Toxoplasma gondii chronic infection decreases visceral nociception through peripheral opioid receptor signaling

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Abstract (149 words)

By eliciting immune activation in the digestive tract, intestinal pathogens may perturb gut homeostasis. Some gastrointestinal infections can indeed increase the risk of developing post-infectious irritable bowel syndrome (PI-IBS). Intriguingly, the prevalent foodborne parasite *Toxoplasma gondii* has not been linked to the development of PI-IBS and the impact of this infection on colon homeostasis remains ill-defined. We show in a mouse model that latent *T. gondii* decreases visceral nociceptive responses in an opioid signaling-dependent manner. Despite the accumulation of Th1 and cytotoxic T cells in the colon of latently infected mice, the selective invalidation of enkephalin gene in T cells ruled out the involvement of T cell-derived enkephalins in hypoalgesia. These findings provide clues about how this widespread infection durably shapes the gut immune landscape and modifies intestinal physiological parameters. They suggest that in contrast to other gut microbes, *T. gondii* infection could be negatively associated with abdominal pain.

Introduction

Most often, infections are rapidly resolved, allowing the organism to return to its initial state. However, in some cases, infections can have long-lasting consequences, thereby negatively or positively regulating the development of chronic diseases [1-3]. As examples, a mouse model suggested that early-life brain viral infection can predispose to the development of brain autoimmune disease during adulthood [4] and in humans, Epstein-Barr virus infection increases by over 30-fold the risk to develop multiple sclerosis [5].

As one of the largest interface between the host and the environment, the gastrointestinal (GI) tract is highly exposed to pathogens, which can profoundly shape the mucosal immune environment [6]. The GI tract is composed of different cellular layers. Below the epithelium which surface is exposed to the gut microbiota, lie (i) a lamina propria containing fibroblasts and immune cells, (ii) muscular layers, and (iii) a neuronal network divided into an intrinsic (enteric nervous system, ENS) and extrinsic peripheral neuronal compartment. These entities exert specific functions that are crucial for tissue homeostasis. The ENS regulates intestinal functions such as gut motility, secretion, permeability, and blood flow. Extrinsic neurons relay peripheral information, such as visceral pain, to the central nervous system (CNS) through afferent projections from neurons (nociceptors) that have their soma in the dorsal root ganglia (DRG). Visceral pain transmitted by nociceptors results from the integration of excitatory (pro-nociceptive) and inhibitory (anti-nociceptive) signals present within the surrounding environment. Pro-nociceptive mediators like pro-inflammatory cytokines or some proteases [7], increase nociceptor firing, leading to visceral hypersensitivity, while anti-nociceptive mediators such as endogenous opioids, decrease nociceptor activity, thus relieving visceral pain [8-10]. As drivers of inflammatory mediators, several intestinal pathogens causing infectious gastroenteritis enhance pro-nociceptive signals and increase the risk of developing Irritable Bowel Syndrome (IBS), a phenomenon known as Post-Infectious IBS (PI-IBS) [11]. According to Rome IV criteria, IBS is a functional chronic disorder characterized by changes in bowel habits and abdominal pain without obvious gut inflammation or macroscopic lesions [12]. In humans, PI-IBS has been reported following infections with pathogens such as bacteria (*Escherichia coli*, *Salmonella*, *Campylobacter jejuni*), parasites (*Giardia intestinalis* [13], *Cryptosporidium* [14]) and viruses (*Norovirus* [15]), suggesting that common immune-mediated mechanisms possibly trigger the development of this syndrome [16]. Interestingly, one study suggested that visceral hypersensitivity in PI-IBS could arise from prolonged nociceptor activation and sensitization due to incomplete resolution of immune responses, thus leading to persistent abdominal pain despite apparent recovery from acute gastroenteritis [17]. Moreover, *Blastocystis*-infected rats exhibit a PI-IBS-like phenotype characterized by a non-inflammatory visceral hypersensitivity [18] and *Citrobacter rodentium* infection of mice evokes hyperexcitability of colonic DRG neurons which persists following resolution of the infection [19], indicating that IBS features can be recapitulated in animal models of infection. Intriguingly, there is currently no epidemiological evidence that the highly prevalent foodborne parasite *Toxoplasma gondii (T. gondii)* is linked to the development of PI-IBS.

T. gondii is an obligate intracellular protozoan parasite able to infect any warm-blooded animal, including humans. It is estimated that around 30% of the world population, reaching up to 50% in some areas, has been exposed to this parasite [20]. Infection occurs after ingestion of contaminated food or water, allowing parasite cysts or oocysts to penetrate the gut and invade the host through the small intestine. Parasites then proliferate as fast-replicating tachyzoites, causing tissue inflammation, bacterial translocation, and strong type 1 immune responses including durable Th1 CD4+ T cell responses [6,21,22]. During this initial acute phase of infection, tachyzoites disseminate systemically and reach the CNS, where chronic infection is established. The long-term chronic phase is characterized by clearance of the parasite from the periphery (including the gut) and persistence of the parasite in the CNS, retina and skeletal muscles in the form of slow-replicating bradyzoïtes located within intracellular cysts [23,24]. While the impact of *T. gondii* chronic infection on brain function has been extensively studied [25,26], much less is known about the potential long-term consequences of *T. gondii* infection on gut homeostasis. One pioneer study has reported that by inducing bacterial translocation during the acute phase, *T. gondii* infection promotes the development of microbiota-specific CD4+ memory T cells with a pro-inflammatory Th1 phenotype. Interestingly, these T cells can persist in the gut for more than 200 days post-infection [22]. More recently, it was shown that chronic *T. gondii* infection exacerbates the severity of intestinal damage caused by chemical injury, due to heightened activation status of monocytes in chronically infected mice [27]. In rats, chronic *T. gondii* infection has been reported to cause damage of the colonic mucosa and induce death of some neurons of the submucosal or myenteric ENS plexi,without altering the gastrointestinal transit time or the fecal pellet output [28,29].

Together, these data suggest that as other intestinal pathogens, by shaping the neuroimmune environment of the gut, *T. gondii* infection could promote the development of PI-IBS. To address this question, we explored the long-term consequences of latent *T. gondii* infection on the colonic microenvironment and on visceral nociceptive responses. Contrary to our expectations, we found that chronic *T. gondii* infection decreases visceral nociception in an opioid receptor signaling-dependent manner, in the absence of macroscopic colonic inflammation. This effect was associated with a long-lasting increase in colonic T cells displaying a Th1 or cytotoxic phenotype, but could not be explained by T cell-produced enkephalins. These findings shed light on the long-term impact of this widespread foodborne infection on the gut neuroimmune landscape and physiology.

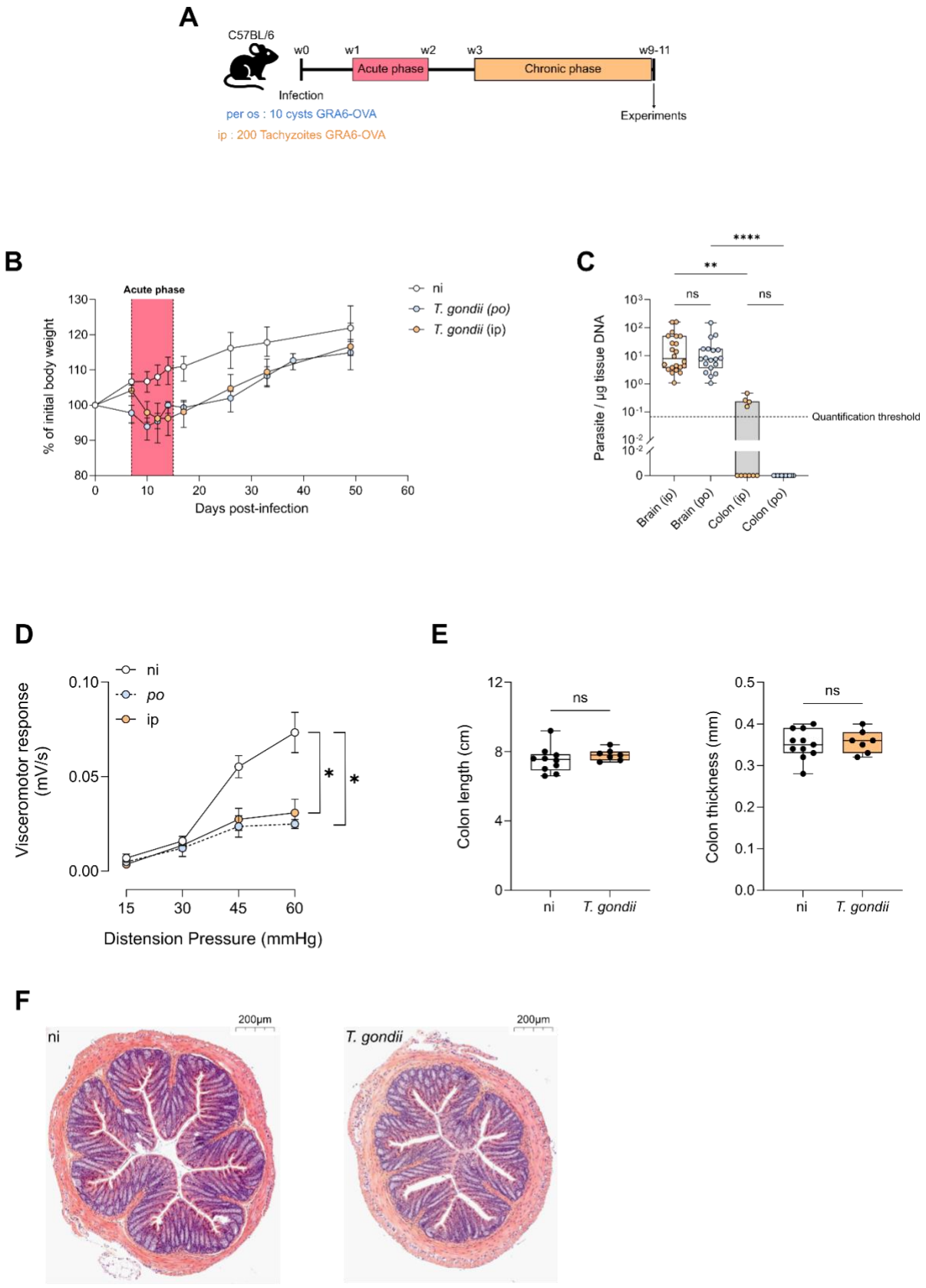
Results

Toxoplasma gondii chronic latent infection decreases visceral nociceptive responses

As C57BL/6 mice are naturally susceptible to *T. gondii* infection and develop encephalitis following infection with a type II strain, we used an established model of infection of C57BL/6 mice with a modified type II strain expressing the immunodominant GRA6-OVA model antigen (Pru.GFP.GRA6-OVA), which elicits a strong CD8+ T cell response able to effectively control the parasite burden during chronic stage [30], thereby mimicking the pathophysiology of latent human infection. To examine the importance of the primary intestinal phase of infection on the pathophysiological outcome, mice were infected either *per os* by administration of 10 cysts or intraperitoneally (ip) by injection of 200 tachyzoites (**Fig. 1A**). As previously reported [31], *T. gondii* infection induced a transient weight loss during the acute phase of infection (**Fig. 1B**), whatever the mode of parasite administration. At 10 weeks post-infection (pi), none of the *per os*-infected mice exhibited detectable parasite burden in the colon while some ip-infected mice still had detectable parasites in the colon. Both models displayed similar parasite loads in the brain (**Fig. 1C**).

To assess visceral sensitivity in non-infected (ni) *versus* chronically infected mice (*per os* or ip, 10 weeks post-infection), we measured the visceromotor response (VMR) elicited by colorectal distension. In this assay, VMR measurements are directly linked to the stimulus intensity evoked by increasing pressures of a balloon inserted into the distal colon. Strikingly, compared to uninfected mice, both *per os* and ip-infected mice exhibited a significant reduction of VMR in response to colorectal distension (**Fig. 1D**). Hence, *T. gondii* chronic infection decreases visceral nociceptive responses regardless of the mode of infection, and thus regardless of local parasite persistence (as parasite load was undetectable in the colon of *per os* chronically infected mice, see **Fig. 1C**). Since visceral hypoalgesia was similarly induced following *per os* and ip routes of infection, and since ip infections avoid the use of 'reservoir' mice required to prepare the cysts, we conducted the rest of the study with ip-infected mice.

To start deciphering the mechanisms explaining this hypoalgesia, we assessed the presence of potential anomalies of the colonic tissue at macroscopic and microscopic levels. Colon thickness and length were not changed at 10 weeks post-infection, showing that this model of chronic latent infection does not induce a macroscopic long-lasting colonic inflammation (**Fig. 1E**). Moreover, H&E stainings of the colon showed that chronically infected mice have a normal architecture and do not display any microscopic damage or massive immune infiltration (**Fig. 1F**). Together, these data indicate that latent *T. gondii* infection decreases visceral nociception independently from the route of infection, and in the absence of apparent gut inflammation.


A
C57BL/6
w0
w1
w2
w3
w9-11
Acute phase
Chronic phase
Infection
Experiments
per os : 10 cysts GRA6-OVA
ip : 200 Tachyzoites GRA6-OVA
B
Acute phase
% of initial body weight
Days post-infection
ni
T. gondii (po)
T. gondii (ip)
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ns
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Parasite / µg tissue DNA
Quantification threshold
Brain (ip)
Brain (po)
Colon (ip)
Colon (po)
D
Visceromotor response (mV/s)
ni
po
ip
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*
Distension Pressure (mmHg)
E
Colon length (cm)
ns
ni
T. gondii
Colon thickness (mm)
ns
ni
T. gondii
F
ni
200µm
T. gondii
200µm

127

Figure 1: *Toxoplasma gondii* chronic infection decreases visceral nociceptive responses in the absence of macroscopic colonic inflammation

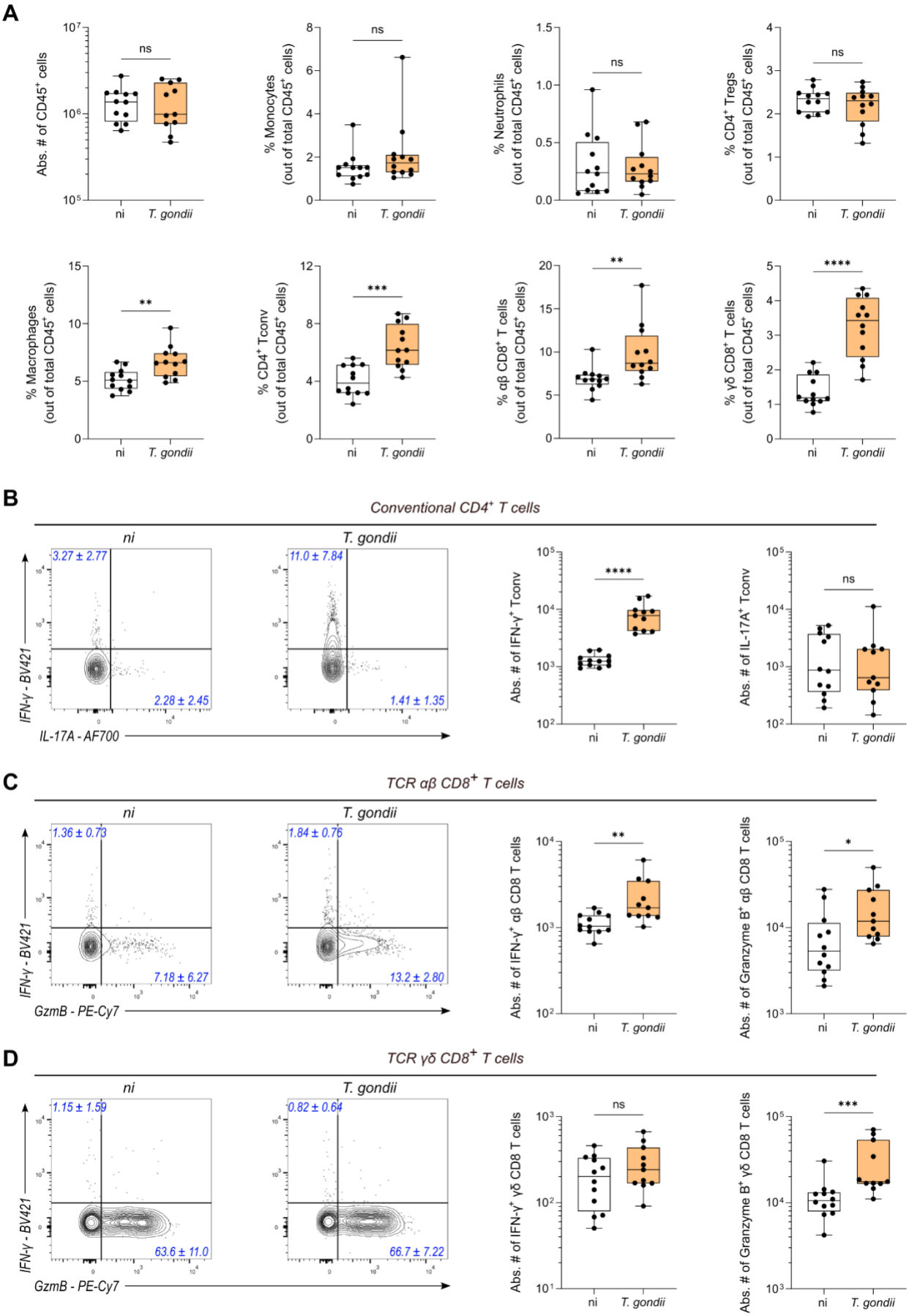
(A) Experimental workflow: C57BL/6 mice were infected with Pru.GFP.GRA6-OVA *T. gondii* either *per os* with 10 cysts or by intraperitoneal injection with 200 tachyzoïtes. Experiments were performed between 9 and 11 weeks post-infection. **(B)** Weight measurements during the course of infection, relative to the initial body weight. Dots represent the median +/- IQR of n = 37 (ni), 22 (ip), or 29 (*per os*) mice. **(C)** Parasite burden per µg of tissue DNA measured by qPCR on genomic DNA extracted from brain and colon. Box and whisker plots show median +/- IQR. Data are from 3 pooled independent experiments with each dot representing one mouse. Statistical analysis was performed with Kruskal-Wallis test and subsequent Dunn's correction for multiple comparisons. **(D)** Visceromotor response (VMR) to increasing colorectal distension pressure (15 to 60 mm Hg) measured in non-infected (ni) mice or mice infected either *per os* or ip. Data are shown as mean +/- SEM and correspond to 3 pooled experiments with n = 15 (ni), 6 (*per os*), and 12 (ip) mice. Statistical analysis was performed with Areas Under the Curve (AUC) using a Kruskal-Wallis test with Dunn's correction for multiple comparisons. **(E)** Colon length was measured and colon was opened longitudinally to measure thickness. Data are from 1 experiment, representative of 2 independent experiments. Statistical analysis was performed using Mann-Whitney test. **(F)** Representative image of Hematoxylin/Eosin staining of distal colon section (5 µm thick) from ni vs. ip-infected mice. Scale bar = 200 µm.

Toxoplasma gondii chronic infection induces long-lasting modifications of the colonic immune landscape

In line with the fact that *T. gondii* infection has not been reported to be a trigger of PI-IBS, mice chronically infected with *T. gondii* exhibited lower visceral response to colorectal distension. This result suggests that analgesic pathways are mobilized in *T. gondii* infected mice, possibly explaining the diminished VMR observed upon chronic infection. Several studies have highlighted the role of immune cells and mediators in modulating visceral sensitivity and pain [32-35]. Therefore, we investigated potential changes in colonic immune infiltration during *T. gondii* infection.

First, we set out to analyze the main immune cell modifications occurring in the colon shortly after infection (i.e. at acute stage). To this aim, we performed an unsupervised flow cytometry analysis of colonic cells extracted from the colon of non-infected mice vs mice infected with *T. gondii* for 14 days. This analysis indicated that the colon of acutely infected mice is enriched in both innate and adaptive immune cells (**Fig. S1**). More specifically, the colon of acutely infected mice exhibited a higher proportion of monocytes (cluster 3) and neutrophils (cluster 1) compared to non-infected mice (**Fig. S1A-B**). In addition, various T cell subsets were enriched (**Fig. S1C-D**), such as IFN-γ-producing CD4+ T cells (clusters 4 & 15), cytotoxic CD8+ T cells (clusters 7 & 2) and T cells producing both IFN-γ and Granzyme B (clusters 5, 6 & 8). In contrast, we found a lower percentage of regulatory (FoxP3+) CD4+ T cells (cluster 3) and of IL-17-producing conventional (FoxP3-) CD4+ T cells (cluster 9) in acutely infected compared to non-infected mice. These data confirmed that acute infection by *T. gondii* is associated with a type 1-polarized immune response, and they allowed us to identify the immune cell subsets of the colon which proportions are most affected (negatively or positively) following *T. gondii* infection.

We next conducted supervised flow cytometry analyses to investigate how the abundance of such immune cell subsets changed throughout chronic infection. The gating strategies used to identify the different immune cell subsets are depicted in **Fig. S2**. First, in contrast to acute infection, we observed no change in the number of leukocytes (i.e. CD45+ Epcam-negative) present in the colon of chronically infected mice compared to non-infected mice, indicating that immune infiltration of the colon has fully resolved at chronic stage (**Fig. 2A**), in agreement with the normal histology described above (see **Fig. 1E-F**). This observation was corroborated by the similar percentages of monocytes, neutrophils and Tregs in the colon of non-infected *vs*. chronically infected mice (**Fig. 2A**). Yet, the composition of the colonic immune compartment remained durably changed upon infection since infected mice showed a significant increase in the proportion of macrophages (F4/80+, CD11b+, Ly6C/G-), conventional CD4+ T cells (Foxp3-) and both TCRαβ+ CD8+ and TCRγδ+ CD8+ T cells (**Fig. 2A**). In addition, T cells from infected mice were more prone to produce effector molecules after PMA/ionomycin restimulation, as demonstrated by the higher number of IFN-γ-positive conventional CD4+ T cells and TCRαβ CD8+ T cells and the higher number of granzyme B-positive (reflecting cytotoxicity) TCRαβ+ CD8+ and TCRγδ+ CD8+ T cells in the colon of *T. gondii*-infected mice (**Fig. 2B-D**). In conclusion, while the proportions of neutrophils, monocytes and Tregs out of CD45+ leukocytes normalized over time, chronic infection by *T. gondii* induced long lasting modifications of the colonic immune landscape, characterized by an enrichment in macrophages, cytotoxic CD8+ T cells and IFN-γ-producing CD4+ and CD8+ T cells, illustrating the prolonged activation of several T cell subsets beyond the acute infection.


A
Abs. # of CD45+ cells
% Monocytes (out of total CD45+ cells)
% Neutrophils (out of total CD45+ cells)
% CD4+ Tregs (out of total CD45+ cells)
% Macrophages (out of total CD45+ cells)
% CD4+ Tconv (out of total CD45+ cells)
% αβ CD8+ T cells (out of total CD45+ cells)
% γδ CD8+ T cells (out of total CD45+ cells)
ns
**
***
****
ni
T. gondii
B
Conventional CD4+ T cells
ni
T. gondii
3.27 ± 2.77
2.28 ± 2.45
11.0 ± 7.84
1.41 ± 1.35
IFN-γ - BV421
IL-17A - AF700
Abs. # of IFN-γ+ Tconv
Abs. # of IL-17A+ Tconv
C
TCR αβ CD8+ T cells
1.36 ± 0.73
7.18 ± 6.27
1.84 ± 0.76
13.2 ± 2.80
GzmB - PE-Cy7
Abs. # of IFN-γ+ αβ CD8 T cells
Abs. # of Granzyme B+ αβ CD8 T cells
*
D
TCR γδ CD8+ T cells
1.15 ± 1.59
63.6 ± 11.0
0.82 ± 0.64
66.7 ± 7.22
Abs. # of IFN-γ+ γδ CD8 T cells
Abs. # of Granzyme B+ γδ CD8 T cells

181

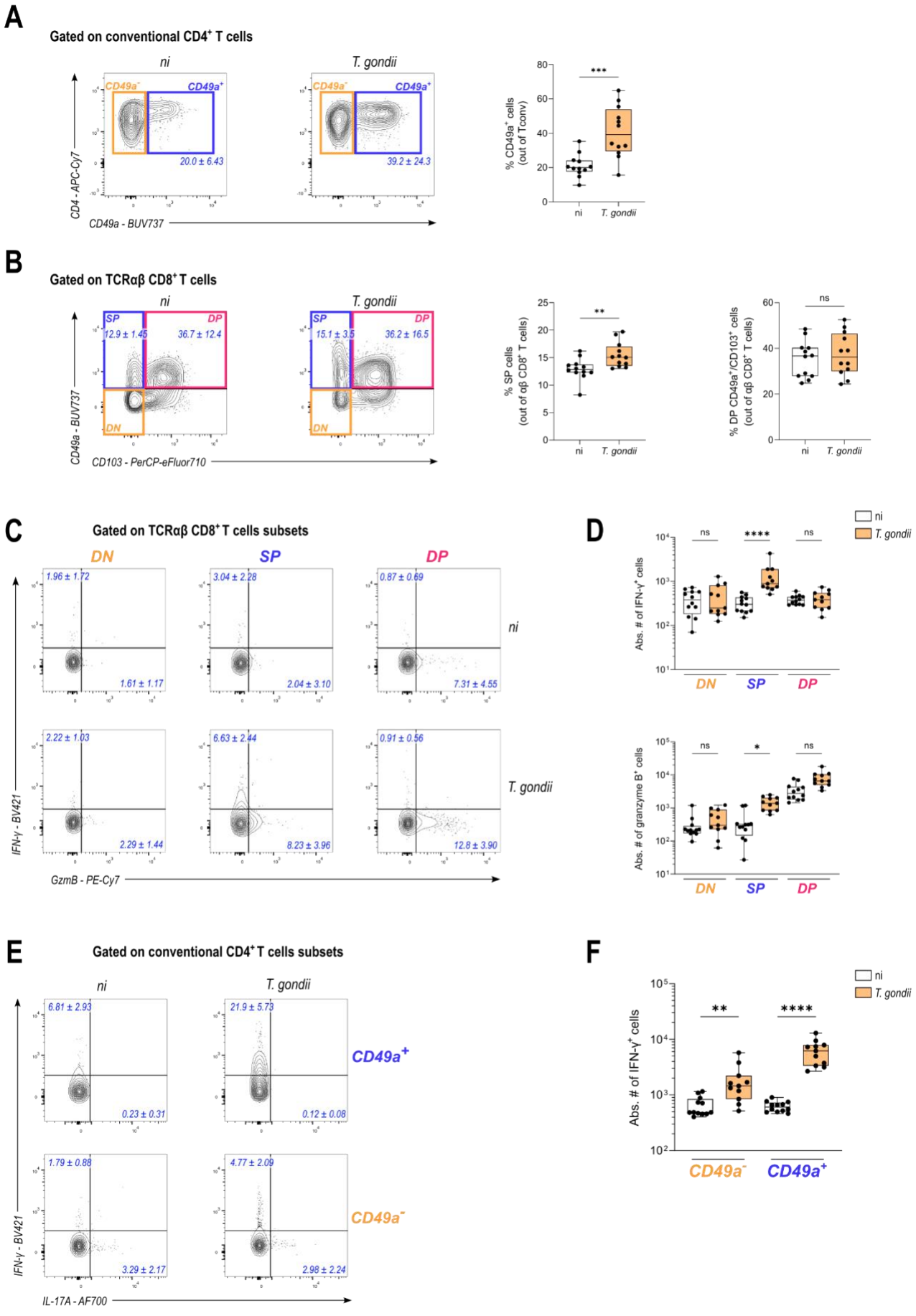
Figure 2: *Toxoplasma gondii* infection induces long-term increase of colonic Th1 CD4+ T cells and cytotoxic CD8+ T cells

(A-D) Flow cytometry analysis of the indicated cell subsets in the colon of non-infected (ni) vs. mice chronically ip-infected for 70 days (*T. gondii*). Box and whisker show median +/- IQR. Data are pooled from 2 independent experiments and each dot represents one mouse with a total of 12 (ni) and 11 (*T. gondii*) mice. Statistical analysis was performed using Mann-Whitney test or unpaired Student's t test depending on the normality of the values. **(A)** Box and whisker plots show absolute number of $CD45^+$ cells and percentage of immune cell subsets out of total $CD45^+$ cells in the colon of mice infected (orange) or not (white) for 70 days. (**B-D**) Representative FACS plot for each condition with numbers (in blue) indicating the median percentage +/- IQR of positive cells among **(B)** conventional (Foxp3-) CD4+ T cells, **(C)** TCRαβ CD8+ T cells and **(D)** TCRγδ CD8+ T cells. Box and whisker plots show the absolute number (median +/- IQR) of each T cell subset in the colon of non-infected (white) vs infected (orange) mice.

Toxoplasma gondii infection induces an accumulation of colon-resident memory T cells (Trm)

The above data indicate that chronic *T. gondii* infection is associated with a long-lasting increase of colonic T cells with enhanced effector functions. Since the parasite is cleared from the colon in most mice during chronic phase (see **Fig. 1C**), these data cannot be explained by continuous stimulation of T cells with parasite-derived antigenic peptides in the colon. Instead, this increase could result from the generation and persistence of resident memory T cells in the colon throughout chronic phase. In the brain, it is now well established that *T. gondii* infection promotes the generation of parasite-specific brain-resident CD8+ T cells, which sequentially acquire expression of residency markers such as CD69, CD49a and CD103, allowing their retention in the tissue [31,36]. To investigate whether T cells persisting in the colon of *T. gondii* chronically infected mice also exhibit a tissue-resident memory phenotype, we examined the expression of CD49a by CD4+ T cells and of CD49a and CD103 by CD8+ T cells. CD103 was not considered for CD4+ T cells since it is more a Treg marker than a meaningful marker of resident CD4+ Tconv, and CD69 could not be used since it is induced by the *in vitro* PMA/ionomycin stimulation step. We found that chronic infection by *T. gondii* leads to an increase in the frequency of CD49a-positive cells among both conventional (Foxp3-) CD4+ and TCRαβ+ CD8+ T cells. However, no difference was found in the frequency of CD49a/CD103 double-positive cells among TCRαβ+ CD8+ T cells (**Fig. 3A-B**). Since we reported above that *T. gondii* chronic infection causes an elevated production of IFN-γ and granzyme B by T cells in the colon, we wondered whether this increased cytokine production could be linked to the increase in colon-resident T cells, as Trm are typically known as fast responders following stimulation. We noticed that infection significantly increased the abundance of resident (CD49a single positive) TCRαβ+ CD8+ T cells able to produce IFN-γ or granzyme B in response to PMA/ionomycin restimulation, while no statistical difference was observed on CD49a-negative or CD49a/CD103 double-positive cells (**Fig. 3C-D**). Similarly, *T. gondii* latent infection caused an increase in conventional CD4+ T cells producing IFN-γ upon PMA/ionomycin restimulation, with the most prominent increase observed among the resident CD49a-positive subset (**Fig. 3E-F**). Combined, these data indicate that chronic infection by *T. gondii* generates CD49a+ Trm that persist in the colon and display heightened effector functions.

Since inflammation is often associated with pain, this increase in 'pro-inflammatory' type 1-polarized resident T cells could appear antagonistic with the observed decreased visceral nociceptive responses of chronically infected mice. Importantly, studies have highlighted that effector T cells, in particular CD4+ T cells, can release endogenous opioid peptides called enkephalins, that are able to alleviate pain in different inflammatory contexts, including colitis. Furthermore, it was reported that enkephalins could be produced by colitogenic T cells, highlighting the dual effect of T cells on inflammation and pain modulation [37,38]. Based on this, we hypothesized that the release of enkephalins by T cells present in the colon of chronically infected mice may be responsible for the decrease in visceral nociceptive responses.



226

227 **Figure 3: *Toxoplasma gondii* infection generates Th1 CD4+ and cytotoxic CD8+ resident T cells**

(A-F) FACS plots or Box and whisker plots with median +/- IQR showing absolute number or percentage of cell subsets in the colon of non-infected (ni) or chronically ip-infected mice (*T. gondii*) for 70 days. Data are pooled from 2 independent experiments and each dot represents one mouse with n = 12 (ni) *vs.* 11 (*T. gondii*). Depending on the normality of the dataset, statistical analysis was performed using Mann-Whitney test or unpaired t test when comparing 2 conditions (A and B), or using Kruskal-Wallis test with Dunn's correction for multiple comparisons (D and F). **(A and B)** Representative FACS plots for each condition with numbers in blue showing median percentage +/- IQR of CD49a-positive cells among **(A)** conventional (Foxp3-) CD4+ T cells and **(B)** TCRαβ+ CD8+ T cells. Corresponding frequency of each T cell subset are represented with box and whisker plots showing median +/- IQR. **(C and D)** Representative FACS plot for each condition with numbers in blue indicating the median percentage +/- IQR of cytokine-positive cells out of TCRαβ CD8+ T cells **(C)** and the absolute number of each subset **(D),** represented as box and whisker plots showing median +/- IQR. **(E and F)** Representative FACS plots for each condition with numbers in blue indicating the median percentage +/- IQR of cytokine-positive cells out of conventional (Foxp3-) CD4+ T cells **(E)** and the absolute number of each subset **(F),** represented as box and whisker plots showing median +/- IQR.

Toxoplasma gondii chronic infection decreases visceral nociceptive responses through peripheral opioid-signaling

To assess the contribution of enkephalins produced by T cells in the hypoalgesic phenotype caused by latent *T. gondii* infection, we took advantage of a new mouse model enabling constitutive T cell-specific deletion of the pro-enkephalin-encoding gene (Penk). To this aim, we crossed Penk-flox mice [39] with CD4-Cre mice. Since most T cells are derived from a thymic precursor stage that co-expressed CD4 and CD8, this CD4-Cre model should allow the deletion of Penk in both mature CD4+ and CD8+ T cells (**Fig. S3A**). We confirmed by FlowFISH that compared to CD4-Cre- mice, CD4-Cre+ mice display a 10-fold decrease in the percentage of T cells expressing Penk mRNA (**Fig. S3B**). Using this model in which T cells are able (Penk T^{WT}) or not (Penk T^{KO}) to produce enkephalins, we assessed visceral nociceptive responses to colorectal distension in chronically infected mice (**Fig. S3C**). Preventing enkephalin production by T cells did not change the visceromotor response of chronically infected mice, suggesting that T cell-released enkephalin peptides are not involved in the regulation of visceral nociception in the context of chronic infection by *T. gondii* (**Fig. S3D**). Notably, this result suggests the potential contribution of other cellular sources of enkephalins, or even other types of opioids, in the hypoalgesic phenotype. To formally assess the implication of opioid signaling, we tested whether the hypoalgesic phenotype could be reversed by blocking opioid receptors. To restrict the inhibition to peripheral opioid receptor, we used naloxone methiodide, an opioid receptor antagonist which cannot cross the blood brain barrier [40]. We assessed visceral nociception in chronically infected mice before and after naloxone methiodide injection (**Fig. 4A**). As shown in **Fig. 4B-C**, the administration of naloxone methiodide to infected mice significantly increased nociceptive responses, indicating that peripheral opioid receptor signaling is involved in the hypoalgesic phenotype induced by *T. gondii* chronic infection. The same naloxone methiodide treatment did not change the visceral nociception of uninfected mice (**Fig. S4**). Therefore, collectively, our data show that peripheral opioid receptor signaling underlies the decreased visceral nociceptive responses observed upon chronic infection by *T. gondii* but that enkephalins produced by T cells do not play a major role in this phenotype.

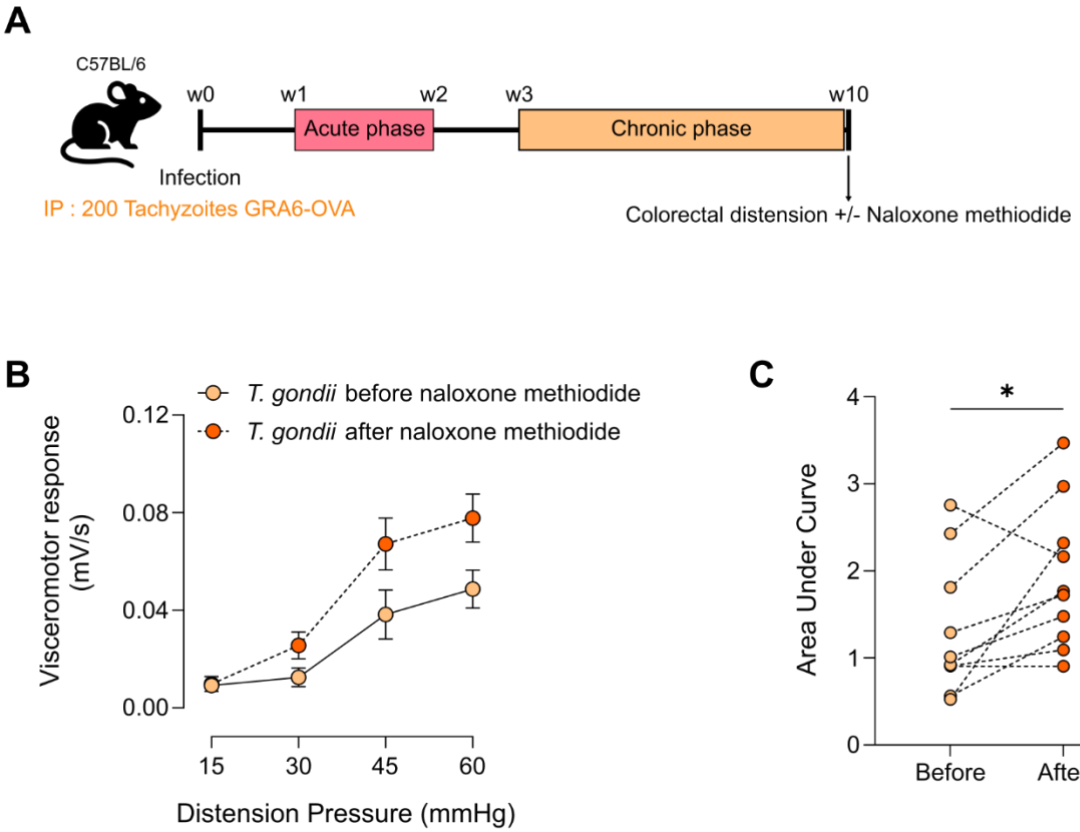


Figure 4: Decreased visceral nociceptive responses induced by *Toxoplasma gondii* chronic infection relies on peripheral opioid signaling

(A) Experimental workflow: C57BL/6 were infected with Pru.GFP.GRA6-OVA *T. gondii* by intraperitoneal injection with 200 tachyzoïtes. At 10 weeks post-infection, colorectal distension was performed to assess visceral nociception of each mice before or 30 min after intraperitoneal injection of naloxone-methiodide. **(B)** Visceromotor response (VMR) to increasing colorectal distension pressure (15 to 60 mmHg) was measured in infected mice before (in orange) or after (in red) naloxone methiodide injection. Data are shown as mean +/- SEM and are from 1 experiment representative of 2 independent experiments with n = 10 mice per experiment. **(C)** Area Under the Curve (AUC) are represented for each mouse with 2 dots corresponding to the AUC before (in orange) and after (in red) naloxone methiodide treatment. Statistical analysis was performed using a paired t test.

Discussion

T. gondii is a widespread parasite that establishes chronic infection in all warm-blooded animals including humans. While *T. gondii* infection is known to have major consequences on brain functions, the long-term impact of this infection on the gut compartment remains ill-defined. Using a mouse model of latent *T. gondii* infection, this study reports that latent infection downregulates basal visceral nociceptive response measured to colorectal distension. This phenotype was observed both after natural infection (*per os*) and following intraperitoneal administration of tachyzoites, suggesting that the systemic dissemination phase of the parasite, rather than the initial intestinal invasion step, is important for the establishment of the hypoalgesic phenotype. Importantly, we observed that this decreased visceral nociceptive response is dependent on peripheral opioid receptor signaling. Whether the parasite is directly responsible for the desensitization of nociceptive fibers was not formally addressed in our work. However, we think it is unlikely since the hypoalgesic phenotype was observed in a context where *T. gondii* has been cleared from peripheral tissues and the only reservoir of *T. gondii* is the CNS. By analogy with the current view that neuroinflammatory pathways more than *T. gondii* cysts themselves, underlie the *T. gondii*–mediated cognitive alterations [25,41,42], we suspect that the consequences of chronic *T. gondii* infection on colon pain perception result from local neuroimmune imprinting, leading to a durable shift of the balance towards antinociceptive signals, rather than from a direct manipulation of infected neurons by *T. gondii* in the colon or DRG.

In our view, this hypoalgesic phenotype was unexpected for at least two reasons. First, *T. gondii* infection has so far been reported to be an aggravating factor rather than a negative modulator of intestinal inflammation and immunopathology, two conditions that are classically associated with pain. Along this line, one study in humans has revealed a higher seroprevalence of *T. gondii* in Crohn's disease patients, although not in Ulcerative Colitis patients [43]. In experimental models, analyses of orally infected C57BL/6 mice showed massive inflammatory lesions of the ileum during acute phase [44,45], suggesting that *T. gondii* could actually be an infectious trigger of inflammatory bowel disease (IBD) [46,47]. Accordingly, chronic *T. gondii* encephalitis was recently found to exacerbate the susceptibility of mice to DSS-induced colitis, an established model of IBD [27]. Data from our paper actually support this hypothesis since we observed that latent *T. gondii* infection mobilizes pro-inflammatory T cells in the colon, including resident T cells, which are known to contribute to chronic intestinal inflammation [48].

Yet, IBD and IBS are notably distinct pathologies. While IBD is characterized by severe and chronic inflammation of the GI tract, IBS is mostly a functional syndrome that is associated with low-grade inflammation or immune activation of the gut, and is generally devoid of overt chronic inflammation. Nevertheless, several gastrointestinal infections have been reported to cause PI-IBS [16,49], which is the second reason why our data are unexpected. To our knowledge, only one case-control study explored the association between *T. gondii* exposure and visceral sensitivity [50]. In this study, the seroprevalence of *T. gondii* infection was higher in subjects with frequent abdominal pain (8%) than in control subjects (4%), suggesting an association between *T. gondii*

infection and frequent abdominal pain. However, the context of abdominal pain (e.g. IBS, pharmacological treatment and/or infection) was not described, thereby hampering interpretation of this work. Moreover, since it is unknown whether seroconversion preceded or not the onset of abdominal pain, our understanding of the underlying mechanisms remains limited. A serodiagnosis of *T. gondii* exposure in well-characterized cohorts of IBS patients, with a detailed description of clinical symptoms and visceral sensitivity, would be needed to establish to which extent our data translate to the human setting.

An important mechanistic achievement of our study is to show that opioid receptor signaling in peripheral neurons underlies *T. gondii*-induced hypoalgesia of the colon but that T cell-produced enkephalins play only a limited or redundant role in this phenotype. These observations raise the question of the nature of the endogenous opioid(s) involved and its cellular source(s). Visceral nociception results from the integration of both pro- and anti-nociceptive signals in the gut, which potentially involve different cell types and opioids, and which could also be modulated by changes in expression of the opioid receptors themselves and/or activity of their signaling pathways. Hence, the hypoalgesic phenotype could involve multiple mechanisms acting on one or several of these processes. As various cell types are able to produce different opioids [33,35,51], an increased opioid receptor activity could arise from a heightened opioid secretion not only by T cells, but also by macrophages, Tuft cells and/or neurons, possibly explaining the lack of effect of a single Penk deletion in T cells. We initially focused on enkephalins since Penk transcripts were the only detectable mRNA for endogenous opioids in the colon tissue (i.e. Pdyn transcripts coding for dynorphin and Pomc transcripts coding for proopiomelanocortin/β–endorphin were undetectable). Quantifying changes in bioactive opioid peptides in the colon microenvironment upon chronic *T. gondii* infection would be useful but the absence of reliable tools to perform this at the single-cell level represents a major roadblock preventing us from tackling this question. Alternatively, it is possible that other (non-opioid) molecules may be involved, provided that such molecules may be capable of modulating the opioid receptor signaling pathway. Of note, serotonin (5-hydroxytryptamine, 5-HT) is a pro-nociceptive mediator able to directly activate neurons [52]. In the GI tract, 5-HT is mainly produced by enterochromaffin cells (EC cells), a subset of enteroendocrine cells located within the epithelium. Importantly, EC cells transduce luminal signals to mucosal nerve endings, producing various effects including nociceptor sensitization leading to visceral hypersensitivity [53]. Moreover, several studies have reported alteration of serotonin metabolism in IBS patients [54,55], suggesting that serotonin signaling could alter pain perception in the gut. In line with these observations, a recent study highlighted a link between serotonin and opioid signaling since both pathways act on PKA activity, though in an opposite manner. More precisely, a decreased serotonin signaling was associated with an enhanced opioid receptor signaling, resulting in a prolonged endogenous analgesia [56]. Serotonin release by EC cells can be triggered by various stimuli, including short chain fatty acids produced by the gut microbiota or neurotransmitters [52], which may be dysregulated upon *T. gondii* chronic infection. Elucidating the potential implication of the serotoninergic pathway in *T. gondii*-induced hypoalgesia could be an interesting avenue for future investigation.

Based on the abundance of the microbiota in the colon and the pleiotropic effects exerted by gut microbiota on the host including the production of 5-HT [57] and the modulation of opioid receptor-mediated analgesia [58], another attractive hypothesis is that changes in visceral nociception caused by chronic *T. gondii* infection may arise from long-lasting alteration of gut commensals. To date, experimental studies have shown that acute *T. gondii* infection causes a clear dysbiosis in the colon, characterized in particular by an increase in *Enterobacteriacea* [59,60]. However, the extent of colon dysbiosis during chronic infection appears more variable, with studies reporting either no impact on gut microbiota [27,59] and studies showing mild modifications in the *Bacteroidetes* and *Firmicute* phyla [61,62]. While *T. gondii* chronic infection does not induce dramatic changes in the microbiota, we cannot rule out the possibility that subtle alterations in the abundance of some specific species might suffice to modulate visceral nociceptive responses in our mouse model. Notably, a study has highlighted the capacity of a particular bacterial species, *L. reuteri*, to upregulate opioid receptor expression, thereby preventing visceral hypersensitivity in a rat model of colon obstruction [58].

In conclusion, our work not only provides the first detailed mapping of immune changes that persist in the colon throughout latent infection, but it also uncovers a new and unexpected consequence of *T. gondii* chronic infection on host intestinal homeostasis. We report that *T. gondii* latent infection elicits a prolonged modification of the colonic T cell compartment as well as a decreased visceral nociceptive response to colorectal distension. This hypoalgesia depends on peripheral opioid receptor signaling but is independent from enkephalins produced by T cells, suggesting that other cell types and/or other opioids may be involved. These data support the notion that *T. gondii* is likely not a driver of post-infectious IBS, and they even suggest that latent *T. gondii* infection could be negatively associated with abdominal pain in humans.

Materials & Methods

Mice

C57BL/6 (B6) and CBA/J mice were purchased from Janvier (France). PenkT^{KO} mice were obtained by crossing Penkfl/fl mice with CD4-Cre mice (B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ from JAX, ref 022071). All non-commercial mouse models were housed and bred under specific pathogen-free conditions at the 'Centre Regional d'Exploration Fonctionnelle et de Ressources Experimentales' (CREFRE-Inserm UMS006). Mice were housed under a 12h light/dark cycle and were experimentally infected between 8 and 10 weeks of age. All mice used in experiments were males. Number of mice and experimental replicates are indicated in the respective figure legends. Animal care and used protocols were carried out under the control of the French National Veterinary Services and in accordance with the current European regulations (including EU Council Directive, 2010/63/EU, September 2010). The protocols APAFIS 20921-2019052311562282 and APAFIS 14513-2018040313435341v6 were approved by the local Ethical Committee for Animal Experimentation registered by the "Comité National de Réflexion Ethique sur l'Experimentation Animale" under no. CEEA122.

Toxoplasma gondii culture and experimental infections

Human Foreskin Fibroblasts (HFF) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with Glutamax, sodium pyruvate, 10% vol/vol FBS (Gibco), 1% vol/vol Penicillin/Streptomycin (Gibco) and 0.1% vol/vol 2-β-mercaptoethanol (Gibco). Pru-GFP.GRA6-OVA tachyzoites were grown *in vitro* on confluent HFF in DMEM supplemented with 1% vol/vol FBS (Gibco). *In vitro* cultured parasites were filtered through a 3 µm polycarbonate hydrophilic filter (it4ip S.A.) and diluted in sterile PBS before infection of mice with 200 tachyzoïtes. For cyst generation, CBA/J mice were infected by intraperitoneal injection with 200 tachyzoïtes. After 2 months, brains of infected CBA mice were collected and homogenized before performing a rhodamine-conjugated Dolichos Biflorus Agglutinin (Vector Laboratories) labelling. Cysts were counted using an inverted fluorescence microscope. After cyst enumeration, brain homogenate was diluted in sterile PBS to orally infect mice by gavage with 10 cysts.

Parasite load quantification (DNA extraction, qPCR)

Genomic DNA (gDNA) was extracted from tissue homogenate using the DNeasy Blood & Tissue Kit (Qiagen). All tissues analyzed were snap frozen in liquid nitrogen directly after isolation before storage at -80°C. For brain and colon, tissues were kept frozen in liquid nitrogen and crushed to obtain a fine powder used to isolate gDNA according to manufacturer's recommendations. gDNA was quantified using a Nanodrop and the number of parasite per µg of tissue DNA was calculated by qPCR using the TOX9/TOX11 primers and a standard curve made

of a known number of corresponding parasites (as described in [63]). The quantification threshold indicates the highest dilution of the standard curve, above which parasite concentration could be extrapolated.

Hematoxylin/Eosin (H/E) stainings

Colonic tissues were fixed for 24h in 4% formaldehyde and then transferred in 70% ethanol at 4°C. Tissues were embedded in paraffin to perform tissue sections (5 µm thickness) and Hematoxylin/Eosin stainings.

Isolation of colonic cells & PMA/ionomycin restimulation

After mouse euthanasia, the colon was collected, opened longitudinally and kept on ice in RPMI supplemented with 10% FBS. Tissue was cut into small pieces before incubation in RPMI supplemented with 5% FBS and 500mM EDTA to remove epithelial cells and intraepithelial lymphocytes (IEL) for 30 min at 4°C under agitation. Supernatant (containing IEL) was filtered through 40 µm cell strainer, washed once and kept on ice until Percoll gradient. The remaining tissue was then digested in RPMI supplemented with 10% FBS, 1 M HEPES, 220 U/mL Collagenase VIII (Sigma) and 10 µg/mL DNase I (Sigma) for 1 hour at 37°C under agitation. The supernatant was collected, filtered through a 70 µm cell strainer, washed and pooled with the IEL fraction, and kept on ice. The remaining tissue was digested a second time with the same digestion buffer for 50 min at 37°C under agitation. At the end, tissue was mechanically dissociated with a 18G needle attached to a 5 mL syringe. Cells were filtered through a 70 µm cell strainer before being pooled with the other fractions. Cells were then isolated using a 30% Percoll gradient diluted in RPMI at 4°C. Cell suspension was used directly for flow cytometry staining, or restimulated for 3.5 h at 37°C 5% CO_2 in complete RPMI supplemented 0.05 µg/mL Phorbol 12-myristate 13-acetate (PMA), 1 µg/mL ionomycin and 3 µg/mL Brefeldin A (BfA). Cells were then washed with FACS buffer (2% FBS and 2mM EDTA) before performing flow cytometry stainings.

Flow cytometry stainings & analysis

For flow cytometry, after Fc receptor saturation and dead cell detection with fixable viability dye, cells were surface stained for 30 min at 4°C. Cells were then washed and fixed using the Foxp3/transcription factor staining buffer set (Invitrogen) before performing intracellular staining to detect cytokines and transcription factors according to the manufacturer's recommendations. Flow cytometry analyses were performed using FlowJo and OMIQ (unsupervised analyses) softwares.

Antibodies used in this study

Antibody	Clone	Fluorochrome	Dilution	Reference	Provider
Anti-mouse CD16/62	93	N/A	50	101302	Biolegend
Fixable Viability Dye	N/A	eFluor780	1000	65-0865-14	eBioscience
CD3ε	145-2C11	BV711	50	100349	Biolegend
CD3ε	145-2C11	APC	100	100312	Biolegend
CD4	GK1.5	APC-Cy7	200	552051	BD Pharmingen
CD8α	53-6.7	BV786	300	563332	BD Horizon
CD11b	M1/70	PE-CF594	2000	562287	BD Horizon
CD11c	HL3	PerCP-Cy5.5	100	560584	BD Pharmingen
CD19	6D5	APC	100	115512	Biolegend
CD45	30-f11	PE	1000	103105	Biolegend
CD45.2	104	AF488	300	109815	Biolegend
CD49a	Ha31/8	BUV737	300	741776	BD OptiBuild
CD90.2	53-2.1	PE-Cy7	500	25-0902-82	eBioscience
CD103	2E7	PerCP-eFluor710	200	46-1031-82	eBioscience
CD117 (c-kit)	2B8	BV785	200	105841	Biolegend
EpCAM	G8.8	APC	500	118213	Biolegend
EpCAM	G8.8	PE-dazzle594	800	118235	Biolegend
F4/80	BM8	AF700	100	123130	Biolegend
FCεRI	MAR-1	APC-Cy7	200	134325	Biolegend
Foxp3	NRRF-30	PE	200	12-4771-82	eBioscience
Gata3	TWAJ	AF488	100	53-9966-42	eBioscience
Granzyme B	QA16A02	PE-Cy7	200	372214	Biolegend
IFNγ	XMG1.2	BV421	200	563376	BD Horizon
IL17	TC11-18H10	AF700	200	560820	BD Pharmingen
Ly6C	HK1.4	BV711	2000	128037	Biolegend
Ly6G	1A8	BV510	200	127633	Biolegend
NK1.1	PK136	BV650	100	564143	BD Horizon
RORγt	Q31-378	BV421	200	562894	BD Horizon
TCRγδ	GL3	BV510	200	118131	Biolegend
CD8a	53-6.7	BV421	200	563898	BD Horizon
CD44	IM7	BV605	200	563058	BD Horizon
CD4	RM4-5	PE-CF594	800	562285	BD Horizon
CD62L	MEL-14	PE-Cy7	300	560516	BD Pharmingen
Penk probes	N/A	AF647	20	PF-204	Invitrogen

Table 1. List of antibodies and conditions used in flow cytometry experiments.

FlowFISH stainings

Spleen from $Penk^{flox/flox}$ / $CD4^{Cre}$ mice was smashed on a 100 µm cell strainer (Falcon) before performing red blood cell lysis. Splenocytes were then stained with the PrimeFlow RNA Assay Kit (Invitrogen) and the anti-mouse Penk probe set (Invitrogen #PF-204) according to the manufacturer's protocol. Briefly, cells were first labelled with antibodies as described above with the reagent provided in the kit. A second fixation step was performed after the intracellular staining before proceeding to the FISH staining. For FISH staining, cells were incubated with the anti-mouse Penk probe set (1:20) for 2h at 40°C. After washes, cells were kept overnight at 4°C in wash buffer containing RNase inhibitors. The day after, amplification steps were performed to increase the signal: cells were first incubated with pre-amplification mix during 1.5 h at 40°C, washed and then incubated with amplification mix for an additional 1.5 h at 40°C. Cells were then incubated with the label probes (Alexa Fluor 647, 1:100) for 1h at 40°C.

Colorectal distension and visceromotor response measurements

Visceral nociception was assessed by measuring the visceromotor response (VMR) to increasing colorectal distension pressures (15, 30, 45, 60 mm Hg) with at least 5 min rest between each distension pressure. Three days before distension, mice were anaesthetized with ketamine (10 mg/kg) and xylazine (1 mg/kg) to implant 2 electrodes (Bioflex insulated wire AS631; Cooner Wire, Chatsworth, CA) into abdominal external oblique muscles. Electrodes were exteriorized at the back of the neck and protected by a plastic tube attached to the skin. After surgery, mice were monitored during the next 3 days for abnormal behavior. The day of the distension, electrodes were connected to a Bio Amp, itself connected to an electromyogram acquisition system (ADInstriments, Inc., Colorado Springs, CO). Ten (10) second-long distensions were performed on conscious animals with a 10.5 mm diameter balloon catheter (Fogarty catheter for arterial embolectomy) gently inserted into the distal colon at 5 mm from the anus (balloon covering a distance of 1cm). Electromyography activity of abdominal muscles was recorded to calculate visceromotor responses using LabChart 8 software (ADInstruments).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 10.1 software. For all panels, normality was assessed with D'Agostino & Pearson tests. When normal, a parametric test was applied as indicated in the figure legend. If not, a non-parametric analysis was performed. * or #: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$. Modalities of data representation and statistical tests are indicated in the respective figure legends.

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CONTRIBUTIONS

AA, FM, NC, GD, CB, NB designed experiments. AA, XMO, LR, MB, EB, NC, CB performed experiments. AA, XMO, LR, MB, EB, FM, NC, GD, CB, NB analyzed experiments. GM contributed mice and expert advice on the Penkfl/fl mouse model. FM provided the CD4-Cre model and expert consulting. AA, NB & CB wrote the manuscript. All co-authors read, edited, and approved the final manuscript.

ETHICAL DECLARATIONS

The authors declare no competing interests.

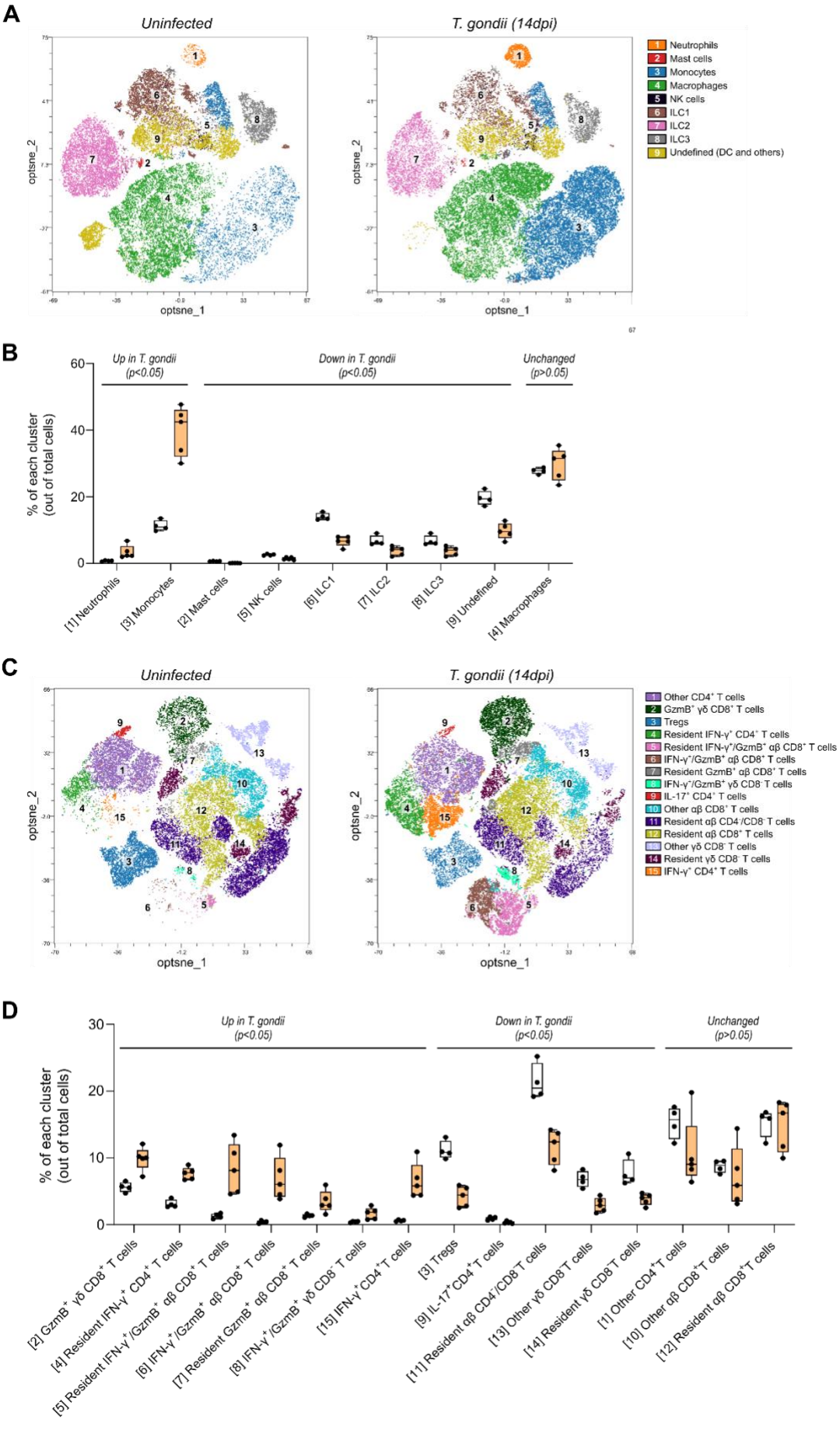


Figure S1: Acute *T. gondii* infection induces recruitment of myeloid cells and T cells in the colon

(**A and B**) Unsupervised analysis with flow cytometry data obtained from the colon of non-infected or ip-infected mice at acute stage (14 days post-infection) was performed using OMIQ software. Statistical analysis was performed using a Mann-Whitney test on each subpopulation and summarized into one single graph. (**A**) Unsupervised analysis was performed on live $CD45^+$ cells negative for CD3ε, CD19 and EpCAM (called Lin-negative cells). Opt-SNE plot of 34 000 Lin-negative cells concatenated from 4 non-infected mice (left) and 42 500 Lin-negative cells concatenated from 5 infected mice (right), divided in 9 clusters using Phenograph algorithm, based on the expression of surface markers associated with each cell subset. Table under opt-sne shows the relative abundance of each cluster in each condition (infected (orange) or not (white)). (**B**) Unsupervised analysis was performed on live T cells using the following gating strategy: $EpCAM^-$ / $CD45^+$ / $CD3^+$. Opt-SNE plot of 36 360 colonic T cells concatenated from 4 non-infected mice (left) and 45 450 colonic T cells concatenated from 5 infected mice (right), divided in 15 clusters using Phenograph algorithm, based on the expression of different markers. Table under opt-sne shows the relative abundance of each cluster in each condition (infected (orange) or not (white)).

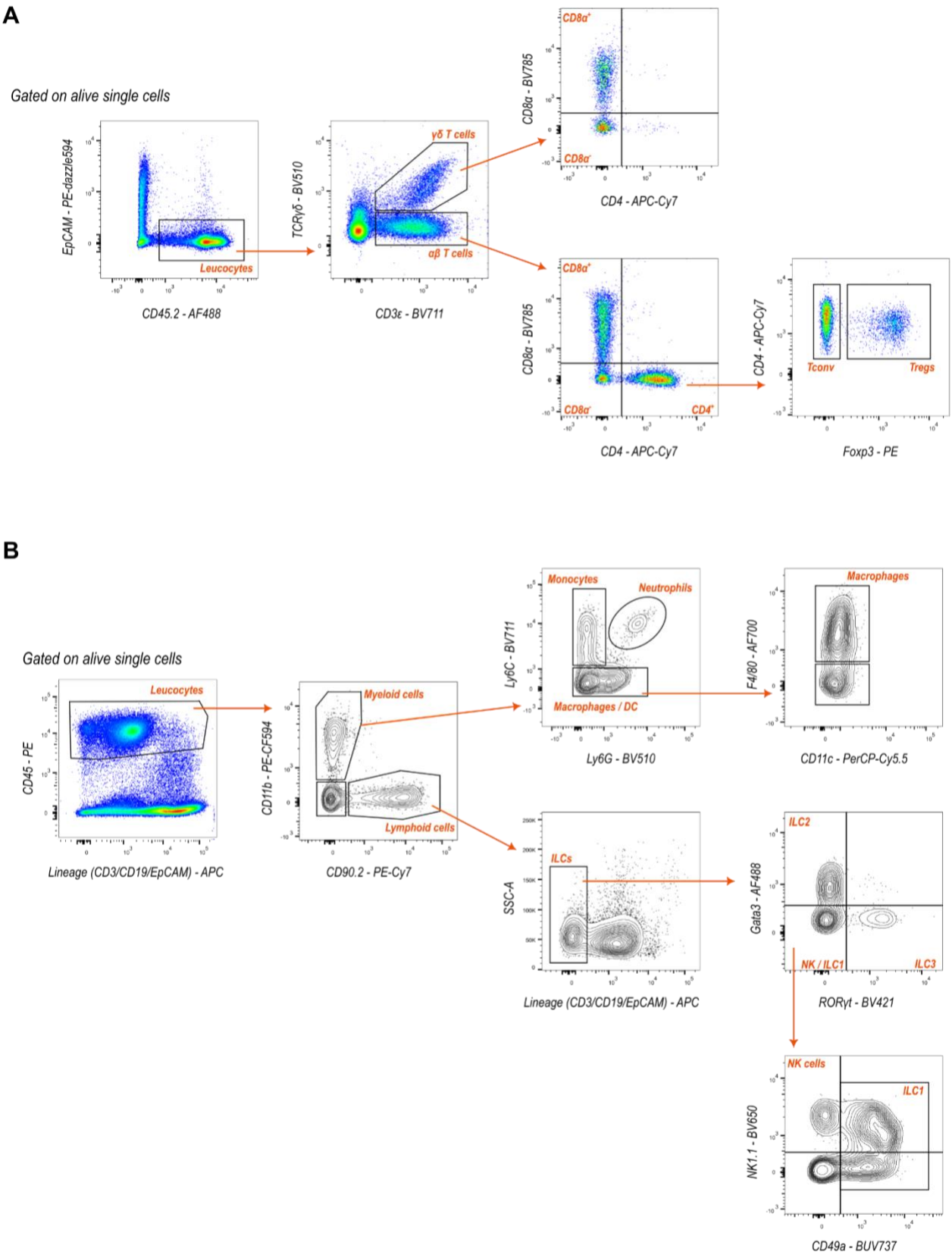
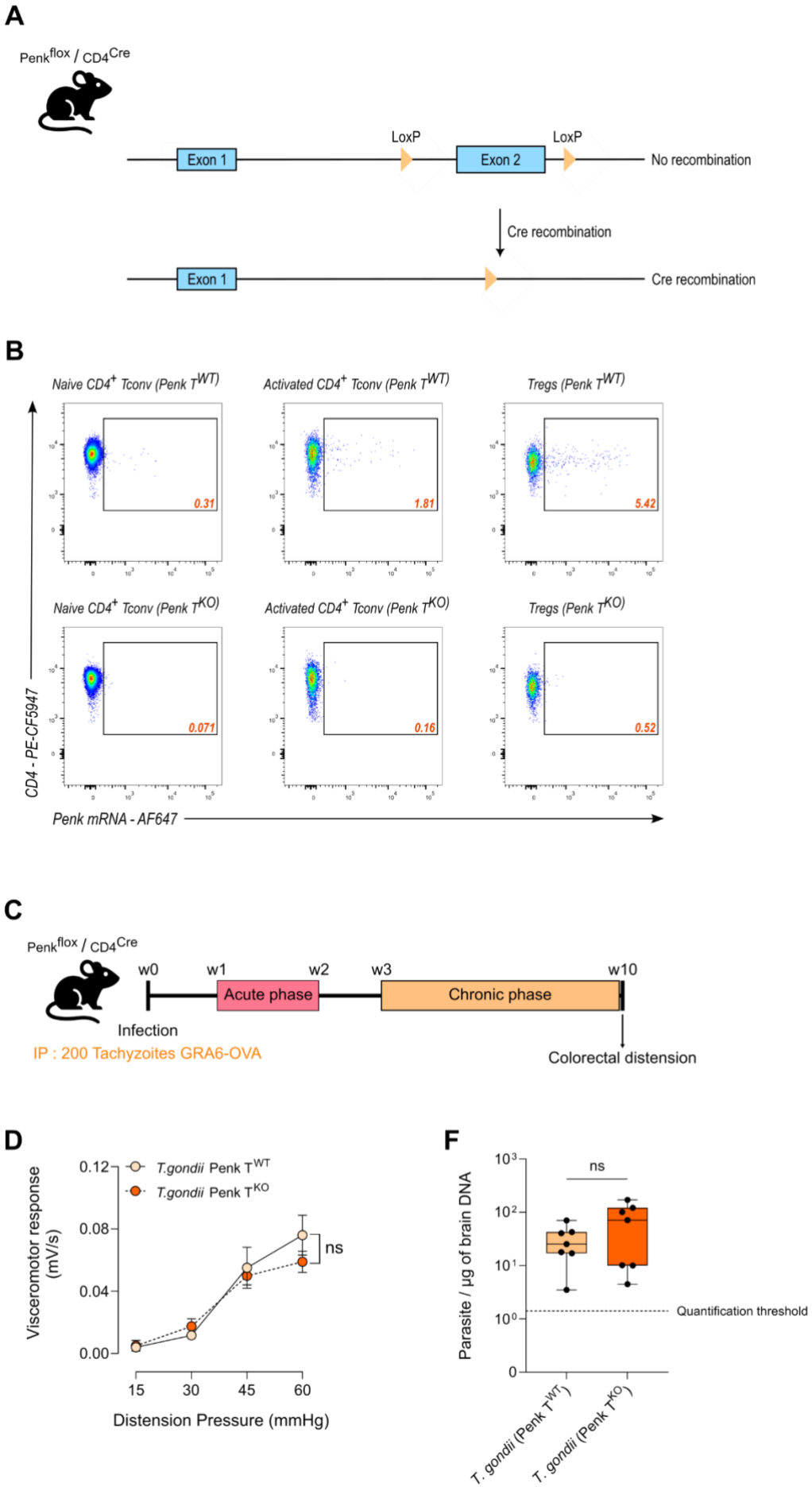


Figure S2 (related to Figure 2): Gating strategies used to identify the immune populations in the colon

(**A and B**) Cell doublets were excluded using double gating on SSC-A vs. SSC-H followed by SSC-W vs. SSC-H exclusion. Dead cells were removed using a viability marker to allow analysis of live cells only. Arrows indicate the order of the gating strategy. (**A**) Gating strategy used to identify the different T cell subsets in the colon. (**B**) Gating strategy used to identify the different cell subsets belonging to the myeloid and Innate Lymphoid Cells (ILC) lineages.


A
Penk flox / CD4 Cre
LoxP
LoxP
Exon 1
Exon 2
No recombination
Cre recombination
Exon 1
Cre recombination
B
Naive CD4+ Tconv (Penk TWT)
Activated CD4+ Tconv (Penk TWT)
Tregs (Penk TWT)
0.31
1.81
5.42
Naive CD4+ Tconv (Penk TKO)
Activated CD4+ Tconv (Penk TKO)
Tregs (Penk TKO)
0.071
0.16
0.52
CD4 - PE-CF5947
Penk mRNA - AF647
C
Penk flox / CD4 Cre
w0
w1
w2
w3
w10
Acute phase
Chronic phase
Infection
IP : 200 Tachyzoites GRA6-OVA
Colorectal distension
D
Visceromotor response (mV/s)
0.12
0.08
0.04
0.00
T.gondii Penk TWT
T.gondii Penk TKO
ns
15
30
45
60
Distension Pressure (mmHg)
F
Parasite / µg of brain DNA
10 3
10 2
10 1
10 0
0
ns
Quantification threshold
T. gondii (Penk TWT)
T. gondii (Penk TKO)

508

Figure S3 (related to Figure 4): *T. gondii*-induced decrease in nociceptive responses is independent of T-cell derived enkephalins

(A) Mouse model for T cell-specific deletion of enkephalin-encoding gene (Penk) in mice. Penk-floxed mice carrying LoxP sites upstream and downstream of Penk exon 2 were crossed with transgenic mice expressing the Cre recombinase under the CD4 promoter (CD4-Cre). **(B)** Genomic DNA was extracted from CD4+ T cells sorted from the spleen of CD4-Cre positive (Penk T^{KO}) or negative (Penk T^{WT}) mice. Deletion of Penk exon 2 was assessed by qPCR using 2 sets of primers to amplify the exons 1 and 2. The set of primer used in each condition is indicated in blue and primers location is represented in panel A. **(C)** Deletion of Penk was confirmed by measuring the expression of Penk mRNA by flow cytometry (FlowFISH) in naïve (CD62L+/CD44-) or activated (CD44+) conventional (FoxP3-) CD4+ T cells, and in regulatory CD4+ T cells (Foxp3+). **(D)** Experimental workflow: C57BL/6 expressing (Penk T^{WT}) or not (Penk T^{KO}) in T cells were infected with the Pru.GFP.GRA6-OVA *T. gondii* by intraperitoneal injection with 200 tachyzoïtes. At 10 weeks post-infection, colorectal distension was performed to assess visceral sensitivity. **(E)** Visceromotor response (VMR) to increasing colorectal distension pressure (15 to 60 mm Hg) was measured in chronically ip-infected mice invalidated (Penk T^{KO}) or not (Penk T^{WT}) for Penk gene in T cells. VMR are represented with mean +/- SEM with n = 7 Penk T^{WT} vs 6 Penk T^{KO} animals. Statistical analysis was performed on Areas Under the Curve (AUC) with a Mann-Whitney test using GraphPad Prism. Data correspond to one experiment. **(F)** Parasite loads in the brain were measured by qPCR on genomic DNA for each mouse, showing no difference between Penk T^{WT} and Penk T^{KO} infected mice.

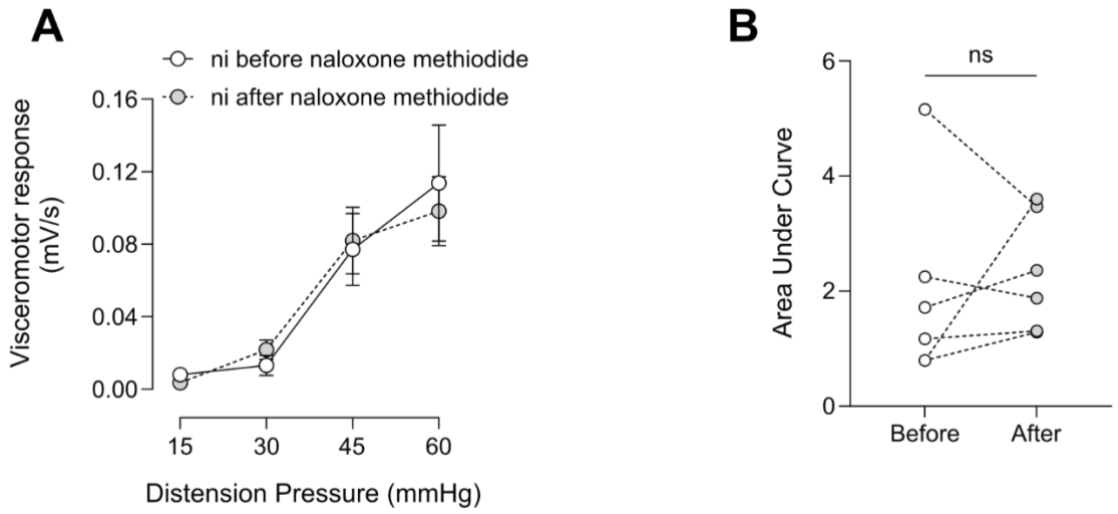


Figure S4 (related to Figure 4) : Naloxone methiodide treatment has no impact on visceral nociceptive responses at steady state

(A and B) Colorectal distension was performed to assess visceral sensitivity of each mice before (in white) or 30 min after (in gray) intraperitoneal injection of naloxone-methiodide. **(A)** Visceromotor response (VMR) in response to increasing colorectal distension pressure (15 to 60mmHg) was measured in uninfected mice (ni) treated or not with Naloxone methiodide. Data are shown as mean +/- SEM and are from 1 experiment representative with n = 6 mice. **(B)** Area Under the Curve (AUC) are represented for each mouse with 2 dots corresponding to the AUC before (in white) and after (in gray) naloxone methiodide treatment. Statistical analysis was performed using a Wilcoxon's test.

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