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# The impact of Toll-like-receptor-9 on intestinal microbiota composition and extra-intestinal sequelae in experimental *Toxoplasma gondii* induced ileitis

Stefan Bereswill<sup>1†</sup>, Anja A Kühl<sup>2†</sup>, Marie Alutis<sup>1</sup>, André Fischer<sup>1</sup>, Luisa Möhle<sup>3</sup>, Daniela Struck<sup>1</sup>, Oliver Liesenfeld<sup>1</sup>, Ulf B Göbel<sup>1</sup>, Ildikò R Dunay<sup>3†</sup> and Markus M Heimesaat<sup>1\*†</sup>

## Abstract

**Background:** Following peroral *Toxoplasma (T.) gondii* infection, susceptible mice develop acute ileitis due to a microbiota-dependent Th1 type immunopathology. Toll-like-receptor (TLR)-9 is known to recognize bacterial DNA and mediates intestinal inflammation, but its impact on intestinal microbiota composition and extra-intestinal sequelae following *T. gondii* infection has not yet been elucidated.

**Methods and results:** Seven days following peroral infection (p.i.) with 100 cysts of *T. gondii* ME49 strain, TLR-9<sup>-/-</sup> and wildtype (WT) mice suffered from comparable ileitis, whereas ileal parasitic loads as well as IFN- $\gamma$  and nitric oxide levels were higher in TLR-9<sup>-/-</sup> compared to WT mice. Locally, TLR-9<sup>-/-</sup> mice exhibited increased ileal CD3+, but not FOXP3+ cell numbers at day 7 p.i.; in mesenteric lymph nodes IFN- $\gamma$ -producing CD4+ cell numbers and TNF- $\alpha$  and IFN- $\gamma$  concentrations were also increased in TLR-9<sup>-/-</sup> compared to WT mice. *T. gondii* DNA levels, however, did not differ in mice of either genotype. Differences in intestinal microbiota were rather subtle except for bifidobacteria that were virtually absent in both, naïve and *T. gondii* infected TLR-9<sup>-/-</sup>, but not WT mice. Extra-intestinally, TLR-9<sup>-/-</sup> mice displayed less distinct systemic immune responses as indicated by lower serum IL-6, and splenic TNF- $\alpha$  and IFN- $\gamma$  levels as compared to WT mice despite higher translocation rates of intestinal bacteria to extra-intestinal compartments such as liver, spleen, kidney, and cardiac blood. Most importantly, brains were also affected in this inflammatory scenario as early as day 7 p.i. Remarkably, TLR-9<sup>-/-</sup> mice exhibited more pronounced inflammatory infiltrates with higher numbers of F4/80+ macrophages and microglia in the cortex and meninges as compared to WT mice, whereas *T. gondii* DNA levels did not differ.

**Conclusion:** We here show that TLR-9 is not required for the development of *T. gondii* induced ileitis but mediates distinct inflammatory changes in intestinal and extra-intestinal compartments including the brain.

**Keywords:** *Toxoplasma gondii*, Acute ileitis, TLR-9, Th1-type immunopathology, Extra-intestinal immune responses, Intracerebral inflammation, Pro-inflammatory cytokines, Intestinal microbiota composition, Bacterial translocation, Systemic inflammatory response, Bifidobacteria, FOXP3, Regulatory T cells, Gut-brain-axis

\* Correspondence: markus.heimesaat@charite.de

†Equal contributors

<sup>1</sup>Department of Microbiology and Hygiene, Charité - University Medicine Berlin, Centrum 5, Campus Benjamin Franklin, Hindenburgdamm 27, D-12203 Berlin, Germany

Full list of author information is available at the end of the article

## Introduction

Seven days following peroral infection with 100 cysts of *Toxoplasma (T.) gondii* ME49 strain, susceptible mice develop massive necrosis in the terminal ileum and succumb to the infection [1]. This fatal scenario is due to a classical Th1-type immunopathology orchestrated by intestinal epithelial cells, granulocytes, macrophages, monocytes, dendritic cells, and lymphocytes [2]. Early upon *T. gondii* infection, parasitic interaction with antigen presenting cells results in activation of CD4+ T cells and overproduction of mediators such as IFN- $\gamma$ , TNF- $\alpha$ , nitric oxide (NO), IL-6, and MCP-1 among others comprising a “pro-inflammatory cytokine storm” [3-7]. Hence, the underlying immunopathogenesis resembles key features of chronic inflammatory bowel diseases (IBD) such as Crohn’s disease in the acute stage [2,8]. Furthermore, we recently showed that lipopolysaccharide (LPS) derived from the intestinal microbiota mediates *T. gondii* induced immunopathology via Toll-like-receptor (TLR) -4 signaling [9-11].

Bacterial unmethylated CpG DNA is the classical ligand for TLR-9 signalling [12-14]. Upon binding, transcription factors such as NF- $\kappa$ B, IFN regulatory factor-7, and AP-1 among others become activated in a MyD88-dependent fashion leading to Th1 type immune responses [15,16]. Initially, TLR-9 was shown to be crucial for an effective Th1-type immune response following oral *T. gondii* infection of mice [17]. However, the direct recognition of *T. gondii* molecules through TLR-9 is controversially discussed [18]. Parasitic DNA and RNA have been shown to activate innate immune responses via TLR-7 and TLR-9, but mice lacking TLR-9 alone were not susceptible to *T. gondii* infection [19]. In addition, TLR-11 and TLR-12 acting as heterodimers were shown to be essentially required for sensing of *Toxoplasma* profilin [19].

Given that bacterial DNA derived from the commensal microbiota provide pivotal immune-stimulatory molecules for effective host defense against parasitic infection [20], the distinct microbiota composition displays an essential determinant for the immunopathogenesis in murine *T. gondii* induced acute ileitis. In the present study we performed a comprehensive survey of quantitative and qualitative changes in the intestinal microbiota composition of TLR-9<sup>-/-</sup> and WT control mice following ileitis induction. Furthermore, we assessed immunopathological sequelae following peroral *T. gondii* infection in intestinal as well as extra-intestinal compartments such as spleen, liver, kidneys, and brain.

## Results

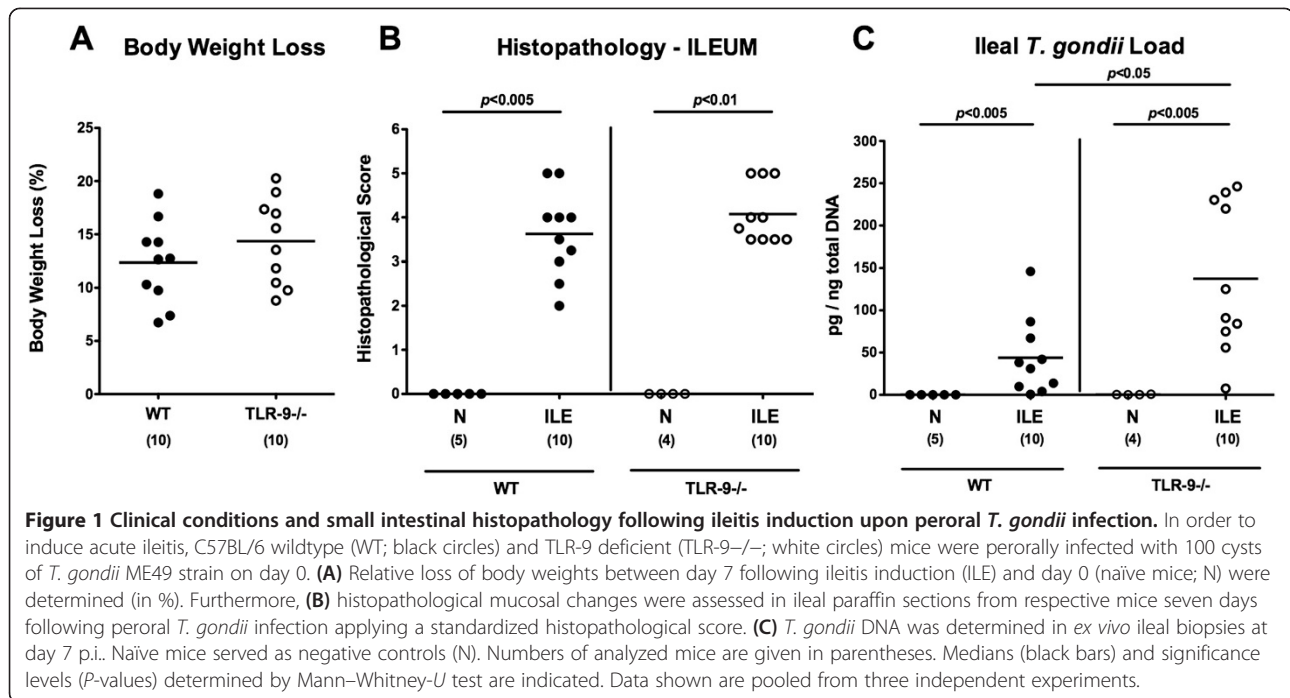
### Acute ileal immunopathology in TLR-9<sup>-/-</sup> mice following peroral *T. gondii* infection

In order to induce acute ileitis TLR-9<sup>-/-</sup> and wildtype (WT) mice were subjected to a peroral infection with 100 cysts of *T. gondii* ME49 strain. Seven days thereafter

mice of either genotype were compromised due to wasting disease and suffered from comparable relative body weight losses (Figure 1A). In addition, TLR-9<sup>-/-</sup> and WT mice displayed similar severity of acute ileitis as indicated by comparable ileal histopathological scores (Figure 1B). Notably, TLR-9<sup>-/-</sup> mice harbored significantly higher parasitic DNA loads in the small intestines as compared to WT controls at day 7 p.i. ( $p < 0.05$ ; Figure 1C).

We next quantitatively assessed ileum mucosal apoptotic cells as well as the influx of distinct immune cell populations into the small intestinal mucosa and lamina propria during acute ileitis applying *in situ* immunohistochemical stainings of ileal paraffin sections. At day 7 p.i., numbers of caspase-3+ apoptotic cells increased to comparable levels in ilea of TLR-9<sup>-/-</sup> and WT mice (Figure 2A), thus further underlining the clinical and histopathological results. In addition, numbers of T cells, the major driving forces of *T. gondii* induced acute ileitis, as well as of neutrophilic granulocytes, monocytes, and macrophages exerting oxidative stress to the intestinal epithelium increased comparably in TLR-9<sup>-/-</sup> and WT mice until day 7 p.i. (Figure 2B-D). Interestingly, FOXP3+ regulatory T cell (Treg) numbers increased in WT, but not TLR-9<sup>-/-</sup> mice upon ileitis induction ( $p < 0.001$ ; Figure 2E). Taken together, TLR-9<sup>-/-</sup> mice were not protected from *T. gondii* induced acute ileitis and were unable to control replication of the parasite.

We next determined pro-inflammatory cytokine levels in *ex vivo* ileal biopsies taken before and seven days after ileitis induction. Until day 7 p.i., intestinal IFN- $\gamma$ , TNF- $\alpha$ , nitric oxide (NO), and IL-6 levels had increased in mice of either genotype ( $p < 0.05-0.001$ ; Figure 3). TLR-9<sup>-/-</sup> mice, however, displayed even higher ileal IFN- $\gamma$  and NO concentrations as compared to WT controls at day 7 p.i. ( $p < 0.05$ ; Figure 3A,C). Notably, small intestinal concentrations of the anti-inflammatory cytokine IL-10 essentially involved in counteracting *T. gondii* induced immunopathology [21] increased to comparable levels in mice of either genotype until day 7 p.i. (Figure 3E). In the following we assessed pro-inflammatory cytokine responses in mesenteric lymph nodes (MLNs) draining the small intestinal tract. Again, IFN- $\gamma$ , TNF- $\alpha$ , and NO protein levels increased multifold upon *T. gondii* infection ( $p < 0.005-0.001$ ; Figure 4A-C), but the former two cytokines were significantly higher in TLR-9<sup>-/-</sup> mice as compared to WT controls ( $p < 0.005$  and  $p < 0.05$ , respectively; Figure 4A,B). Furthermore, flow cytometry analysis of lymphocytes isolated from MLNs revealed a higher abundance of IFN- $\gamma$  producing CD4+ cells in TLR-9<sup>-/-</sup> versus WT mice at day 7 p.i. (2.84% versus 0.88%; Figure 5), whereas naïve mice of either genotype exhibited similar frequencies of CD4+ IFN- $\gamma$  + cells. Furthermore, relative frequencies of CD3+ CD4+ cells were comparable in MLNs of naïve and *T. gondii* infected mice of either



genotype, whereas percentages of activated T lymphocytes (i.e. CD69<sup>+</sup> CD4<sup>+</sup> cells) increased comparably upon *T. gondii* infection in both, TLR-9<sup>-/-</sup> and WT animals (Figure 5).

#### Intestinal microbiota changes and bacterial translocation to extra-intestinal compartments in TLR-9<sup>-/-</sup> mice with acute ileitis

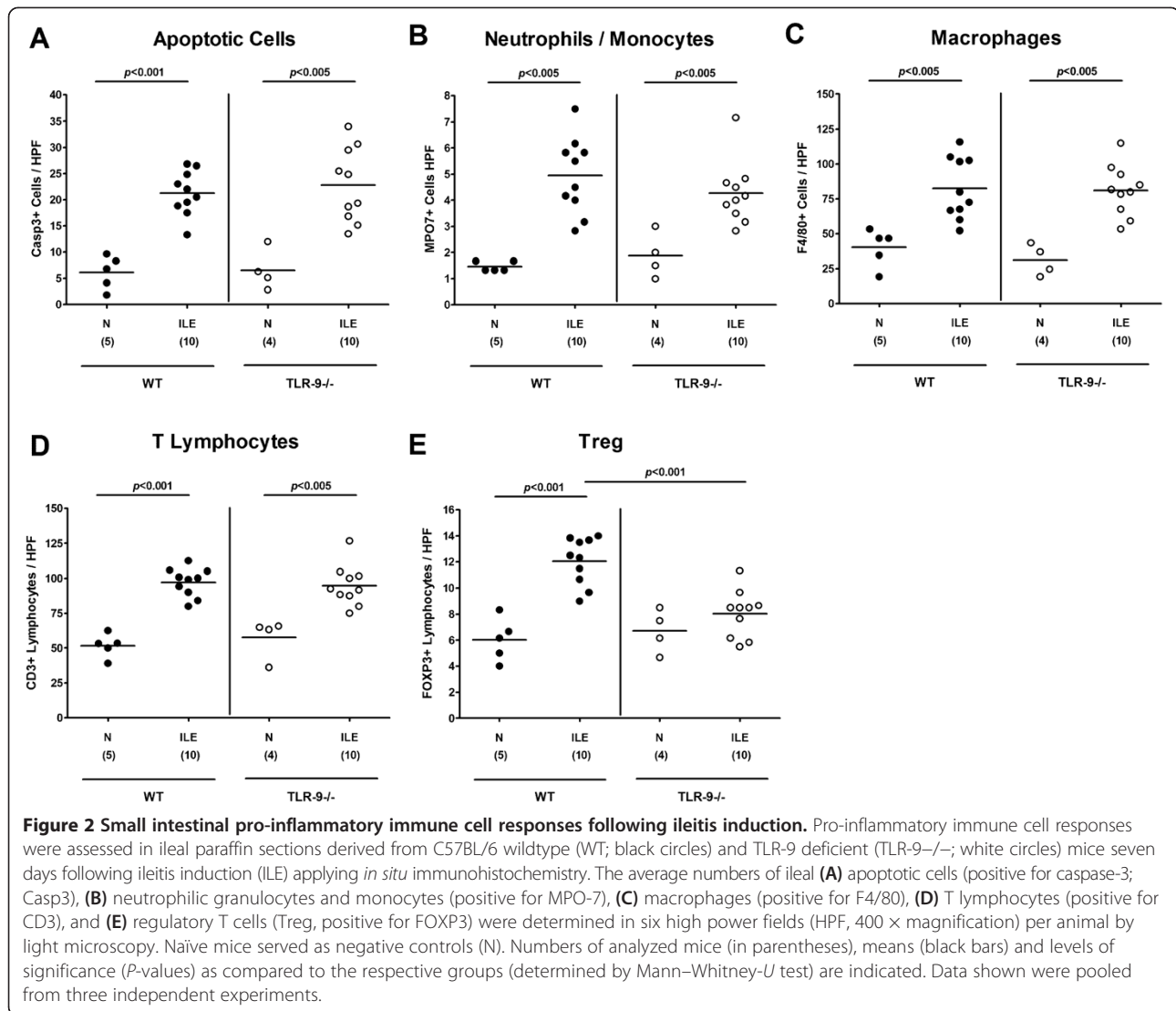
Given that the commensal intestinal microbiota composition mediates induction and perpetuation of intestinal immunopathogenesis [2,9,10,22], we next performed a comprehensive quantitative molecular survey of the main fecal bacterial groups derived from TLR-9<sup>-/-</sup> and WT mice, before and seven days after ileitis induction. Until day 7 p.i., the total eubacterial load increased slightly, but only reached statistical significance in TLR-9<sup>-/-</sup> mice ( $p < 0.001$ ; Figure 6A). Higher total bacterial loads at day 7 as compared to day 0 were due to increases in enterobacteria, enterococci, *Bacteroides/Prevotella* spp. loads in both, WT and TLR-9<sup>-/-</sup> mice (Figure 6B,C,F), and of Mouse Intestinal *Bacteroides* in the latter (Figure 6I). Notably, naïve TLR-9<sup>-/-</sup> mice harbored slightly lower fecal enterobacteria and Mouse Intestinal *Bacteroides* DNA, whereas *Clostridium leptum* loads were higher compared to naïve WT mice (Figure 6B, I, G). Remarkably, bifidobacteria were virtually absent in TLR-9<sup>-/-</sup>, but not WT mice at either time point (Figure 6E).

Given that a compromised epithelial barrier facilitates bacterial translocation from the intestinal lumen to sub-epithelial and further extra-intestinal sites, we next assessed translocation rates of viable bacteria. Of interest, whereas

commensal intestinal bacteria could be cultured from MLNs of TLR-9<sup>-/-</sup> and WT mice at identical frequencies (80%), mean translocation rates to spleen (20% vs 10%, respectively), liver (40% vs 20%, respectively), and kidneys (20% vs 10%, respectively) were higher in TLR-9<sup>-/-</sup> as compared to WT mice (Figure 7). Furthermore, even 10% of cardiac blood samples derived from TLR-9<sup>-/-</sup> mice were culture-positive whereas all blood cultures were negative in WT animals at day 7 p.i. (Figure 7). Cultured translocated bacteria comprised commensal intestinal species such as *E. coli*, *Enterococcus* spp. and *Lactobacillus* spp., but no obligate anaerobic bacteria (not shown). Notably, respective translocation frequencies were identical in independent experiments. Taken together, these data point towards a more severe damage to the ileal epithelial barrier in diseased TLR-9<sup>-/-</sup> mice as compared to WT controls, thus facilitating translocation of live commensal bacteria originating from the intestinal lumen to extra-intestinal compartments.

#### Extra-intestinal immunopathology in TLR-9<sup>-/-</sup> mice with acute ileitis

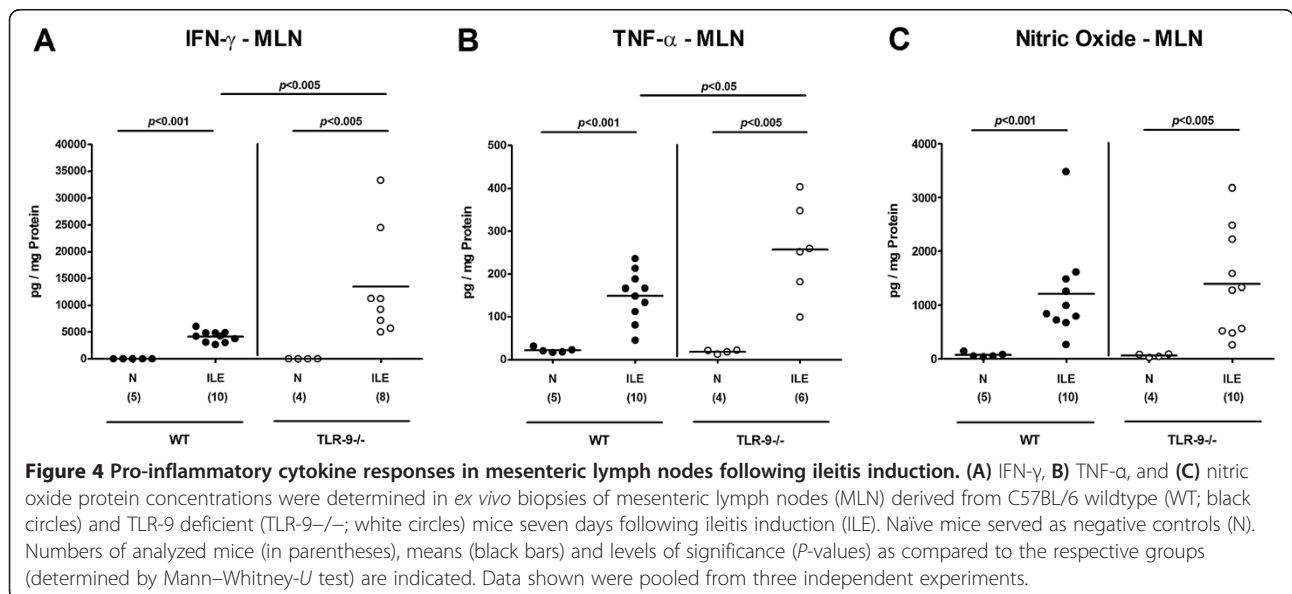
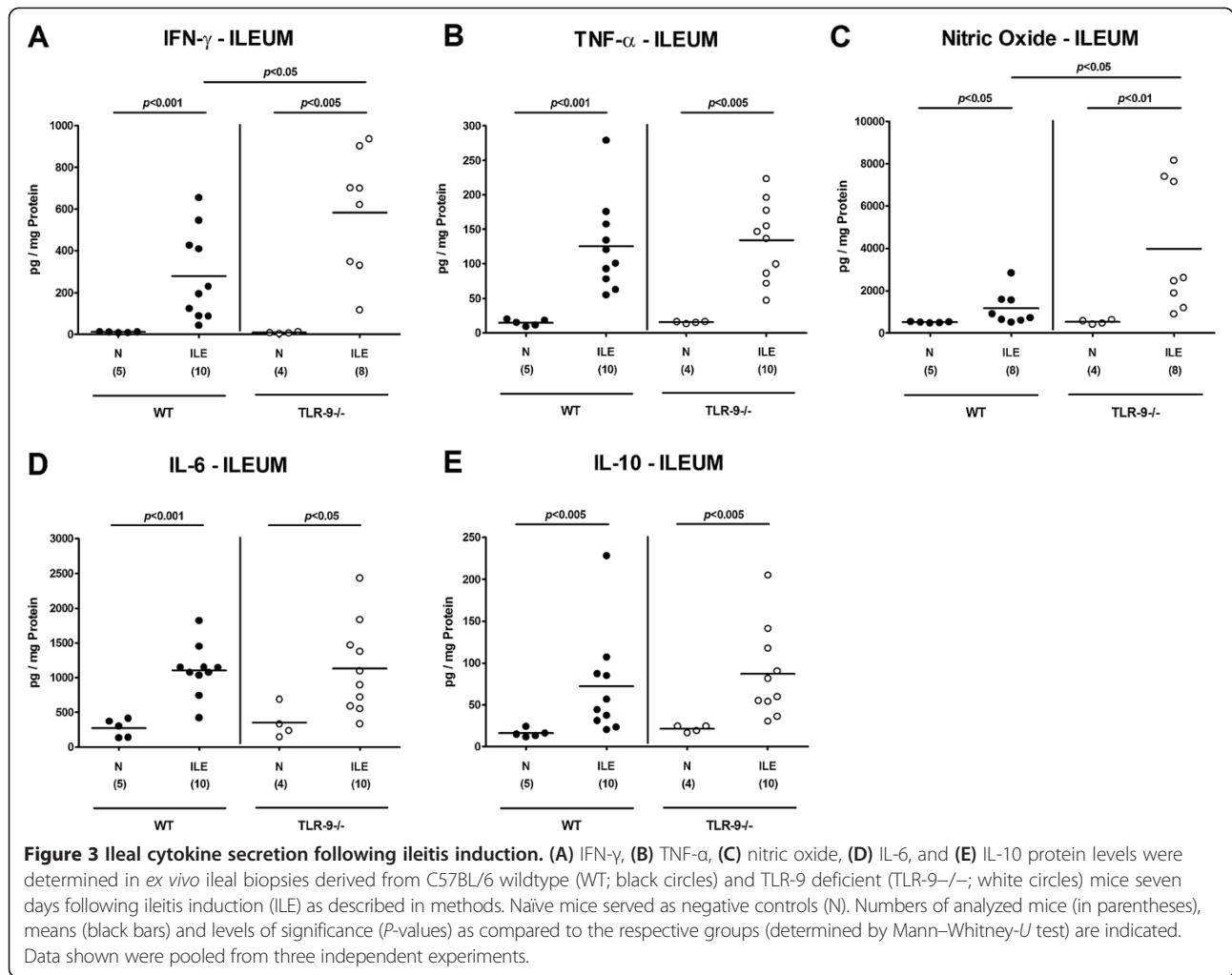
We next investigated systemic immune responses and potential pro-inflammatory sequelae in extra-intestinal organs following ileitis induction. Irrespective of the genotype of mice, IL-6 and IFN- $\gamma$  serum levels (Figure 8A,B), splenic TNF- $\alpha$  and IFN- $\gamma$  (Figure 8C,D) as well as hepatic TNF- $\alpha$  and IL-6 concentrations (Figure 8E,F) increased multifold until day 7 following infection. Remarkably, pro-inflammatory cytokine levels in the respective extra-intestinal compartment were significantly lower in

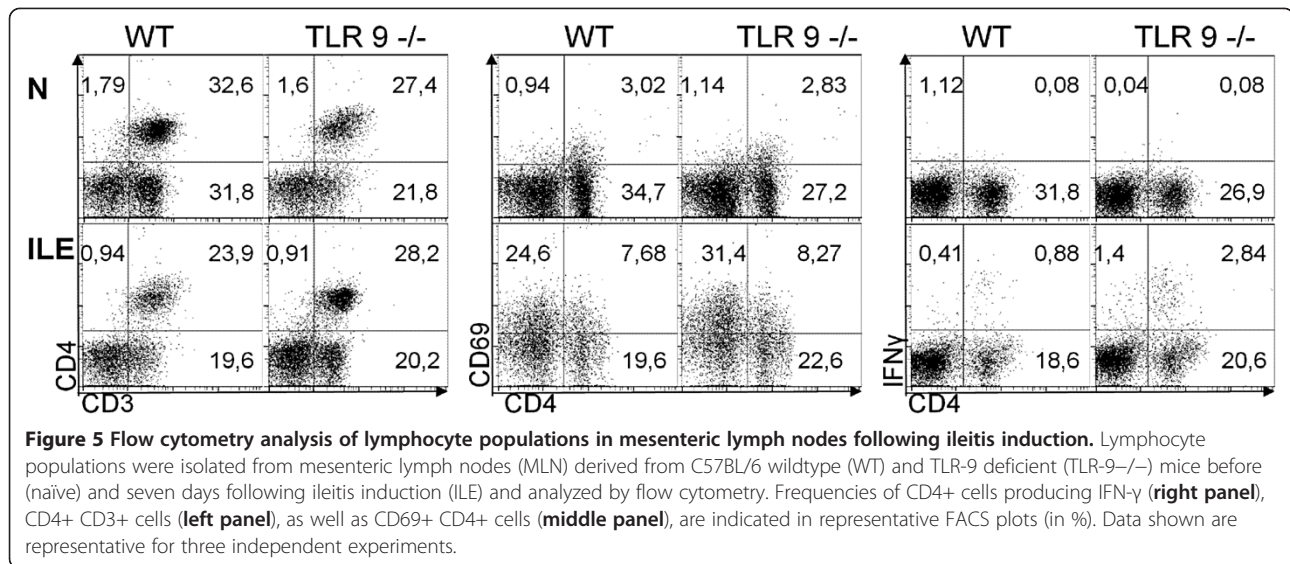


TLR-9<sup>-/-</sup> as compared to WT mice at day 7 p.i. ( $p < 0.05$ - $0.005$ ; Figure 8). Furthermore, TLR-9<sup>-/-</sup> mice exhibited approximately 50% lower IL-6 serum concentrations as compared to WT controls at day 7 p.i. ( $p < 0.05$ ; Figure 8A). Taken together, the absence of TLR-9 does not prevent the development of *T. gondii* induced acute ileitis. However, intestinal parasite loads and local immune responses are more distinct in infected TLR-9<sup>-/-</sup> as compared to WT mice. In contrast, the absence of TLR-9 results in lower levels of extra-intestinal and systemic inflammation.

Next we analyzed a potential intracerebral inclusion in *T. gondii* induced acute small intestinal immunopathology. Most importantly, *T. gondii* induced acute ileitis and brain inflammation could be observed in mice of either genotype as early as day 7 p.i. (Figure 9); the cortex as well as the meninges of infected, but not naïve animals were affected (Figure 9A). These inflammatory responses, however, were more pronounced in TLR-9<sup>-/-</sup>

as compared to WT mice as indicated by higher histopathological scores (assessing both, cortical and meningeal inflammatory foci) in the former ( $p < 0.05$ ; Figure 9A). *In situ* immunohistochemical analyses revealed higher numbers of F4/80+ inflammatory monocytes, macrophages and residential microglia in TLR-9<sup>-/-</sup> as compared to WT mice (Figure 9B). Interestingly, comparable parasitic DNA loads could be detected in brain tissues derived from mice of either genotype seven days following *T. gondii* infection (Figure 9C). Cerebral IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 mRNA expression levels increased multifold upon *T. gondii* infection, but did not differ between TLR-9<sup>-/-</sup> and WT mice at day 7 p.i. (Figure 10A-C). Taken together, pro-inflammatory immune responses develop not only in intestinal, but also in extra-intestinal compartments including the brain as early as 7 days after peroral infection with *T. gondii*. In the absence of TLR-9, *T. gondii* induced small intestinal and intracerebral pro-inflammatory cytokine responses





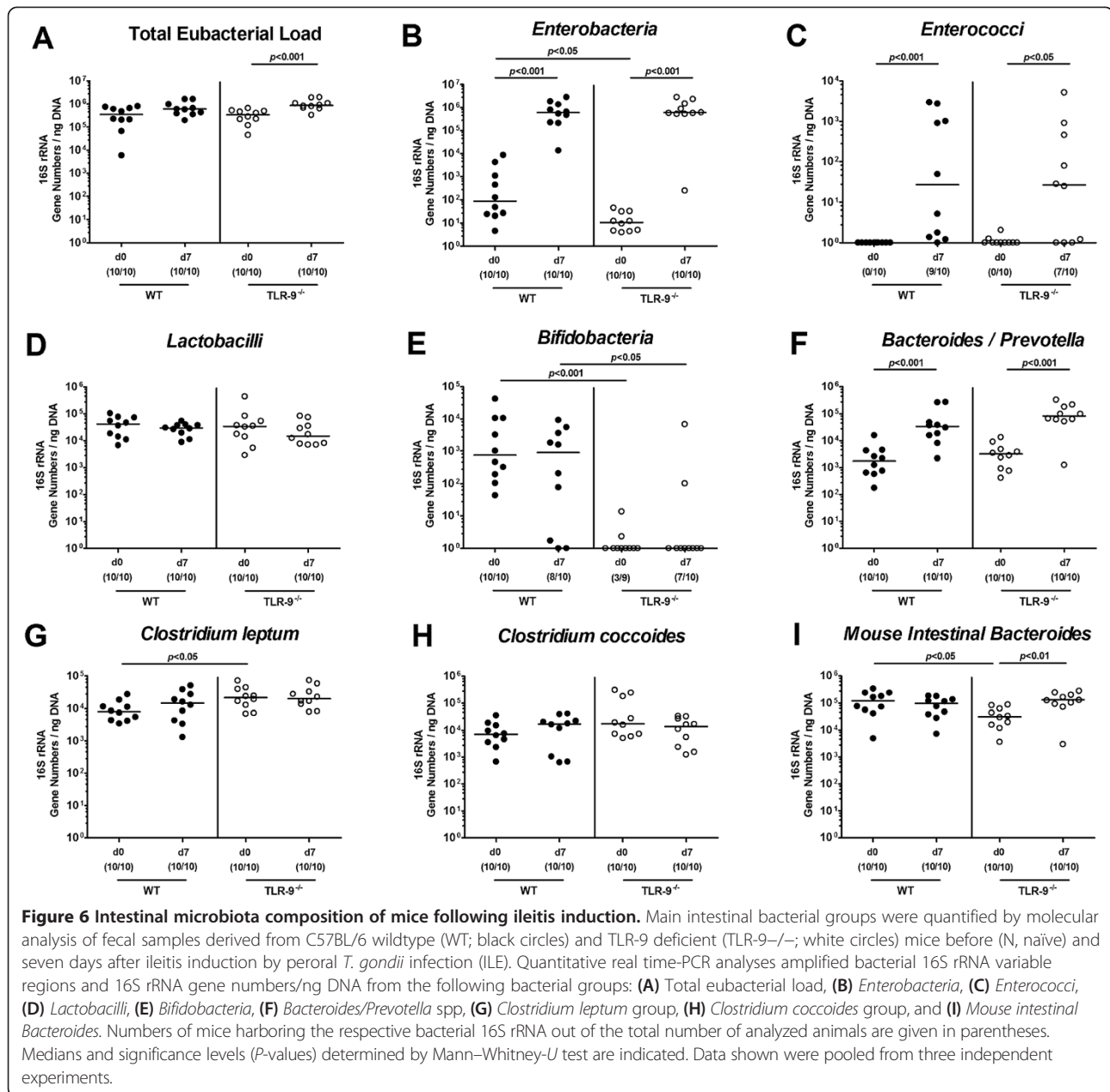
are higher, whereas systemic (i.e. serum and splenic) levels are lower as compared to WT mice at day 7 p.i.

## Discussion

In the present study we show that the absence of TLR-9 does not protect mice from the development of acute ileitis following peroral infection with *T. gondii*. TLR-9<sup>-/-</sup> mice succumbed to intestinal immunopathology as did WT mice. Despite a comparable clinical and intestinal (e.g. histopathological) outcome of infection we observed several TLR-9-mediated effects. For instance, TLR-9<sup>-/-</sup> mice displayed higher parasitic loads in the small intestines as compared to WT mice, which was accompanied by higher levels of pro-inflammatory cytokine such as IFN-γ and NO in ilea and IFN-γ and TNF-α in MLNs in the absence of TLR-9. The *T. gondii* induced Th1 type immune response in TLR-9<sup>-/-</sup> mice was further supported by a higher abundance of IFN-γ producing T cells in MLNs derived from TLR-9<sup>-/-</sup> as compared to WT mice at day 7 p.i. Our results are in contrast to a previous study by Minns and colleagues demonstrating that *T. gondii* infected TLR-9<sup>-/-</sup> mice were protected from disease due to a diminished Th1-type immune response [17]. TLR-9 deficiency had resulted in approximately 50% reduction of IFN-γ production in infected mice, which in turn was insufficient to combat the parasite [17]. Consequently and in agreement with our study, infected TLR-9<sup>-/-</sup> mice harbored more parasites as compared to WT animals. It is unclear, however, why the overwhelming (local) Th1 response in our report was still insufficient to control parasite replication. One reason could be that a down-regulated systemic expression of pro-inflammatory cytokines as shown by lower splenic IFN-γ and TNF-α and serum IL-6 might contribute to insufficient parasite control in *T. gondii* infected TLR-9<sup>-/-</sup> mice. Notably,

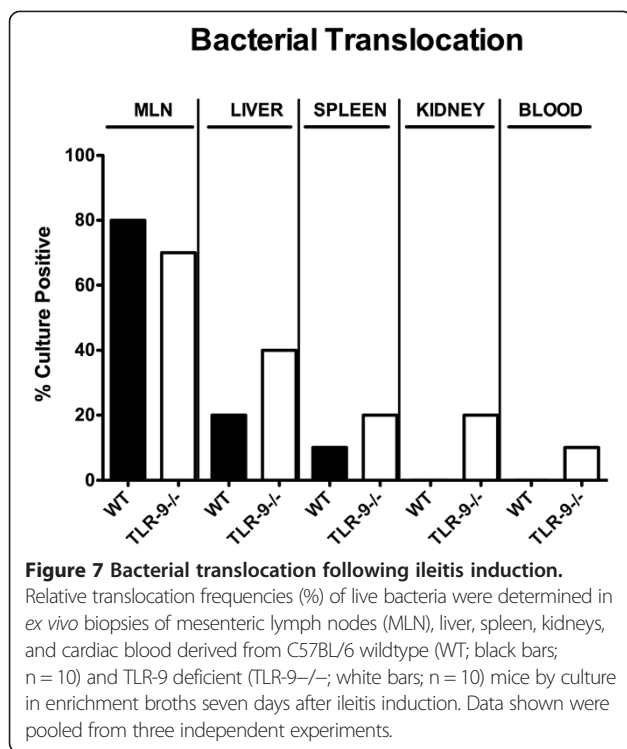
expanding intestinal IL-10 producing FOXP3<sup>+</sup> Treg populations exert important anti-inflammatory defense mechanisms against invading pathogens [23]. Given that WT, but not TLR-9<sup>-/-</sup> mice exhibited increased numbers of ileal FOXP3<sup>+</sup> cells upon infection, reduced Treg numbers might be indicative for compromised ileal mucosal regulatory properties in the absence of TLR-9, which in turn contributes to a more devastating inflammatory scenario. Interestingly, a previous study revealed a TLR-9 dependent induction of intestinal α-defensins comprising a conserved heterogenous group of antimicrobial peptides upon *T. gondii* infection [24]. In infected TLR-9<sup>-/-</sup> mice, however, secretory granules of α-defensin producing Paneth cells failed to degranulate and, in turn, to elicit a potential local anti-parasitic effect [24]. Furthermore, we showed that in the absence of TLR-9 viable bacterial species originating from the commensal intestinal microbiota were more frequently capable of translocating through the inflamed epithelial barrier to extra-intestinal organs such as liver, spleen, kidney, and even cardiac blood. It is somewhat surprising, however, that even during more pronounced ileal disease TLR-9 deficient mice exhibited less extra-intestinal and systemic pro-inflammatory cytokine in liver, spleen, and serum as compared to WT mice at day 7 p.i.

Several factors might be responsible for the conflicting results reported by Minns et al. such as differences in *T. gondii* strain and numbers of applied cysts. Minns et al. perorally infected mice with 35 cysts of the 76 K strain, whereas in our study mice were challenged with three times more (i.e. 100) cysts of the ME49 strain. Hence, the induction of the Th1-type immunopathology was more pronounced in our present study due to the higher amount of applied *T. gondii* cysts. Furthermore, differences in the genetic background (complete versus



incomplete backcrossings into the same genetic background), sex and age as well as housing conditions (e.g. diet, hygienic conditions in the animal facility) and the colonization status of the applied mice might also impact host resistance against parasitic infection [25-27]. A plethora of recent reports highlight the pivotal role of the intestinal microbiota in immunity and inflammation, and even subtle differences in the microbiota composition might have a major biological impact on the initiation and perpetuation of immunopathology [23,28-31]. This encouraged us to perform a comprehensive comparative survey of the intestinal microbiota composition in naive and *T. gondii* infected TLR-9<sup>-/-</sup> as well as WT mice.

Interestingly, naive TLR-9<sup>-/-</sup> mice harbored approximately one order of magnitude lower enterobacteria (such as commensal *E. coli*) and Mouse Intestinal *Bacteroides* as compared to WT mice, whereas bifidobacteria were virtually absent in the former. One needs to take into consideration that bifidobacterial species are considered probiotic bacteria with anti-inflammatory capacities. In a previous *in vitro* study, for instance, CD4<sup>+</sup> T cells co-cultured with bifidobacteria stimulated dendritic cells resulted in an increase of CD25<sup>+</sup> FOXP3<sup>+</sup> Tregs [32]. It is tempting to speculate that due to the absence of bifidobacteria in TLR-9<sup>-/-</sup> mice we did not observe increased numbers of ileal FOXP3<sup>+</sup> Treg



populations upon *T. gondii* infection, which additionally contributed to a compromised host resistance against the parasite. However, it is currently virtually impossible to decipher whether the observed immune responses are mainly due to signaling of DNA signals derived from the intestinal microbiota and/or the parasite.

Experimental chronic encephalitis develops within several weeks following intraperitoneal low dose (i.e. 1 to 10 cysts) *T. gondii* infection of mice [2,33,34]. To our surprise, in the acute ileitis model presented here overt inflammatory changes were not restricted to the small intestinal tract, but could also be observed within the central nervous system affecting both, the brain cortex and meninges as early as one week following peroral high dose *T. gondii* infection in WT as well as TLR-9<sup>-/-</sup> mice. Remarkably, brains of *T. gondii* infected TLR-9<sup>-/-</sup> mice were even more distinctly affected by the induced pro-inflammatory immune responses as compared to WT control animals given that higher numbers of inflammatory foci and higher abundance of F4/80+ cells comprising recruited macrophages, inflammatory monocytes, and residential microglia were detectable. Interestingly, intracerebral parasitic loads and mRNA expression levels of pro-inflammatory cytokines such as IFN- $\gamma$ , TNF- $\alpha$ , and IL-6 were similar in mice of either genotype at day 7 p.i. Even though we did not address the underlying mechanisms of this collateral damage within the hyper-

acute intestinal inflammatory disease, several factors might explain the concomitant intracerebral inflammatory response. It is well described that pro-inflammatory cytokines and active molecules such as IFN- $\gamma$ , TNF- $\alpha$ , IL-6, and reactive oxygen species are up-regulated during acute *T. gondii* infection [1,2,8,35]. TNF- $\alpha$  is mainly produced by Ly6C+ inflammatory monocytes that regulate parasite control [35]. Importantly, systemic TNF- $\alpha$  has been shown to activate cerebral microglia in infection-induced encephalopathy [36] and murine neurocysticercosis [37], and subsequently up-regulates other inflammatory mediators and neurotransmitters. Furthermore, matrix-degrading mediators such as matrix metalloproteinases (MMP), e.g. gelatinases, are up-regulated in the small intestinal tract during acute *T. gondii* induced ileitis [22] and ischemic injury [38]. As a consequence, immune cells become activated [39,40] and further biologically active TNF- $\alpha$  and IL-6 are released from the surfaces of macrophages [25,41,42] which might lead to blood brain barrier breach and consequent influx of immune cells from the blood to the CNS [38]. Enhanced intracerebral F4/80+ monocyte recruitment might exacerbate oxidative stress to the brain parenchyma to perpetuate the inflammatory response [36].

## Conclusion

TLR-9 impacts the intestinal microbiota composition and mediates immunopathological responses during acute ileitis following peroral high dose *T. gondii* infection. Furthermore, acute *T. gondii* induced ileitis is accompanied by distinct TLR-9 dependent inflammatory sequelae in extra-intestinal compartments including the brain as early as 7 days p.i. In the absence of TLR-9 local (i.e. small intestinal) as well as intracerebral inflammatory changes are more pronounced, whereas systemic pro-inflammatory immune responses are down-regulated upon *T. gondii* infection.

## Methods

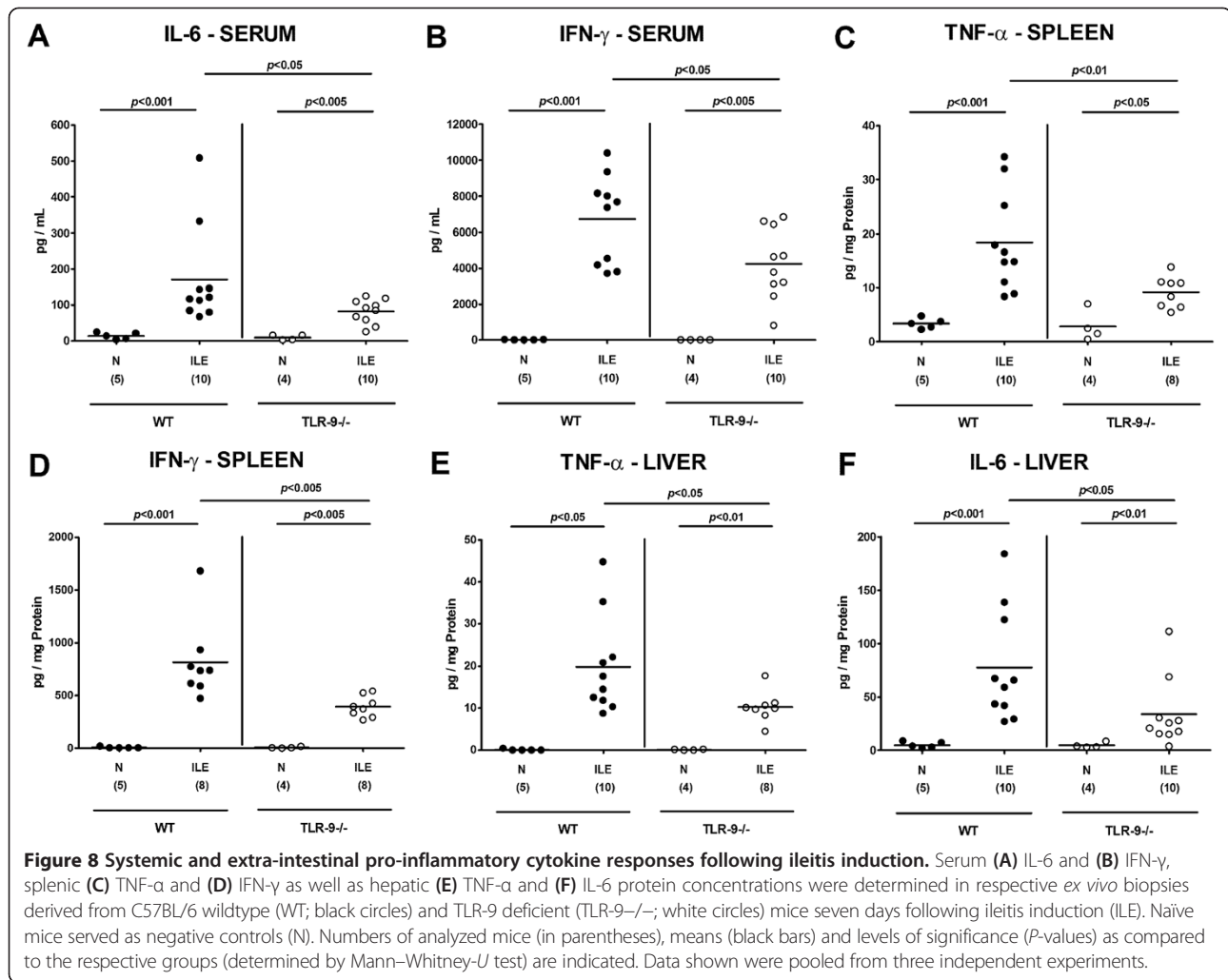
### Ethics statement

All animal experiments were conducted according to the European Guidelines for animal welfare (2010/63/EU) with approval of the commission for animal experiments headed by the "Landesamt für Gesundheit und Soziales" (LaGeSo, Berlin; registration number G0146/10). Animal welfare was monitored twice daily by assessment of clinical conditions and weight loss of mice.

### Mice and induction of acute ileitis

TLR-9<sup>-/-</sup> mice (in C57BL/6 background; described elsewhere [43]) and wildtype controls were bred and housed under specific pathogen-free (SPF) conditions in the Forschungseinrichtung für Experimentelle Medizin (FEM, Charité – University Medicine Berlin, Germany). For induction of acute ileitis, age matched 3 months old female





mice were infected perorally by gavage with 100 *T. gondii* cysts (ME49 strain) from homogenized brains of intra-peritoneally infected NMRI mice in a volume of 0.3 mL phosphate-buffered saline (PBS), as described previously [9,10,44].

#### Sampling procedures and histopathology

Mice were sacrificed by isofluran treatment (Abbott, Germany). Cardiac blood and tissue samples from brain, spleen, liver, kidneys, mesenteric lymph nodes, and ileum were removed under sterile conditions. Small intestinal samples from each mouse were collected in parallel for histopathological, immunohistochemical, microbiological, and immunological analyses. Immunohistopathological changes were determined in samples derived from ileum and brain that were immediately fixed in 5% formalin and embedded in paraffin. Sections (5  $\mu$ m) were stained with hematoxylin and eosin (H&E), examined by light microscopy (magnification 100  $\times$  and 400  $\times$ ) and histopathological changes quantitatively

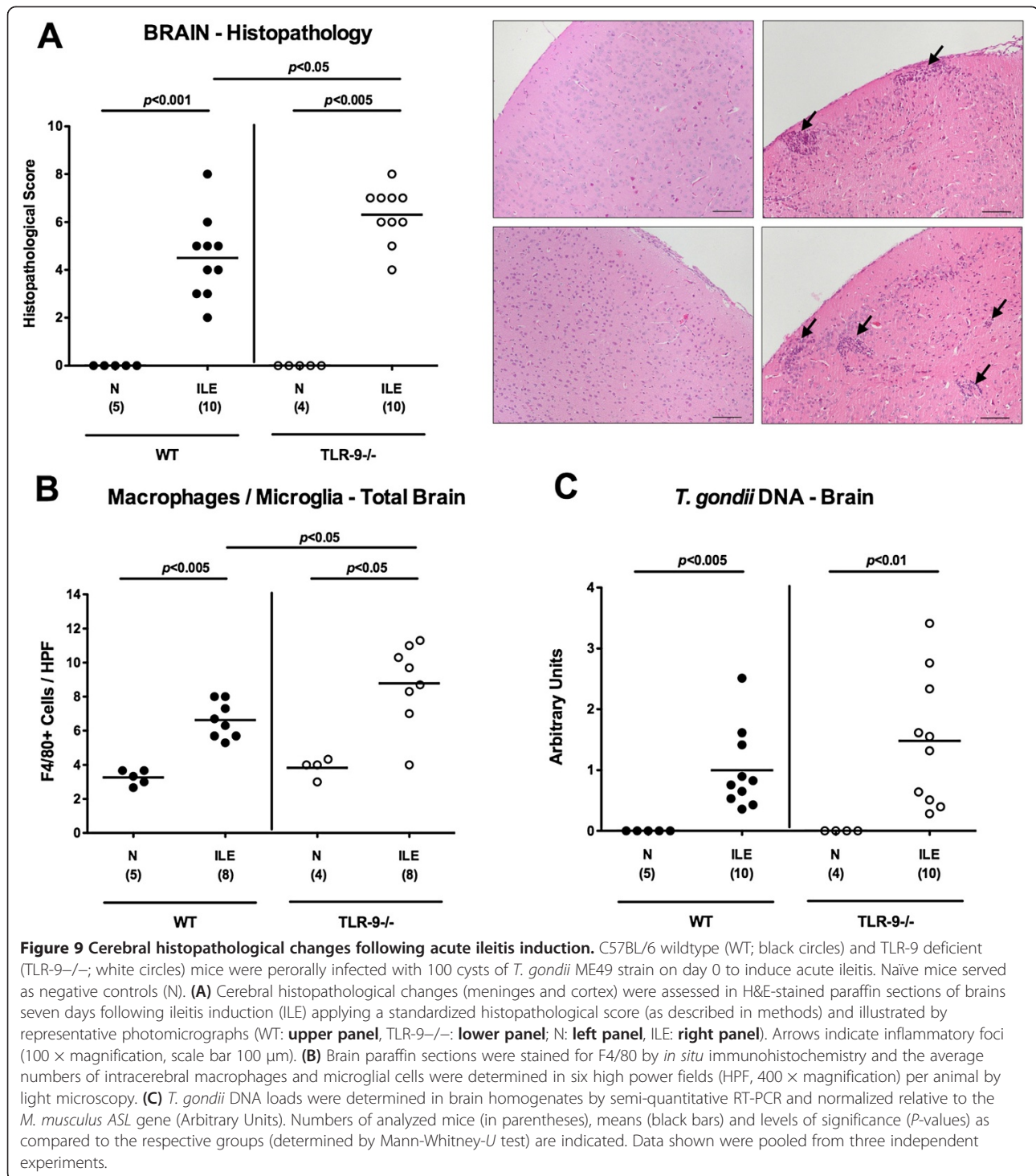
assessed applying respective histopathological scoring systems.

**Ileal histopathology** was determined as described previously [9].

**Brain histopathology** (max. 10 points; according to [33-35] with minor modifications): The numbers of inflammatory foci per HPF (100  $\times$  magnification) were assessed in cortex and meninges (separately) and the sum of the resulting scores indicated (maximum score of 10). 0, healthy brain structure with no inflammatory foci; 1, single inflammatory foci [1-3]; 2, inflammatory foci [3-6]; 3, inflammatory foci [7-10]; 4, inflammatory foci [11-15]; 5, inflammatory foci (>15).

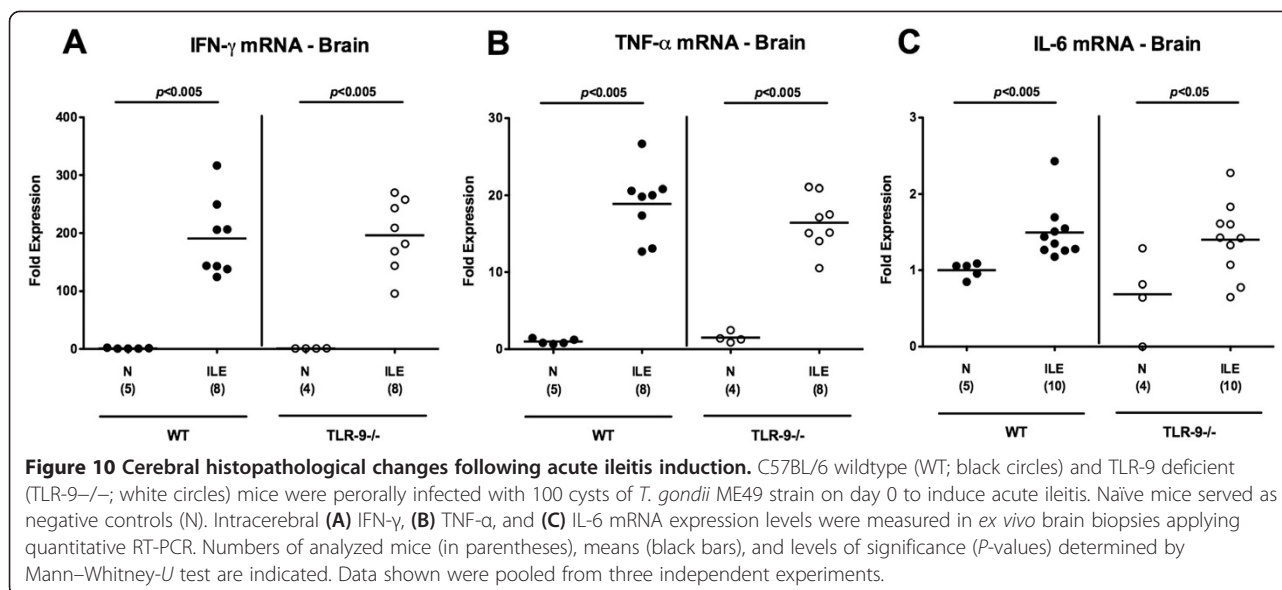
#### Immunohistochemistry

*In situ* immunohistochemical analyses of 5  $\mu$ m thin ileal paraffine sections were performed as described previously [43,45-47]. Primary antibodies against cleaved caspase-3 (Asp175, Cell Signaling, USA, 1:200), CD3 (M20, Santa Cruz, 1:1000), myeloperoxidase-7 (MPO-7, #



A0398, Dako, 1:10000), F4/80 (# 14–4801, clone BM8, eBioscience, 1:50), and FOXP3 (FJK-16 s, eBioscience, 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<sup>2</sup>; 400 × magnification) was determined microscopically by two independent double-blinded investigators.

**Ileal *T. gondii* DNA detection and cytokine measurement**  
 Ileal *ex vivo* biopsies were cut longitudinally and washed in PBS. In approximately 1 cm<sup>2</sup> of homogenized ileal tissue *T. gondii* DNA was measured as described previously [22]. Spleen, liver, mesenteric lymph nodes or strips of approximately 1 cm<sup>2</sup> ileal tissue were placed in 24-flat-bottom well culture plates (Nunc, Wiesbaden,



Germany) containing 500  $\mu$ L serum-free RPMI 1640 medium supplemented with penicillin (100 U/mL) and streptomycin (100  $\mu$ g/mL; PAA Laboratories). After 18 h incubation at 37°C, culture supernatants or serum samples were tested for IFN- $\gamma$ , TNF- $\alpha$ , IL-6, and IL-10 by the Mouse Inflammation Cytometric Bead Assay (CBA; BD Biosciences) on a BD FACSCanto II flow cytometer (BD Biosciences). Nitric oxide (NO) was determined by Griess reaction as described earlier [9].

#### Lymphocyte isolation, sorting and flow cytometry

Mesenteric lymph nodes were removed and subsequently minced through a 70  $\mu$ m filter. For intracellular staining, cells were stimulated for 4 h with 50 ng/mL 12-O-tetradecanoylphorbol-13 acetate (Sigma, Missouri, USA), 750 ng/mL ionomycin (Sigma) and Golgi Stop (BD Biosciences, San Diego, USA) at 37°C. Stainings and cell sorting with anti-CD3 (clone 145-2C11, isotype hamster IgG1; BD Pharmingen), anti-CD4 (clone GK1.5 isotype rat IgG2b; BD Pharmingen), anti-CD69 (clone H1.2 F3, isotype Hamster IgG1\*, Lamda 3; BD Pharmingen), and anti-IFN- $\gamma$  (clone 145-2C11, isotype hamster IgG1; BD Pharmingen) were performed. Cells were analyzed with a FACSCalibur or LSR II Flow Cytometer (BD Biosciences).

#### Molecular analysis of the intestinal microbiota

DNA from fecal samples was extracted as described previously [9]. Briefly, DNA extracts and plasmids were quantified using Quant-iT PicoGreen reagent (Invitrogen, UK) and adjusted to 1 ng per  $\mu$ L. Then, main bacterial groups abundant in the murine conventional intestinal microbiota were assessed by quantitative RT-PCR with group-specific 16S rRNA gene primers (Tib MolBiol, Germany) as described previously [43,45,48]. The number

of 16S rRNA gene copies per ng DNA of each sample was determined and frequencies of respective bacterial groups calculated proportionally to the eubacterial (V3) amplicon.

#### Bacterial translocation

For qualitative detection of bacterial translocation, entire MLNs, liver, spleen, kidneys, and cardiac blood were transferred into a thioglycolate broth each and incubated for maximum seven days at 37°C [49]. Bacterial growth was monitored daily by turbidity assessment. Aliquots of turbid broths were cultivated on respective solid media under aerobic, microaerobic, and obligate anaerobic conditions. Bacterial species identification was performed as described earlier [9].

#### Cerebral cytokine and parasitic DNA detection

Brain tissue preparation and measurement of pro-inflammatory cytokine mRNA expression by quantitative real time-PCR were performed as described previously [34]. Perfused brain tissue samples were snap-frozen and kept at -80°C. 30 mg of brain tissue were used for nucleic acid purification using the spin column based AllPrep DNA/RNA/Protein Mini Kit (QIAGEN, Hilden, Germany) and following the manufacturer's instructions. On-membrane DNase I digestion (peqGOLD, Erlangen, Germany) was performed during RNA purification. RNA and DNA purity and concentration were determined by absorbance at 230, 260 and 280 nm in a NanoDrop spectrophotometer (Fisher Scientific, Germany).

Semi-quantitative real time-PCR analyses were performed to determine parasite loads in brains. FastStart Essential DNA Green Master (Roche, Grenzach-Wyhlen, Germany) was used with 90 ng genomic DNA in a reaction

volume of 20  $\mu$ L. Triplicate reactions were developed in a LightCycler<sup>®</sup> 480 Instrument II (Roche, Grenzach-Wyhlen, Germany). After an initial activation step (95°C for 10 min), 45 amplification cycles were run, comprising of denaturation at 95°C for 15 sec, annealing at 60°C for 15 sec and elongation at 72°C for 15 sec. The following primers were manufactured by Tib MolBiol (Berlin, Germany) and used at a final concentration of 0.3  $\mu$ M: *Toxoplasma gondii* B1: (Forward) 5'-TCCCCTCTgCTggCgAAAAgT-3' and (Reverse) 5'-AgCgTTCgTggTCAAC TATCgATTg-3' [50]. *Mus musculus* argininosuccinate lyase (ASL) gene: (Forward) 5'-TCTTCgTTagCTggCAAACCTACCT-3' and (Reverse) 5'-ATgACCCAgCagCTAAgCagATCA-3' [51]. Parasite loads (target: *Toxoplasma gondii*, B1 gene) were measured relative to mouse cell number (reference: *Mus musculus*, argininosuccinate lyase (ASL) gene), that is the target/reference ratio calculated with the LightCycler<sup>®</sup> 480 Software release 1.5.0 (Roche, Grenzach-Wyhlen, Germany).

To determine relative gene expression, SuperScript<sup>®</sup> III Platinum<sup>®</sup> One-Step Quantitative RT-PCR System (life technologies, Darmstadt, Germany) was used with 300 ng total RNA in a reaction volume of 10  $\mu$ L. Triplicate reactions were developed in a LightCycler<sup>®</sup> 480 Instrument II (Roche, Grenzach-Wyhlen, Germany). Reverse transcription was performed for 15 min at 50°C followed by 2 min at 95°C. Subsequently, 45 amplification cycles were run, comprising of denaturation at 95°C for 15 sec and annealing/elongation at 60°C for 30 sec. TaqMan<sup>®</sup> Gene Expression Assays (life technologies, Darmstadt, Germany) were used for amplification of the house-keeping gene HPRT, IFN- $\gamma$  TNF- $\alpha$ , and IL-6. HPRT expression was chosen as reference for normalization and target/reference ratios were calculated with the LightCycler<sup>®</sup> 480 Software release 1.5.0 (Roche, Grenzach-Wyhlen, Germany). Resulting data were further normalized on values of control groups.

### Statistical analysis

Medians, means and levels of significance were determined using Mann–Whitney *U*-Test. Two-sided probability (*P*) values  $\leq 0.05$  were considered significant. All experiments were performed three times.

### Competing interests

The authors have declared that no competing interests exist.

### Authors' contributions

Conceived and designed the experiments: IRD, MMH. Performed the experiments: AAK, MA, AF, LM, DS, IRD, MMH. Analyzed the data: AAK, AF, LM, DS, OL, IRD, MMH. Contributed reagents/materials/analysis tools: SB, AAK, OL, UBG. Wrote the paper: SB, AAK, AF, IRD, MMH. All authors read and approved the final manuscript.

### Authors' information

Ildikó R Dunay and Markus M Heimesaat shared last authorship.

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### Author details

<sup>1</sup>Department of Microbiology and Hygiene, Charité - University Medicine Berlin, Centrum 5, Campus Benjamin Franklin, Hindenburgdamm 27, D-12203 Berlin, Germany. <sup>2</sup>Department of Internal Medicine, Rheumatology and Clinical Immunology/Research Center ImmunoSciences (RCIS), Charité - University Medicine Berlin, Berlin, Germany. <sup>3</sup>Department of Microbiology and Hygiene, University of Magdeburg, Magdeburg, Germany.

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