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Breakdown of intestinal homeostasis by mucosal infections triggers adaptive immune responses against antigens from commensal bacteria

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SUMMARY

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ACRONYMS AND ABBREVIATIONS

GALT: gut associated lymphoid tissues

mLn: mesenteric lymph nodes

siLP: small intestine lamina propria

Sp: spleen

Pp: Peyer's patches

IEL: intraepithelial lymphocyte

LivLn: liver-draining lymph node

T-bet: T-box transcription factor

Foxp3: forhead transcription factor 3

Treg cell: T regulatory cell

DC: dendritic cell

gfDNA: gut flora DNA

TLR: toll like receptor

NOD: nucleotide oligomerization domain

IBD: inflammatory bowel disease

sIgA: secretory IgA

SFB: segmented filamentous bacteria

ABSTRACT

The gastrointestinal tract of mammals is inhabited by thousands of distinct species of commensal microorganisms that exist in a mutualistic relationship with the host. It has previously been shown that these gut microbes play an important role in modulating host immune responses. On the other hand, commensals can also contribute to pathology in the context of acute infection. For instance, oral infections with *Toxoplasma gondii* in certain inbred strains of mice lead to an exacerbated intestinal inflammation that is accompanied by a loss of diversity within the gut flora. Furthermore, the microbiota was shown to aggravate the immunopathology of the disease. The mechanisms underlying this phenomenon still remain poorly understood. In order to study how the recognition of innocuous microbes can influence immune responses and pathological consequences during acute mucosal infections we treated mice a cocktail of antibiotics. Treated mice showed decreased inflammatory responses and lower parasite load. Germfree mice infected with *T. gondii* displayed less severe disorder with reduced parasite burden and lower levels of liver enzymes. Systemic translocation of gut bacteria was observed at the peak of infection in *T. gondii*-infected mice as well as temporal changes in diversity of the gut microbial community. Three different bacteria that were abundant in the gut of *T. gondii*-infected mice at the peak of infection were isolated and used for investigation of specific immune responses against commensal bacteria. *T. gondii* acute infection induced specific antibody responses towards antigens from the microbiota and adaptive cellular responses indicated by a strong DTH reaction against antigens from one of the isolated bacteria. Moreover,

Moreover, CBir 1 TCR Tg cells that only respond to a specific peptide from flagellin become activated after oral infection with *T. gondii.* We also showed that antibody responses against the microbiota occur also after a less intense inflammatory response induced by Microsporidia infection or after *Citrobacter rodentium* colitis. Furthermore, vaccination of mice against *E. coli* led to a more efficient clearance of *T. gondii* parasites but did not worsen the immunopathology. All together our findings suggest that mucosal acute infections can trigger an adaptive immune response against gut commensals that in turn contributes to the protection of the host against subsequent infections.

RESUMO

O trato gastrointestinal de mamíferos é colonizado por uma diversa comunidade microbiana que co-existe mutualisticamente com seu hospedeiro. Estudos recentes têm demonstrado o papel importante destes microrganismos na modulação de respostas imunes. Por outro lado, bactéria da microbiota podem também contribuir para a patologia no contexto de infecções agudas. Por exemplo, infecções orais com *Toxoplasma gondii* em certas linhagens murinas levam a uma inflamação intestinal exacerbada que é acompanhada por perda de diversidade da microbiota. Além disso, a microbiota agrava a imunopatologia da toxoplasmose. Os mecanismos que explicam este fenômeno ainda não são completamente compreendidos. No presente estudo utilizamos de camundongos tratados com antibióticos para estudar como o reconhecimento de microrganismos da microbiota pode influenciar respostas imunes e a patologia de infecções agudas de mucosa. Camundongos tratados mostraram menor resposta inflamatória e menor quantificação de parasita após infecção com *T. gondii.* Camundongos germfree também infectados com *T. gondii* têm menor carga parasitária e níveis reduzidos de enzimas do fígado. Translocação sistêmica de bactérias intestinais foi observada no pico da infecção assim como mudanças temporais de diversidade dentro da comunidade microbiana intestinal. Três bactérias mais encontradas no intestine de camundongos infectados com *T. gondii* foram usadas para o estudo de respostas específicas contra a microbiota. A infecção com *T. gondii* foi capaz de induzir respostas humorais específicas contra antígenos da microbiota e respostas adaptativas celulares indicadas por uma forte reação de DTH contra uma das

bactérias isoladas. Além disso, células CBir TCR transgênicas que somente respondem a um peptídeo de flagelina se tornam ativadas após a infecção oral com *T. gondii.* O presente estudo também demonstrou que uma infecção menos intensa, com Microsporidia, também induz anticorpos contra antígenos microbiamos assim como a colite induzida pela bactéria *Citrobacter rodentium*. A vacinação de camundongos contra uma bacteria commensal levou a um controle mais eficiente de *T. gondii* mas não agravou a imunopatologia da doença. Em conjunto, nossos resultados sugerem que infecções agudas de mucosa podem ativar respostas adaptativas imunes contra bactérias da microbiota intestinal que por sua vez contribuem para a proteção contra infecções subsequentes.

If a man will begin with certainties, he shall end in doubts,
but if he will content to begin with doubts, he shall end in certainties

Francis Bacon

INTRODUCTION

The intestinal microbiota

Mammalian barrier surfaces host complex microbial communities whose combined membership outnumbers our own cells by at least a factor of ten. Although beneficial bacteria colonize all mammalian epithelial surfaces, the gastrointestinal tract has the largest bacterial burden, with more than 100 trillion individual organisms (Xu and Gordon, 2003). The bacterial communities of the mammalian intestine are also some of the best characterized. For decades, our understanding of the composition of intestinal microbial communities was based on the enumeration and characterization of culturable organisms. However, as most gut organisms are resistant to culture by available methods, this approach left substantial gaps in the catalogue of intestinal bacterial species.

Recently, there has been an increased understanding of the importance of intestinal microbiota for human physiology, evidenced by large endeavors such as the MetaHIT project (Qin et al., 2010) and the Human Microbiome Project (Nelson et al., 2010). The recent development of molecular profiling methods, including molecular advances in DNA bar coding and 454 pyrosequencing of 16S ribosomal RNA gene segments, has revolutionized the understanding of the intestinal microbiota through culture-independent analyses of microbial community composition. The pyrosequencing technology introduced by 454 Life Science (Margulies et al., 2005), consists of amplicons of partial 16S ribosomal RNA gene sequences that are attached and sequenced on microscopic beads placed separately in picoliter-sized wells. For the

Genome Sequencer 454 FLX system, this generally produces around 400,000 reads with average lengths of 250 base pairs (bp) and an average quality score of greater than 99.5% accuracy rate. These read sizes are sufficient to cover most of the variable regions in the 16S rRNA gene (Claesson et al., 2009). These recent high-throughput methods have allowed unprecedented insight into the diversity of intestinal microbial communities, and have even led to the identification of new bacterial species. Currently, new insights into non culturable bacterial communities are placing species estimates from conservative numbers of 1000–2000 to numbers as high as 15,000–40,000 individual members (Hill and Artis, 2010).

The vast majority of these microbes are bacteria, although eukaryotes, viruses, and even archaea are also represented. Molecular profiling of the human intestinal microbiota has revealed a high level of variability between individuals at the bacterial species level. The species variability among individuals has important implications for understanding intestinal immune system function, as it indicates that the mucosal immune system must be able to rapidly adapt to a microbiota. Nevertheless, common patterns emerge when microbial communities are compared at higher-level taxa. Firmicutes and Bacteroidetes are the predominant intestinal phyla across all vertebrates. The intestinal Firmicutes are Gram-positive bacteria, dominated by species belonging to the Clostridia class, but also include Enterococcaceae and Lactobacillaceae families and *Lactococcus* spp. Intestinal Bacteroidetes are Gram-negative bacteria comprised of several Bacteroides species, including *Bacteroides thetaiotaomicron*, *Bacteroides fragilis* and *Bacteroides ovatus*. The remaining intestinal

bacteria, accounting for less than 10% of the total population, belong to the Proteobacteria, Fusobacteria, Actinobacteria, Verrucomicrobia and Spirochaetes phyla and a bacterial group that is closely related to Cyanobacteria. Thus the gastrointestinal tract constitutes an exceptionally diverse and dynamic microbial ecosystem (Wang et al., 2003; Eckburg et al., 2005; Ley et al., 2008).

A large body of evidence has now been gathered confirming the fundamental role of gut commensal microbes in the maintenance of host's homeostasis. For instance, commensals can play a major role in the control of host defense. Work from our laboratory has previously demonstrated that the microbiota can act as adjuvants providing immunostimulatory signals that improve resistance against infections. This study revealed that the gut flora DNA (gfDNA) plays a major role in intestinal homeostasis through Toll like receptor 9 (TLR9) signaling acting as a natural adjuvant for priming intestinal responses. TLR9-/- mice were shown to be more susceptible to a parasitic infection. Moreover, treatment with antibiotics impaired Th1 and Th17 immune responses in infected wild-type (WT) mice but addition of gfDNA was sufficient to restore the immune responses (Hall et al., 2008). Both mucosal innate and adaptive immune responses to *Toxoplasma gondii* rely on the indirect stimulation of dendritic cells by normal gut microbiota in absence of TLR 11 signaling (Benson et al., 2009). Another study showed that the commensal microbiota composition critically regulates the generation of virus-specific $CD4^+$ and $CD8^+$ T cells and antibody responses following respiratory influenza virus infection in mice (Ichinohe et al., 2011).

Commensal microbiota can also influence the metabolism of the host. The commensal microorganism *Bacteroides thetaiotaomicron*, component of the normal mouse and human gut microbiota, was shown to modulate expression of genes involved in several important intestinal functions, including nutrient absorption, mucosal barrier fortification and xenobiotic metabolism (Hooper et al., 2001). Gut microbes can also modulate the bile-acid metabolism (Swann et al., 2011), carbohydrate metabolism, energy production and synthesis of cellular components (Gosalbes et al., 2011). A recent study reported that gut commensal bacteria can positively influence immune responses and protect against the development of inflammatory diseases by regulating the production of short-chain fatty acids (SCFAs), which are produced by fermentation of dietary fiber by intestinal microbiota (Maslowski et al., 2009). Gut microbiota has been shown to also contribute metabolic syndrome cause by deficiency in Toll-like receptor 5 (TLR 5) (Vijay-Kumar et al., 2010).

Important evidence that gut microbes are important for the proper formation of the immune system came, specially, from experiments with germfree mice that are born and raised in sterile isolators. The development of gut-associated lymphoid tissue (GALT), the first line of defense for the intestinal mucosa, is defective in these mice. Germ-free mice display fewer and smaller Peyer's patches (Pp), smaller and less cellular mesenteric lymph nodes, and less cellular lamina propria (LP) of the small intestine relative to animals with a microbiota (Falk et al., 1998; Macpherson and Harris, 2004). The cellular and molecular profile of the intestinal immune system is also compromised in the absence of commensals. In germ-free mice, intestinal epithelial

cells (IECs), which line the gut and form a physical barrier between luminal contents and the immune system, have lower expression of Toll-like receptors (TLRs). Interspersed between epithelial cells is a specialized population of T cells known as intraepithelial lymphocytes (IELs). IELs from germ-free mice are reduced in number, and their cytotoxicity is compromised (Lee and Mazmanian, 2010). Germ-free mice also have reduced numbers of γδ T cells and $CD4^+$ T cells in the lamina propria. The development of isolated lymphoid follicles, specialized intestinal structures made of mostly dendritic cells and B cell aggregates, is also dependent on the microbiota (Bouskra et al., 2008; Duan et al., 2010). Therefore, multiple populations of intestinal immune cells require the microbiota for their proper development and function. The absence of microbiota also leads to several extra-intestinal defects, including reduced numbers of $CD4^+$ T cells in the spleen, fewer and smaller germinal centers within the spleen, and reduced systemic antibody levels, which suggests that the microbiota is capable of also shaping systemic immunity (Mazmanian et al., 2005). Beyond development, the microbiota also influences functional aspects of intestinal and systemic immunity, including pathogen clearance. Germ-free mice are more susceptible to infectious agents such as *Leishmania, Listeria monocytogenes, Shigella flexneri, Bacillus anthracis* (Inagaki et al., 1996; de Oliveira et al., 1999; Smith et al., 2007).

Several studies also highlight the importance of gut commensals for proper development of the immune system. Introduction of commensal bacteria leads to increase in CD4+ T cell numbers, induction of secretory IgA (sIgA) and development of organized gut associated lymphoid tissues (GALT) (Macpherson and Harris, 2004).

Intestinal microbes have been demonstrated to influence the manifestation of gut-associated lymphoid tissues through CCR7 signaling (Pabst et al., 2006). Colonization of germfree mice with the human symbiont *Bacteroides fragilis* directs the development of the immune system, including the expansion and differentiation of splenic $CD4^+$ T cells. Moreover, treatment of germfree mice with purified polysaccharide A (PSA), a product of *B. fragilis,* has several immunomodulatory activities such as correcting systemic T cell deficiencies, restoring balance between Th cell subsets and directing lymphoid organogenesis (Mazmanian et al., 2005). Another report revealed that a group of gut microbes, the segmented filamentous bacterium (SFB), a non-culturable Clostridia-related host-specific species that strongly adheres to the ileal mucosa and to Peyer's patches (Pp), was able to recapitulate the immune-inducing effects of a complete conventional microbiota suggesting, unexpectedly, that only a restricted number of microbiota members have shaped host-immune T cell interactions during evolution (Gaboriau-Routhiau et al., 2009). In return to all these positive effects, resident microorganisms derive benefit from association with their hosts by inhabiting a protected, nutrient-rich environment. Thus, these host-microbial associations constitute a mutually beneficial symbiosis.

A generally accessible alternative to using germ-free animals for studying host-microbe *interaction in vivo* is to deplete animals of their intestinal microbiota by using a combination of broad spectrum antibiotics administered orally. Antibiotics have been used effectively as a means to treat bacterial infections in humans and animals for over half a century (Willing et al., 2011). Antibiotic treatment in animal model systems has

identified complex pro-inflammatory and immunoregulatory roles for intestinal communities in modulating intestinal cytokine responses and altering resistance to enteric pathogens (Hall et al., 2008; Garner et al., 2009) and in maintaining mucosal homeostasis (Rakoff-Nahoum et al., 2004). Depletion of gut microbiota by treatment with a broad spectrum antibiotic cocktail produces a macroscopically germfree-like phenotype. Macroscopically, germfree mice display hypoplastic secondary lymphoid organs, significantly fewer Peyer's patches, smaller spleen, enlarged ceca, and reduced epithelial cell turnover (Reikvam et al., 2011). Recent studies have shown that treatment with antibiotics causes changes within the gut microbiota composition. The route of administration as well as the duration of the treatment appear to have different effects (Hill et al., 2010).

However, being a member of the resident intestinal microbial community does not necessarily imply that a particular species has an entirely benign disposition towards its host. Although many gut microorganisms establish mutually beneficial relationships with their hosts, specific members of the microbiota may exist at plays parts in both mutualism and pathogenicity (Hooper and Macpherson, 2010). These bacteria also are known as opportunistic pathogens or pathobionts, symbiont microorganisms that does not normally elicit an inflammatory response but under particular conditions (environmentally induced) has the potential to cause deregulated inflammation and lead to disease (Round and Mazmanian, 2009). For example, *Enterococcus faecalis* is a gram-positive ubiquitous lactic acid bacterium and common member of the human intestinal microbiota of humans and many animals. The intrinsic ability of this bacterium

to resist strongly against stressing environments may allow the bacterium to persist in hospital environments and to survive host defenses. In the last decades, *Enterococci* have been recognized as one of the most common bacteria involved in hospital-acquired infections. Indeed, these microorganisms can opportunistically invade mucosal tissues and cause sepsis, urinary tract infections, peritonitis and endocarditis. The species *E. faecalis* is still responsible for the majority of human enterococcal infections (Hanin et al., 2010). Proteobacteria are a major group of gram-negative bacteria that include a variety of pathogens such as *Escherichia coli, Salmonella* and *Helicobacter.* The subgroup γ of Proteobacteria comprise the most common human pathobionts. *Proteus mirabilis,* a facultative anaerobic, urea-hydrolyzing bacterium is a member of the γ-Proteobacteria subgroup and an opportunistic pathogen of the human urinary tract that infects patients with indwelling urinary catheters or with postoperative wound infections (Nielubowicz and Mobley, 2010). Similarly, *Bacteroides fragilis* is a prominent gram-negative member of the microbiota that closely associates with mucosal surfaces and opportunistically invades intestinal tissues (Redondo et al., 1995). Although *E. faecalis, P. mirabilis* and *B. fragilis* are controlled in healthy people, they pose a serious threat of invasion and disease in immunodeficient individuals.

Probiotics

In the early 1900s, Ilya Mechnikov was the first to propose the use of live microorganisms to maintain bowel health and prolong life. Now, the term probiotic is

used to describe dietary microorganisms that are beneficial to the health of the host. Many individual or combinations of bacterial species have been shown to help protect against pathogens and ameliorate the symptoms of Inflammatory Bowel Disease (IBD) in humans and mouse models (Round and Mazmanian, 2009). Although many of these probiotic strains decrease toxic microbial metabolic activities, more recent evidence shows that these organisms can modulate intestinal immune responses and protect mice against infections. The probiotic *Lactobacillus rhamnosus* augmented intestinal IgA responses and protected mice against infection with *Escherichia coli* O157:H7 (Shu and Gill, 2002). Two strains of *Lactobacillus* were able to stimulate the immune system and protect mice against a lethal pneumovirus infection (Gabryszewski et al., 2011). Studies by our group have shown that the probiotic candidate *Lactobacillus delbrueckii bulgaricus* can induce the production of pro-inflammatory cytokines as well as anti-inflammatory cytokines conferring protection against *Listeria monocytogenes* infection in germfree mice (Dos Santos et al., 2011).

The Intestinal Homeostasis

Intestinal host–microorganism homeostasis comprehends minimizing the adverse effects of intestinal microorganisms, even during environmental perturbations such as shifts in microbial community structure, changes in host diet or pathogenic challenge. This involves ensuring that resident bacteria breach the barrier as rarely as possible; those that do invade are killed rapidly and do not penetrate to systemic sites.

There are several mechanisms that together help contain penetrant bacteria within the mucosal immune compartment that could lead to aberrant responses against commensal antigens and consequently, pathology.

Mechanisms that prevent translocation of intestinal bacteria

The small intestine is organized into crypts and villi to increase the surface area for absorption of nutrients. The entire epithelium is renewed approximately every 5 days in humans because of proliferation and differentiation of the pluripotent stem cells residing in the crypts. The most abundant intestinal surface cell is the enterocyte, an epithelial cell lineage. Enterocyte membranes, together with the tight junctions that they form with their neighboring cells, are essential for preventing bacterial penetration while allowing nutrient flux into host tissues. Besides providing an important physical barrier, enterocytes play a more active role in promoting luminal compartmentalization of symbiotic bacteria by secreting a variety of antimicrobial proteins. These natural antibiotics are members of several distinct protein families such as defensins, cathelicidins, and C-type lectins, and they promote bacterial killing by targeting the integrity of bacterial cell walls (Mukherjee et al., 2009). Antimicrobial proteins are produced either constitutively or are inducible by bacteria (Hooper et al., 2003).

Other less abundant epithelial lineages that helps limit bacterial penetration are goblet cells and Paneth cells. Goblet cells secrete large quantities of mucin that form a protective layer of mucus over the surface epithelium. The mucus is organized in two

layers: the outer layer is colonized with bacteria whereas the inner layer is resistant to bacterial penetration probably due to the antibacterial factors that are secreted by epithelial cells and retained there (Johansson et al., 2008). Paneth cells are specialized cells that harbor secretory granules containing a number of microbicidal proteins including α-defensins, lysozyme and RegIIIγ. When Paneth cells sense bacterial signals, they react by discharging their microbicidal granule contents into the gut lumen (Ayabe et al., 2000). In a recent study genetic ablation of Paneth cells caused increased translocation of bacteria to peripheral tissues indicating that these cells are for controlling mucosal penetration of bacteria (Vaishnava et al., 2008).

The mucosal adaptive immune system has evolved mechanisms for precisely monitoring and controlling bacterial interactions with mucosal surfaces. In addition to the various epithelial cell lineages that defend mucosal surfaces from bacterial invasion, subepithelial adaptive immune cells play an essential role in sequestering bacteria in the gut. IgA-producing B cells are among the most abundant and best-characterized of the adaptive immune cell populations in the intestinal mucosa. These cells populate the intestinal lamina propria and secrete bacteria-specific IgA, which is transcytosed across the epithelium and deposited on the apical surface of epithelial cells. IgA against intestinal bacteria is produced with the aid of dendritic cells that sample bacteria at various mucosal sites. Dendritic cells located beneath the Peyer's patches sample bacteria that penetrate the overlying epithelium. Lamina propria dendritic cells also actively sample the small numbers of bacteria that are present at the apical surfaces of epithelial cells, allowing them to monitor bacteria that have penetrated the inner mucus

layer and are in close association with the mucosal surface (Rescigno et al., 2001). The bacteria-laden dendritic cells interact with B and T cells in lymphoid tissues such as the Peyer's patches (Pp), isolated lymphoid follicles (ILF), or in the intestinal lamina propria inducing B cells to differentiate into plasma cells that produce IgA directed against intestinal bacteria (Macpherson et al., 2000). IgA is essential in maintaining luminal compartmentalization of intestinal bacteria, as shown by the fact that IgA deficiency leads to increased penetration of symbiotic bacteria into the host tissues (Macpherson and Uhr, 2004b). Also, specific pathogen free mice have abundant IgA whereas germfree animals do not, arguing that commensals direct IgA production and that the major role of IgA is in maintaining the balance between the host and its microbiota (Peterson et al., 2007).

Another adaptive immune cell type that plays an important role in defending mucosal surfaces is the γδ T cell receptor bearing intraepithelial lymphocytes (IEL). These cells intercalate between intestinal epithelial cells on the basolateral side of epithelial tight junctions and contribute in several ways to restore mucosal homeostasis after epithelial injury. First, they contribute to epithelial repair by secreting epithelial growth factors (Chen et al., 2002). Second, they express a number of pro-inflammatory and antimicrobial factors in response to signals from the microbiota (Ismail et al., 2009). Consistent with both of these functions, γδ T cells have been shown to play an essential role in limiting bacterial penetration across injured mucosal surfaces.

Finally, symbiotic bacteria that breach the mucosal surface are quickly phagocytosed and killed by macrophages in the lamina propria (Macpherson and Harris, 2004). This is in contrast to pathogens, that actively interfere with macrophage microbicidal mechanisms, allowing survival and replication of these bacteria in host tissues (Sansonetti, 2004). The susceptibility of symbionts to the microbicidal mechanisms of macrophages likely represents an evolutionary coadaptation with their hosts, because suppression or evasion of phagocytic killing would compromise the host health (Macpherson et al., 2005).

Regulation of intestinal balance by innate immunity mechanisms

The detection of pathogens by the host is achieved through the families of pattern recognition receptors (PRRs) that recognize conserved molecular structures known as pathogen-associated molecular patterns (PAMP) and induce production of innate effector molecules. Because these structures are also found on nonpathogenic microorganisms, the term microbe-associated molecular patterns (MAMP) is increasingly used, particularly in the context of host–commensal interactions. These signaling receptors can be divided into three families: Toll-like receptors (TLR), retinoic acid inducible gene I (RIG-I)-like receptors (RLR), and nucleotide oligomerization domain (NOD)-like receptors (NLR). TLRs have been shown to be the main receptors to mediate the recognition of molecular patterns associated with commensal microbes (Rakoff-Nahoum et al., 2004; Round et al., 2011).

Although various mechanisms are in place to dampen PRR signaling in the healthy gut, there is clear evidence from work in TLR knockout mice that a tonic level of signaling is necessary to maintain intestinal homeostasis. For example, mouse knockouts in TLR9, TLR-4, TLR-2, and the adaptor protein MyD88 all showed increased susceptibility to DSS-induced colitis (Lee et al., 2006). A gut commensal microorganism, *Bacteroides fragiles,* activates the TLR pathway signaling through TLR2 directly on Foxp3+ regulatory T cells (Tregs) to promote immunologic tolerance therefore actively suppressing immunity (Round et al., 2011). Additionally, germ-free mice and mice treated with multiple antibiotics to reduce the microbial content of the intestine are also more susceptible to DSS-induced colitis but can be protected by administration of agonists for TLR2 and TLR-4 (Rakoff-Nahoum et al., 2004).

Adaptive immune responses regulating intestinal homeostasis

Several studies revealed the importance of the adaptive immunity for maintaining intestinal homeostasis. It has been reported that in lack of TLR signaling, control of microbial mucosal translocation is impaired. However, commensal-specific serum IgG responses induced in response to these bacteria can restore effective bacterial clearance to wild-type levels (Slack et al., 2009). Thus, innate and adaptive immune mechanisms can complement each other to establish and maintain mutualism.

The $CD4^+$ T cell compartment, including T helper 1 (Th1), Th17, and $Foxp3^+$ regulatory T (Treg) cells, is important for intestinal immune homeostasis in the presence

of a nonpathogenic commensal flora (Barnes and Powrie, 2009). The importance of regulatory T (Treg) cells in the regulation of intestinal homeostasis is best illustrated by the finding that these cells can prevent the induction of experimental colitis following transfer into diseased hosts (Powrie et al., 1994a). Foxp3+ Treg cells are abundant in the small intestine and colon, where they control potentially deleterious responses to dietary and microbial stimuli. In addition to thymic-derived Treg cells, the intestine is also a preferential site for TGF-β-dependent induction of $Foxp3^+$ Treg cells from naive $CD4^+$ T cell precursors. Conditioning of gut dendritic cells by food antigen and the gut microbiota induces a $CD103^+$ retinoic acid-dependent dendritic cell that induces Tregs (Sun et al., 2007; Coombes et al., 2007). Intestinal $CD103^+$ DCs are capable of intralymphatic migration through expression of CCR7 (Pabst et al., 2006). Once settled in the mLN, LP-derived CD103+ DCs cooperate with mLN stromal cells to shape a unique environment that critically determines the signature of T cell responses induced here. The antigen-induced Treg cells are further expanded in the lamina propria in models of oral tolerance and can control local and systemic antigen-induced hypersensitivity responses (Izcue et al., 2009; Hadis et al., 2011). In addition, indigenous Clostridia are potent inducers of mucosal and systemic Treg cell responses (Atarashi et al., 2011).

Induced Treg cell and Th17 cell populations seem to be reciprocally regulated in the intestine. The cytokine TGF-β converts naïve T cells into regulatory T (Treg) and in the presence of interleukin-6 (IL-6), has also been found to promote the differentiation of naïve T lymphocytes into pro-inflammatory IL-17-producing T cells. Although TGF-β is

required for the differentiation of both populations, the presence of STAT3-mediated signals (such as IL-6 or IL-23) promotes Th17 cells at the expense of $Foxp3^+$ Treg cells (Zhou et al., 2008). The regulation of TGF-β-dependent immune responses seems also to be dependent of retinoic acid that inhibits Th17 immune responses while promoting $Foxp3^+$ cell differentiation and regulating pro- and anti-inflammatory responses (Mucida et al., 2009). Promotion of effector T cell responses by retinoic acid has been shown to occur via the retinoic acid receptor alpha (RARα). Furthermore, immune responses that are abrogated in absence of vitamin A can be rescued by treatment with retinoic acid (Hall et al., 2011). Such mechanisms contribute to keep the balance of immune responses in the gastrointestinal tract. Recent evidence suggests that bacterial components differentially affect this balance, providing potential therapeutic strategies to influence tolerance and immunity in the gut (Littman and Rudensky, 2010).

Little is known about the antigen specificity of intestinal Treg cells that control microbiota-driven responses. Recent studies have begun to reveal the mechanisms of intestinal immune modulation by the microbiota. A recent study has shown that germ-free animals have defective Th17 cell development in the small intestine and that the reduction in IL-17 production is associated with a reciprocal increase in the number of $CD4^+Foxp3^+$ Treg cells in the colon of these mice. Reconstitution of these animals with a complex and diverse microbiota that does not contain the prominent phyla Bacteroidetes does not restore proper immune balance, suggesting again that distinct organisms might have the capacity to modulate immune responses in the gut (Mazmanian and Kasper, 2006).

Flagellins have recently been identified as immunodominant antigens of the microbiota, using serologic expression cloning (Lodes et al., 2004). Flagellin is both a potent antigen for an adaptive response and is also able to stimulate innate response through binding its receptor, TLR5, on innate cells (Smith et al., 2003). These microbiota flagellin antigens provide an opportunity to study the host immune response to its microbiota and the role of antigen-specific IgA on intestinal immune homeostasis. A T cell receptor transgenic (Tg) mice specific for one of these flagellins, denoted CBir1 was generated and has been used as a tool for studying specific immune responses against microbiota (Lodes et al., 2004). Studies using this transgenic mouse strain showed that intestinal IgA regulates the activation of peripheral, flagellin-specific $CD4^+$ T cells. Moreover, Tregs control such intestinal IgA B cell responses in an antigen-specific manner, via production of TGF-β. The depletion of $CD4^+CD25^+$ Tregs substantially reduced intestinal IgA levels within days, in part due to interruption of survival signals to LP IgA B cells provided by Tregs. These data are consistent with Tregs being the major helper T cells for induction and maintenance of intestinal IgA B cell responses (Cong et al., 2009).

An important component of Treg cell-mediated control of intestinal homeostasis is their ability to survive and compete with effector T cells in the intestinal niche. In addition, Treg cells can express several transcription factors associated with particular effector responses, including T-bet, IRF4 and GATA3 (Littman and Rudensky, 2010). Under homeostatic conditions these allow Treg cell-mediated control of distinct effector modules. However, the system is delicately poised and can sometimes lead to Treg-cell

instability. For example, high-level T-bet expression in the presence of acute intestinal infection drives Treg cells into an inflammatory IFN-γ-secreting phenotype (Oldenhove et al., 2009).

Intestinal inflammation

Inflammatory bowel disease (IBD) refers to chronic inflammatory disorders that affect the gastrointestinal tract. There are two main clinical forms of IBD: Crohn's disease, which can affect any part of the gastrointestinal tract, and ulcerative colitis, in which pathology is restricted to the colonic mucosa. The precise etiology of (IBD) remains unclear, but several factors that make a major contribution to disease pathogenesis have been identified. Among these factors, IBD is thought to result from inappropriate and ongoing mucosal immune responses to normal intestinal bacteria (Kaser et al., 2010). Tolerance to intestinal bacteria is broken in IBD leading to inappropriate local and systemic immune responses to intestinal communities that may contribute to pathogenesis (Duchmann et al., 1995). With few exceptions, murine models of intestinal inflammation are attenuated in germ-free animals (Strober et al., 2002). In humans, both antibiotic treatment and diversion of the fecal stream can similarly attenuate disease in some Crohn's disease patients (Rutgeerts et al., 1991; Perencevich and Burakoff, 2006). Additionally, bacterial communities from the intestine of IBD patients have a reduced diversity compared with those from healthy individuals and IBD patients display aberrant cytokine production, T cell activation, and IgG

antibody responses to intestinal bacteria (Sartor, 2008; Hill and Artis, 2010). Patients with IBD have increased numbers of epithelial cell surface-associated bacteria, suggesting a failure of mechanisms that normally limit direct contact between the microbiota and the epithelium (Swidsinski et al., 2005).

Genetic susceptibility loci have been identified for the inflammatory bowel diseases Crohn's and ulcerative colitis, including mutations in the pattern-recognition receptor nucleotide-binding oligomerization domain-containing protein 2 (NOD2) a component of the innate immune system that is important for immune recognition and responses to intracellular bacteria. These findings implicate altered immune responses to intestinal bacteria in the pathogenesis of IBD (Ogura et al., 2003).

The only microorganism reported to be strongly associated with Crohn's disease is adherent-invasive *E. coli*. However, it seems that inflammatory responses in human and experimental IBD are directed towards certain subsets of commensal organisms that have pathogenic potential, such as *Helicobacter*, *Clostridium* and *Enterococcus* species. Nevertheless, these organisms are abundant in the microbiota and are not typically pathogenic. As the microbiota of all mammals contains these potentially harmful species, known as pathobionts, it is not entirely clear why inflammation ensues only in subjects affected by IBD (Scanlan et al., 2006; Round and Mazmanian, 2009).

There are a multitude of animal models of IBD that either arise spontaneously or are induced by various experimental manipulations, which reproduce distinct features of

human IBD. However, there is no perfect experimental model, because patients with IBD present a heterogeneous spectrum of pathological features that reflect the participation of a diverse range of innate and adaptive immune effectors. This heterogeneity is further underscored by the recent observations that around 100 distinct genetic loci may contribute to IBD susceptibility, and the key target of these aberrant immune responses, the gut microbiota, is unique to each individual2. It is therefore likely that there will be several etiologies of human IBD, and these may reflect aberrant expression of distinct immune modules (Powrie and Uhlig, 2004).

Thus, the current view is that constitutive sensing of commensals plays an important homeostatic role while active responses against them is believed to be associated to pathogenesis. However, this distinction is clearly not absolute and need to be revisited in light of the observation that healthy human serum normally contains antibodies against commensals. This would suggest that commensal recognition is a common occurrence and in most circumstances, is not associated with pathogenic responses.

Models of intestinal inflammation

Oral infection with *Toxoplasma gondii*

Toxoplasma gondii is an intracellular parasite that is globally distributed and can be found within many different species of mammals and birds. It is estimated that up to a third of the world's population are infected with *T. gondii*. Sexual stages of the parasite

occur within gut epithelial cells of the cat, and the products of gamete fusion, the oocysts, are shed in the feces. As in most coccidia, the sexual stages of *Toxoplasma* are highly specific, occurring in no other known hosts than those of feline species. Nevertheless, in contrast to other coccidia, *Toxoplasma* has evolved to infect a wide variety of vertebrate species, including humans. In the intermediate host, after infection of intestinal epithelial cells, the infective stages (oocysts or bradyzoies) transform into tachyzoites. When the cells become packed with tachyzoites, the host cell plasma membrane ruptures and parasites are released into the extracellular milieu. The free tachyzoites can then infect virtually any nucleated cell they encounter, and they continue to replicate, spreading throughout host tissues. If not controlled by the immune system, tachyzoites are highly virulent and cause a generalized toxoplasmosis which is always fatal (Denkers and Gazzinelli, 1998).

Induction of T-cell-mediated immune responses and resistance to the tachyzoite stage is a key step in the *T. gondii* life cycle, determining the survival of the intermediate host and the parasite itself. After development of immunity, the tachyzoite stage is cleared from host tissues, and bradyzoites, the slowly multiplying, essentially dormant and harmless forms of the parasite, persist. The bradyzoites survive within cysts and are effectively isolated from the host immune system by the cyst wall. The ability of bradyzoites to escape the host immune response and persist in a quiescent form within the host is therefore another key event in the *T. gondii* life cycle.

T. gondii infection in inbred mice has become a major model to elucidate the basis of protective immunity against intracellular pathogens in general and to examine the regulation of immunopathologic. One of the most distinctive immunologic features of *T. gondii* infection is the strong and persistent cellular-mediated immunity elicited by the parasite, resulting in host protection against rapid tachyzoite growth and consequent pathologic changes. Thus, athymic nude mice, which lack functional T cells, are extremely susceptible to both virulent and avirulent parasite strain. More importantly, adoptive transfer of immune T cells to naive mice protects animals against challenge with virulent *T. gondii* strains. Immunogenetic studies also point to a major influence of major histocompatibility complex (MHC) class I and II on resistance and susceptibility to the parasite, consistent with the idea that T lymphocytes are crucial in determining the outcome of infection. Virtually all mouse strains develop a strong Th1 immune response to *T. gondii*, regardless of whether they possess resistant or susceptible MHC haplotypes. Thus, cytokines such as IFN-γ and TNF-α (which activate macrophage functions) are important for controlling tachyzoite replication during both acute and chronic phases of infection (Gazzinelli et al., 1994; Gazzinelli et al., 1996).

Remarkable differences in mortality following acute infection via the peroral route have been observed among inbred strains of mice (McLeod et al., 1984; McLeod et al., 1989). The peroral route is the natural route of infection with this parasite. The genetically susceptible C57BL/6 mice develop necrosis of the villi and mucosal cells in their small intestines within 8 days following peroral infection with *T. gondii (*Liesenfeld et al., 1996*)*. This infection model has been used as an experimental model of IBD

considering that is very similar to human ileitis with regard to disease localization, histological findings, and immunologic imbalance . Death of mice in this model is not related to an uncontrolled parasite replication but is due to an overwhelming immune response characterized by a Th1-type immunopathology, characterized by a $CD4^+$ T cell-mediated increase in pro-inflammatory mediators including IFN-γ, TNF-α, and NO. Activation of inducible NO synthase (iNOS) by IFN-γ and TNF-α is critical for intestinal pathology (Rachinel et al., 2004).

The gut represents one of the primary sites of exposure to pathogenic microbes. In this environment, the pro-inflammatory properties of commensals can directly contribute to the pathogenesis of mucosal infection. Recent studies have shown that the oral model of *T. gondii* infection is likely to be one of these scenarios in which pathology is associated with exuberant sensing of commensals. Analysis of the intestinal microflora revealed that *T. gondii* ileitis is accompanied by increasing bacterial load, decreasing species diversity, and bacterial translocation. Gram-negative bacteria identified as *Escherichia coli* and *Bacteroides*/*Prevotella* spp. accumulated in inflamed ileum at high concentrations. Antibiotic treatment ameliorated immunopathology and reduced intestinal NO and IFN-γ levels indicating that gram-negative bacteria, in this case *E. coli* in special, aggravated pathogen-induced intestinal Th1-type immunopathology (Heimesaat et al., 2006).

Citrobacter rodentium colitis

Citrobacter rodentium is a natural mouse extracellular enteric pathogen that mimics human enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *Escherichia coli* (EHEC), all of which use attaching and effacing lesion formation, initially on gut epithelial cells, as a major mechanism of tissue targeting and infection (Mundy et al., 2005). *C. rodentium* is highly infectious, causing colitis and transmissible colonic hyperplasia. Following ingestion, *C. rodentium* colonizes the intestines of mice, residing predominantly in the cecum and colon. Colonization of *C. rodentium* peaks 10 days after infection and is usually eradicated by day 28 after oral administration in immunocompetent mice. Bacterial colonization is mostly limited to the intestinal mucosa, with a few bacteria reaching systemic sites. The infected mice exhibit a loss of body weight and diarrhea in association with crypt hyperplasia, a loss of goblet cells, and mucosal infiltration of the epithelium with lymphocytes, macrophages, neutrophils, and mast cells.

The lymphocytic host response to *C. rodentium* is characterized by a large infiltration of $CD4^+$ T cells into the colonic lamina propria and a highly polarized Th1 response (Higgins et al., 1999) . Recent reports have shown that Th17 cells are also involved in the host defense against *C. rodentium* infection. Antigen-presenting cells (APCs) from *C. rodentium*-infected mice, when stimulated with microbial products such as lipopolysaccharide, peptidoglycans, and zymosan, produce significant amounts of IL-23. Furthermore, IL-17 A -/- mice and IL-23 A-/- mice are more susceptible to *C. rodentium* infection. These studies indicate that Th17 cytokines play an important role in eradicating *C. rodentium* infection (van Beelen et al., 2007; Shiomi et al., 2010). $CD4^+$ T

cell and B cell deficient mice also fail to eradicate *C. rodentium* infection. Among the factors produced by these cells, antibacterial IgG is particularly important. In contrast, mice lacking $CD8^+$ T cells, IgA, secreted IgM and proteins required for transport of IgA and IgM into the lumen (polymeric Ig receptor and J chain) clear *C. rodentium* normally (Maaser et al., 2004).

Recent analysis of 16S rRNA 454 pyrosequencing revealed that *C. rodentium* infection is followed by alterations in the *Proteobacteria*, *Deferribacteres*, *Clostridia*, and *Lactobacillus* lineages and their distributions differ in time post-infection and in space within the gut (Hill et al., 2010). Another study showed that antibiotic treatment alters the colonic mucus layer and predisposes the host to exacerbated *C. rodentium*-induced colitis (Wlodarska et al., 2011). These data suggest that the gut microbiota may have a role during *C. rodentium* infection.

Encephalitozoon cuniculi infection

Microsporidial infections of humans (including *Encephalitozoon cuniculi*) occur worldwide with prevalence rates of up to 50% depending on geographic region, method of diagnosis, socioeconomic and immune status of the population studied. *Encephalitozoon cuniculi* is the best studied microsporidia, yet little is known regarding

host immunity against these infectious agents. *E. cuniculi* infects epithelial and endothelial cells, fibroblasts, and macrophages in a variety of mammals. In murine *E. cuniculi* infection, ascitis develops and then clears in immunocompetent mice (Moretto et al., 2004a). Initial studies showed that cytokines released by sensitized T cells activate macrophages to kill *E. cuniculi in vitro* (Didier, 2000). It is now known that *E. cuniculi* infection results in a strong burst of IL-12 production by host macrophages or dendritic cells. Early IL-12 release leads to polarization towards Th1 cytokines manifested by high levels of IFN-γ in the circulation and tissues, immune response that is required for protection against oral as well as intraperitoneal (IP) challenge. Indeed, treatment of *E. cuniculi*-infected mice with neutralizing antibody against IFN-γ or IL-12 resulted in increased mortality for these animals. γδ T cells, which are increased at early stages of infection, are probably important sources of IFN-γ production (Khan and Moretto, 1999; Khan et al., 2001; Moretto et al., 2004b).

Adaptive immune responses against commensal bacteria

Host response to pathogens is characterized by rapid innate recognition combined with strong adaptive immune responses, causing microbial eradication often at the cost of significant tissue damage. Response to the symbiotic microbiota is characterized by a process that encompasses a complex integration of microbial

recognition and tightly controlled innate and adaptive immune responses (Sansonetti, 2011). It is believed that lymphoid structures such as mesenteric lymph nodes (mLns) are heavily influenced by the milieu of commensal bacterial molecules that penetrate host tissues, although penetration of live commensal bacteria and the induction of adaptive immune responses are absent or limited in immunocompetent hosts (Macpherson and Uhr, 2004a).

Small numbers of commensal bacteria are probably continuously penetrating the intestinal epithelial cell layer, but they usually never reach the threshold of priming a systemic adaptive immune response because of effective phagocytosis and microbicidal activity by phagocytic cells in the lymphoid tissues. The result is that there is a way for the luminal microbial contents to be immunologically sampled and for relevant protective adaptive immune responses to be induced in the intestinal mucosa (Hadis et al., 2011). The induction is confined to the mucosal immune system, but the recirculation of B and T cells through the lymph and blood to home back to mucosal tissues ensures that the local response is disseminated and averaged over the mucosal surfaces as a whole. Indeed, this adaptive response is lost in specific pathogen-free (SPF) mice in which mesenteric lymph nodes have been surgically removed. Therefore, it seems that although the mucosal immune system is primed by intestinal microorganisms, the adaptive systemic immune system remains ignorant rather than tolerant to commensal bacteria (Hooper and Macpherson, 2010).

However, all the recent data demonstrating that disruption of intestinal homeostasis by infection with mucosal pathogens alter the composition and numbers of indigenous microbiota and that this microbiota can aggravate such infections suggest that mucosal infections could lead to a breakdown of the systemic immune 'ignorance' towards commensal bacteria. In fact, it has long been reported by several groups that the systemic adaptive immune system can indeed be primed against gut bacterial antigens. Reports date from 1966 when it was detected significant amounts of immunoglobulins G and M reactive with gram negative bacteria in human adult sera (Cohen and Norins, 1966). Several others studies have demonstrated the presence of bacteria-specific antibodies in human and mice (Scott et al., 1985; Allan et al., 1995; Manukyan et al., 2008; Haas et al., 2011). Bacteria-specific T cells were found in normal intestine and increased in inflamed IBD intestine (Duchmann et al., 1996; Duchmann et al., 1999). Moreover, it has also been shown that anti-commensal microbe IgG responses initiated in mice with deficiencies in innate immunity can also have a protective role (Slack et al., 2009)

Therefore it seems that at barrier sites responses against pathogens are entwined with reactivity against the flora. How this dual response also leads to priming of effector responses against innocuous microbes and how such responses could relate to the outcome of infections has not yet been addressed. Gaining further insight of how recognition of bacteria in the gut influences immune responses could help understand how intestinal inflammatory disorders occur but also permit the development of new strategies to prevent the onset of such syndromes. In the present study, using various

models of oral infection, we explored the circumstances and consequences of adaptive responses against commensal bacteria. We established that surprisingly, priming of both cellular and humoral responses against commensal bacteria parallels pathogen exposure and that regardless to the virulence of the later. Furthermore, we found that adaptive responses against commensals enhanced the capacity of the host to respond against subsequent mucosal infections.

OBJECTIVES

General objective:

The aim of the present study was to investigate the consequences of the disruption of the homeostatic relationship between the host and the microbiota influence systemic immune responses.

Specific objectives:

- To examine the contribution of gut bacteria to immune responses during the acute phase of *Toxoplasma gondii* infection using an animal models of depletion or absence of intestinal microbiota;
- To identify the contribution of commensal bacteria to the immunopathology during *T. gondii* infection using of germfree mice;
- To determine the role of the microbiota in the clearance of *T. gondii* parasites during the acute phase of infection;
- To investigate whether *T. gondii* infection affects the diversity of the gut microbial community;
- To determine whether *T. gondii* infection induces systemic translocation of commensal bacteria;
- To investigate whether *T. gondii* acute infection activates humoral and cellular adaptive immune responses towards commensal bacterial antigens;

- To evaluate whether a mild mucosal infection such as *Encephalitozoon cuniculi* infection induce systemic translocation of gut bacteria;
- To examine humoral immune responses against gut bacterial antigens during *E. cuniculi* infection;
- To determine whether *Citrobacter rodentium* infection activates microbiota-specific adaptive humoral responses in the host;
- To examine the consequences to the host of adaptive immune responses towards the microbiota.

MATERIALS AND METHODS

Mice

C57BL/6 (WT) and B6.SJL specific pathogen free (SPF) mice were purchased from Taconic Farms and Jackson Laboratory or bred in house. Germfree mice were purchased from Taconic Farms or bred in house. CBir1 transgenic mice were obtained from Dr. Charles Elson (Cong et al., 2009). All mice were bred and maintained at an American Association for the Accreditation of Laboratory Animal Care-accredited animal facility at the NIAID-NIH and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal approved by the NIAID Animal Care and Use Committee. Germfree mice were housed in semi-rigid isolators (Park Bioservices LLC, Groveland, MA, USA). Sterility was confirmed once a week by microbiological methods. Mice between 8 and 12 weeks of age were used. Mice were gender, vendor and age-matched for each experiment.

Antibiotic treatment

Mice were treated for 4 weeks prior the infection with *T. gondii* with a cocktail of antibiotics that consisted of: Metranidazole (1 g/L), Ampicilin (1 g/L), Neomycin (1 g/L) and Vanconmycin (0.5 g/L) all mixed in the drinking water. Mice were checked twice a day to be certain that they were not dehydrated as previous evidences have suggested that the antibiotic treatment regimen in drinking water could cause dehydration (Hill et al., 2010).

Parasite and infection protocol

ME-49 type II strain (ATCC # 50840) (American Type Culture Collection, Manassas, VA, USA) of *T. gondii* was used for production of tissue cysts in C57BL/6 mice. Parental ME-49 was electroporated with RFP and selected for red fluorescence. ME-49 C1 clone were used throughout this study. Tissue cysts used in experiments were obtained from mice that were inoculated 1–3 months previously with ten cysts by gavage. Animals were sacrificed, the brains were removed and homogenized in 1 ml of phosphate buffer saline (PBS), pH 7.2, and tissue cysts were counted on the basis of 3 or more aliquots of 20 µL. For all studies mice used were 8 to 12 week old. Mice were infected by oral route.

A rabbit isolate of *E. cuniculi* obtained from Waterborne Inc. (New Orleans, USA) was used throughout the study. The parasites were maintained by continuous passage in rabbit kidney (RK13) cells obtained from the American Type Culture Collection (ATCC #CCL37) and maintained as previously described (Bouladoux et al., 2003). Spores were ressuspended in sterile PBS, and immediately used for inoculation of mice or cell cultures. Mice were infected by gavage with 5×10^6 fresh spores in a volume of 200 µl. For inoculations, *C. rodentium* was grown with shaking (250 r.p.m.) in Luria–Bertani (LB) broth at 37℃ for 18 h. Mice were infe cted with 1×10^9 CFUs by oral gavage. Bacterial culture was serially diluted and plated after each inoculation so that inoculum administered could be confirmed.

Quantitation of parasite tissue loads

Parasite load was quantitated by using two different methods. Human fibroblast (Hs27; ATCC nº. CRL-1634) cultures were used for parasite burden quantification as previously described (Roos et al., 1994). Briefly, tissue single-cell suspensions (10^4 to 10^6 cells) were added to the fibroblast monolayer cultures and titrated by plaque formation. The results of these titrations are reported in Plaque Forming Units (PFUs).

Alternately, parasite burden quantificantion was evaluated by number of cells expressing red fluorescein protein (RFP) which indicates number of cells infected with the ME49 C1 strain of *T. gondii*. Cells from spleen (Sp), mesenteric lymph nodes (mLNs) and small intestine lamina própria (siLP) were collected and single-cell suspensions were incubated with the LIVE/DEAD Fixable Blue Dead cell stain kit (Invitrogen, Carlsberg, CA, USA) for exclusion of dead cells. Cell acquisition was performed on an LSRII machine using FACSDiVa software (BD Biosciences, San Diego, CA, USA). For each sample, at least 300,000 events were collected. Data were analyzed using FlowJo software (TreeStar).

Phenotypic analysis

Single-cell suspensions of spleen, mLNs, liver draining lymph nodes (LivLn) or siLP stained with fluorochrome-conjugated antibodies as described previously against CD4 (RM4-5), CD8-α (53-6.7), TCR-β chain (H57-597), CD45.1 (A20), CD45.2 (104), in

PBS containing or not 1% FBS for 20 min on ice and in the presence of anti-Fc-γIII/II receptor and then washed. LIVE/DEAD Fixable Blue Dead cell stain kit (invitrogen) was used to exclude dead cells. For Foxp3 staining, cells were subsequently stained using the Foxp3 staining set (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. Nuclear Ki-67 was performed using anti-Ki-67 (B56) from BD Pharmingen. For T-bet staining anti-mouse/human T-bet (eBio4B10) from eBioscience was used. Cell acquisition was performed on an LSRII machine using FACSDiVa software (BD Biosciences). For each sample, at least 300,000 events were collected. Data were analyzed using FlowJo software (TreeStar).

In vitro **restimulation and intracellular cytokine detection**

RPMI 1640 supplemented with 10% FBS, penicillin, streptomycin, gentamicin, HEPES, glutamine, nonessential amino acids, and 50 mM of β-mercaptoethanol was used for in vitro restimulation. For basal cytokine detection, spleen, mLNs and siLP and single cell suspensions were cultured in triplicate at $1x10^6$ cells/ml in a 96-well U-bottom plate and stimulated with 50 ng/ml PMA (Sigma, St. Louis, MO, USA) and 5 µg/ml ionomycin (Sigma), in the presence of brefeldin A (GolgiPlug, BD Biosciences). After 4 hrs, samples were stained LIVE/DEAD Fixable Blue Dead cell stain kit, washed twice with PBS and fixed using 2% paraformaldehyde (Electron Microscopy Sciences) solution. Cells were then stained with fluorochrome-conjugated antibodies against TCR-β chain (H57-597), CD4 (RM4-5), CD8-α (53-6.7), IFN-γ (XMG1.2), IL-10 (JES5-16E3), IL-17A (eBio17B7) or isotype controls: rat IgG1 (eBRG1), rat IgG2a (eBR2a), rat IgG2b

(eB149/10H5) in the presence of anti-Fc-γIII/II receptor for 60 min in FACS buffer containing 0.5% saponin. All antibodies were purchased from eBioscience or BD Biosciences.

Pathology assessment

Mice were euthanized 9 days post oral infection with 40 cysts of ME-49 of *T. gondii* or 11 days after oral infection with *E. cuniculi*. Their small intestines were removed and immediately fixed in a solution containing 10% formalin. Paraffin embedded sections were cut at 0.5 µm and stained with hematoxylin and eosin. Sections of *T. gondii*-infected guts were stained with endotoxin-core antibody evaluated for the numbers of inflammatory loci and necrosis. Liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in serum samples, using commercially available kits (Boehringer Mannheim).

E. coli **antibody staining**

Mice were euthanized 9 days post oral infection with 40 cysts of ME-49 of *T. gondii.* Their small intestines were removed and immediately fixed in a solution containing 10% formalin. Paraffin embedded sections were cut at 0.5 µm stained with endotoxin-core antibody (EndoCAb, Eskia Inc., Edinburgh, Scotland).

Isolation and identification of bacteria

Mice were infected orally with 25 cysts of ME49 C1. At day 9 post infection fresh fecal pellets were collected and serial dilutions were plated on Tryptic Soy agar (Sigma-Aldrich, St. Louis, MO, USA), MacConkey agar (Himedia, Mumbai, India) for isolation of gram negative bacteria or Bile Esculin Agar with azide (Hardy Diagnostics, Santa Maria, CA, USA) for isolation of enterococci strains. Single colonies were then cultured in Tryptic Soy broth and overnight cultures were used for identification of bacteria by morphological aspects of colonies and commercially available biochemical methods. The three isolates were identified as *Escherichia coli, Enterococcus faecalis* and *Proteus mirabilis*.

Evaluation of systemic translocation

Mice were infected with 10 cysts of *T. gondii* ME49 II strain. At day 9 of infection mLNs, Sp and liver were collected and homogeneized in PBS using a tissue grinder. Serial dilutions of each sample were perfomed and plated on Tryptic Soy agar. Plates were incubated at 37º C in an aerobic environment. After 48 hours of incubation colonies were counted. The result was reported as the Log of the number of colony forming units (CFU) per organ.

454 Sequencing

Total DNA from stool samples was extracted from frozen samples by using a QIAamp DNA stool minikit (Qiagen Inc., Valencia, CA, USA) according to the

manufacturer's protocol for pathogen detection. The 16S rRNA gene fragment used in the present study was obtained as previously described. Briefly, each sample was amplified by using a bar-coded primer pair based on the universal bacterial primers BSF8 and BSR357. These primers were chosen on the basis of extensive optimization studies that compared segments of the 16S gene for (i) the ability to recover known clustering among bacterial communities and (ii) support accurate phylogenetic placement. In addition to the eight-nucleotide bar code, the primers used to obtain the 16S rRNA gene fragment were also modified to contain the 454 Life Sciences primers A and B.

A 50-µl PCR was carried out using in the AmpliTaq System (Applied Biosystems). The amplification system used AmpliTaq Buffer II and contained 1.5 mM $MgCl_2$, 0.05% Triton X-100, 100 µM concentrations of each deoxyribonucleoside triphosphates, 0.4 µM concentrations of each primer, 0.1 µg of bovine serum albumin/µl, and 2.5 U of AmpliTaq DNA polymerase. A total of 100 ng of template DNA was used. Amplification was carried out using the following cycling parameters: 5 min denaturing at 95℃, followed by 20 cycles of 30 s at 95℃ dena turing), 30 s at 56℃ (annealing), and 90 s at 72℃ (elongation), with a final extensi on at 72℃ for 7 min. Four independent PCRs were performed for each sample, along with a no template negative control. Sequencing was subsequently performed on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, West Sussex, UK) according to 454 protocols. Operational taxonomic units (OUT) clustering and analysis were carried out using Mothur (Department of Microbiology & Immunology at The University of Michigan).

Mothur workflow uses OTU-based approaches to analyze the frequency distribution of sequences found in bins using a variety of methods. It allows calculation of richness, diversity, and similarity. OTU based method workflow consisted of processing of raw sequence reads to create a file of unique sequences. Sequences were aligned to an aligned set of ~15 K 16S RNA sequences and filtered for removal of chimeras. OUT analyses (Alpha, Beta and taxonomic classification) were performed in preclustered sequences. Phylogenetic analyses were carried out using UniFrac analysis.

Measurement of bacterial specific antibodies in serum

The levels of antibodies in the serum that react with bacteria were determined by a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, to prepare the bacterial lysates overnight cultures were washed in PBS twice and resuspended in cold PBS with 1 mg/ml of chicken egg white lysozyme (SIGMA L7651). Bacterial suspensions were incubated for 30 minutes at 37º C and then overnight at 4º C. After overnight incubation, suspensions were submitted to 10 cycles of freeze/thaw in liquid nitrogen and water bath. The debris was removed by 30 minute centrifugation at 12,000 *g* and the supernatants were used as coating antigen in ELISA assay. The protein concentration in the lysates was determined using a standard Bicinchoninic acid (BCA) protein assay (Pierce, UK). For the assay, the cell-free bacterial extracts (1 µg/µl) were coated onto a 96-well microtiter plate (Maxisorp, Nunc) and kept at 4º C overnight. The plate then was washed with PBS and blocked with 1% BSA in PBS for 1 h at room

temperature. Serum samples were serially diluted in PBS and loaded onto the plate. After overnight incubation at 4º C, the plate was washed and the following peroxidase-conjugated goat anti-mouse antibodies were added: polyvalent IgG, IgM and IgA (Sigma) (1:3000), IgG (1:3000), IgM (1:2000), IgG2b (1:2000), IgG2c (1:2000), IgA (1:1000) (Southern Biotech) in PBS were added to each well. The plate was then left at room temperature for 1 h, washed and Peroxidase Substrate Solution for ELISA (KPL) was added. Color development was allowed for 20 minutes and absorbance was measured immediately after at 405 nm using a plate reader. Antibody levels were either reported as O.D. or titers. Titer values were calculated taking the highest dilution which gave reading.

Delayed Type Hypersensitivity response

Mice were infected orally with 10 cysts of ME49 C1. On Day 45 post infection 10 µg of bacterial lysate was inoculated intradermally into both ear dermis of mice using a 27½-gauge needle in a volume of 10 µl. Naïve mice of same age and gender were also injected with the lysate. After 48 hours, ears were harvested and stained for intracellular cytokine detection.

Bacterial FACS

Bacterial FACS was performed as described previously (Slack et al., 2009). Bacteria were grown in LB broth overnight at 37º C with or without shaking.

Bacterial cultures were gently pelleted for 2 minutes at 8000 rpm in an Eppendorf minifuge and washed 3x with sterile-filtered PBS/1%BSA/Azide before resuspending at approximately 10^7 bacteria per ml. Mouse serum was diluted 1:10 in PBS/1%BSA/Azide and heat-inactivated at 60ºC for 30 mins. Serum solution was then spun at 13000 rpm for 10 minutes to remove any bacteria-sized contaminants and the supernatant was used to perform serial dilutions. 25 µl serum solution and 25 µl bacterial suspension were then mixed and incubated at 4ºC for 1h. Bacteria were washed twice before resuspending in monoclonal anti-mouse IgM, IgG1, IgG2b or IgA (Ball BD Pharmingen). After a further hour of incubation the bacteria were washed once with PBS/1% BSA/Azide and then resuspended in 2% PFA/PBS for acquisition.

T cell purification and transfer of transgenic cells and infection of recipient mice

Single cell suspensions of spleens extracted from CBir1 transgenic (CD45.2) naïve mice or naïve OT-II (CD45.2) mice were enriched for $CD4^+$ T cells by negative selection using an autoMACs (Miltenyi Biotec). The enriched fraction was further labeled with fluorochrome-conjugated antibodies, including CD4 (RM4-5), CD25 (7D4), CD44 (IM7) (all from eBioscience) and sorted by flow cytometry on a FACSVantage or FACSAria (BD Biosciences). Positive cells used for transfer were $CD4^+$ $CD25^-$ $CD44^-$. Purified Cbir1 cells or OT-II cells were transferred intravenously into host congenic B6.SJL (CD45.1) mice at a total amount of ~$5x10^6$ cells/mouse. Host congenic mice that

received purified cells were infected with 10 cysts of *T. gondii* ME49 II strain. A few mice that received the Cbir 1 cells were not infected and were used as control. Immune responses were evaluated at day 9 after infection.

Vaccination protocol

C57BL/6 mice were immunized intraperitoneally with 200 µg of bacterial lysate on day 0 and 7. On day 14 mice were orally infected with 10 cysts of ME49 C1 and euthanized 9 days post infection.

Statistical analysis

Groups were compared with Prism software (GraphPad) using the unpaired Student's t test.

RESULTS

Pro-inflammatory immune responses are down-regulated in antibiotic-treated mice orally infected with *T. gondii*

We have previously demonstrated that commensal bacteria can be beneficial to the host enhancing resistance against a lethal bacterial infection. A normal member of the microbiota and probiotic candidate was capable of modulate immune responses and protect otherwise susceptible germfree mice against lethal infection with the bacteria *Listeria monocytogenes*. Monoassociation with this commensal bacteria prevented death of mice and enhanced production of IFN-γ and TNF-α, cytokines that are crucial for resistance against *L. monocytogenes,* which reflected on a more efficient clearance of the pathogen and less severe immunopathology (Dos Santos et al., 2011). On the other hand, commensals can also contribute to pathology in the context of acute intestinal inflammation. Intestinal bacteria have been shown to contribute to inflammatory bowel diseases (IBD). In fact, it is still not conclusive whether IBD is triggered or not by intestinal bacteria (Kaser et al., 2010).

T. gondii-induced intestinal immunopathology resembles the inflammatory responses seen in IBD. Depletion of gut microbiota by antibiotics has been shown to prevent and even ameliorate the immunopathogenesis of *T. gondii*-induced ileitis. Evidences suggest that this event is related to the capacity of modulation of immune responses by gut commensals (Heimesaat et al., 2006). In order to further study the mechanisms of which the microbiota controls immunopathology in an acute mucosal infection we treated C57BL/6 mice with a cocktail of antibiotics for 4 weeks. After this

period of time mice were infected with 10 cysts of *T. gondii* strain ME49 C1. Immune responses were evaluated at day 9 of infection (peak of infection). Our data revealed that mice that were treated with antibiotics prior to the infection have reduced pro-inflammatory responses in the mesenteric lymph nodes (fig. 1) and the small intestine lamina propria (fig. 2). Accordingly, a large proportion of the $CD4^+$ T cells within the mLNs and small intestine lamina propria (siLP), which are the dominant responding T cell subset during the acute stage of infection, expressed the transcription factor T-bet, which mediates the differentiation to a Th1-type response and consequent IFN-γ production. After treatment with a cocktail of antibiotics, we found that the expression of T-bet was significantly reduced in the mLNs (Fig. 1 A). Furthermore, the expression of the nuclear proliferation antigen, Ki-67, was also diminished in ATB mice, indicating decreased T cell activation/proliferation (Fig. 1 A). Effector $CD4^+$ T cells ($Foxp3^-$) from mLNs of ATB mice produced lower levels IFN-γ upon polyclonal stimuli, confirming the state of decreased activation of the infectious environment (Fig. 1 B). Expression of T-bet by $CD4^+$ T cells from siLP was also lower after treatment with antibiotics (Fig. 2 A) as well as production of IFN-γ (Fig. 2 B). These data indicate that the intestinal microbiota is involved in the immune responses against *T. gondii* infection.

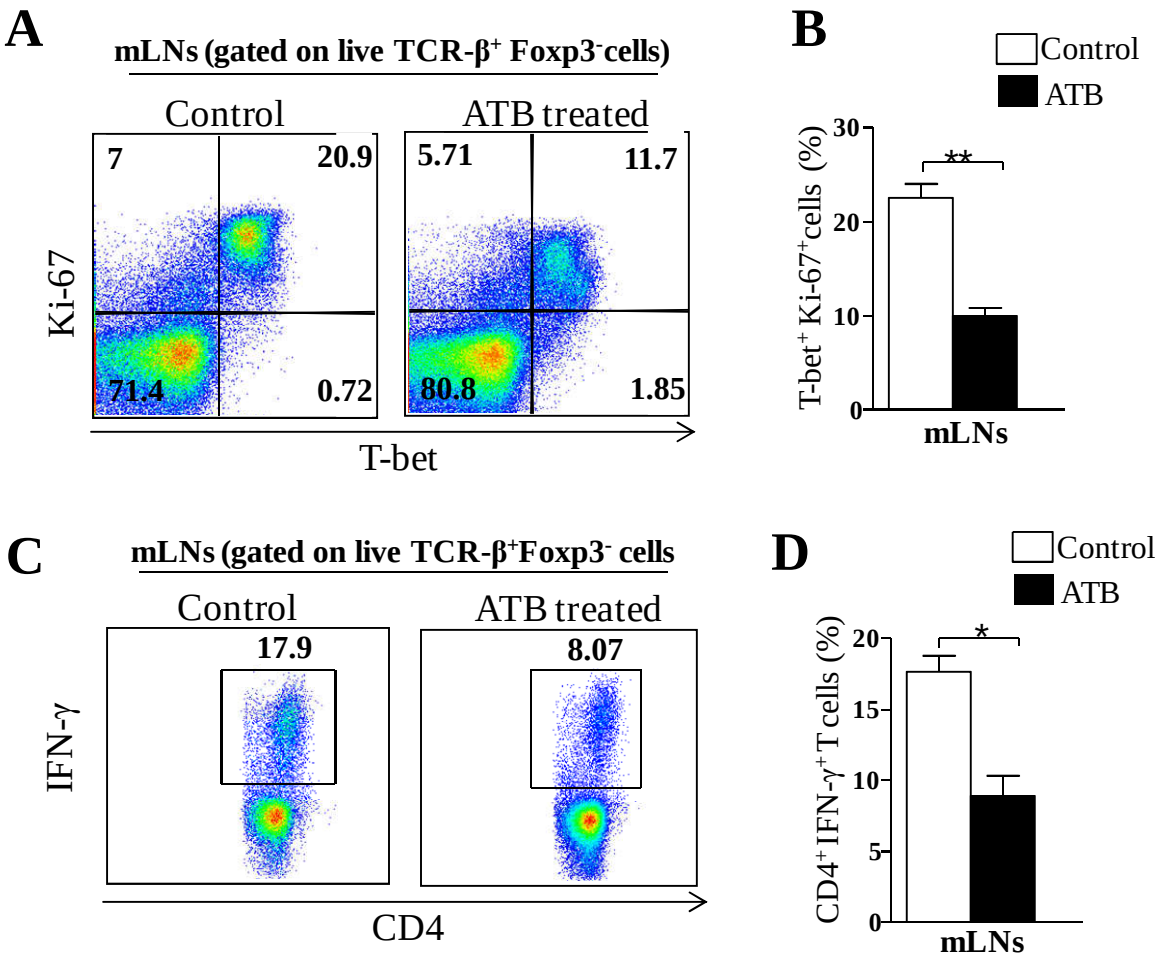


Fig 1. Immune responses against *T. gondii* are down-regulated in the mLNs of ATB mice. Mice were treated with a cocktail of antibiotics (ATB) for 4 weeks and then infected with 10 cysts of *T. gondii* strain ME49 C1. Control mice were also infected. At day 9 of infection, mesenteric lymph nodes (mLNs) cells were harvested and single-cell suspensions were either immediately stained or cultured with polyclonal stimuli. After 4 hours, stimulated cells were stained for flow cytometry assessment. **A**: representative plot of T-bet and Ki-67 co-expression in live $CD4^+$ T cells. **B**: Bar graph showing quantification of A. **C**: representative plot of IFN-γ expression in live $CD4^+$ T cells. **D:** Bar graph showing quantification of C. The data are representative of 3 different experiments performed with 4 mice each * $P< 0.05$. ** $P < 0.01$.

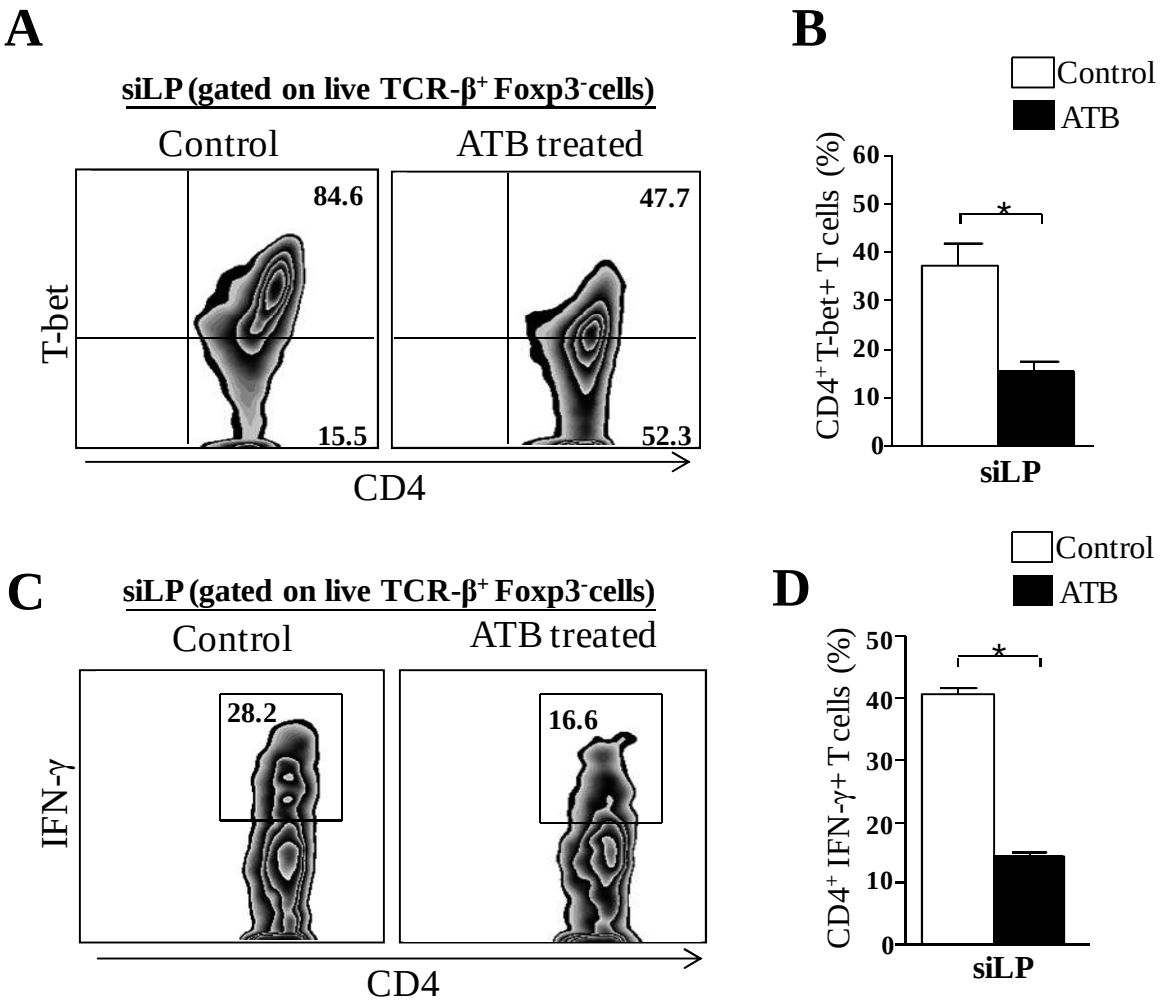


Fig 2. Reduced inflammatory immune responses in the small intestine of ATB treated mice infected with *T. gondii*. Mice were treated with a cocktail of antibiotics for 4 weeks and then infected with 10 cysts of *T. gondii* strain ME49 C1. Control mice were also infected. At day 9 of infection, small intestine lamina propria (siLP) cells were harvested and single-cell suspensions were either immediately stained or cultured with polyclonal stimuli. After 4 hours, stimulated cells were stained for flow cytometry assessment. **A**: representative plot of T-bet expression in live $CD4^+$ T cells. **B**: Bar graph showing quantification of A. **C**: representative plot of IFN-γ expression in live $CD4^+$ T cells. **D:** Bar graph showing quantification of C * $P< 0.05$. The data are representative of 3 different experiments performed with 4 mice each

Regulatory responses induced by *T. gondii* oral infection are not affected by antibiotic treatment

Regulatory response and in particular IL-10 are crucial during *T. gondii* infection for they help prevent the pathological effects of an exacerbated inflammatory response (Jankovic et al., 2010). Several studies also have shown that the microbiota can control the induction of regulatory responses particularly contributing to the Th17/Treg cell balance. Investigating the regulatory responses during the acute infection, we found that the treatment with the cocktail of antibiotics did not have any effect on the frequency of regulatory T cells in the gut or lymphoid tissues (fig 3). Our group has previously demonstrated that during the acute phase of *T. gondii*-induced ileitis regulatory T (Treg) cell numbers collapse via multiple pathways including blockade of Treg cell induction and disruption of endogenous Treg cell homeostasis (Oldenhove et al., 2009). In conformation with these previous studies, frequency of Treg cells in the siLP 9 days after infection was reduced after infection with *T. gondii*. Depletion of gut microbiota by antibiotic treatment did not have an effect on Treg induction since both groups of mice have similar frequencies of these cells (Fig. 3 B). The production of IL-10 by lamina propria $CD4^+$ T effector cells ($Foxp3^-$) as well as Treg cells ($Foxp3^+$) was also not altered by the treatment with antibiotics. Thus, our data suggest that the gut microbiota does not contribute directly to the onset of regulatory responses.

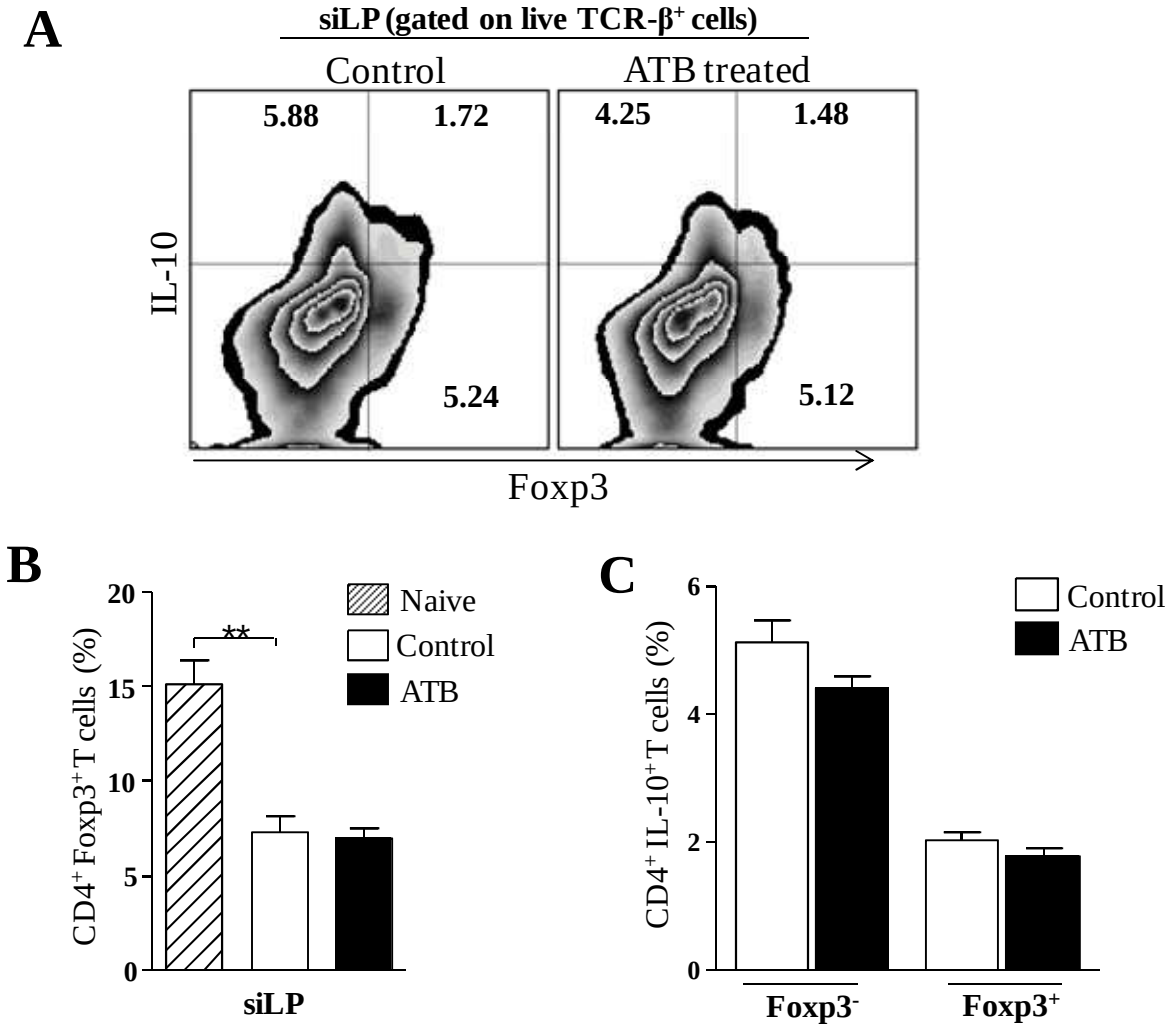


Fig 3. Regulatory responses are not affected by depletion of gut microbiota. Mice were treated with a cocktail of antibiotics for 4 weeks and then infected with 10 cysts of *T. gondii* strain ME49 C1. Control mice were also infected. At day 9 of infection, small intestine lamina propria cells (siLP) were harvested and single-cell suspensions were either immediately stained or cultured with polyclonal stimuli. After 4 hours, stimulated cells were stained for flow cytometry assessment.. **A**: representative plot of Foxp3 and IL-10 co-expression in live $CD4^+$ T cells. **B**: Bar graph showing frequency of Treg cells. **C**: frequency of IL-10 expression in effector (Foxp3-) $CD4^+$ T cells and Treg cells. The data are representative of 3 different experiments with 4 mice each ** $P< 0.01$.

Reduced parasite load in the gut of antibiotic treated mice

Proper clearance of *T. gondii* from the gut is dependent on an efficient Th1-type response (Gazzinelli et al., 1994). We examined whether the treatment with antibiotics would affect the clearance of the parasite during the acute phase of infection. Parasite burden quantification was performed by titration of plaque formation in a fibroblast monolayer and indicated as the number of plaque forming units (PFU). In figure 4 we found that the parasite burden in the small intestine lamina propria after 9 days of infection is around 4 x 104 PFUs, in according to our previous observations (Oldenhove et al., 2009). Surprisingly, despite the decreased immune responses shown by us in figs. 1 and 2, mice treated with antibiotics showed reduced parasite load when compared with the control group suggesting a more efficient clearance of the parasite. Our results indicate that although the microbiota contributes to the immune responses during *T. gondii* infection, this effect seems not to reflect on parasite clearance. It is possible that factors other than immune responses could be involved in this phenomenon.

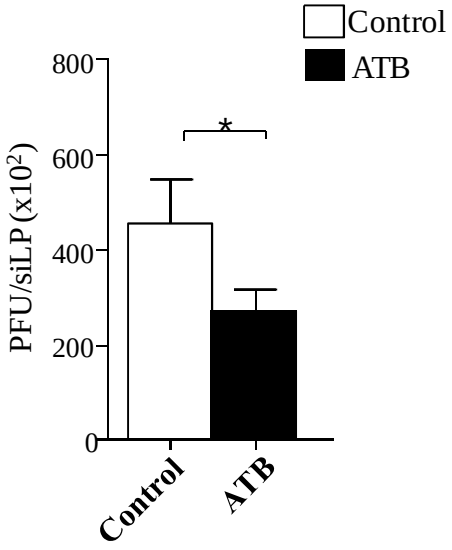


Fig 4. Lower parasite burden at day 9 of infection in ATB treated mice infected with *T. gondii*. Mice were treated with a cocktail of antibiotics for 4 weeks and then infected with 10 cysts of *T. gondii* strain ME49 C1. Control mice were also infected. At day 9 of infection, small intestine lamina propria (siLP) cells were harvested and single-cell suspensions (10^4 to 10^6 cells) were added to fibroblast monolayer cultures and titrated by plaque formation. The results of these titrations are reported in Plaque Forming Units (PFUs). The data are representative of 3 different experiments with 4 mice each. *. $P< 0.05$.

Less severe immunopathology in germfree mice infected with *T. gondii*

Treatment of mice with antibiotics leads to a reduction rather than total elimination of bacteria from the gut (Hill et al., 2010). To further examine the role of gut microbiota in the intestinal inflammation induced by oral infection with *T. gondii,* we utilized the germfree animal model. Germfree mice have no live microorganisms and therefore are likely to be ignorant of commensal bacterial antigen. We infected germfree mice with 10 cysts of the ME49 C1 strain of *T. gondii* and examined the pathogenesis, parasite burden and immune responses at day 9 of infection. To evaluate the immunopathological damages caused by the infection, we assessed the levels of liver Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) from sera of both germfree (GF) and specific-pathogen free mice (SPF). Elevated levels of these two enzymes indicate hepatic dysfunction cause by sever necrosis of the liver tissue. As shown in fig. 5 and according to previous reports, the levels of ALT and AST at day 9 of infection are highly increased when compared to the ones found in naïve mouse sera. However, the levels detected in GF sera are significantly lower when compared to SPF mice. Our results indicate that *T. gondii*-infected germfree mice display less severe immunopathology during the acute phase of infection.

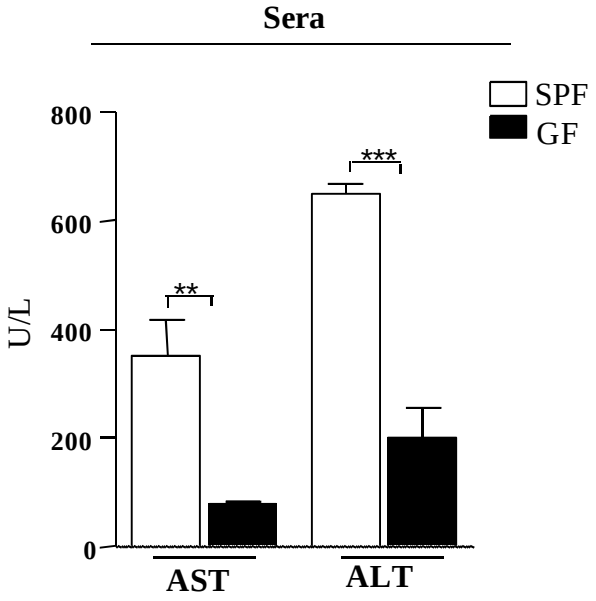


Fig 5. Lower levels of liver enzymes in sera from germfree mice infected with *T. gondii.* Germfree mice (GF) and specific-pathogen free mice (SPF) were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, sera were collected and used for detection of the hepatic enzymes Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST). The results of are reported as Units/ml of serum. The data are representative of 2 different experiments with 3 mice each **. $P< 0.01$; *** $P< 0.001$..

Reduced immune responses in germfree mice infected with *T. gondii*

We next evaluated the adaptive response against the parasite. Expression of T-bet in effector $CD4^+$ T cells was found to be similar between SPF and GF mice (fig. 6 A). Production of IFN-γ by effector $CD4^+$ T cells at day 9 of infection was slightly but no significantly reduced in GF mice (fig. 6 B). In agreement with previous reports associating intestinal Th-17 immune response to a restricted group of bacteria, the segmented filamentous bacteria (SFB) (Ivanov et al., 2008), we could not detect IL-17 production by $CD4^+$ T cells in the lamina propria of the small intestine of GF mice when compared to SPF mice (fig. 6 C).

GF mice displayed increased regulatory responses at day 9 of infection. Frequency of Treg cells were increased in GF mice in gastrointestinal lymphoid tissues such as the lamina propria (siLP) and the Peyer's patches (Pp) but similar to SPF mice in non-gastrointestinal related tissues such as spleen and liver draining lymph nodes (LivLn) (Fig. 7 A and B). Production of IL-10 by effector $CD4^+$ T cells was augmented in the siLP but not at the Pp of GF mice. Therefore, immune responses in germfree mice also seem to be altered by the lack of microbiota.

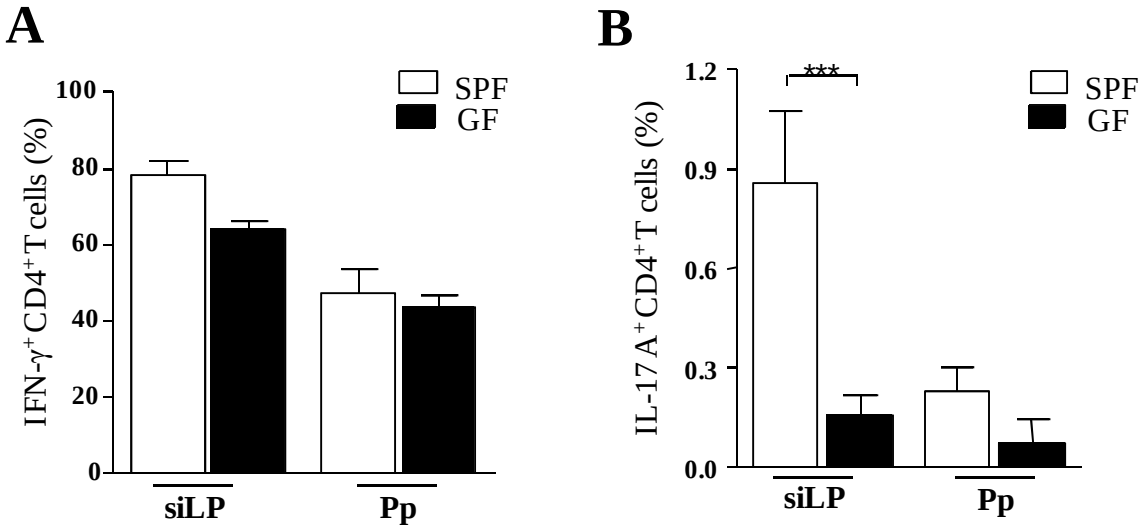


Fig 6. Reduced inflammatory immune responses in the small intestine of germfree mice infected with *T. gondii*. GF and SPF mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, small intestine lamina propria (siLP) cells and Peyer's patches (Pp) cells were harvested and single-cell suspensions were cultured with polyclonal stimuli. After 4 hours, stimulated cells were stained for flow cytometry assessment. **A**: expression of IFN-γ in live $CD4^+$ T cells. **B**: expression of IL-17 A in live $CD4^+$ T cells. *** $P< 0.001$. The data are representative of 2 different experiments with 3 mice each

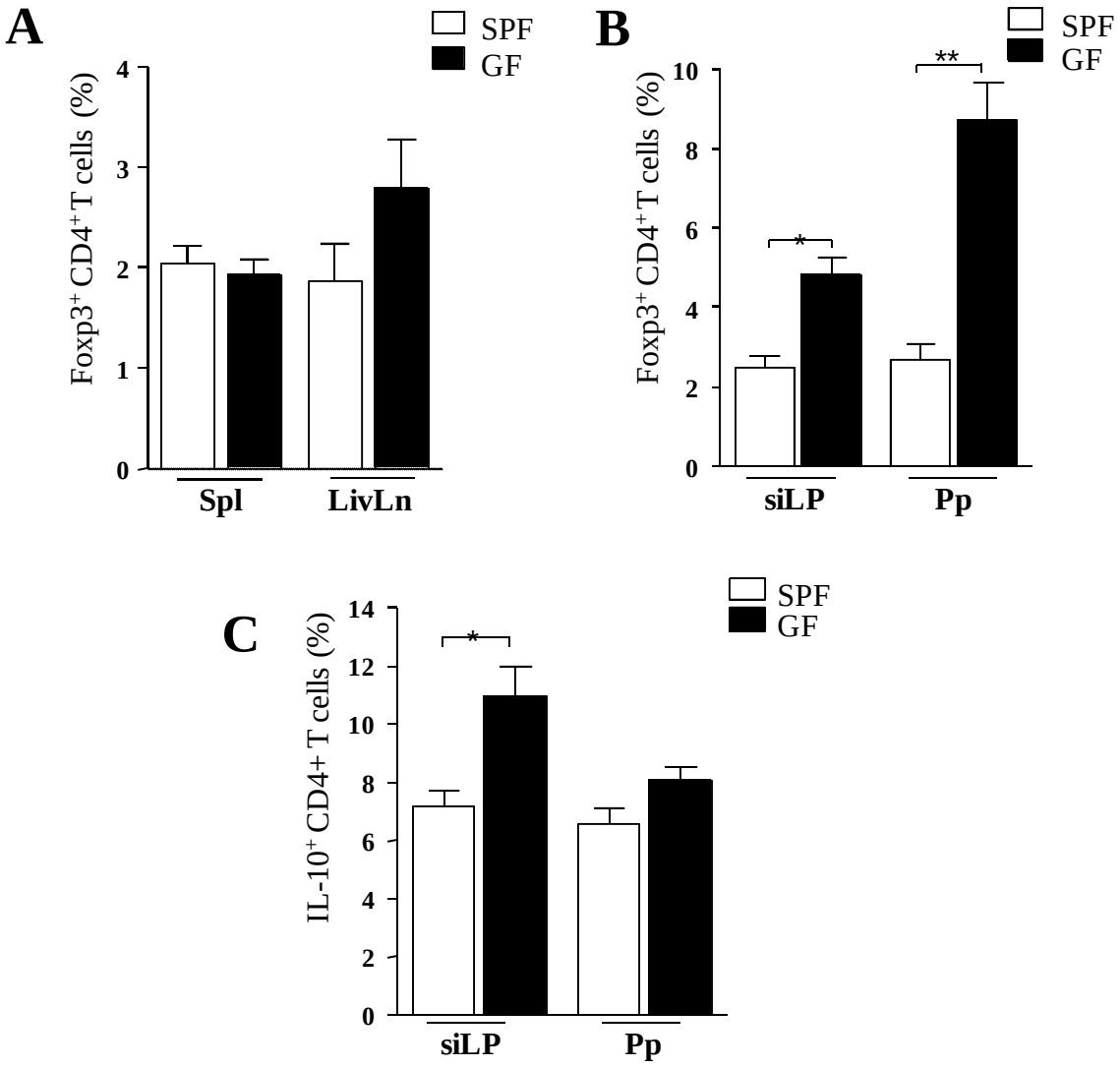


Fig 7. Germfree mice infected with *T. gondii* have increased regulatory responses in the gut. GF and SPF mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, small intestine lamina propria cells (siLP) were harvested and single-cell suspensions were either immediately stained or cultured with polyclonal stimuli. After 4 hours, stimulated cells were stained for flow cytometry assessment.. **A**: frequency of Treg cells in spleen (Sp) and liver lymph nodes (LivLn). **B**: expression of IL-10 in live effector ($Foxp3^-$) $CD4^+$ T cells from small intestine lamina propria (siLP) and Peyer's patches (Pp). * $P< 0.05$; ** $P< 0.01$. The data are representative of 2 different experiments with 3 mice each

Reduced parasite load in the gut of germfree mice

As seen in ATB treated mice, germfree mice also showed reduced parasite burden at day 9 of infection although changes in inflammatory immune responses in the gut were shown to be similar to SPF mice (fig. 8). Parasite burden was assessed by flow cytometry since the ME49 C1 strain expresses constitutively the red fluorescein protein (RFP). We found no difference in frequency of cells expressing RFP between SPF and GF mice in the spleen. On the other hand, parasite load was reduced in the lamina propria and significantly reduced in mLn and Pp of GF mice.

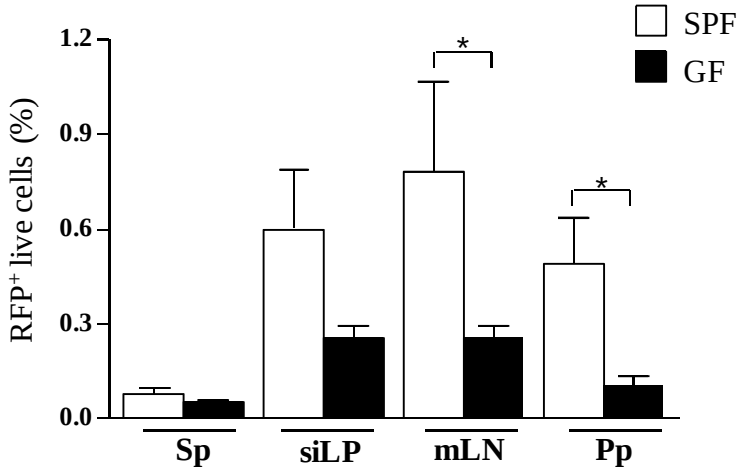


Fig 8. Lower parasite burden at day 9 of infection in germfree mice infected with *T. gondii*. Germfree mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, small intestine lamina propria (siLP), spleen (Sp), mesenteric lymph nodes (mLN) and Peyer's patches (Pp) were harvested and single-cell suspensions were assessed by flow cytometry for detection of expression of RFP (infected cells).. The results are reported as frequency of live cells expressing RFP. The data are representative of 2 different experiments with 3 mice each. *. $P < 0.05$.

Changes in composition of the microbiota during *T. gondii*-induced ileitis

Previous work showed that *T. gondii*-induced ileitis is accompanied by decreasing species diversity and increasing bacterial load within the gut microbiota. This phenomenon has been shown to aggravate the pathogenesis of the disease (Heimesaat et al., 2006). To further explore these changes we used 454 pyrosequencing of 16S rRNA genes from fecal samples to survey the bacterial diversity of the gut microbiota during *T. gondii* infection. We sampled fecal pellets from naïve mice, mice at day 9 of infection (peak of acute phase) and at day 45 of infection (chronic phase). Figure 9 represent the taxonomic classification of OTUs found in all samples at the phylum level. Overall, Firmicutes and Bacteroidetes predominated in the gut of naïve mice. Firmicutes, that include *Lactobacillus* species for example, were reduced during the acute phase of infection in comparison with naïve mice (Fig. 10 A). Bacteroidetes, on the other hand, were increased in samples from acute phase of infection (Fig. 10 B). Verrucomicrobia, a recent describe phylum of bacteria, were found mostly during the acute phase but not in naïve mice or chronic mice (Fig. 10 C). Proteobacteria which includes gamma-proteobacteria considered pathogenic bacteria or pathobionts such as *Escherichia coli* were found only during the acute phase of infection confirming recent reports that state that the accumulating bacteria that aggravates intestinal inflammation are mainly Enterobacteria such as *E. coli* and *Enterococcus* species (Fig. 10 D). Interestingly, the composition of the microbiota after resolution of intestinal inflammation (chronic) was not restored to the profile seen in

naïve mice indicating that the changes caused by parasitic infection might persist beyond the resolution of the infection (Fig. 9).

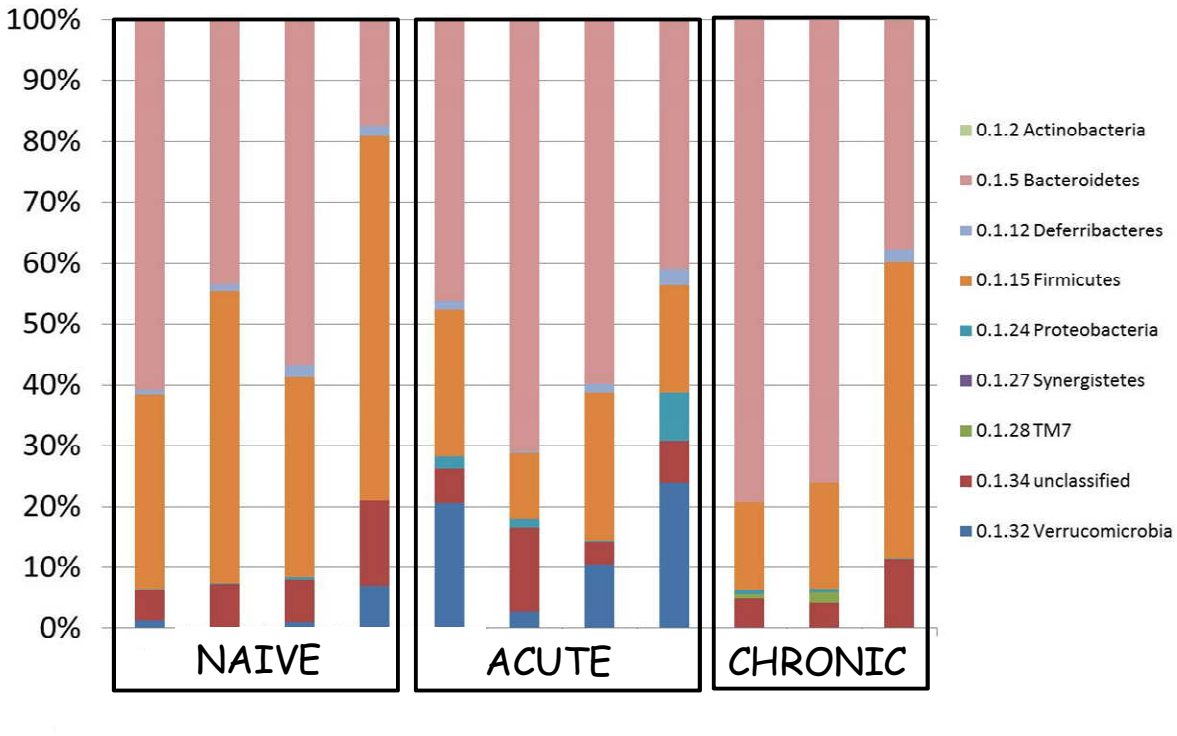


Fig 9. Composition of the gut microbiota at different time-points during *T. gondii* infection. Mice were infected with 25 cysts of *T. gondii* strain ME49 C1. Fresh fecal pellets from naïve, day 9 of infection and 45 day of infection mice were collected and bacterial DNA was extracted and used for 16S rRNA sequencing. Results are reported as percentage of OTUs of each phylum within total OTUs.

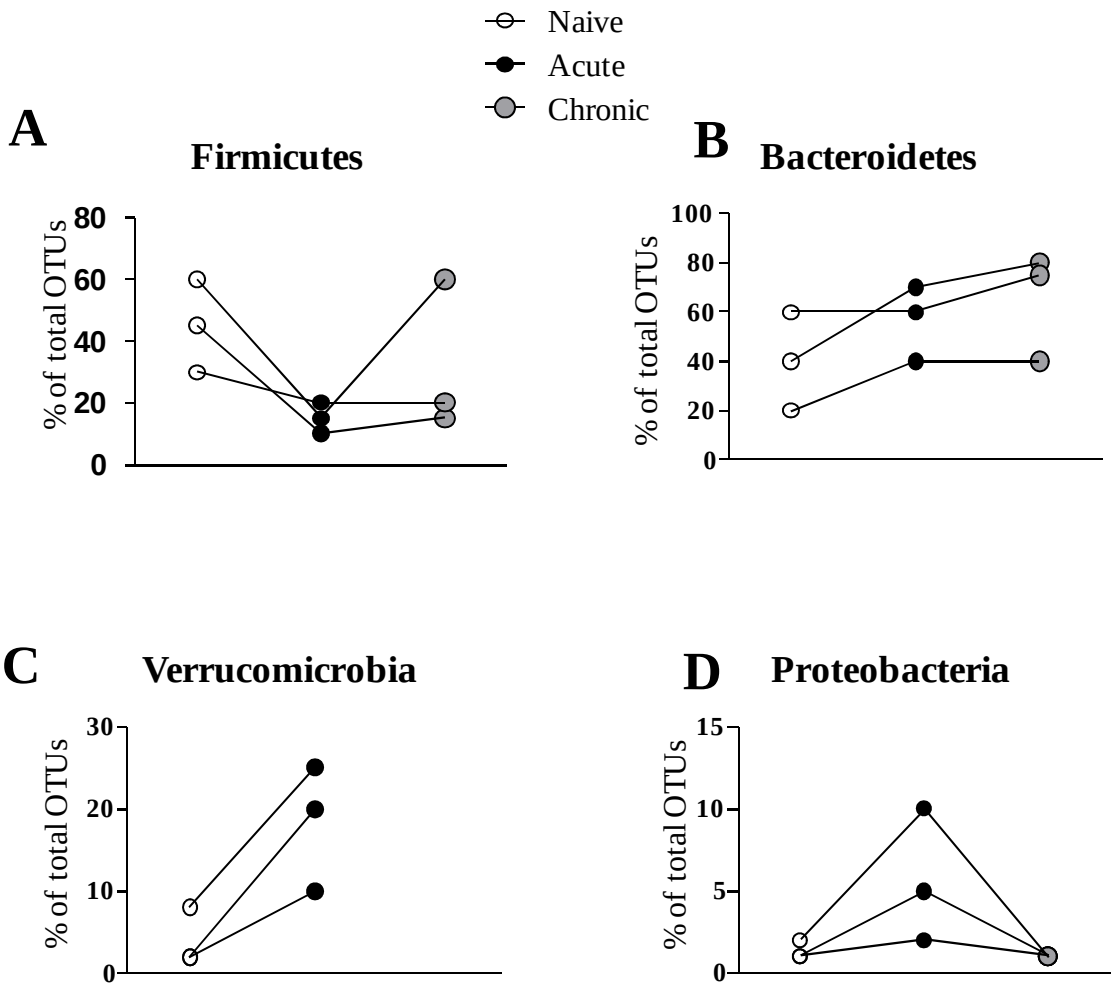


Fig 10. Changes of microbiota during *T. gondii* infection. Mice were infected with 25 cysts of *T. gondii* strain ME49 C1. Fresh fecal pellets from naïve, day 9 of infection and 45 day of infection mice were collected and bacterial DNA was extracted and used for 16S rRNA sequencing. Results are reported as percentage of OTUs of each phylum within total OTUs for individual mice. Open circle: naïve mice; closed circle: day 9; grey circle: day 45.

Temporal intestinal immune responses against *T. gondii*

As it was observed by analyzing the composition of the gut microbiota at different time-points during the infection with *T. gondii*, changes that are triggered by the breakdown of the intestinal balance induced by the infection are long-lasting.. We decided then to investigate whether these changes reflect changes in the immune response throughout the infection. According to the literature and our own observations, during *T. gondii* infection, immunocompetent individuals are able to completely clear the parasite from the gastrointestinal tract, and consequently resolve intestinal inflammation, after 25 to 30 days of infection. However, when we examined the production of IFN-γ in the small intestine lamina propria of mice 45 days after infection we observed strikingly high levels of this cytokine in chronic mice compared to naïve mice although histopathological analysis did not reveal the presence of pathological features of inflammation (fig. 11 A and B). These results suggest that not only *T. gondii*-induced ileitis provokes changes in the gut microbiota that are never restored to profiles seen before infection but the steady-state mucosal immune responses are also permanently shifted towards an inflammatory profile.

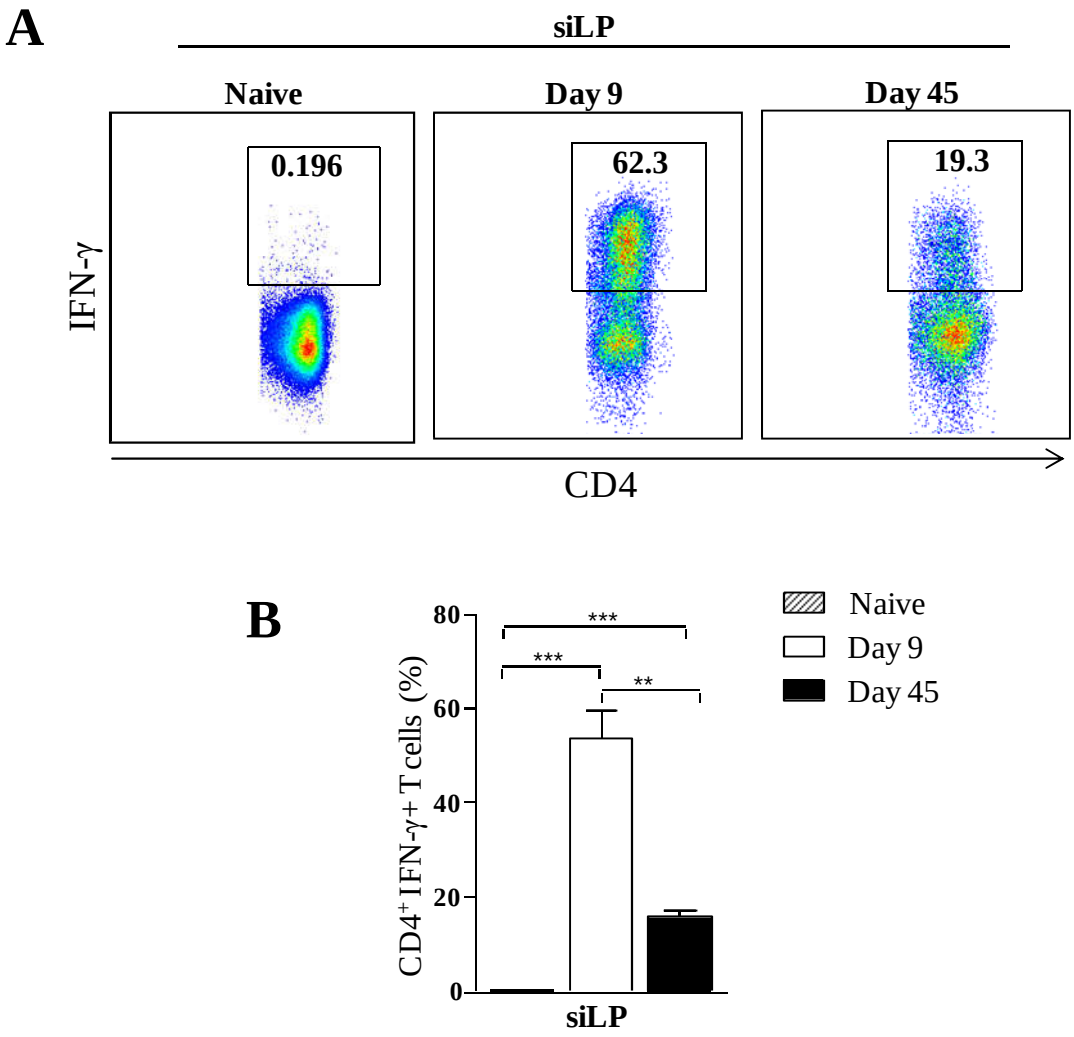


Fig 11. Temporal immune responses during *T. gondii* infection. Mice were infected with *T. gondii* strain ME49 C1. At day 9 of infection, small intestine lamina propria (siLP) cells were harvested and single-cell suspensions were cultured with polyclonal stimuli. After 4 hours, cells were stained for flow cytometry assessment. **A**: representative plot of IFN-γ expression in live $CD4^+$ T cells **B**: bar graph showing quantification of A. The data are representative of 2 different experiments with 3 mice each ** $P< 0.01$; *** $P< 0.001$.

T. gondii-induced ileitis induces systemic translocation of commensal bacteria

T. gondii-induced ileitis promotes a deregulation of intestinal mucosal homeostasis that could result in translocation of gut bacteria or bacterial products to systemic sites leading to priming of immune responses against gut bacteria. To begin to address our hypothesis, we infected C57BL/6 with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, translocation to peripheral tissues was evaluated by microbiological methods. Indeed, we found that gut bacteria translocate to spleen, liver and mLNs during acute *T. gondii* infection (fig. 12 A). We were able to detect bacteria in the order of thousands of colonies in all three tissues examined. Prevalent bacteria were found to be mostly gamma-proteobacteria such as *Escherichia coli* and *Proteus mirabilis.* To confirm that commensal bacteria cross the intestinal epithelial barrier during the acute phase of infection, small intestine sections were stained with an LPS core antigen specific antibody. As shown in figure 12 B bacteria were found inside of the small intestinal villi, indicating that the acute infection with *T. gondii* induces systemic translocation of commensal bacteria.

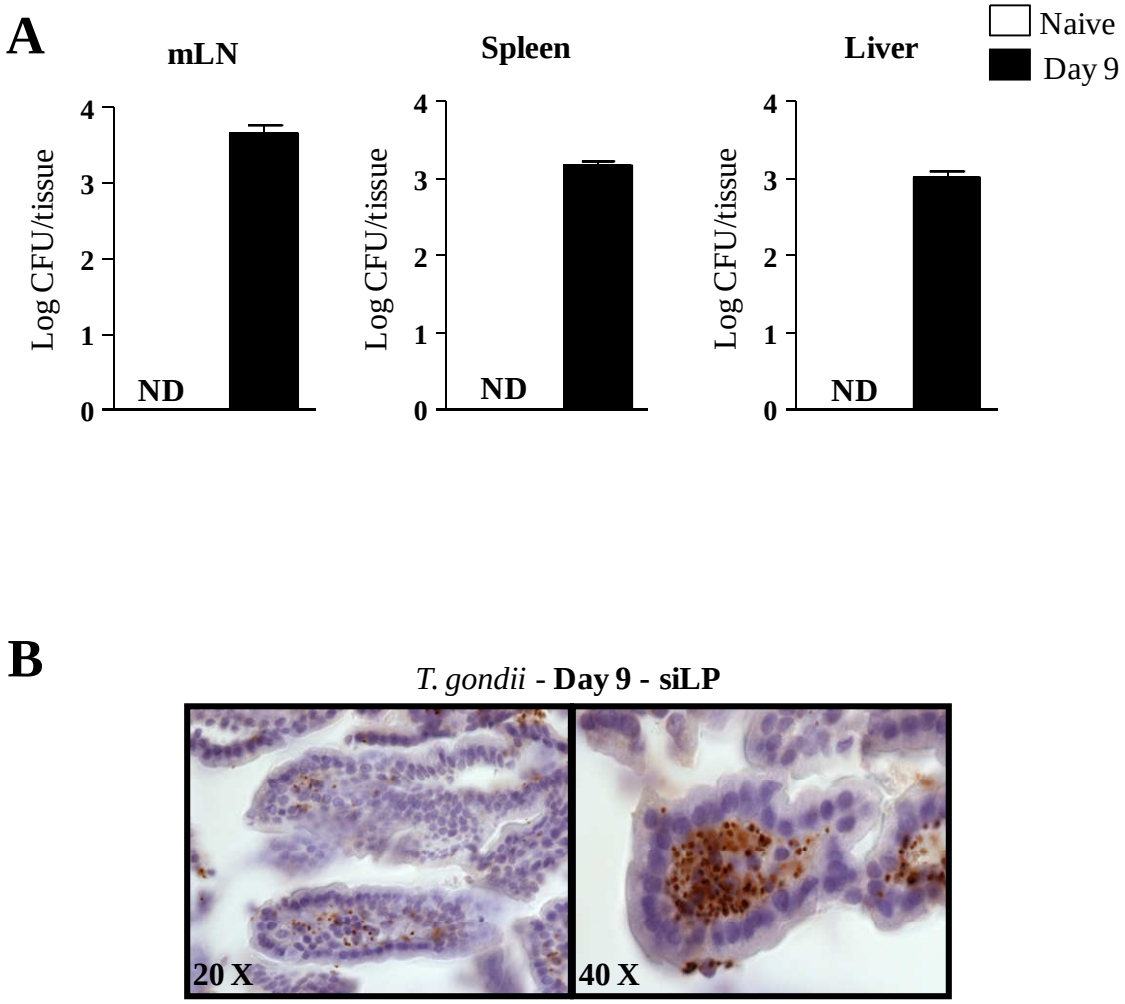


Fig 12. Systemic translocation of bacteria in *T. gondii* oral infection. Mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, different tissues were collected. **A**: mesenteric lymph nodes (mLns), spleens and livers were homogeneized in saline and serial dilutions were plated in Tryptic Soy agar plates. Colonies were counted after 48 hours of culture. The result was reported as the number of colony forming units (CFU) per organ. **B**: Small intestine of infected mice were fixed in 10% formalin and 5 μm slides were prepared and stained with anti-LPS core antigen. The data are representative of 2 different experiments with 4 mice each. ND: not detected..

Antibody responses against commensal bacteria are triggered during the acute mucosal *T. gondii* infection

Given that we can readily observe systemic translocation in response to parasitic infection we decided to investigate the adaptive immune response to commensal bacteria and the consequences of such responses. In order to study whether translocation of commensal bacteria during acute *T. gondii* infection can activate adaptive immune responses against commensal bacterial antigens, C57BL/6 mice were infected orally with the type II *T. gondii* strain ME 49 and fresh fecal pellets were collected at day 9 of infection. The fecal pellets were homogeneized and serial dilutions were plated in a rich media and incubated in aerobic conditions. Single colonies obtained from plates of higher dilutions were expanded in broth and plated in several differential media. Two gram-negative bacteria and one gram-positive were isolated and identified by chemical and morphological methods. The three bacteria that accumulated and were found in higher quantities during the acute phase of the infection were identified as *E. coli, Enterococcus faecalis* and *P. mirabilis.* These three strains were used as whole bacterial lysate to evaluate specific B cell responses.

Sera was collected during the peak of acute infection (day 9) and after 45 days of infection at the chronic phase of the infection and used in ELISA assays. Figure 13 shows the antibody titers detected in sera in response to antigens of our *E. coli* isolate. At day 9 of infection we found increased Pan Ig titers *E. coli*-specific in infected mice when compared to naïve mice. These titers were maintained even after 45 days of

infection indicating that the response is long-lasting (fig 13 A). The total IgG levels in response to *E. coli* antigens were also higher in infected mice in both time points analyzed (fig 13 B) as well as IgM levels (fig 13 C). Interestingly, we did not find detectable levels of *E. coli*-specific IgA at the peak of infection (day 9). The IgA titers were restored after 45 days of infection to levels comparable to those observed in naïve mice. We did not find significant levels of any of the antibodies in germfree mice sera.

We also examined B cell responses against *E. faecalis* and *P. mirabilis.* The Pan Ig titers against *E. faecalis* are increased at days 9 and 45 after infection in comparison to naïve levels although they are lower than the titers observed for *E. coli* responses (fig. 14 A). The Pan Ig titers after 45 days of infection are significant higher than the titers found at day 9. There was also an increased in *E. faecalis*-specific IgG2c and IgG1 levels in response to *T. gondii* infection (fig. 14 B and C). We also found increased antibody responses against *P. mirabilis* after *T. gondii* infection (fig. 15 A and B). Our data shows that there is an induction of B cell response against commensal bacteria during acute phase of *T. gondii* oral infection.

The whole lysate used in our ELISA assays is a complex mix of bacterial components which makes it difficult to study antigen-specific immune responses. To better examine the B cell response against commensal antigens activated by oral infection with *T. gondii* we used sera from infected mice to perform bacterial FACS assays according to previously optimized protocol (Slack et al., 2009). This way we can detect antibodies that are specific to molecules expressed in the surface of our three

commensal bacteria. Figure 16 confirms that there is an induction of antibody production in response to commensal bacterial antigens during the acute phase of *T. gondii* infection. Using this approach we were able to detect IgM, IgG1, IgA and IgG2b specific against surface antigens from our *E. coli* isolate.

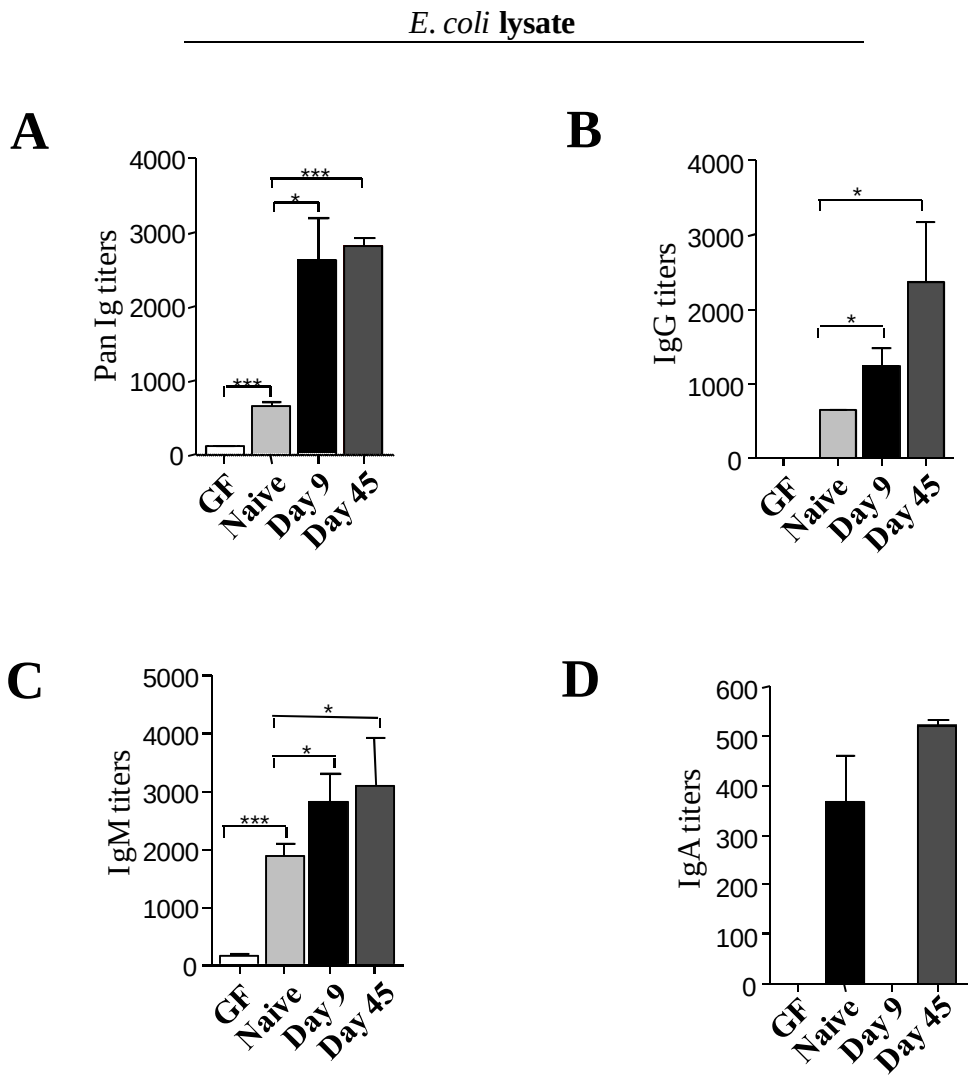


Fig 13. Antibody responses against commensal *E. coli* after acute parasitic infection. Mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At days 9 and 45 of infection, sera were collected and used for detection of antibodies specific for *E. coli* by ELISA. Sera from naïve mice and from germfree (GF) mice were used as controls. **A**: Pan Ig titers. **B**: IgG titers. **C**: IgM titers. **D**: IgA titers. The data are representative of 3 different experiments with 4 mice each * $P< 0.05$. *** $P<0.001$.

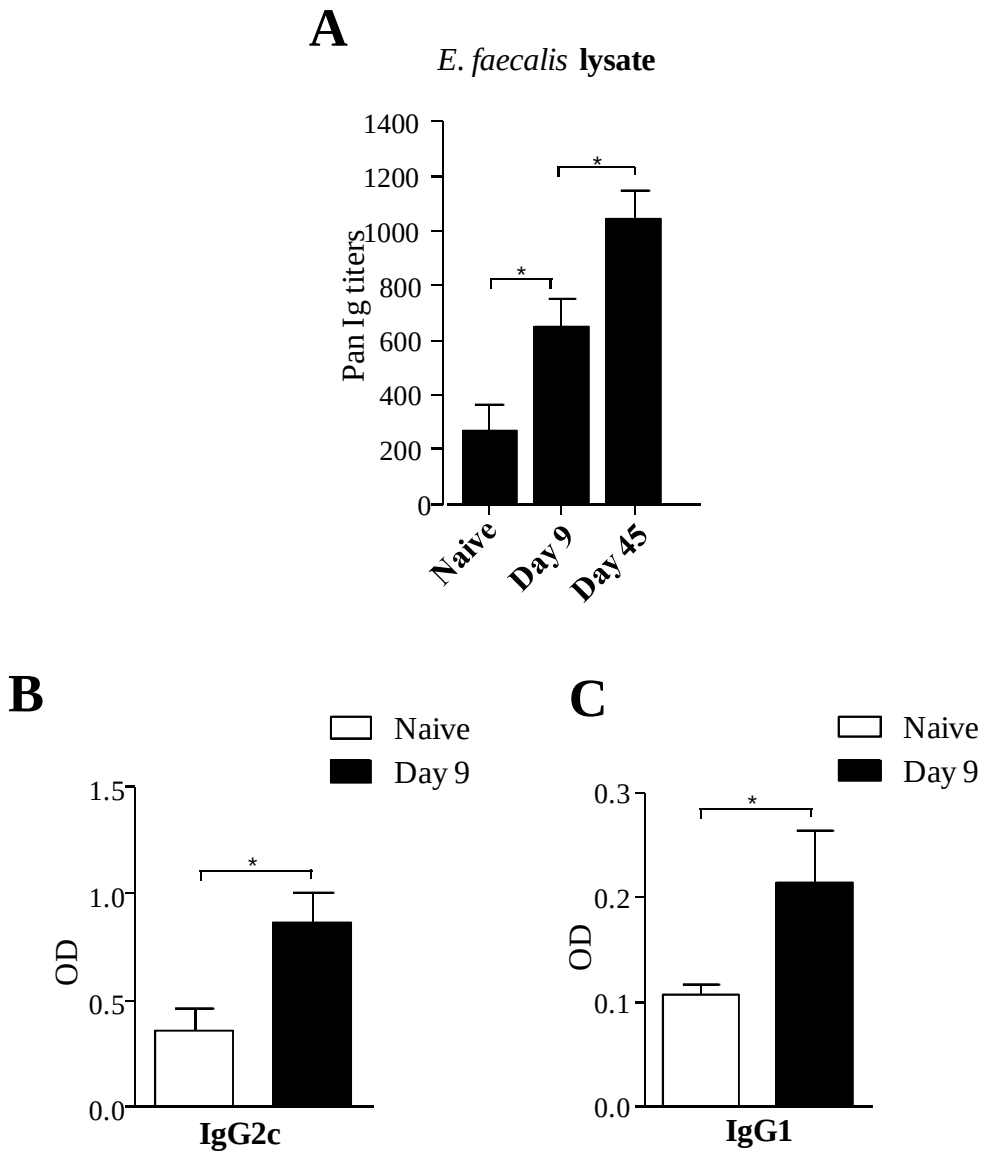


Fig 14. Antibody responses against commensal *E. faecalis* after acute parasitic infection. Mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At days 9 and 45 of infection, sera were collected and used detection of antibodies specific for *E. coli* by ELISA. Sera from naïve mice was used as controls. **A**: Pan Ig titers. **B**: IgG2c **C**: IgG1. The data are representative of 3 different experiments with 4 mice each * $P < 0.05$.

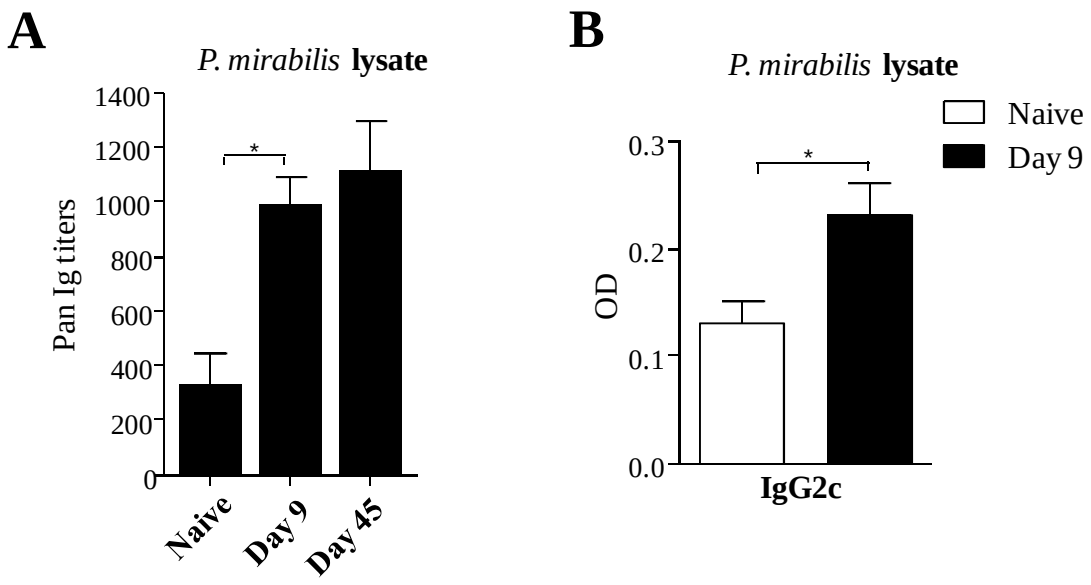


Fig 15. Antibody responses against commensal *P. mirabilis* after acute parasitic infection. Mice infected were with 10 cysts of *T. gondii* strain ME49 C1. At days 9 and 45 of infection, sera were collected and used detection of antibodies specific for *E. coli* by ELISA. Sera from naïve mice was used as controls. **A**: Pan Ig titers. **B**: IgG2c. The data are representative of 3 different experiments with 4 mice each. * $P < 0.05$.

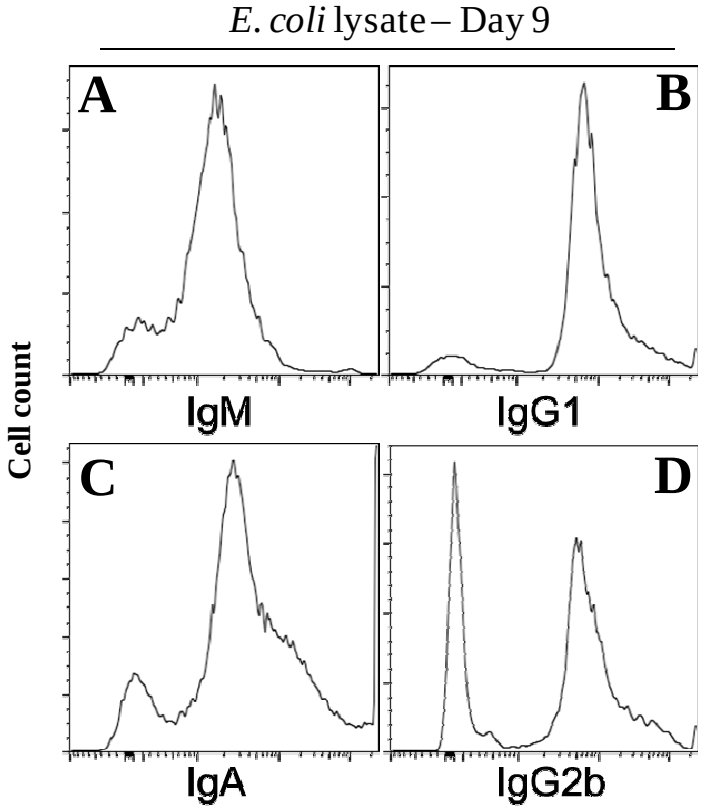


Fig 16. Bacterial FACS for detection of antibodies against commensal *E. coli*. Mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection sera were collected and used for detection of antibodies specific for *E. coli* by FACS. **A**: IgM. **B**: IgG1. **C**: IgA. **D**: IgG2b. The data are representative of 2 different experiments with 3 mice each.

T. gondii-induced ileitis triggers long term T cell immune responses against commensal bacterial antigens that can be recalled

Priming of immune responses towards commensal bacteria activates specific humoral responses against bacterial antigens. To investigate whether acute mucosal infections could also induce cellular memory responses against the microbiota, we challenged chronically infected C57BL/6 mice in both ears with the whole lysate of our isolate of *E. coli.* After 48 hours memory responses were evaluated by detection of a delayed-type hypersensitivity reaction. As shown in figure 17 A, we observed a strong DTH response in both ears of mice that had been infected 45 days before the challenge with the lysate characterized by redness and swelling of the site of inoculation. DTH reaction was accompanied by a robust cellular immune response with eduction of frequency of Treg cells and accumulation of effector $CD4^+$ T cells ($Foxp3^-$ cells) (fig. 17 B). Furthermore, IFN-γ expression by $CD4^+$ T cells was higher in the ears of infected mice (fig 17 C). Thus, our data indicate that mice infected with *T. gondii* have previously been sensitized against *E. coli* antigens.

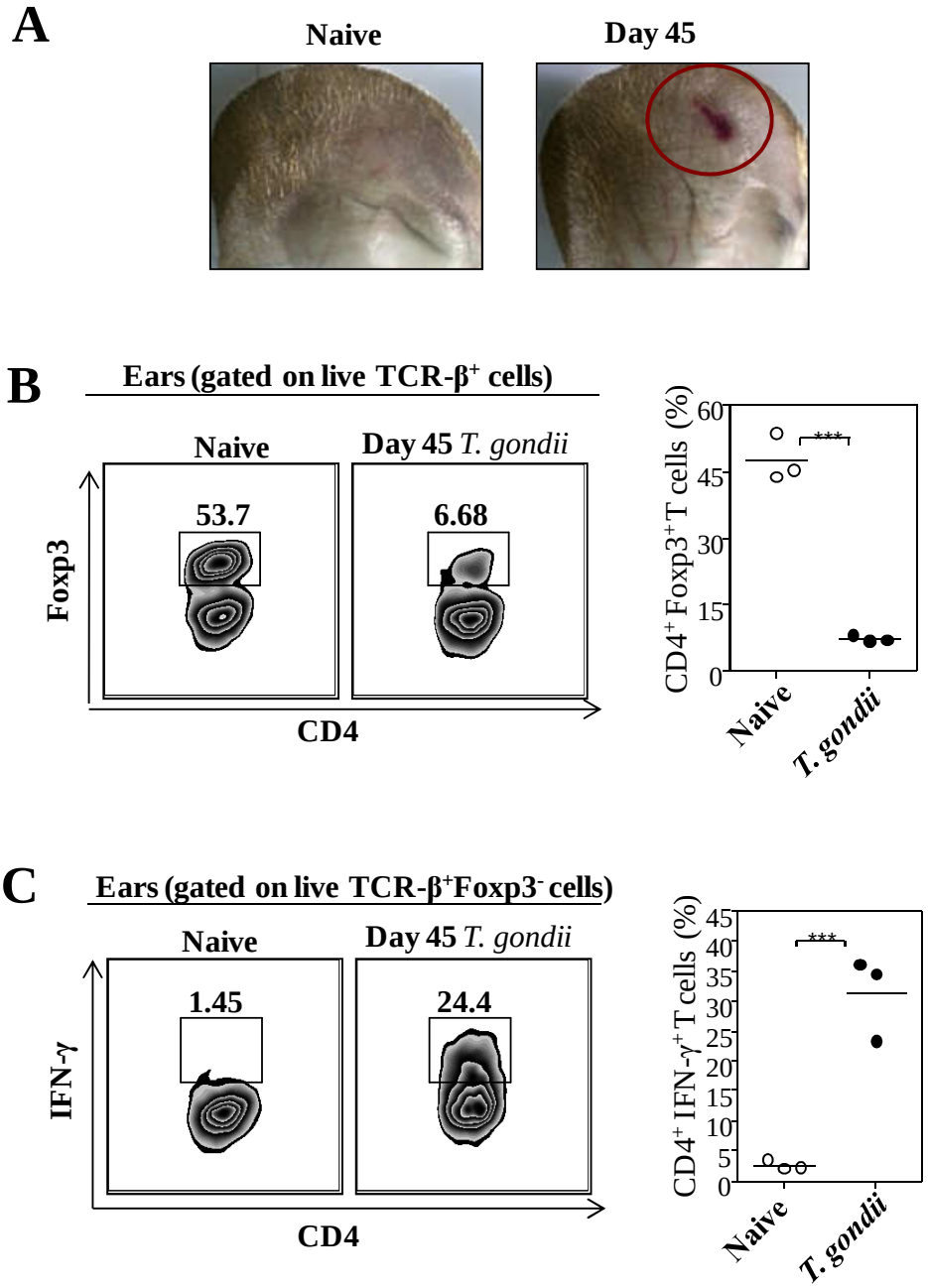


Fig 17. DTH response to *E. coli* whole lysate in mice infected with *T. gondii*. Mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 45 of infection, *E. coli* whole lysate was injected intradermally in both ears. DTH response was analyzed after 48 hours. **A**: Presence of inflammatory process in the ear of an infected mouse. **B**: Frequency of Treg cells in the ears. **C**: Frequency of $CD4^+$ T cells expressing IFN-γ in the ears. The data are representative of 3 different experiments with 4 mice each. *** $P<0.001$.

Antigen-specific CD4 T cell activation and differentiation in response to commensal antigens activated by *T. gondii* oral infection

To further investigate the antigen-specific responses against commensal bacteria triggered during acute mucosal gastrointestinal infection we used Cbir TCR Tg mice in an adoptive transfer system, where we can track the activation and differentiation of CBir Tg T cells during the infection. When ~$5x10^6$ CBirTg T cells (Ly5.2) were transferred to naïve congenic mice (Ly5.1) (Fig 18 A and B) they remained largely naïve in the spleen and mesenteric LN, as tolerance to commensal bacteria is intact (Cong et al. 2009). However when the mice are subsequently infected with *T. gondii* post adoptive transfer, the majority (75%±1) of CBirTg T cells are driven to proliferate, as shown by up-regulation of Ki-67 and induce T-bet. Significantly, the proportion of differentiated CBir Tg cells, as measured by T-bet expression, is comparable to the proportion of T-bet expressing cells amongst the total effector T cell subset activated in response to *T. gondii* infection. OT-II TCR transgenic T cells, specific to a peptide from chicken ovalbumin, are not significantly activated following infection with *T. gondii* indicating that the CBirTg response to infection is dependent upon a commensal-specific T cell receptor response and is not due to expression of a second cross-reactive TCR. Thus commensal-specific CD4 T cells become activated during infection with a mucosal infection.

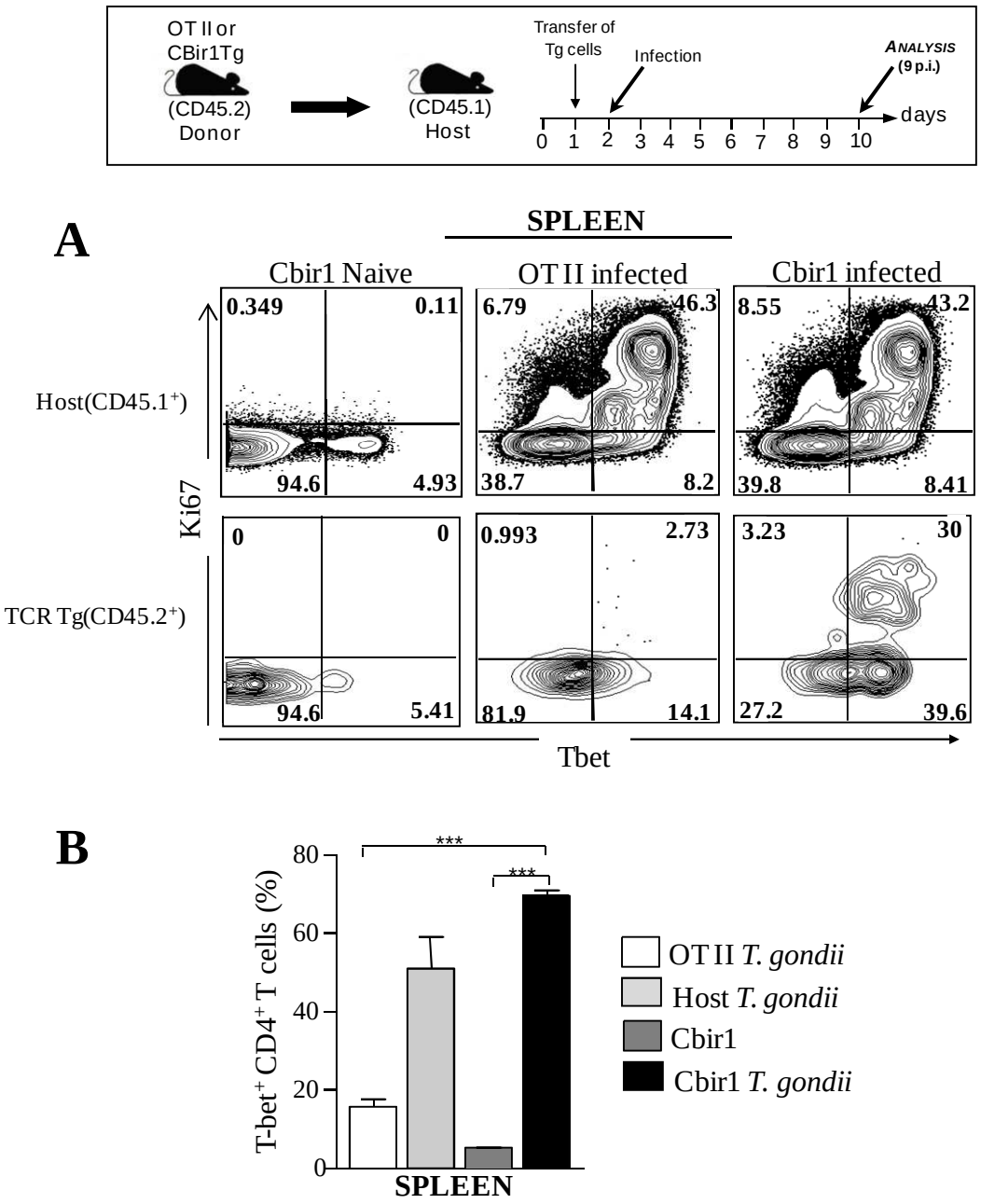


Fig 18. Antigen-specific CD4 T cell activation and differentiation in response to commensal antigens during *T. gondii* infection. Cbir1 T cells (CD 45.2) or OT II T cells (CD 45.2) were purified and ~ 5 x 10^6 cells were injected i.v. into host BSJ.L congenic C57BL/6 mice (CD 45.1). After 24 hours mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, immune responses were evaluated. **A**: Representative plots of co-expression of Ki-67 and T-bet in $CD4^+$ T cells in the spleen. **B**: Frequency of T-bet expression in $CD4^+$ T cells in the spleen. The data are representative of 2 different experiments with 3 mice each. *** $P<0.001$.

A non-lethal gastrointestinal infection also induce translocation of commensal bacteria

In order to investigate whether adaptive immune responses against the microbiota only occur under intense inflammation or not we chose the parasite *Encephalitozoon cuniculi* as a model of a non-lethal mucosal infection. This parasite leads to a Th1-type immune response in gastrointestinal tract but it does not lead to any kind pathologic process causing no death of mice (fig. 19 A). We found that even a mild infection as the one caused by *E. cuniculi* can induce translocation of commensal bacteria to peripheral tissues such as mLns, spleen and liver (fig. 19 B).

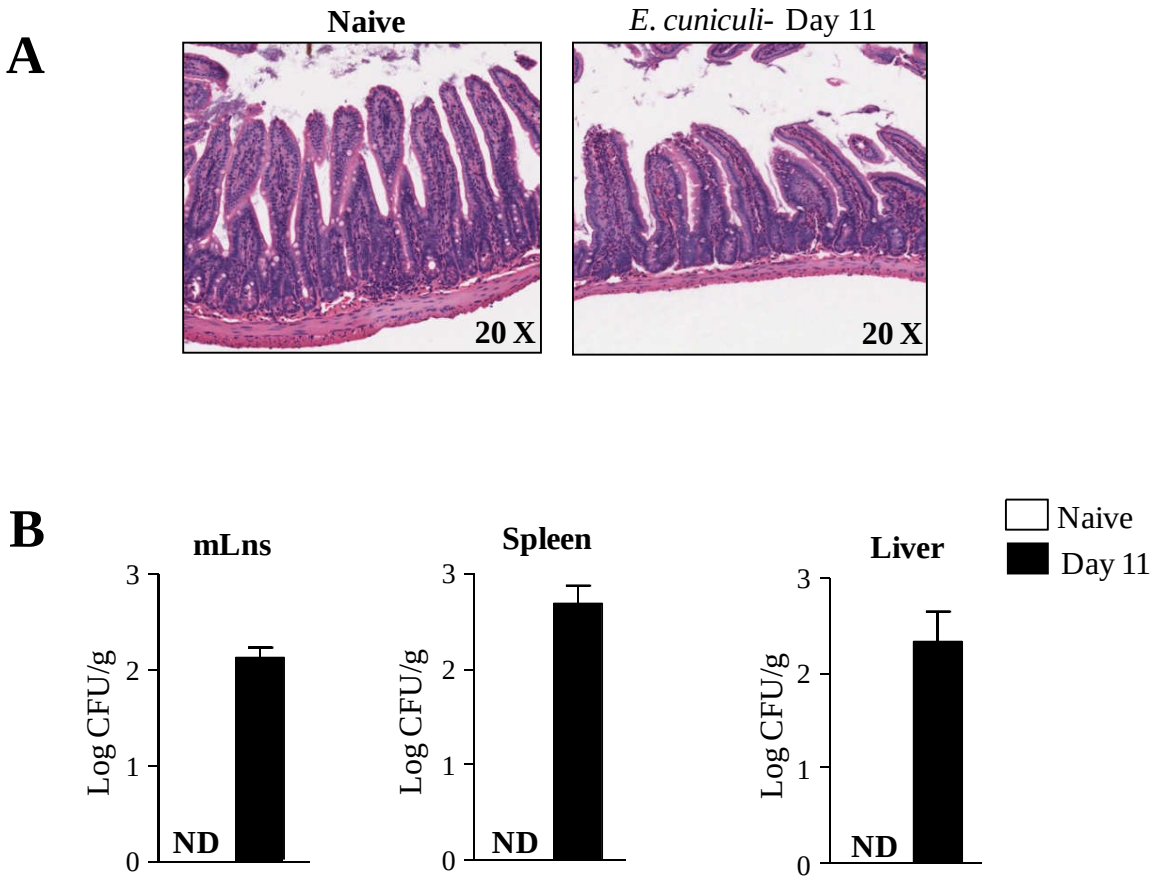
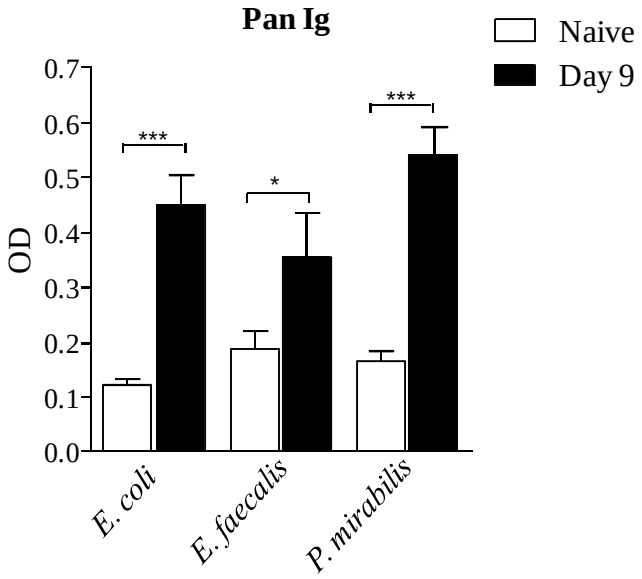


Fig 19. Systemic translocation of bacteria in *E. cuniculi* infection. Mice were infected with 5,000 spores of *E. cuniculi*. At day 11 of infection, different tissues were collected. A: histological aspect of small intestine. B: translocation of bacteria to peripheral tissues. Mesenteric lymph nodes (mLns), spleens and livers were homogeneized in saline and serial dilutions were plated in Tryptic Soy agar plates. Colonies were counted after 48 hours of culture. The result was reported as the number of colony forming units (CFU) per organ. The data are representative of 2 different experiments with 4 mice each. ND: not detected.

E. cuniculi infection activates adaptive immune responses against antigens from the microbiota

Sera of *E. cuniculi*-infected mice collected at the peak of infection (day 11) were used in ELISA assays with whole lysate of our three commensal bacteria. Sera of infected mice showed increased titers of Pan Ig against *E. coli, E. faecalis* and *P. mirabilis* when compared to naïve mice (fig. 20 A). The levels of total IgG were also higher for all three bacteria in infected mice (fig. 20 B). Our data indicates that a mucosal infection can trigger adaptive immune responses specific to commensal bacterial antigens by leading to systemic translocation of bacteria even though it is a non-pathological, non-lethal disease.

A



B

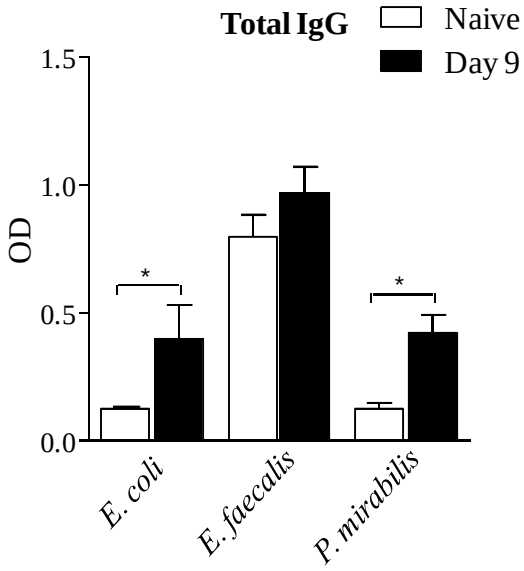
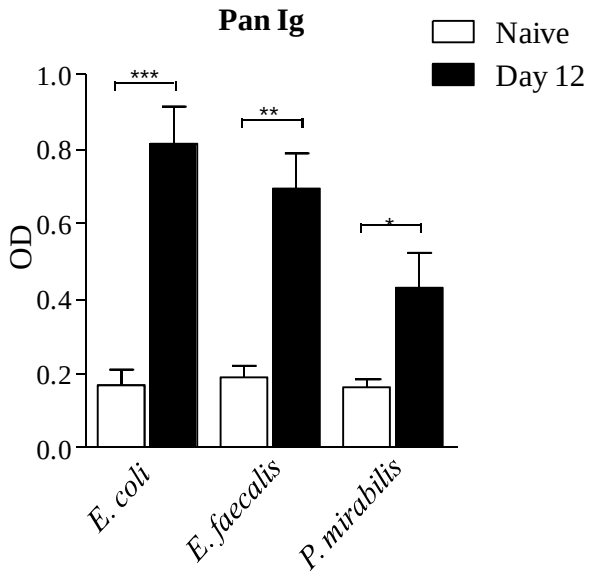


Fig 20: Antibody responses against commensal bacteria after *E. cuniculi* infection. Mice were infected with 5,000 spores of *E. cuniculi*. At day 11 of infection sera were collected and used for detection of antibodies against commensal bacteria lysate by ELISA. Sera from naïve mice was used as controls. **A**: Pan Ig titers. **B**: Total IgG. The data are representative of 2 different experiments with 4 mice each. * $P< 0.05$. *** $P<0.001$.

Commensal specific antibody responses during *C. rodentium* **colitis**

We also used a model of *C. rodentium*-induced colitis to examine immune responses against commensal bacteria. Sera were collected at day 12 after infection (peak of inflammatory process). We found significantly increased levels of antibodies specific to the three bacteria isolated by us during the peak of *C. rodentium* infection (fig. 21). Pan Ig (fig. 21 A) and IgG (fig. 21 B) responses against *E. coli* were shown to be the stronger among the three bacteria. IgG response towards *E. faecalis* were not significantly different when comparing naïve and infected mice.

A



B

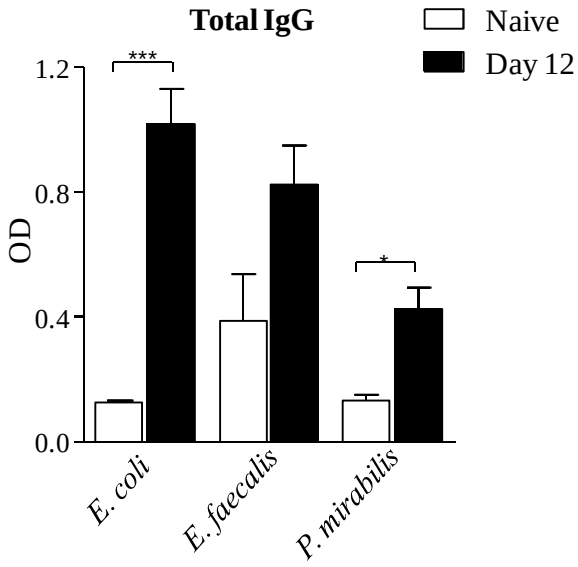


Fig 21: Antibody responses against commensal bacteria after *C. rodentium* infection. Mice were infected with 5,000 spores of 10^9 *C. rodentium*. At day 12 of infection sera were collected and used for detection of antibodies against commensal bacteria lysate by ELISA. Sera from naïve mice was used as controls. **A**: Pan Ig titers. **B**: Total IgG. The data are representative of 2 different experiments with 3 mice each. * $P< 0.05$. *** , $P<0.01$, $P<0.001$.

Vaccination with *E. coli* lysate protects mice against subsequent mucosal infection

To determine the role of the adaptive immune responses generated by acute mucosal infections we immunized C57BL/6 mice with the whole lysate of our *E. coli* lysate. After two immunizations, mice were infected with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection immune responses, parasite burden and pathology parameters were evaluated. Our vaccine strategy was able to elicit an antibody response against *E. coli* antigens as shown by figure 22. We observed increased levels of *E. coli*-specific Pan Ig in sera of vaccinated mice.

We observed no differences in the in immune responses at the gastrointestinal mucosa between vaccinated and control mice (fig. 23 A and B). T-bet expression by $CD4^+$ T cells was comparable in both groups (fig. 23 A). There was also no significant difference in the expression of IFN-γ by $CD4^+$ T cells between the two groups of mice (fig. 23 B). Additionally, the levels of liver enzymes, that indicate the severity of the pathological process, were similar in both groups (fig 24 A and B). Interestingly, when we evaluated the parasite burden we found that vaccinated mice have smaller parasite loads in the small intestine, mLns and spleen when compared with the non-vaccinated group (fig 25). Our results indicate that priming of immune responses against commensal bacterial antigen can help protect mice from an acute mucosal infection without aggravating the pathogenesis of the disease.

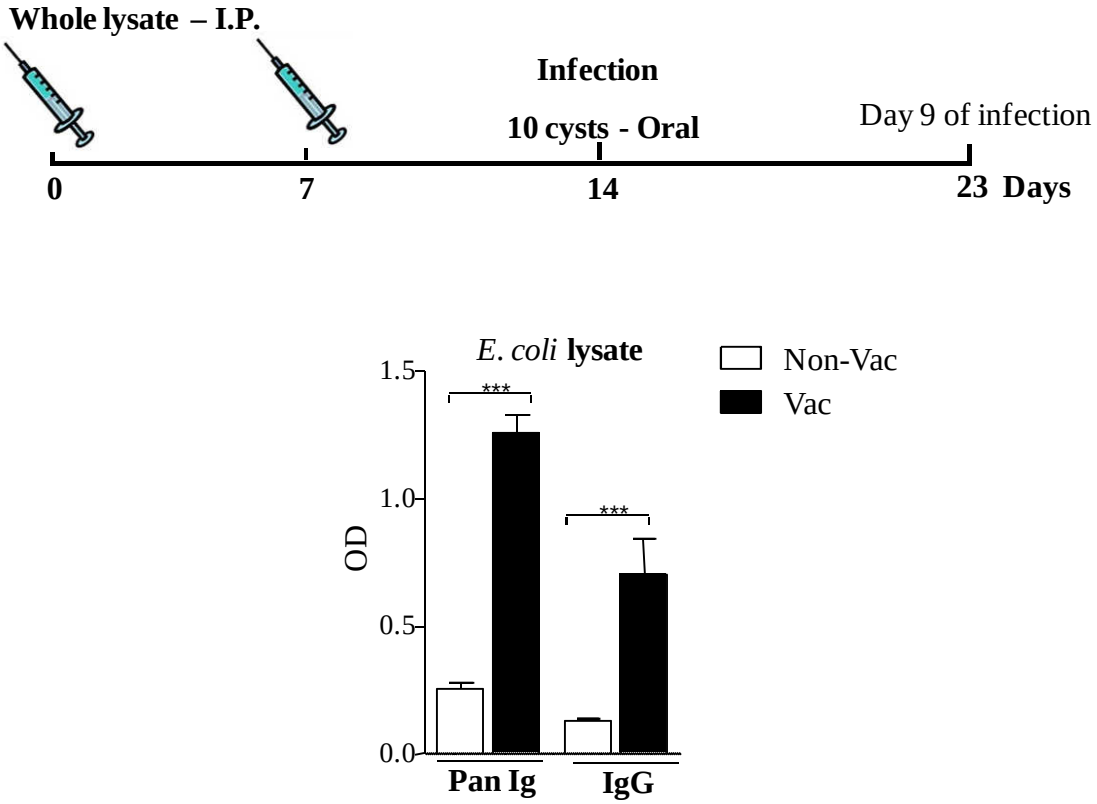


Fig 22. Vaccination of mice against commensal *E. coli* during *T. gondii* infection. Mice were injected intraperitoneally twice with lysate of *E. coli.* After two weeks mice were with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, antibody titers were evaluated. The data are representative of 3 different experiments with 4 mice each. *** $P<0.001$.

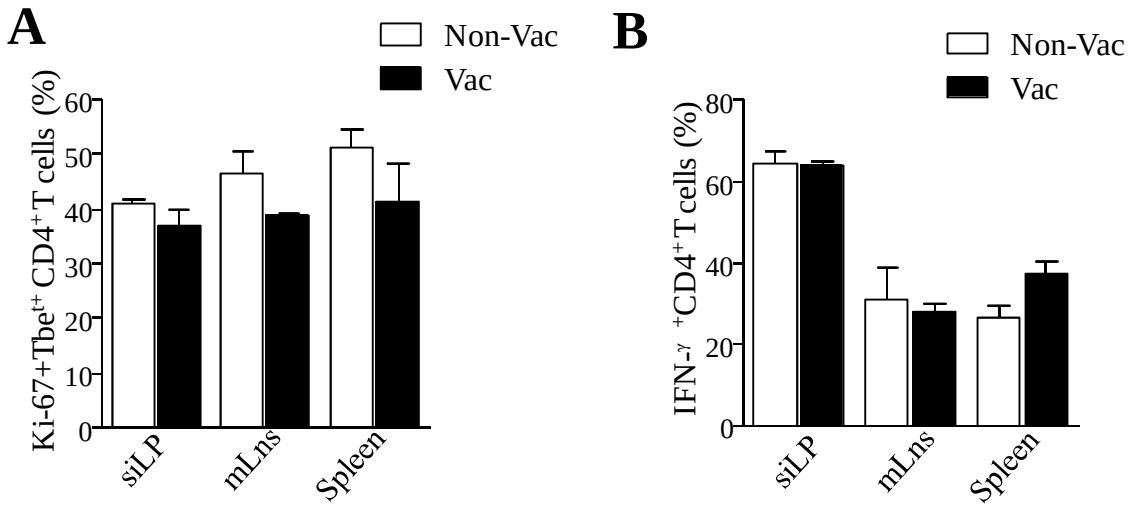


Fig 23. Vaccination of mice against commensal *E. coli* during *T. gondii* infection. Mice were injected intraperitoneally twice with lysate of *E. coli.* After two weeks mice were with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, immune responses were evaluated. A: . T-bet expression in CD4+ T cells in different tissues. B: frequency of CD4+ T cells expressing IFN-γ in different tissues. The data are representative of 2 different experiments with 3 mice each.

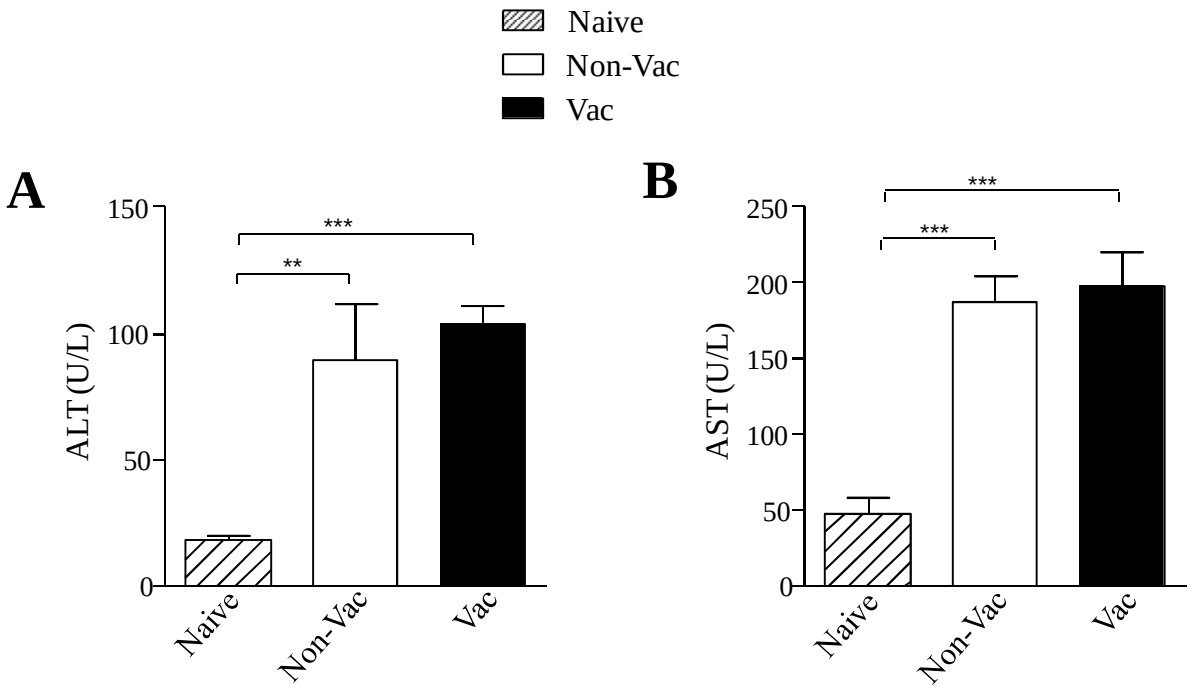


Fig 24. Pathogenesis after vaccination of mice against commensal *E. coli* during *T. gondii* infection. Mice were injected intraperitoneally twice with lysate of *E. coli.* After two weeks mice were with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, sera were collected and the level of liver enzymes were measured. A: Alanine Transferase (ALT). B: Aspartate Transferase (AST). The data are representative of 2 different experiments with 3 mice each. ** $P<0.01$. *** $P<0.001$.

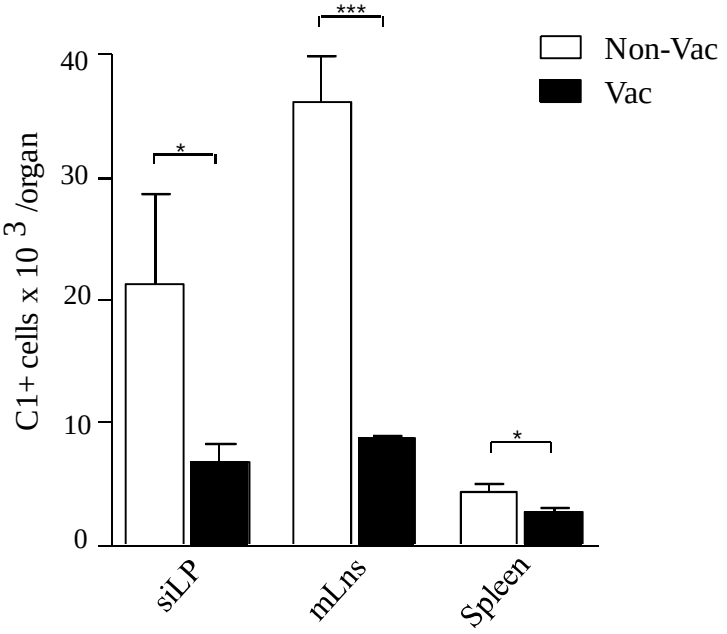


Fig 25. Parasite load after vaccination of mice against commensal *E. coli* during *T. gondii* infection. Mice were injected intraperitoneally twice with lysate of *E. coli.* After two weeks mice were with 10 cysts of *T. gondii* strain ME49 C1. At day 9 of infection, siLP, mLns and spleen cells were extracted and RFP expression (infected cells) was evaluated by flow cytometry. The results are reported as the absolute number of cells expressing RFP. The data are representative of 2 different experiments with 3 mice each. * $P<0.05$. *** $P<0.001$.

DISCUSSION

The gastrointestinal tract is the primary site of interaction between the host immune system and microorganisms, both symbiotic and pathogenic. Intestinal host–microorganism homeostasis involves minimizing the adverse health effects of intestinal microorganisms, even during environmental perturbations such as shifts in microbial community structure and changes in host diet. This homeostatic relationship with the microbiota does not imply that microbes are not continually sensed by the host innate system. Indeed, maintenance of responsiveness against pathogenic microbes as well as protection against commensal translocation relies on the establishment of a dynamic equilibrium maintained in part by the stimulatory capacity of the flora. Breakdown of intestinal homeostasis by such perturbations in the gut environment causes deregulation of the intestinal immune responses. In the present study we suggest that overt pathogenic challenge can disrupt the balanced interactions between host and microbiota leading to systemic translocation of bacteria and consequently, exuberant sensing of commensal antigens. Recognition of bacteria from the microbiota activates long-lasting immune responses that help protect the host against subsequent infections.

Oral infections with *Toxoplasma gondii* in certain strains of mice induces a severe form of intestinal inflammation referred to as the lethal ileitis model (Liesenfeld, 2002). Recent studies have demonstrated the microbiota has an important role during the *T. gondii* infection. These studies showed that during the acute phase of infection, changes can be seen in the composition and numbers of bacteria from the microbiota. More specifically, gram-negative bacteria, in special enterobacteria, accumulate in the gut and cross the intestinal epithelial barrier. Moreover, depletion of bacteria was able to

prevent the immunopathology associated with with the disease (Heimesaat et al., 2006). These studies were performed in antibiotic-treated mice which alone causes shifts in frequencies in the bacterial community (Hill et al., 2010; Dethlefsen and Relman, 2011). Our data utilizing this animal model showed that, indeed, the microbiota is involved in the development of the immune response and pathological consequences after infection with *T. gondii.* Other studies have highlighted the role of intestinal microbiota in shaping immune responses. Antibiotic treatment alone alters the mucosal immune responses homeostasis with reduction of the production of pro-inflammatory cytokines (Hill et al., 2010). Here, we showed that treatment of mice with a cocktail of antibiotics before challenge with *T. gondii* reduces pro-inflammatory immune responses in the gut and gut associated lymphoid tissues. Bacterial involvement in activating immune responses in the gut explains the less severe pathogenesis observed in antibiotic treated mice.

Regulatory T cells are known to limit microbe-triggered intestinal inflammation and the $CD4^+$ T cell compartment is shaped by the presence of particular microbes or bacterial compounds. Recent studies show that alterations of commensal populations can determine the Treg/Th17 cell balances in the GALT (Ivanov et al., 2008; O'Mahony et al., 2008). However, our findings here revealed that regulatory responses were not affected by reduction of microbiota in antibiotic treated mice. It is possible that the did not significantly affect bacteria groups that directly or indirectly control intestinal regulatory immune responses. Treg cell induction in the gut was probably maintained by the normal homeostatic mechanisms dependent on retinoic acid from antigen presenting cells (APC) in the gut and TGF-β (Sun et al., 2007; Mucida et al., 2009).

Germfree mice have several immune responses defective or lacking (Round and Mazmanian, 2009). To further examine the contribution of microbiota to immune responses during acute infections we infected germfree mice with *T. gondii.* Pro-inflammatory responses were found to be similar to the ones found in SPF mice. However, regulatory responses seemed to be enhanced in the small intestine and lymphoid tissues associated with the gut. Improved regulatory responses in germfree mice when compared to SPF mice could explain the reduced pathogenesis observed in germfree mice revealed by lower levels of liver enzymes which indicate lower pathology in the liver. Lower production of IL-17A after depletion of the microbiota or in germfree mice is in accordance with studies that showed that a group of bacteria present within the microbiota are necessary and sufficient for induction of Th17 responses in the gut (Ivanov et al., 2008; Gaboriau-Routhiau et al., 2009).

Germfree mice were found to have higher frequency of regulatory T cells in the gut and gut associated lymphoid tissues (GALT) but collapse of Tregs caused by acute infection with *T. gondii* was similar to SPF mice. Production of IL-10, an important regulatory cytokine, was also increased in the siLP of germfree mice. Overall, our data did not showed an important role for the microbiota in controlling regulatory responses other than an indirect inhibitory effect by stimulation of inflammatory responses. A recent study showed that intestinal Treg cells are induced and activated by benign commensal colonization but Treg cell activation is intrinsic and not due to differences in the flora composition (Geuking et al., 2011).

Innate immunity towards gut microbiota has been shown to be essential for protection of the host. Work from our group recently demonstrated that sensing of gut bacterial DNA through TLR9 is critical for protection of mice against infectious diseases (Hall et al., 2008). Another study reported that both mucosal innate and adaptive immune responses to *T. gondii* rely on the indirect stimulation of dendritic cells by normal gut bacteria indicating that recognition of bacterial antigens during the acute phase of infection helps to boost the immune responses against the parasite(Benson et al., 2009). It has also been reported that the microbiota are a source of peptidoglycan that systemically primes the innate immune system through signaling via the pattern recognition receptor nucleotide-binding, oligomerization domain-containing protein-1 (NOD-1), enhancing killing by bone marrow-derived neutrophils of two major pathogens (Clarke et al., 2010). It would be reasonable to say that depletion of total absence of microbiota most likely causes impaired immune responses against *T. gondii* that would reflect on defective clearance of the parasite. Surprisingly, we found that depletion or total lack of microbiota in the intestine led to smaller parasite load in antibiotic-treated mice and germfree mice in spite of the decreased immune responses observed in these mice. The paradox of lower *T. gondi* protective immune responses associated with more efficient systemic clearance of the parasite remains to unexplained. The reduced parasite burden could be a result of impaired growth or infectivity of the parasite in the beginning of the infection rather than enhanced stronger Th1 response. To answer that, parasite load in early times during the infection would have to be evaluated.

It is possible that the presence of the microbiota would directly, through interactions bacteria-parasite, or indirectly, by supplying essential metabolites, provide a more fitting environment for proliferation of *T. gondii.* Direct interaction between parasite and bacterium has been shown to interfere with the ability of infection of parasites. A recent study showed that the successful establishment of the parasitic nematode *Trichuris muris* is in the large intestine dependent on the microbiota. *In vitro* studies showed that the hatching of eggs from this parasite requires an interaction between bacteria and the egg, via type 1 fimbriae. Moreover, treatment of mice with the antibiotic enrofloxacin reduced the worm burden and increased Th2 response suggesting that the smaller number of worms found in antibiotic treated mice was due to an enhanced protective immune response against the parasite. However, treatment of immunocompromised mice with antibiotic also reduced the number of worms demonstrating that the increased Th2 response was not solely responsible for the smaller worm burden (Hayes et al., 2010).

A few studies have investigated the mechanism by which gram negative bacteria from the microbiota contribute for inflammatory processes in the gut. Recognition of bacterial antigens and signaling through TLR2 and TLR4 seems to be critical for the bacterial effects. These studies show that the absence of TLR 2, TLR4 or both can ameliorate intestinal inflammation but also accumulation of bacteria in deficient mice is limited and never reach levels comparable to WT mice (Heimesaat et al., 2007a; Heimesaat et al., 2007b).

T. gondii infection has been reported before to cause translocation of bacteria through the intestinal epithelial barrier (Heimesaat et al., 2006). In fact, translocation and consequently, increased sensing of gut bacterial products during *T. gondii*-induced ileitis has been associated with exacerbation of disease immunopathology (Heimesaat et al., 2007a). Our data here showed that indeed, bacteria that accumulates during the acute phase of *T. gondii* infection reaches peripheral tissues and contribute to local and systemic immune responses, aggravating inflammation and pathogenesis of the disease.

In our work, to establish whether exuberant recognition of gut normal bacteria and bacterial products during acute mucosal infections could generate a substantial systemic immune response against the microbiota, we initially isolated and identified a number of intestinal bacteria from *T. gondii*-infected mice. The rich media used for isolation allowed us to isolate the predominant intestinal bacteria while selective media was used to represent a broader selection of bacterial diversity. According to morphological and biochemical methods, the prevalent bacteria found in stool pellets from infected mice were the gram negative *E. coli*, *P. mirabilis,* and the the gram positive cocci *E. faecalis.* All three bacteria are part of the Enterobacteria group, which are known to be the primary accumulating bacteria during the acute phase of infection with *T. gondii.*.

We found that acute infection with *T. gondii* causes translocation of bacteria from the intestinal lumen to peripheral tissues such as spleen, mesenteric lymph node and liver. Evidences have been gathered that disruption of intestinal homeostasis can cause

systemic translocation of gut bacteria in different settings. Bacterial translocation from the gut and subsequent immune activation are hallmarks of HIV infection and are thought to determine disease progression. Intestinal barrier integrity is impaired early in acute retroviral infection, but levels of plasma lipopolysaccharide (LPS), a marker of bacterial translocation (Brenchley et al., 2006). In a “humanized” mice model, treatment with dextran sodium sulfate (DSS) of irradiated RAG2-/- mice reconstituted with human cord blood cells induced bacterial translocation to spleen and mLNs (Hofer et al., 2010).

Since only a small proportion of the gut bacteria can be quantified using traditional plate culturing methods, culture-independent approaches are required for determining the structure of complex microbial communities. High-throughput molecular technologies are now being used to elucidate microbial community structure at much higher resolution than was previously possible. The target for these methods is the 16S ribosomal RNA gene that is present in all prokaryotes. Previous studies have revealed that acute infections can induce spatial and temporal changes in the intestinal microbiota community. *T. gondii* infection has been associated with increase of gram-negative bacteria within the gut, especially enterobacteria (Heimesaat et al., 2006). In the present study we used 454 pyrosequencing for analysis of temporal and diversity changes in the microbiota community during *T. gondii* infection. Our data showed *T. gondii* infection caused a shift in the diversity of the bacterial community during the acute phase of infection when compared to naïve mice. Proteobacteria, in special *E. coli,* that has previously been associated with inflammatory diseases in the gut (Barnich et al., 2007) are present in higher frequencies in mice infected with *T. gondii* at day 9 of

infection. *E. coli* and other proteobacteria are known to be common opportunistic pathogens. The fact that these bacteria accumulate in the gut of infected mice suggest that they are probably the group of microorganisms that contribute most to aggravate the immunopathology during *T. gondii*-induced ileitis.

Other infections have been shown to alter the microbiota community. A significant shift in the abundance and relative distribution of bacterial species in the ileum of mice is associated with *Heligmosomoides polygyrus* infection for example (Walk et al., 2010). *C. rodentium*-induced colitis has also been shown to cause increased abundance of enterobacteria, although *C. rodentium* being itself a member of this group, it is hard to be conclusive whether the higher numbers of gram-negative bacteria really reflected higher numbers of commensal microrganisms (Hill et al., 2010).

Changes in diversity and frequency of bacterial communities in sites other than the intestine such as the skin have also been investigated. The skin is the human body's largest organ, colonized by a diverse milieu of microorganisms. The complexity and stability of the microbial community in the skin are dependent on the specific characteristics of the skin site and physiologically comparable sites harbor similar bacterial communities (Grice et al., 2009). A study observed longitudinal selective shift in microbiota present in non-healing wounds of diabetic mice that coincides with impaired healing (Grice et al., 2010).

Interestingly, we found that *T. gondii* had been cleared from the gut and migrated to brain and intestinal balance had been supposedly restored (after 45 days of infection), the profile of the community of the microbiota was not the same as the one observed before the infection in naïve mice. Shifts in bacterial community caused, for example, by antibiotic treatment in mice and humans have been previously investigated. The ability of the gut microbial community to recover to baseline following the cessation of antibiotic administration seems to differ according to the antibiotic regimen administered and changes have been shown to be somewhat permanent (Dethlefsen et al., 2008; Antonopoulos et al., 2009; Dethlefsen and Relman, 2011).

Furthermore, the altered microbiota during the chronic phase of *T. gondii* infection corresponded to the production of high levels of IFN-γ in the small intestine after 45 days of infection indicating that, even though histopathological aspects of the gut did not show signs of pathological damage (data not shown), abnormal sensing of commensal bacteria antigens is still ongoing and therefore intestinal homeostasis was never completely restored after infection.

E. cuniculi infection also led to translocation of bacteria to peripheral sites indicating that even a small and very transient disruption of the intestinal balance is sufficient to cause translocation. Levels of bacteria translocated were lower when compared to levels seen during the acute phase of *T. gondii* infection. This was probably due to the fact that although *E. cuniculi* infection induces a robust immune

response in the gut, this does not occasion in pathological damage to the epithelial barrier of immunocompetent mice.

Our data suggest that during inflammation, innate cells in the lamina propria exhibit heightened sensitivity to the physiologically normal concentrations of bacterial ligands entering from the gut lumen which aggravates the inflammatory response. One of the consequences of such continuous stimulation of the innate immunity resulting in inflammation may be the priming of adaptive immune responses directed towards the antigens of gut commensal bacteria. The present work presents evidences that acute mucosal infections can prime the immune system against gut bacteria antigens, eliciting adaptive immune responses specific to the microbiota. Disruption of intestinal homeostasis by intestinal inflammatory disorders such as IBD has been previously linked with newly acquired responsiveness against antigens from normal gut bacteria (Duchmann et al., 1995). The role of acute mucosal infections had not been in this scenario had not been fully addressed before. Given the extensive data that highlights the remarkable compartmentalization of the mucosal immune system, our data comes to provide a better understanding of the commensal-host-pathogen trialogue that contribute to host homeostatic mechanisms that protect against aberrant activation of the innate immune system in response to a pathogen.

Delayed-type hypersensitivity (DTH) and contact sensitivity (CS) are prototypic skin examples of Th1 reactivity mediated by Ag-specific effector T cells. At the time of immunization, DTH-effector T cells are activated by binding to complexes of Ag

peptides and MHC molecules on APCs. Subsequently, at Ag skin challenge to elicit DTH, immunized T cells are recruited into the tissues from the circulation and then interact again with Ag/peptide-MHC complexes on presenting cells to elicit characteristic late 24- to 48-h effector responses. The T cells release proinflammatory cytokines like IFN-γ, which induce local tissue cells to produce chemokines that recruit and activate an infiltrate of bone marrow-derived leukocytes(Ptak et al., 1991). We found that inoculation of *T. gondii*-chronically infected mice with antigens from our *E. coli* isolate induced a strong DTH response with production of IFN-γ at the site of inoculation. It is possible that some of this reactivity was due to response against some of *T. gondii* antigens although it is not likely.

We observed robust systemic humoral immune responses against *E. coli* antigens in mice infected with *T. gondii.* The specificity of this response was corroborated by the fact that we did not find detectable levels of any of the antibodies in sera from germfree mice. The persistence of specific antibody titers can also be used to assess immunological memory. Memory serum antibody responses may benefit from nonspecific stimulation, such as commensal bacterial products, but it is unclear whether this could apply to specific responses in the mucosal immune system (Bernasconi et al., 2002). We found high levels of PanIg, IgM and IgG against *E. coli* even after 45 days of infection which could indicate that heightened recognition of bacteria during *T. gondii* infection activate memory, long-lasting B cell responses against commensal bacteria. However, 45 days could be a short period of time to assess a memory B cell response.

Longevity of most of mucosal B cells have been described to be up to 6 weeks (Bernasconi et al., 2002).

The bacterial lysate ELISA could be dominated by responses to internal components of the bacteria and cross-reactivity could be happening. In order to try to avoid all these issues we performed bacterial FACS in sera of *T. gondii*-infected mice. This assay allows the detection of specific antibodies against bacterial surface antigens. Our results obtained with the bacterial FACS confirmed the ELISA results for most of the antibody isotypes analyzed.

The role for innate immunity, in special TLR stimulation, in B cell responses has been previously discussed. Memory B cells respond to TLR9, which recognises CpG DNA, and start to proliferate and secrete Ig independently of B cell receptor (BCR) triggering Increased levels of LPS, however, may be excluded as a direct contributor to TLR-mediated polyclonal B cell activation since B cells do not express TLR4 along with the majority of other TLRs (Bernasconi et al., 2003). To investigate whether TLR signaling is involved in the generation of antibody responses against the microbiota, studies are currently being made with knock-out mice models.

Immunoglobulin (Ig) A is the dominant antibody produced in mammals, mostly secreted across mucous membranes, especially in the intestine. We found high levels of IgA specific to our *E. coli* isolate in naïve mice sera but these levels were decreased to almost non-detectable titers at day 9 of infection with *T. gondii.* After 45 days of

infection, IgA levels in the sera of infected mice were restored to levels comparable to the ones observed in naïve mice sera. Dendritic cells interact with both T cells and B cells to induce IgA responses and this plays an important role in intestinal homeostasis (Coombes et al., 2007). Treg cells, in special, are known to regulate secretion of intestinal IgA in an antigen-specific manner via TGF-β signaling. Our group has previously reported that Treg cell numbers collapse during highly polarized Th1-type mucosal immune response such as the one seen in the acute phase of infection with *T. gondii* (Oldenhove et al., 2009). The low numbers of Treg cells could explain the lack of IgA response during the acute phase of *T. gondii* infection. Therefore, it would seem that *T. gondii* infection does not induce IgA response against commensal bacteria. However, when evaluating specific IgA responses by FACS, we observed IgA staining in sera from day 9-infected mice, contradicting the data obtained with the lysate ELISA. It is possible that the ELISA is a less sensitive method than the bacterial FACS and therefore the levels of IgA were not detected.

Specific antibody responses against antigens from *E. faecalis* and *P. mirabilis* were also activated by acute infection with *T. gondii*, only in lower levels when compared with responses against *E. coli.* This bacterium has been shown to be the primary bacteria that accumulates and translocates which could explain the lower B cell response towards the other two bacteria. Increased levels of microbiota-specific antibodies were also found after infection with *E. cuniculi* and *C. rodentium*. Lower numbers of bacteria translocated reflected on lower B cell responses against commensal bacteria antigens in mice infected with *E. cuniculi* when compared to

humoral responses activated after *T. gondii* infection. Reactivity against common molecules shared by both *E. coli* and *C. rodentium* could explain the high levels of antibodies against the *E. coli* lysate found during *C. rodentium.*

A few studies have found evidences of antibody specific responses against bacteria from the microbiota. IgG antibodies against antigens of *Bacteroides, Parabacteroides, Escherichia* and *Enteroccocus* were significantly increased in sera from patients with an autoinflammatory condition called Familial Mediterranean Fever (FMF) compared to healthy individuals (Manukyan et al., 2008). Commensal bacteria specific antibodies and T cell clones have been found in patients with IBD. Together with our findings, these data correlates exacerbated inflammatory processes and concomitant systemic translocation of bacteria with priming of immune responses against the microbiota (Duchmann et al., 1999; Haas et al., 2011).

Surprisingly, we and others have found high levels of antibodies specific to indigenous microbiota in naïve mice or healthy individuals (Duchmann et al., 1999). Oral tolerance has been classically defined as the specific suppression of immune responses to an antigen administered by oral route. Tolerance induction to dietary proteins as well as to commensal bacteria has been considered a major immunological event taking place in the gut in physiological conditions since most of the contact with foreign antigen occurs via the mucosal surfaces (Faria and Weiner, 2006). On the other hand, mucosal immune responses to commensals have been said to be highly compartmentalized, never reaching the systemic immunity at steady-state suggesting that the immune

system remains ignorant of the microbiota rather than tolerant (Macpherson and Uhr, 2004a). Adaptive immune responses in mice that supposedly had not been previously exposed to mucosal infections could suggest that response against the normal bacteria in the gut is, in fact, a common event and part of a homeostatic mechanism.

Microbiota stimulation of innate immune pathways is required for T cell spontaneous proliferation and chronic intestinal inflammation. Given the involvement of innate immune mechanisms in the modulation of T cell responses, the bacterial dependence of intestinal inflammation is likely to involve bacterial products such as lipopolysaccharide (LPS), peptidioglycan (PG), and other TLR ligands, and specific bacterial antigens capable of stimulating $CD4^+$ T cell responses. $CD4^+$ T lymphocytes have been identified as the crucial effector cells in experimental models of intestinal inflammation, and these pathogenic $CD4^+$ T cell responses are directed against the enteric microbiota (Powrie et al., 1994b; Feng et al., 2011). The *in vitro* data to date suggest that there are a relatively small number of immunodominant antigens that stimulate the pathogenic T cell responses, but the complexity of the intestinal microbiota has posed a significant challenge to their identification. Here we utilized a T cell receptor transgenic mouse line to further evaluate the generation of adaptive immune responses towards commensal bacteria and specificity of these responses. Flagellin is both a potent antigen for an adaptive response and is also able to stimulate innate responses through binding its receptor, TLR5, on innate cells. Cbir1 TCR transgenic T cells are specific for the CBir1 peptide, one of the immunodominant commensal bacterial antigens, present in the flagellin of bacteria common to the gastrointestinal

tract of laboratory mice. These transgenic mouse line allows the study of the host immune response to a defined commensal bacterial antigen (Cong et al., 2009). Defining the specificity of the adaptive immune response to the gut microbiota and the impact of this specificity on the gut barrier function is still limited by lack of comprehensive data. Our results showed that not only adaptive immune responses towards commensal bacteria antigens are activated by mucosal infection as was demonstrated by the DTH response but these responses are highly specific since $CD4^+$ T cells from CBir1 Tg mice were activated after infection with *T. gondii,* probably in response to translocation and activation of dendritic cells in the GALT.

Several recent studies have shown that specific organisms have defined effects on mucosal immune function. Commensal *Bacteroides fragilis* was able to induce intestinal regulatory responses through production of polysaccharide A. (Mazmanian et al., 2008; Round and Mazmanian, 2010). Colonization of GF animals with *B*. *fragilis* mediated the development of regulatory T cells that produced IL-10. Importantly, *B. fragilis*-induced Treg cells protected mice from colitis initiated by *Helicobacter hepaticus* infection via IL-10. Furthermore, commensal bacteria are required for induction of Th17 cells, as these cells are absent in GF mice. Colonization of GF mice with normal members of the microbiota, specifically the segmented filamentous bacteria (SFB), restored mucosal Th17 cells, indicating that SFB stimulate mucosal Th17 cell development (Gaboriau-Routhiau et al., 2009). Promotion of Th17 cells by SFB has been implicated in the pathogenesis of arthritis and experimental autoimmune

encephalomyelitis (EAE), as both diseases were attenuated in mice housed under GF conditions.

The present work also brought evidences that adaptive immune responses towards commensal bacteria that are triggered by acute mucosal infections can be protective against subsequent infections. Priming of immune responses against bacteria from the microbiota did not aggravate the pathological consequences of a secondary infection. Moreover, mice sensitized with antigens from *E. coli* were able to clear more efficiently the parasite after challenge with *T. gondii.* Several studies have already demonstrated the importance of commensal bacteria to protection of the host (Hall et al., 2008; Benson et al., 2009; Dos Santos et al., 2011). Our group has shown that monoassociation of germfree mice with the probiotic *Lactobacillus delbrueckii bulgaricus,* a normal inhabitant of the human gut, can protect against infection with *Listeria monocytogenes.* Monoassociated mice had higher survival rate, less sever pathology in the liver and produced higher levels of IFN-γ and TNF-α that are critical for resistance against *L. monocytogenes* and IL-10 to help control the immunopathological damages caused by the infection (Dos Santos et al., 2011). The mechanism by which *L. delbrueckii* protects the host against *L. monocytogenes* is still unknown and studies are being conducted in this regard. Consumption of a single commensal microorganism, *Bifidobacterium infantis* 35624, drives the generation and function of Treg cells which control excessive NF-κB activation *in vivo* following infection with *Salmonella typhimurium* or injection with LPS (O'Mahony et al., 2008).

Nevertheless, the idea of mucosal infections triggering adaptive immune responses against gut bacteria that are beneficial to the host had not been explored before. Here, mice sensitized with antigens from *E. coli* were able to clear more efficiently the parasite after challenge with *T. gondii.* An enhanced immune response against *T. gondii* was not observed after priming the immune system towards commensal bacteria. However, the more efficient clearance of the parasite could be related to a faster response. To evaluate that, immune responses would have to be analyzed at earlier time points during the infection with *T. gondii.*

It has been previously shown that immune responses to *T. gondii* depend on the direct stimulation of dendritic cells provided by both epithelial cell-derived cytokines and commensal bacteria (Benson et al., 2009). It is likely that the microbiota functions as a molecular adjuvant during parasitic infections and that activation of dendritic cells by commensal bacteria plays a major role in the regulation of the innate and adaptive immune responses to intestinal pathogens. Importantly, our data showed that priming of immune responses against bacteria from the microbiota did not aggravate the pathological consequences of *T. gondii* infection. the priming of the host mucosal immune system towards intestinal microbiota assure balanced immune responses that do not result in intestinal immunopathology, but are fully sufficient for initiating protective Th1 immunity against *T. gondii* infection.

We used the intraperitoneal route of delivery of antigen for immunization of mice against *E. coli* antigens. It has been shown that the normal route by which antigens are

taken up by the GALT is via the epithelial surface but antigen presented in this way via villus epithelial cells predominantly results in oral tolerance and generation of a suppressor response. Strategies designed to overcome this effect include accessing the GALT via the serosal surface by administration of intraperitoneal antigen (Husband, 1993).

CONCLUSION

In the present study we showed that acute mucosal infections can trigger adaptive immune responses against gut commensal bacteria. This new response was shown to be robust and of both humoral and cellular types. Furthermore, the adaptive immune response against commensal bacteria improved the ability of the host to respond against subsequent mucosal infections. Our findings shed new light in the host-microbiota interactions and the consequences of breakdown of this homeostatic relationship. Comprehending fully how perturbations in the intestine can affect this balance leading to aberrant reactivity against commensal bacteria could contribute to a better understanding of the etiology of inflammatory diseases and to the development of new strategies of prevention and therapy of these disorders.

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