Peroral Low-Dose *Toxoplasma gondii* Infection of Human Microbiota-Associated Mice — A Subacute Ileitis Model to Unravel Pathogen–Host Interactions

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Within 1 week following high-dose *Toxoplasma gondii* infection, mice develop lethal necrotizing ileitis. However, data from a subacute *T. gondii*-induced ileitis model are scarce. Therefore, mice harboring a human gut microbiota were perorally infected with one cyst of *T. gondii*. Within 9 days post-infection, the intestinal microbiota composition shifted towards higher loads of commensal enterobacteria and enterococci. Following *T. gondii* infection, mice were clinically only mildly affected, whereas ≈60% of mice displayed fecal blood and mild-to-moderate ileal histopathological changes. Intestinal inflammation was further characterized by increased apoptotic intestinal epithelial cells, which were accompanied by elevated proliferating gut epithelial cell numbers. As compared to naive controls, infected mice displayed elevated numbers of intestinal T lymphocytes and regulatory T-cells and increased pro-inflammatory mediator secretion. Remarkably, *T. gondii*-induced apoptotic and pro-inflammatory immune responses were not restricted to the gut, but could also be observed in extra-intestinal compartments including kidney, liver, and lung. Strikingly, low-dose *T. gondii* infection resulted in increased serum levels of pro- and anti-inflammatory cytokines. In conclusion, the here presented subacute ileitis model following peroral low-dose *T. gondii* infection of humanized mice allows for detailed investigations of the molecular mechanism underlying the "ménage à trois" of pathogens, human gut microbiota, and immunity.

Keywords: host–pathogen interactions, *Toxoplasma gondii*, subacute ileitis mouse model, host immunity, human microbiota, fecal microbiota transplantation, secondary abiotic mice, intestinal, extra-intestinal, systemic immune responses

Introduction

Upon peroral infection with a high dose (i.e., >50 cysts) of the intracellular parasite *Toxoplasma gondii*, susceptible mice develop non-self-limiting acute necrotizing ileitis within 1 week [1–3]. This fatal inflammatory scenario is characterized by a CD4+ T lymphocyte dependent pro-inflammatory mediator storm with excessive secretion of tumour necrosis factor (TNF), interferon gamma (IFN-γ), and nitric oxide (NO), whereas anti-inflammatory interleukin (IL) 10 production constitutes counter-regulatory measures in order to limit immunopathological sequelae [3–7]. We have recently shown that the commensal gut microbiota plays a pivotal role during ileitis development [8]. Whereas mice with a depleted microbiota (i.e., secondary abiotic mice) following broad-spectrum antibiotic treatment were unaffected from high-dose *T. gondii* infection, secondary abiotic mice with a reconstituted murine gut microbiota following fecal microbiota transplantation (FMT) developed full-blown disease following ileitis induction and succumbed to infection [9]. Ileitis development was furthermore accompanied by profound shifts in gut microbiota composition (i.e., dysbiosis) as indicated by ileal overgrowth with Gram-negative aerobic (i.e., *Escherichia coli*) and anaerobic commensals (such as *Bacteroides* or *Prevotella* species), whereas lactobacilli and clostridia were virtually undetectable in the ileal lumen [9, 10]. *T. gondii*-induced ileitis was further exaggerated by Toll-like receptor (TLR)-4-dependent signaling of the lipopolysaccharide (LPS) derived from the Gram-negative commensal species overgrowing the ileal lumen [11]. Taken together, the high-dose *T. gondii*-infected mouse model resembles key features of inflammatory bowel diseases (IBDs) in humans such as acute Crohn's disease [3, 9, 12].

Very recently, we generated (with respect to their gut microbiota composition) "humanized" mice to mimic human gut microbiota conditions when dissecting the molecular mechanisms of the interplay between pathogens, commensal microbiota, and host immunity during infection and inflammation [13]. To accomplish this, secondary abiotic mice were subjected to human FMT, and the human microbiota could stably establish within the intestinal tract of the murine host for more than 6 weeks [13]. When perorally challenged with a high dose of 50 cysts of *T. gondii*, humanized mice also developed non-self-limiting T cell-driven ileitis within 7 days and exhibited not only intestinal but also extra-intestinal and systemic immunopathological sequelae of infection [14]. Whereas the high-dose *T. gondii* infection model does not allow for investigations of the pathogen–commensal microbiota–host immunity interplay beyond day 7 post-ileitis induction due to the fatal course of infection, data in a less acute (i.e., low dose) *T. gondii* infection model are scarce, but desirable. Therefore, in the present study, we surveyed intestinal, extra-intestinal, and systemic immune responses upon peroral infection of human microbiota-associated (hma) mice with only one cyst of *T. gondii* ME49 strain resulting in subacute and non-lethal ileitis.

Materials and Methods

Generation of Human Microbiota-Associated Mice. Female C57BL/6j wild-type mice were reared and maintained in the facilities of the "Forschungseinrichtungen für Experimentelle Medizin" (FEM, Charité — Universitätsmedizin, Berlin, Germany) under specific pathogen-free (SPF) conditions.

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Eight-week-old mice were transferred to sterile cages and treated with a quintuple antibiotic cocktail for 6 weeks in order to remove the murine gut microbiota as previously described [9]. Cultural and culture-independent (i.e., 16S ribosomal RNA [rRNA]-based molecular) quality control measures revealed the virtual absence of bacteria in the fecal samples derived from the generated secondary abiotic (i.e., gnotobiotic) mice as reported earlier [13, 15].

Fresh fecal samples free of enteropathogenic bacteria, viruses, and parasites were collected from five individual healthy human volunteers, dissolved in sterile phosphate buffered saline (PBS; Gibco, Life Technologies, UK), aliquoted, and stored at −80 °C as described earlier [13, 16, 17].

Three days before association of the microbiota-depleted mice with a complex human gut microbiota by FMT, the antibiotic cocktail was replaced by autoclaved tap water (ad libitum). Immediately before FMT, individual fecal aliquots were thawed and pooled [13, 16, 17], and secondary abiotic mice were subjected to the human fecal donor suspension by gavage (0.3 mL) on three consecutive days. Bacterial groups varied less than 0.5 logarithmic orders of magnitude between independent experiments (Figure 1) as assessed by both cultural and molecular analyses that had been described elsewhere [9, 13, 18]. To assure proper establishment of the human microbiota in the murine host, mice were kept for 2 weeks until ileitis induction.

Induction of Ileitis. In order to induce ileitis, hma mice were infected perorally with *T. gondii* (ME49 strain) by gavage as described previously [9, 11, 19]; however, the dose was reduced to one cyst in 0.3-mL brain suspension.

Sampling Procedures. Mice were sacrificed by isofluran treatment (Abbott, Greifswald, Germany) at day (d) 9 post-ileitis
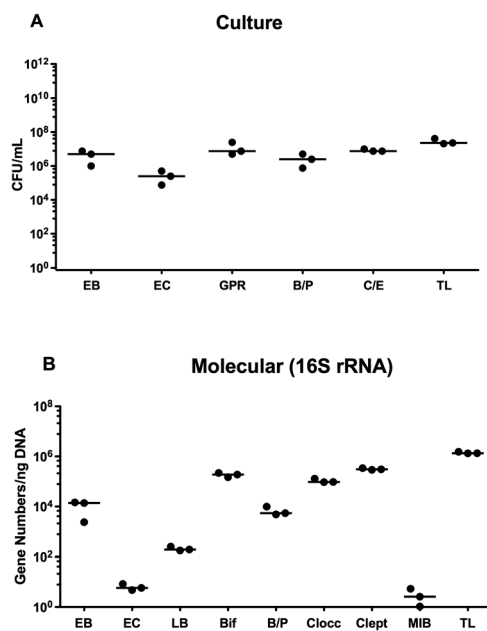

Figure 1. Microbiota composition of human donor feces. Before fecal microbiota transplantation of secondary abiotic (i.e., gnotobiotic) mice for three consecutive days, main intestinal bacterial groups were quantitatively assessed in human donor fecal suspensions. (A) Numbers of viable enterobacteria (EB), enterococci (EC), Gram-positive rods (GPR), *Bacteroides/Prevotella* species (B/P), *Clostridium/Eubacterium* species (C/E), and the total bacterial load (TL) were determined by culture and expressed as colony-forming units (CFU) per milliliter suspension. (B) 16S rRNA of the main intestinal bacterial commensals including enterobacteria (EB), enterococci (EC), lactobacilli (LB), bifidobacteria (Bif), *Bacteroides/Prevotella* species (B/P), *Clostridium coccoides* group (Clocc), *Clostridium leptum* group (Clept), *Mouse Intestinal Bacteroides* (MIB), and the total eubacterial load (TL) were analyzed by quantitative RT-PCR and expressed as gene numbers per ng DNA. Data shown are representative for three independent experiments.

induction. Cardiac blood, ileal, and colonic luminal samples, as well as ex vivo biopsies derived from spleen, liver, kidney, lung, mesenteric lymph nodes (MLN), ileum, and colon, were taken under sterile conditions and collected from each mouse in parallel for immunological, microbiological, and immunohistochemical analysis. For immunohistopathological analyses, tissue samples were immediately fixed in 5% formalin and embedded in paraffin.

Histopathology. Ex vivo biopsies derived from the terminal ileum were immediately fixed in 5% formalin and embedded in paraffin. Sections (5 μm) were stained with hematoxylin and eosin (H&E) and subjected to a standardized histopathological scoring system ranging from 0 to 6 as described earlier [9, 11].

Immunohistochemistry. In situ immunohistochemical analyses of paraffin sections from respective ex vivo biopsies were performed as reported earlier [11, 18, 20, 21]. In brief, primary antibodies against cleaved caspase-3 (Asp175, #9661, Cell Signaling, Leiden, Netherlands; 1:200), Ki67 (clone 16A8, #652401, BioLegend/Biozol, Eching, Germany; 1:200), CD3 (#IR50361-2, Dako, Santa Clara, CA, USA; 1:5) and FOXP3 (clone FJK-165, #14-5773, eBioscience, Frankfurt, Germany; 1:100) were used. For each animal, the average number of positively stained cells within at least six high power fields (HPF, 0.287 mm^2, 400× magnification) was determined microscopically by an independent blinded investigator.

Cytokine Measurements. Ileal and colonic samples were cut longitudinally and washed in PBS (Gibco, Life Technologies, Paisley, UK). Ex vivo biopsies derived from MLN, kidney (one half after longitudinal cut), liver (approximately 1 cm^2), lung, spleen (one half), or strips of approximately 1 cm^2 ileal or colonic tissue were placed in 24 flat-bottom well culture plates (Nunc, Wiesbaden, Germany) containing 500 μL serum-free RPMI 1640 medium (Gibco, Life Technologies) supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL; PAA Laboratories). After 18 h at 37 °C, culture supernatants and serum samples were tested for IFN-γ, TNF, IL-6, monocyte chemoattractant protein-1 (MCP-1), IL-6, and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences, Heidelberg, Germany) on a BD FACSCanto II flow cytometer (BD Biosciences) as described earlier [13, 22, 23]. NO concentrations were determined by the Griess reaction as stated elsewhere described earlier [9, 21].

Cultural Survey of Human Gut Microbiota Composition and Bacterial Translocation to Extra-Intestinal Compartments. For comprehensive quantitative and qualitative survey of the microbiota composition in fecal human donor suspensions and small as well as large intestinal luminal contents and, furthermore, of viable bacteria translocating from the intestines to extra-intestinal compartments including MLN, spleen, liver, kidney, and lung, respective intestinal samples and ex vivo biopsies were homogenized in sterile PBS (Gibco, Life Technologies, UK) and analyzed in serial dilutions on respective solid media as described earlier [9, 11, 24]. Cardiac blood was directly streaked onto solid media. Bacteria were grown at 37 °C for at least two days under aerobic, microaerobic, and anaerobic conditions.

Molecular Analysis of Gut Microbiota. Fresh ileal and colonic luminal samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further processing. DNA was extracted from intestinal luminal samples as described earlier [9, 25]. In brief, DNA was quantified by using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng/μL. Then, the main bacterial groups abundant in the murine intestinal microbiota including enterobacteria, enterococci, lactobacilli, bifidobacteria, *Bacteroides/Prevotella* spp., *Clostridium coccoides* group, *Clostridium leptum* group, *Mouse Intestinal Bacteroides* (MIB), as well as total eubacterial loads, were assessed by quantitative real-time polymerase chain reaction (qRT-PCR) with

species-, genera-, or group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as reported previously [13, 17, 18, 26–28], and the numbers of 16S rRNA gene copies per ng DNA of each sample were determined.

Statistical Analysis. Medians, means, standard deviations, and significance levels using Mann–Whitney U test were determined using GraphPad Prism Software v6 (La Jolla, CA, USA). Two-sided probability (p) values ≤ 0.05 were considered significant. All experiments were repeated twice.

Ethics. All animal experiments were conducted according to the European Guideline for animal welfare (2010/63/EU) with approval from the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin, Germany). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice.

Results

Generation of Human Microbiota-Associated Mice by Fecal Microbiota Transplantation. To counteract physiological colonization resistance exerted by the complex host specific gut microbiota, thereby providing protection from bacteria including pathogenic colonization and infection, conventional mice were subjected to broad-spectrum antibiotic treatment for 8 weeks. Thereby generated secondary abiotic mice were then associated with human microbiota by peroral FMT on three consecutive days. As quantitatively assessed by both culture and culture-independent (i.e., molecular 16S rRNA based) methods, bacterial groups were comparable in the respective human microbiota

suspensions (Figure 1). Three weeks thereafter (and, hence, immediately before ileitis induction), the human microbiota had stably established within the murine small and large intestines (Figure 2).

Microbiota Shifts Following Low-Dose *T. Gondii* Infection of Human Microbiota-Associated Mice. On day 0, "humanized" mice were perorally challenged with one cyst of *T. gondii* by gavage. Until day 9 post-infection (p.i.), the small as well as large intestinal microbiota composition shifted towards higher luminal loads of commensal enterobacteria such as *E. coli* and *Enterococcus* spp. ($p < 0.01$–0.001; Figure 2), whereas clostridia and *Mouse Intestinal Bacteroides* decreased in the ileal and colonic lumen, respectively ($p < 0.01$–0.001; Figure 2B, D). Hence, low-dose *T. gondii* infection resulted in profound shifts in the small as well as large intestinal microbiota composition.

Macroscopic and Microscopic Changes in Low-Dose *T. Gondii*-Infected Human Microbiota-Associated Mice. At the day of necropsy (i.e., day 9 p.i.), hma mice were clinically, if at all, only moderately compromised (less than 5% body weight loss; not shown), whereas 57.8 ± 12.6% of *T. gondii*-infected mice, but none of the uninfected hma control animals displayed microscopic or even macroscopic blood in their feces (Figure 3A). Furthermore, microscopic analyses of ileal paraffin sections applying a standardized histopathological scoring system [9, 11] revealed that low-dose *T. gondii* infection induced rather mild-to-moderate small intestinal mucosal changes such as edematous blubbing, leukocytes infiltrating the lamina propria, cell-free exudate into the ileal lumen with intact epithelium, and, in some cases, additionally cellular shedding,
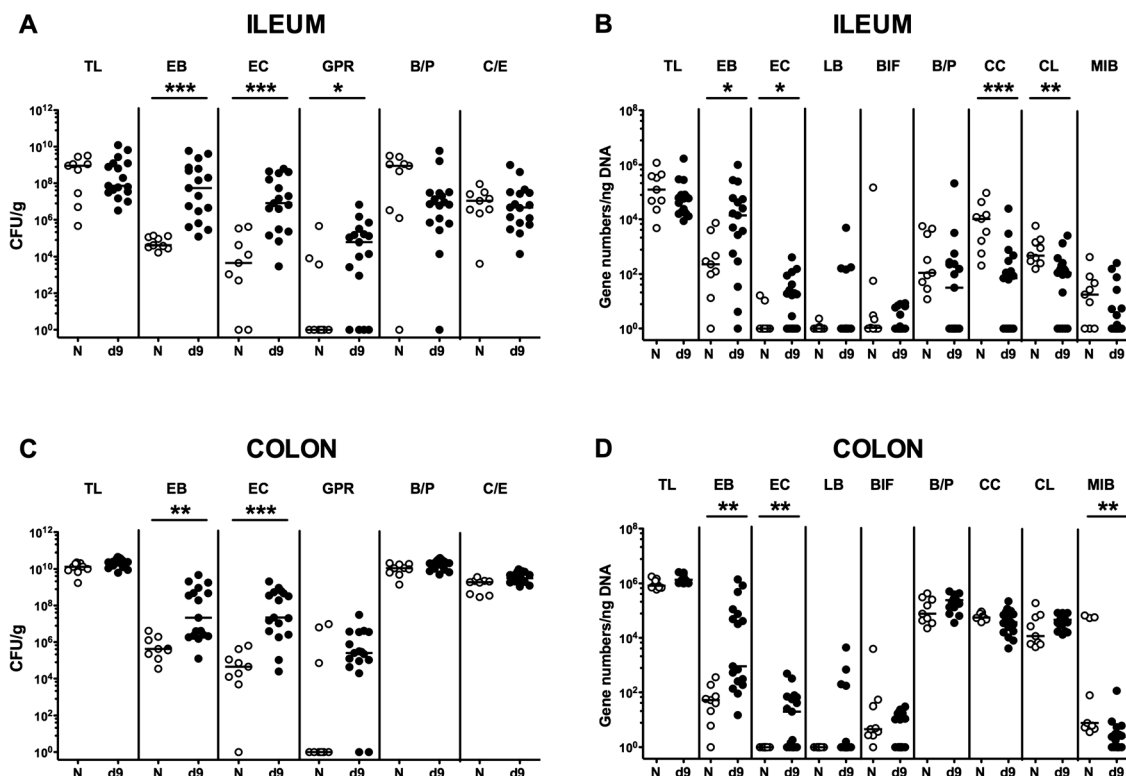

Figure 2. Intestinal microbiota composition in low-dose *T. gondii*-infected human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods. Main intestinal bacterial groups abundant in the ileum (A, B) and the colon lumen (C, D) of hma mice were quantitatively assessed applying both culture (A, C) and culture-independent (i.e., molecular 16S rRNA based; B, D) methods 9 days following ileitis induction (ILE, filled circles; $n = 17$). Noninfected hma mice served as controls (N, open circles; $n = 9$). Total bacterial loads (TLs) as well as numbers of enterobacteria (EB), enterococci (EC), Gram-positive rods (GPR), *Bacteroides/Prevotella* species (B/P), and *Clostridium/Eubacterium* species (C/E) are expressed as colony-forming units per gram feces (CFU/g). 16S rRNAs of the total eubacterial loads (TL), as well as of the main intestinal bacterial groups including enterobacteria (EB), enterococci (EC), lactobacilli (LB), bifidobacteria (Bif), *Bacteroides/Prevotella* species (B/P), *Clostridium coccoides* group (Clocc), *Clostridium leptum* group (Clept), and *Mouse Intestinal Bacteroides* (MIB), are expressed as gene numbers per ng DNA. Medians (black bars) and significance levels (p-values) determined by Mann–Whitney U test are indicated. Data shown are pooled from three independent experiments.
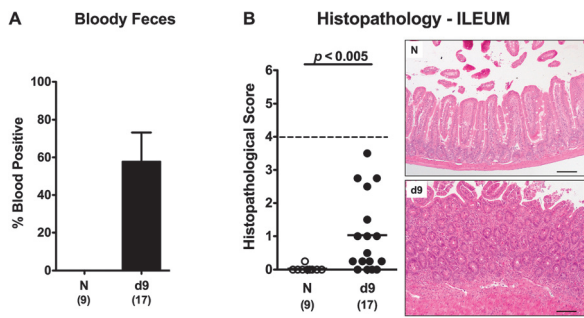
Figure 3. Clinical and histopathological sequelae following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). Clinical and microscopic intestinal changes were assessed at day 9 following ileitis induction. (A) Abundance of blood was determined in fecal samples by the Guajac (Haemoccult) method. Means, standard deviations, and numbers of analyzed mice are indicated. (B) Histopathological changes were determined in H&E-stained ileal paraffin sections applying a standardized scoring system (see Materials and Methods). Scores ≥ 4 (dotted line) indicate severe inflammation with necrosis. Numbers of animals (in parentheses), means, and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.

whereas severe small intestinal sequelae such as necrosis could not be observed ($p < 0.005$ vs naive mice; Figure 3B).

We further substantiated inflammatory responses of low-dose *T. gondii* infection applying in situ immunohistochemical staining of intestinal paraffin sections. At day 9 p.i., a multi-fold increase in small intestinal epithelial numbers of caspase3+ cells could be detected ($p < 0.001$; Figure 4A), indicative of *T. gondii*-induced apoptosis. Remarkably, inflammatory responses upon *T. gondii* infection were not restricted to the small intestines, given that elevated apoptotic cell numbers could also be observed in the colon of hma mice at day 9 p.i. ($p < 0.001$; Figure 4C). Since Ki67 is known as a nuclear factor essential for cellular proliferation [29], we additionally stained paraffin sections of intestinal ex vivo biopsies with Ki67 antibodies to determine potential proliferative and hence regenerative measures of the intestines counteracting *T. gondii*-induced intestinal mucosal damage. In fact, in both small and large intestine, numbers of Ki67+ epithelial cells substantially increased until day 9 p.i. ($p < 0.001$; Figure 4B, D). Hence, low-dose *T. gondii* infection of hma mice resulted in increased abundance of fecal blood and mild-to-moderate (but not severe) intestinal mucosal sequelae, indicative of subacute ileitis that were accompanied by pronounced counter-regulatory cell-regenerative measures.

Intestinal Inflammatory Immune Responses in Low-Dose *T. Gondii*-Infected Human Microbiota-Associated Mice. Given that non-self-limiting acute ileitis following peroral high dose (i.e., >50 cysts) *T. gondii* infection is T cell dependent, we next quantitatively assessed intestinal T lymphocytes and, additionally, regulatory T cells (Treg), applying in situ immunohistochemistry. At day 9 following low-dose *T. gondii* infection of hma mice, numbers of T lymphocytes substantially increased in the mucosa and lamina propria of the ilea and, remarkably, also in the large intestines ($p < 0.005$; Figure 5A, C). Subacute ileitis induction additionally resulted in elevated Treg numbers in both small and large intestines at day 9 p.i. ($p < 0.001$ and $p < 0.01$, respectively; Figure 5B, D). Increased T cell counts were further accompanied by increased secretion of pro-inflammatory cytokines such as IFN-γ in ex vivo biopsies derived from ileum, colon, and MLN of *T. gondii*-infected hma mice ($p < 0.01$–0.001; Figure 6A–C), whereas additionally elevated TNF and NO concentrations could be measured in MLN at day
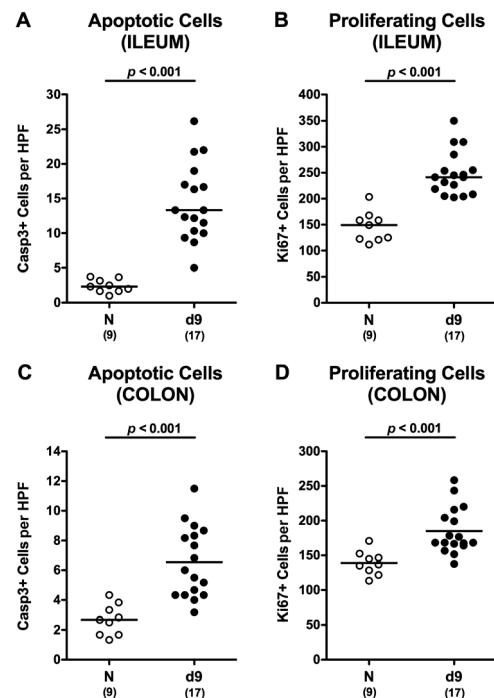

Figure 4. Intestinal apoptotic and proliferating cell responses following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). The average numbers of (A, B) ileal and (C, D) colonic apoptotic cells (positive for caspase3 [Casp3]; A, C) and proliferating cells (positive for Ki67; B, D) from six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained intestinal paraffin sections at day 9 post-ileitis induction. Numbers of animals (in parentheses), means, and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.

9 p.i., as compared to naive mice ($p < 0.001$; Figure 6D, E). Hence, low-dose *T. gondii* infection of hma mice resulted in distinct pro-inflammatory immune responses in the intestinal tract.

Extra-Intestinal Inflammatory Immune Responses in Low-Dose *T. Gondii*-Infected Human Microbiota-Associated Mice. We next addressed whether the inflammatory sequelae of low-dose *T. gondii* infection could also be observed in extra-intestinal compartments of hma mice. Like in the intestinal tract, subacute ileitis induction resulted in increased numbers of caspase3+ cells in ex vivo biopsies derived from the kidney, liver, and lung of infected hma mice, indicative of *T. gondii*-induced extra-intestinal apoptosis ($p < 0.001$; Figures 7A, 8A, and 9A). Remarkably, a massive infiltration of all three organs with T lymphocytes could be observed until day 9 p.i. ($p < 0.001$; Figures 7B, 8B, and 9B), which was accompanied by elevated Treg numbers in kidney, liver, and lung of infected hma mice ($p < 0.001$, Figures 7C, 8C, and 9C).

We further assessed pro-inflammatory cytokine secretion in respective extra-intestinal compartments. In fact, multi-fold increased IFN-γ and TNF concentrations could be measured in respective extra-intestinal tissue sites ($p < 0.01$–0.001; Figures 7D, 7E, 8D, 8E, 9D, and 9E). In addition, elevated MCP-1 and IL-6 levels could be determined in the kidney of infected mice ($p < 0.001$; Figure 7F, G); pulmonary MCP-1 concentrations were higher at day 9 p.i. as compared to uninfected mice ($p < 0.001$; Figure 9F). Of note, secretion of the anti-inflammatory cytokine IL-10 in liver and lung was more pronounced in infected mice as compared to naive hma mice ($p < 0.005$ and $p < 0.001$, respectively; Figures 8F and 9G).
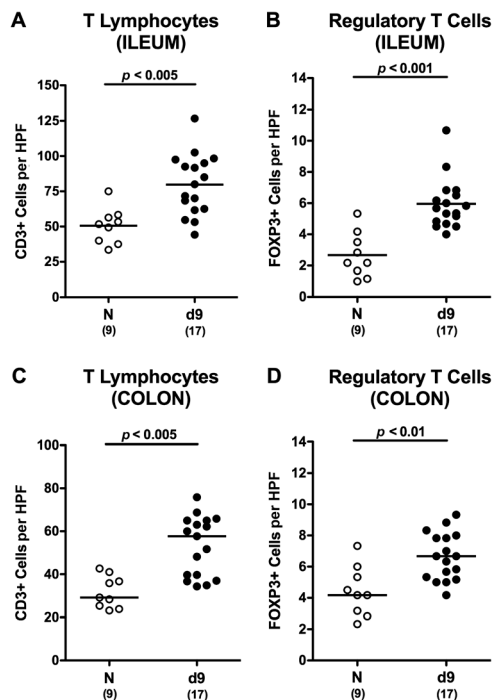
Also of note, no viable bacteria that might have translocated from the intestinal tract could be cultured from respective extra-intestinal compartments. Hence, low-dose *T. gondii*-induced inflammatory sequelae were not restricted to the intestinal tract, but could also be observed in extra-intestinal organs such as kidney, liver, and lung. Furthermore, anti-inflammatory (i.e., counter-regulatory) responses could be determined in liver and lung of infected hma mice.

Systemic Inflammatory Immune Responses in Low-Dose *T. Gondii*-Infected Human Microbiota-Associated Mice. We further addressed whether low-dose *T. gondii* infection also resulted in systemic immune responses. At day 9 p.i., multi-fold elevated IFN-γ, TNF, and NO concentrations could be measured in splenic ex vivo biopsies ($p < 0.01$–0.001; Figure 10A, B, D). Strikingly, subacute ileitis induction was accompanied by excessively increased serum levels of pro-inflammatory mediators such as IFN-γ, TNF, MCP-1, and IL-6 ($p < 0.01$–0.001; Figure 11A–D) and also by increased systemic secretion of anti-inflammatory IL-10 ($p < 0.05$; Figure 11E). As for extra-intestinal organs, no bacterial commensals originating from the intestines could be isolated from cardiac blood. Hence, low-dose *T. gondii* infection of hma infection resulted in marked systemic pro-inflammatory cytokine responses, whereas anti-inflammatory counter-regulatory measures were activated.

Discussion

The peroral high-dose *T. gondii* infection model for an acute T helper cell (Th) 1-type immunopathology affecting the distal small intestine (i.e., ileum) has been proven suitable to unravel the interactions between the parasitic pathogen and host immunity [3, 9, 22, 30]. Of note, the extent of the non-self-limiting and within only 1 week fatal disease does not follow a direct (i.e., linear) relationship to the numbers of *T. gondii* cysts as one might assume [3]. It is rather an infectious threshold of approximately 50 cysts, which needs to be reached to induce the full-blown disease and, hence, is rather irrelevant whether the inoculum reaches 50, 100, or 150 cysts, for instance.
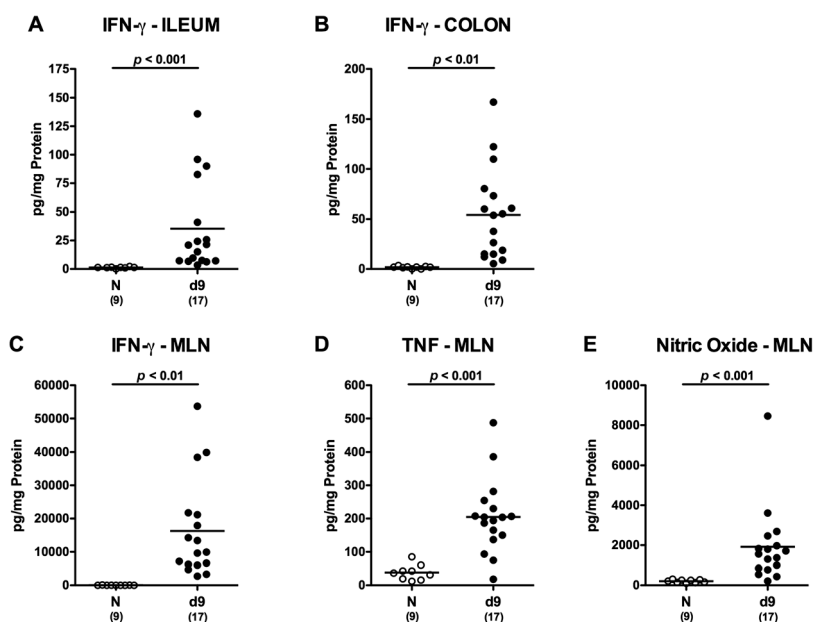
Figure 5. Intestinal T cell responses following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). The average numbers of (A, B) ileal and (C, D) colonic T lymphocytes (positive for CD3; A, C) and regulatory T cells (positive for FOXP3; B, D) from six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained intestinal paraffin sections at day 9 post-ileitis induction. Numbers of animals (in parentheses), means, and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.

Figure 6. Intestinal pro-inflammatory mediator responses following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). At day 9 post-ileitis induction, secretion of distinct pro-inflammatory mediators (as indicated) were determined in ex vivo biopsies derived from distinct intestinal compartments such as (A) ileum, (B) colon, and (C, D, and E) mesenteric lymph nodes (MLN). Numbers of animals (in parentheses), medians, and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.
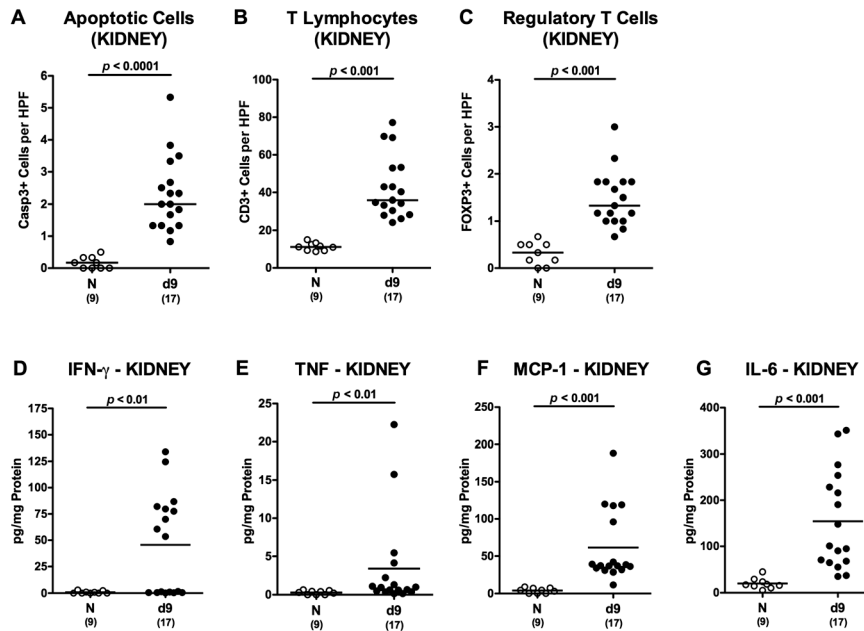
Figure 7. Apoptosis, T cells, and cytokines in kidneys following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). At day 9 post-ileitis induction, the average numbers of (A) apoptotic cells (positive for caspase3 [Casp3]), (B) T lymphocytes (positive for CD3), and (C) regulatory T cells (positive for FOXP3) from six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained paraffin sections of ex vivo kidney biopsies. Furthermore, secretion of pro-inflammatory cytokines such as (D) IFN-γ, (E) TNF, (F) MCP-1, and (G) IL-6 were determined in ex vivo kidney biopsies. Numbers of animals (in parentheses), means, and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.

Nevertheless, the high-dose *T. gondii* infection model mimics key features of the acute phase of Crohn's disease in humans ("ileitis terminalis") with (1) the predilection site of the terminal ileum, (2) the underlying Th1-type immunopathology, (3) the dysbiosis observed during disease development and furthermore, (4) the dependence of the commensal gut microbiota, and, particularly, (5) the Gram-negative commensal species further deteriorating the immunopathology in an TLR-4 dependent fashion [3]. The lethal outcome within 1 week post-infection, however, needs to be considered as a limitation and disadvantage of the gut inflammation model under certain circumstances. For instance, in a recent study, we addressed whether infection with a multidrug-resistant (MDR) *Pseudomonas aeruginosa* strain, an opportunistic pathogen that can cause
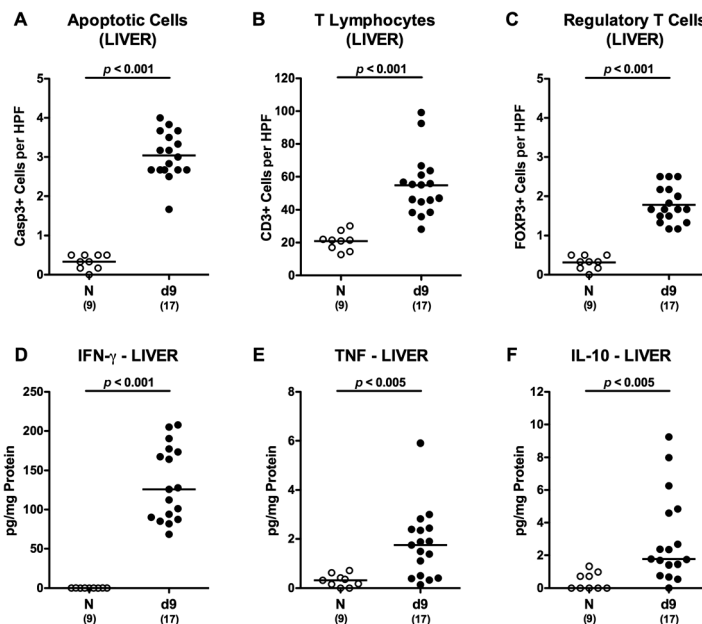

Figure 8. Apoptosis, T cells, and cytokines in livers following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). At day 9 post-ileitis induction, the average numbers of (A) apoptotic cells (positive for caspase3 [Casp3]), (B) T lymphocytes (positive for CD3), and (C) regulatory T cells (positive for FOXP3) from six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained paraffin sections of ex vivo liver biopsies. Furthermore, secretion of pro-inflammatory cytokines such as (D) IFN-γ and (E) TNF as well as of the anti-inflammatory cytokine (F) IL-10 were determined in ex vivo liver biopsies. Numbers of animals (in parentheses), means, and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.
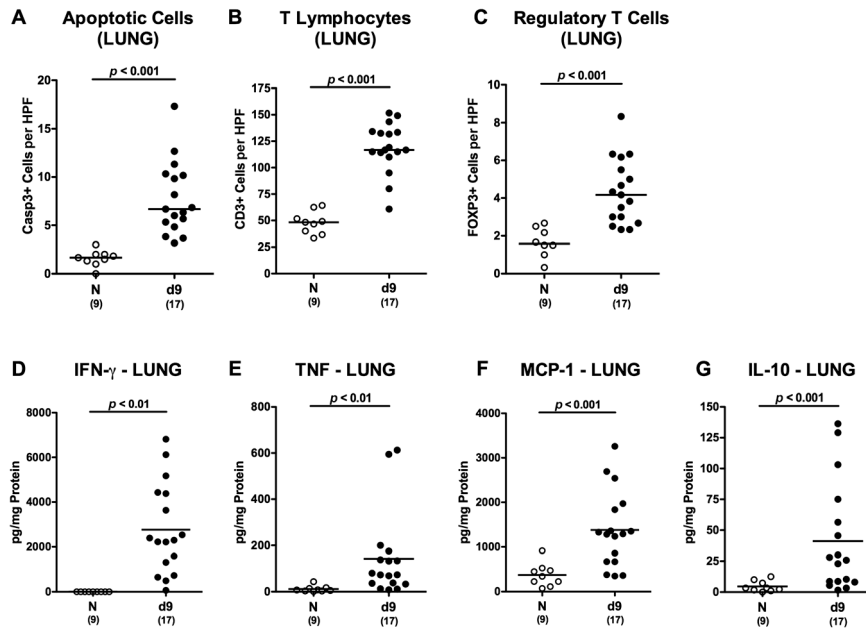
Figure 9. Apoptosis, T cells, and cytokines in lungs following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in Materials and Methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). At day 9 post-ileitis induction, the average numbers of (A) apoptotic cells (positive for caspase3 [Casp3]), (B) T lymphocytes (positive for CD3), and (C) regulatory T cells (positive for FOXP3) from six high power fields (HPF, 400× magnification) per animal were determined microscopically in immunohistochemically stained paraffin sections of ex vivo lung biopsies. Furthermore, secretion of pro-inflammatory cytokines such as (D) IFN-γ, (E) TNF, and (F) MCP-1, as well as of the anti-inflammatory cytokine (G) IL-10 were determined in ex vivo lung biopsies. Numbers of animals (in parentheses), means and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.

severe infections in health care settings, particularly in immuno-compromised patients [31, 32], might worsen *T. gondii*-induced ileitis [33]. However, given that the hma control mice (i.e., without MDR *P. aeruginosa* infection) were already exhibiting the hyper-acute inflammatory scenario following peroral infection

Figure 10. Splenic pro-inflammatory mediator responses following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). At day 9 post-ileitis induction, secretion of (A) IFN-γ, (B) TNF, (C) MCP-1, and (D) nitric oxide were determined in splenic ex vivo biopsies. Numbers of animals (in parentheses), means and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.

with 50 *T. gondii* cysts, potential additional *P. aeruginosa*-induced inflammatory effects in the intestinal tract and beyond "on top" might be masked by the underlying induced immuno-pathology and thus not be discriminated [33].

This prompted us in the present study to establish a less acute and non-lethal ileitis model following low-dose *T. gondii* infection. In fact, infected mice were suffering from only mild-to-moderate small intestinal histopathology with ileal epithelial apoptosis, but not transmural intestinal necrosis as seen following high-dose infection [9], thus indicative of subacute ileitis that was accompanied by pronounced counter-regulatory cell-regenerative measures. Of note, apoptotic and pro-inflammatory T cell dependent immune responses, but also cell regenerative counter-regulatory measures could be observed not only in the terminal ileum but also in the large intestinal tract of diseased mice. These results are well in line with our previous reports about intestinal, extra-intestinal, and systemic sequelae of acute ileitis in hma mice following high-dose *T. gondii* infection [14, 33].

Like following high-dose *T. gondii* infection [9, 14, 23, 33–35], pro-inflammatory mediators such as IFN-γ, TNF, and NO were increased in the intestinal tract of mice suffering from subacute ileitis as shown here. Remarkably, low-dose *T. gondii*-induced T cell-dependent inflammatory sequelae were not restricted to the intestinal tract, but could also be observed in extra-intestinal organs such as the kidney, liver, and lung. These results are supported by our recent high-dose *T. gondii* infection study displaying increased secretion of pro-inflammatory mediators in ex vivo biopsies derived from the liver and kidney of mice with fatal acute ileitis [33]. Furthermore, anti-inflammatory (i.e., counter-regulatory) responses could be determined in the liver and lung of low-dose *T. gondii*-infected hma mice, which was not the case in hma mice suffering from lethal ileitis [14, 33].

Remarkably, alike in the lethal acute ileitis model [14, 33], low-dose *T. gondii* infection of hma mice resulted in marked systemic pro-inflammatory cytokine responses, whereas also anti-inflammatory IL-10 secretion as counter-regulatory
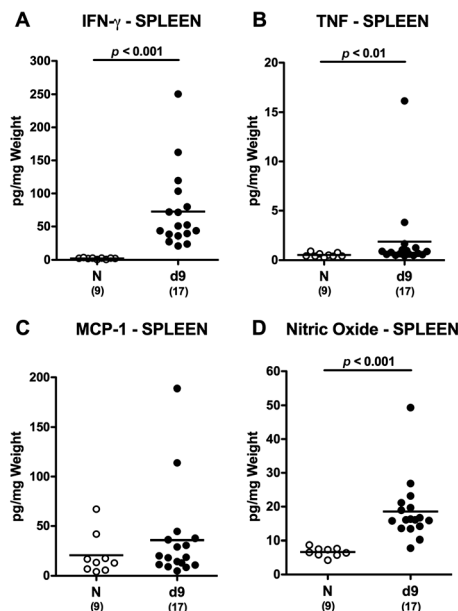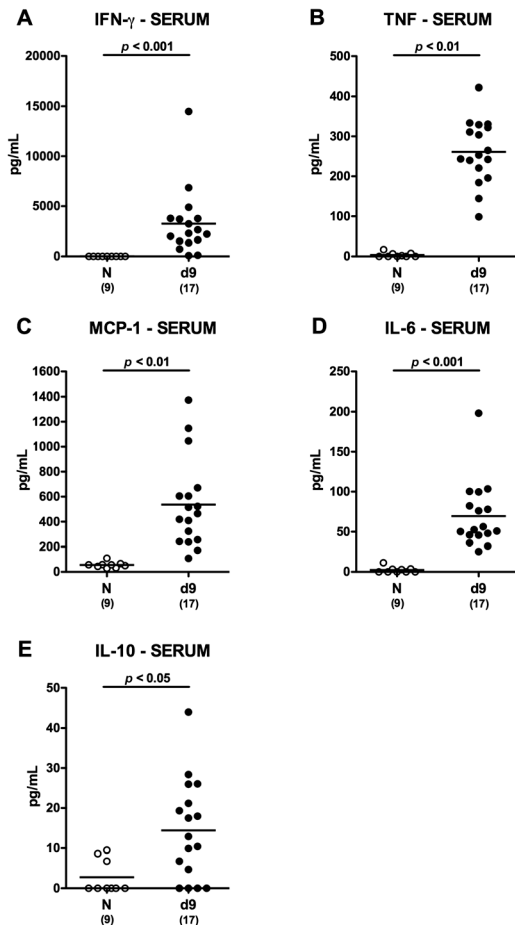
Figure 11. Systemic cytokine responses following low-dose *T. gondii* infection of human microbiota-associated mice. Human microbiota-associated (hma) mice were perorally infected with one cyst of *T. gondii* strain ME49 to induce non-lethal subacute ileitis as described in methods (ILE; filled symbols). Noninfected hma mice served as controls (N, open symbols). At day 9 post-ileitis induction, systemic secretion of (A) IFN-γ, (B) TNF, (C) MCP-1, (D) IL-6, and (E) IL-10 were determined in serum samples. Numbers of animals (in parentheses), means, and significance levels (*p*-values) determined by Mann–Whitney *U* test are indicated. Data shown were pooled from three independent experiments.

measure had been activated. Of note, in the present study, no viable bacteria originating from the intestinal commensal microbiota could be isolated from extra-intestinal compartments including the systemic circulation, whereas, in single cases of diseased mice suffering from acute ileitis following high-dose *T. gondii* infection, translocated bacteria could be detected [14, 33].

Given the importance of the (gut) microbiota in initiating and perpetuating immunopathology in the intestinal tract and beyond [8, 13, 36], we also assessed gut microbiota shifts in the intestinal tract of mice suffering from subacute ileitis applying both cultural and molecular approaches. Low-dose *T. gondii* infection resulted in profound shifts in the microbiota composition of the inflamed small intestinal and large intestinal tract, as indicated by elevated enterobacteria and enterococci in either intestinal lumen, whereas obligate anaerobic clostridia were markedly reduced in the ilea of diseased hma mice at day 9 p.i. These patterns in microbiota shifts could also be observed in our previous experiments, applying high-dose *T. gondii* infection [8–11].

One of the pitfalls of the low-dose infection model is to narrow the peroral parasitic infection dose down to one *T. gondii* cyst per recipient animal only. Depending on the virulence of the respective *T. gondii* strain and the age and sex of the susceptible

mice [3], an accidental increase in cyst number might result in a much more pronounced induced immunopathology as expected and (in the worst case) even in a lethal phenotype. Hence, the well functioning, the reliability, and the reproducibility of the low-dose *T. gondii* infection model depend on the experienced hands of the researchers involved.

More commonly, the murine low-dose *T. gondii* infection model is applied to induce inflammation of the central nervous system. Three weeks following intraperitoneal application of less than 10 *T. gondii* cysts of the ME 49 strain, susceptible mice developed chronic encephalitis [37–41]. To the best of our knowledge, data regarding the intestinal immunopathological responses during chronic *T. gondii*-induced cerebral inflammation are scarce, however, and should be investigated in the future to gain deeper insights into the interactions within the gut–brain axis in health and disease.

Conclusions

In conclusion, we here present a low-dose *T. gondii*-induced non-lethal subacute ileitis model in mice harboring a human gut microbiota that presents with immunopathological sequelae of infection not only in the intestinal tract but also in extra-intestinal including systemic compartments. This murine infection model will help to further elucidate the interplay between pathogens, the human commensal gut microbiota, and host immunity during subacute intestinal inflammation in the future.

Authors' Contribution

S.B. and M.M.H. conceived and designed the experiments. U.E., A.G., U.F., and M.M.H. performed the experiments. U.E., A.G., U.F., and M.M.H. analyzed the data. M.M.H. wrote the article. A.G. and S.B. coedited the article.

Conflict of Interest

Markus M. Heimesaat and Stefan Bereswill are editorial board members.

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