 weeks post-infection (p.i.). Mean fold-change of mRNA expression was measured in caecal-colonic tissue for interleukin-1β (IL-1β), tumour necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and transforming growth factor-β (TGF-β) from mice infected with *L. reuteri*, *H. hepaticus* or co-infected with both for 8–11 weeks p.i. Co-infected mice had significant up-regulation in all four mediators compared with mono-associated mice (Lr or Hh) or germ-free controls ($P < 0.05$). SD = standard deviation which is shown for controls (baseline values set at zero) and for experimental groups as error bars. Significant comparisons are indicated by asterisk (*).

Levels of IgA were similar at both 8–11 and 20 weeks p.i. and, as anticipated, germ-free and *L. reuteri*-infected mice had no detectable IgA to *H. hepaticus*.

Typhlocolitis from *L. reuteri* and *H. hepaticus* co-infection of IL-10^{-/-} mice was associated with elevated mRNA expression for innate and adaptive immune system genes

An expression array for innate and adaptive immune system gene expression was used to characterize the host inflammatory response to *L. reuteri* and *H. hepaticus* as independent and co-infections at 8–11 weeks p.i. With mRNA expression levels in germ-free control mice as a baseline for comparison, pro-inflammatory IL-1β, TNF-α, inducible nitric oxide synthase (iNOS) and anti-inflammatory transforming growth factor-β (TGF-β) were significantly elevated in caecal-colonic tissues obtained from IL-10^{-/-} mice co-infected with *L. reuteri* and *H. hepaticus* compared with germ-free controls or mice mono-associated with *L. reuteri* or *H. hepaticus* ($P < 0.05$) (Fig. 6). The fold change increase was highest for TNF-α, followed by IL-1β, iNOS and TGF-β expression in response to the co-infection. Expression of mRNA for all four mediators was similarly low in *L. reuteri* or *H. hepaticus* mono-associated IL-10^{-/-} mice.

Compared with levels measured in germ-free mice, Toll-like receptor (TLR) gene expression was elevated less than twofold for TLR1, TLR2 or TLR3 in any of the *L. reuteri* and/or *H. hepaticus* infected IL-10^{-/-} mice. There was a twofold or more increase for TLR4, TLR6, TLR8 and TLR9 in response to co-infection with *L. reuteri* and *H. hepaticus* ($P < 0.05$) (Fig. 7). The TLR4 gene expression also increased threefold in response to *L. reuteri* mono-association, which was similar to the fourfold

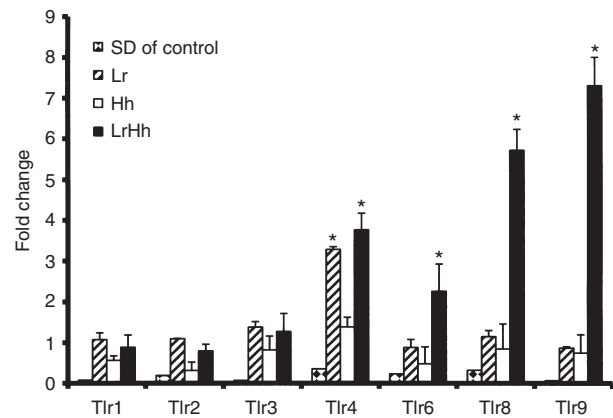


Figure 7. Germ-free B6.129P2-IL-10^{tm1Cgn} (IL-10^{-/-}) mice were colonized at 6–8 weeks of age with *Lactobacillus reuteri* (Lr), *Helicobacter hepaticus* (Hh) or *L. reuteri* followed in 1 week by *H. hepaticus* (LrHh) then killed and examined post-mortem at 8–11 weeks post-infection (p.i.). Mean fold-change of mRNA expression in caecal-colonic tissue for select toll-like receptors (TLR) from mice infected with *L. reuteri*, *H. hepaticus* or co-infected with both for 8–11 weeks p.i. TLR4, TLR6, TLR8 and TLR9 were up-regulated at least twofold by co-infection with LrHh compared with other infection groups ($P < 0.05$). Significant comparisons are indicated by asterisks (*). TLR4 gene expression increased threefold in response to *L. reuteri* mono-association which was similar to the fourfold increase noted in co-infected mice.

increase noted in co-infected mice. Expression of TLR4 in response to *H. hepaticus* mono-infection was elevated less than twofold, so was low compared with *L. reuteri* mono-infection or *L. reuteri* and *H. hepaticus* co-infection ($P < 0.02$). The TLR9 mRNA expression was elevated to the greatest degree in *L. reuteri* and *H. hepaticus* co-infected mice, followed by TLR8 and TLR6. Notably there were no significant TLR responses other than the

TLR4 response to *L. reuteri* in intestinal tissues of mice mono-infected with *L. reuteri* or *H. hepaticus*.

A broad variety of anti-microbially related genes of the immune system were activated twofold or more by *L. reuteri* and *H. hepaticus* co-infection (Table 1). Notably, genes that were up-regulated many-fold at the caecal-colonic junction of the co-infected mice included bactericidal virulence factors such as lactotransferrin, chemokine ligands for recruitment of inflammatory cells, transcription factors belonging to the IL-1 superfamily

and lymphoid cell receptors that amplify the Th1 response such as Trem1 and IL-12 β .

Discussion

In this study, mono-association of gnotobiotic B6.129 IL-10^{-/-} mice with *H. hepaticus* 3B1, the prototypic *H. hepaticus* strain containing CDT¹⁹ and a putative pathogenicity island,²⁴ did not induce significant typhlocolitis, consistent with an earlier report that gnotobiotic,

Table 1. Innate and adaptive immune system genes activated twofold or more by *Lactobacillus reuteri* and *Helicobacter hepaticus* co-infection

Gene	<i>L. reuteri</i>	<i>H. hepaticus</i>	<i>L. reuteri</i> and <i>H. hepaticus</i>	Biological relevance
<i>Casp4</i> (Caspase 4, apoptosis related cysteine peptidase)	1.5	1.0	3.0	Enzyme component of inflammasome-mediated apoptosis
<i>Ccl2</i> [Chemokine (C-C motif) ligand 2]	0.7	0.8	15.7	Monocyte chemotaxis
<i>Ccr3</i> Chemokine (C-C motif) receptor 3]	1.1	0.9	19.0	Mediates granulocyte activation and pro-inflammatory cytokine production
<i>Cd14</i> (CD14 antigen)	1.4	1.0	2.6	Binds lipopolysaccharide and other PAMPs
<i>Cfp</i> (Complement factor properdin)	1.1	0.9	4.2	Alternative pathway for complement activation, stimulates phagocytosis
<i>Clec7a</i> (C-type lectin domain family 7, member α)	1.1	0.7	3.8	PAMP receptor
<i>Cxcr4</i> (Chemokine receptor 4)	0.8	0.5	4.8	Receptor of CXCL12 mediating inflammatory cell recruitment
<i>Cybb</i> (Cytochrome β -245, β polypeptide)	1.1	0.7	8.5	Superoxide anion production by neutrophils
<i>IL-12β</i> (IL-12 receptor β 2)	1.4	1.0	3.7	Subunit of interleukin-12 binding receptor, up-regulated by interferon- γ
<i>IL-1α</i> (Interleukin 1 α)	1	0.8	3.6	Pro-inflammatory cytokine secreted by macrophages
<i>IL-1β</i> (Family member 6)	0.5	0.5	79.5	Structural and functional homology with IL-1 superfamily
<i>IL-1δ</i> (Family member 8)	1.5	5.2	4.4	Structural and functional homology with IL-1 superfamily
<i>IL-1ρ</i> (Family member 9)	0.8	0.7	3.3	Structural and functional homology with IL-1 superfamily
<i>Il1r2</i> (Interleukin 1 receptor, type II)	1.1	0.7	4.0	Member of IL-1 receptor family
<i>IL-1β</i> (Interleukin 1 receptor-like 2)	1.1	0.7	2.3	Member of IL-1 receptor family
<i>IL-1β</i> (Receptor antagonist)	1.0	1.0	4.7	Blocks binding of IL-1 α and IL-1 β
<i>Irf1</i> (Interferon regulatory factor 1)	1.2	1.0	2.9	Transcription factor regulating pro-inflammatory IL-18
<i>Ltf</i> (Lactotransferrin)	1.6	0.3	97.0	Bactericidal, binds iron, inhibits bacterial adhesion
<i>Lyz1</i> (Lysozyme 1)	1.4	1.4	3.6	Anti-bacterial muramidase released from neutrophils
<i>Ncf4</i> (Neutrophil cytosolic factor 4)	0.9	0.8	4.4	Regulator of NADPH-oxidase production of superoxide by phagocytes
<i>NfκBα</i> (NF- κ B inhibitor, α)	1.1	0.9	2.1	Binds and inhibits nuclear factor- κ B
<i>Pglyrp1</i> (Peptidoglycan recognition protein 1)	1.8	0.9	3.7	Neutrophil amidase targeting bacterial peptidoglycans
<i>Pglyrp2</i> (Peptidoglycan recognition protein 2)	1.7	0.9	4.4	Neutrophil amidase targeting bacterial peptidoglycans
<i>Prg2</i> (Proteoglycan 2, bone marrow)	0.5	0.3	19.2	Natural killer cell activation, constituent of eosinophil granules
<i>Ptafr</i> (Platelet activating factor receptor)	0.9	1.2	4.9	Binds PAF to signal recruitment of neutrophils
<i>Serpina1a</i> (Serine peptidase inhibitor, clade A, member 1a)	0.8	0.6	3.4	Serine protease inhibitor, protects host tissues from proteases
<i>Stab 1</i> (Stablin 1)	0.9	0.7	2.3	Cell-cell and cell-matrix interactions in vascular function and inflammatory processes
<i>Trem1</i> (Triggering receptor on myeloid cells 1)	2.0	2.0	7.1	Expressed on macrophages and amplifies T helper type 1 cytokine responses

IL-1, interleukin-1; PAF, platelet-activating factor; PAMP.

colitis-susceptible C57BL/6 × 129/Ola or 129/SvEv IL-10^{-/-} mice developed minimal colitis when mono-associated with *H. hepaticus* 3B1 for 16 weeks.¹⁵ The lack of intestinal lesions from *H. hepaticus* mono-association may reflect the *in vitro* ability of *H. hepaticus* to suppress innate immune responsiveness of murine epithelial cells.³¹ Additionally, a type 6 secretion system in *H. hepaticus*³² has been shown to have a protective, anti-inflammatory activity that suppresses innate and adaptive immune responses during intestinal colonization. Nonetheless, with a complex microbiota in an SPF IL-10-deficient host, the same *H. hepaticus* 3B1 inoculum caused significant typhlocolitis within 11 weeks. Our study is similar to a report of gnotobiotic IL-10^{-/-} mice that developed aggressive pancolitis when co-infected with non-pathogenic strains of *Enterococcus faecalis* and *Escherichia coli* that individually caused only mild inflammatory responses.⁵ Minimal inflammatory response to *H. hepaticus* in mono-associated IL-10^{-/-} mice appears reproducible¹⁵ but nonetheless is striking given the deficiency of IL-10, an anti-inflammatory cytokine important for T regulatory cell function.³³

This is a first report of mono-associated *L. reuteri* PTA-6475 persistently colonizing gnotobiotic IL-10^{-/-} mice without producing intestinal lesions; however, *L. reuteri* co-colonized with *H. hepaticus* caused severe typhlocolitis. These findings contrast with the attenuation of intestinal lesions in *H. hepaticus* infected, SPF B6.129 IL-10^{-/-} male mice when experimentally co-colonized with murine-derived *L. paracasei* 1602 and *L. reuteri* 6798.⁶ In the current study, typhlocolitis, epithelial defects, hyperplasia and dysplasia at the caecal-colonic junction lesions in co-infected mice were associated with elevated serum levels of Th1-associated IgG2c specific for *H. hepaticus*, possibly the result of an adjuvant effect of *L. reuteri* infection on the inflammatory response to *H. hepaticus*. Mucosal IgA responses to *H. hepaticus* were similar in mice mono-associated with *H. hepaticus* or co-infected with *H. hepaticus* and *L. reuteri*. Hence, *H. hepaticus* mono-associated mice developed a mucosal IgA response that was not accompanied by pro-inflammatory responses as demonstrated in co-infected mice that had elevated IL-1 β , TNF- α , iNOS and TGF- β gene expression in addition to increased numbers of T cells and macrophages at the caecal-colonic junction, the main site of inflammation. These data are consistent with a report that IL-10^{-/-} mice developed more robust gastritis and were colonized with higher levels of *Helicobacter pylori* when the gene for IgA was functionally inactivated,³⁴ supporting the theory that IgA responses protected both the host and bacteria against inflammation.

Lactobacillus reuteri PTA-6475 was originally isolated from human milk, suggesting a role in GALT development in human babies.³⁵ Until recently, only *in vitro* suppression of nuclear factor- κ B signalling, reduced TNF- α

activity and lymphoid apoptosis have been demonstrated for this *L. reuteri* strain.^{6,22,36} The *L. reuteri* may have suppressed nuclear factor- κ B signalling and so suppressed innate and adaptive immune responses to *H. hepaticus* during early co-infection. In a neonatal rat model, *L. reuteri* PTA-6475 significantly reduced intestinal mucosal levels of KC/GRO (\sim IL-8) and IFN- γ and intestinal lesions in neonatal rats fed formula containing lipopolysaccharide.³⁷ As *H. hepaticus* colonization became established, *L. reuteri* may have had an impact on the expression of virulence genes important for *H. hepaticus* colonization or virulence. Genome analysis²⁴ and infection studies with *H. hepaticus* strains containing sequence mutations in the cytolethal distending toxin gene *cdt*^{17,18} or deletions in a putative pathogenicity island (*HHGI*)^{2,16} have demonstrated a genetic basis for the virulence variability of various *H. hepaticus* isolates. Hence, *L. reuteri* may have promoted the inflammatory response to *H. hepaticus* by enhancing expression of *H. hepaticus* CDT or genes within the putative *HHGI* pathogenicity island or possibly down-regulating the type 6 secretion system of *H. hepaticus*.

Paradoxically, select strains of probiotic lactobacilli have been associated with stimulating the innate immune response that can progress into a Th1-mediated inflammation^{38,39} in contrast to other lactobacilli that promote expansion of T regulatory cells through an adjuvant-like effect mediated by TLR9 signalling.^{40,41} Thus, in contrast to suppression of innate immunity, *L. reuteri* PTA-6475 may have alternatively primed an immune response to subsequent *H. hepaticus* infection, as noted in another *L. reuteri* strain (100–23) that was reported to provoke pro-inflammatory cytokine responses early in the course of colonizing mice.^{14,38,42} The order and timing of the serial infections of *L. reuteri* and *H. hepaticus* may have been important as early immune priming by *L. reuteri* may have led to further promotion of inflammatory responses to *H. hepaticus* in co-infected mice, particularly when superimposed on an IL-10-deficient tissue environment.

Typhlocolitis developed only in response to co-colonization of *L. reuteri* and *H. hepaticus* and both bacteria were found in significant numbers in the same ecological niche of the caecal-colonic junction. Although some *L. reuteri* strains release known and putative anti-microbial factors that may inhibit proliferation of important gastrointestinal pathogens,⁴³ *L. reuteri* PTA-6475 is a relatively low producer of reuterin⁴⁴ and co-infected mice did not have *H. hepaticus* colonization levels different from those found in mono-associated mice. Colonization of *L. reuteri* in co-infected mice was reduced compared with *L. reuteri* mono-associated mice at 8–11 weeks p.i. and reduction of *L. reuteri* levels may have been secondary to typhlocolitis.

Chronic *H. pylori* gastritis and hepatitis in mice induced by a novel enterohepatic helicobacter species (MIT

96-1001) have been associated with *de novo* formation of organized tertiary lymphoid tissue.^{45,46} In this study, the *H. hepaticus* and *L. reuteri* induced typhlocolitis was accompanied by increased cellularity of GALT, observable as well-developed Peyer's patches in the small intestine accompanied by variably sized lymphoid aggregates, and was most evident at the caecal-colonic junction where lymphoid follicles were expanded by B and T cells. Intra-epithelial T lymphocytes plus B cells, T cells and macrophages were also increased in the surrounding lamina propria. Consistent with higher scores for epithelial hyperplasia induced by co-infection, Ki67 activity was prominently increased in the glands as well as within lymphoid follicles in the mucosa and submucosa. Comparison of GALT sections between germ-free mice and mice mono-associated with *H. hepaticus* or *L. reuteri* showed that the mono-associated groups had variably increased but comparatively low numbers of B cells, T cells and macrophages in contrast to the higher numbers in co-infected mice.

Messenger RNA expression levels of genes reflecting activation of the innate and adaptive immune responses were elevated at least twofold in IL-10^{-/-} mice co-infected with *L. reuteri* and *H. hepaticus*, reflecting a Th1-promoted inflammatory bowel disease as demonstrated for helicobacter-infected IL-10^{-/-} mice with complex microbiota.^{2,3,6,47} In support of *L. reuteri* promoting the inflammatory response to *H. hepaticus*, IL-10^{-/-} mice mono-associated with *H. hepaticus* exhibited no enhanced mRNA expression for inflammatory mediators and developed a higher anti-inflammatory-like IgG1-IgG2c ratio of isotype responses to the single infection compared with co-infected mice. Gene array data suggest that typhlocolitis was probably promoted principally by bacterial breakdown products, such as bacterial DNA CpG motifs activating TLR9 expression,⁴⁸ which was the TLR gene most activated by co-infection. Although DNA from commensal microbiota, including probiotic species, promotes T regulatory cell development through TLR9 signalling, commensal DNA also has natural adjuvant effects on intestinal innate immune responses.^{40,49} A TLR9 polymorphism in humans linked to increased risk of *H. pylori*-induced pre-malignant gastric pathology has been associated with increased TLR9 transcription secondary to enhanced binding of nuclear factor- κ B and subsequent increased host sensitivity to *H. pylori* CpG DNA.⁵⁰ The increased TLR9 mRNA expression noted in our study may reflect enhanced intestinal permeability reported for IL-10^{-/-} mice⁵¹ or enhanced sensitivity of the inflamed IL-10^{-/-} tissue environment to CpG motifs released from both *L. reuteri* and *H. hepaticus*. In addition, the balance between the pro-inflammatory and anti-inflammatory effects of probiotics probably depends on host genetics and the type and breadth of bacterial species comprising the intestinal microbiota. For example, TLR9 transcriptional activity and increased nuclear factor- κ B activation

appeared higher in C57BL/6 mice compared with BALB/c.⁵² *Helicobacter felis*-infected TLR9^{-/-} mice had lower lymphoproliferative and IFN- γ responses and reduced neutrophilic gastritis, supporting our link between significantly up-regulated expression of TLR9 and other pro-inflammatory host genes in response to *L. reuteri* and *H. hepaticus* co-infection.

Gene expression levels of TLR4, TLR6, and TLR8 were also elevated in *L. reuteri* and *H. hepaticus* co-infected IL-10^{-/-} mice. Although TLR4 mRNA expression was highest in co-infected mice, TLR4 mRNA was elevated in *L. reuteri* mono-associated mice even though TLR4 activation is most often associated with lipopolysaccharide activation. A role for TLR4 in T regulatory cell-mediated homeostasis in the *H. hepaticus*-infected IL-10^{-/-} colitis model was suggested by earlier onset of colitis in *H. hepaticus*-infected TLR4^{-/-} IL-10^{-/-} mice that had aberrant T regulatory Foxp3⁺ cells in the lamina propria that secreted IFN- γ , IL-17, and RANTES (regulated on activation, normal, T-cell expressed and secreted).⁵³ Little is known about the role of TLR6 in *H. hepaticus*-induced inflammation except that TLR6 complexed with TLR2 mediated IL-8 release from TLR2-transfected HEK293 cells incubated with *H. hepaticus*.⁵⁴ Notably, typhlocolitis in the co-infected IL-10^{-/-} mice may have been partially mediated through TLR8, as TLR8 activation has been shown in mice to reverse T regulatory cell suppression.⁵⁵ Importantly, gene expression for TLRs was measured on whole thickness samples of the caecal-colonic junction and phenotypic evaluation of these receptors by cell type or signalling activity may yield more information on the significance of the mRNA elevation noted here in response to *L. reuteri* and *H. hepaticus*. Additionally, extension of these studies into evaluation of germ-free, TLR knockout mice should reveal whether these TLR-mediated signals are inflammatory or regulatory in the response to co-infection.

These data support that the development of typhlocolitis in *H. hepaticus* infected IL-10^{-/-} mice required co-colonization with other microbiota and in this study, required only *L. reuteri*. Although the effects other microbiota may have on *H. hepaticus* virulence properties remain speculative, further investigations using this gnotobiotic model are now possible.

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Disclosure

The authors have no conflicts of interest to declare.

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