Immune response to *Toxoplasma gondii* at the choroid plexus and the immunomodulatory impact of the neuropeptide PACAP

Thesis

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PREAMBLE

The work presented here is a cumulative dissertation based on the following scientific publications of the author:

Publication 1

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Publication 2

FIGUEIREDO, C.A., Dusedau, H.P., Steffen, J., Gupta, N., Dunay, M.P., Toth, G.K., Reglodi, D., Heimesaat, M.M., and Dunay, I.R. (2019). *Immunomodulatory Effects of the Neuropeptide Pituitary Adenylate Cyclase-Activating Polypeptide in Acute Toxoplasmosis*. Front Cell Infect Microbiol *9*, 154.
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ABSTRACT

Toxoplasma gondii (*T. gondii*) is a highly successful parasite able to cross all biological barriers of the body. The parasites invade through the intestinal epithelium the body, where they cause acute infection, thereafter they cross the borders of the central nervous system (CNS), entering the brain and inducing neuroinflammation.

Previous studies have described the critical involvement of the CNS borders during *T. gondii* invasion and the development of neuroinflammation. While many studies have focused on the blood-brain barrier (BBB), the contribution of the choroid plexus (CP), the main structure forming the blood-cerebrospinal fluid (CSF)-barrier (BCSFB) has remained largely unknown. In this present study, confocal imaging of immuno-stained brain sections, tissue whole mount preparations, and determination of *T. gondii* *g*DNA levels were applied to reveal that CP is infected by *T. gondii*. In addition, flow cytometric and gene expression analysis showed that CP elicits an immune response prior to the BBB. Moreover, imaging and gene expression of tight junctions (TJ) in CP and primary cultured CP epithelial cells indicate a loss of function and integrity of the BCSFB, which was confirmed by *in vivo* FITC-dextran permeability assay **[Publication 1]**.

Generally, albeit the elicited immune response during infection is essential for parasite control, continuous activation and recruitment of immune cells to infected organs entail tissue damage and cause detrimental alterations to their function. To mitigate these alterations, this study additionally set out to investigate the effects of the neuropeptide PACAP upon acute **[Publication 2]** and chronic *T. gondii* infection **[Publication 3]**, as PACAP has shown immunomodulatory and neuroprotective properties in other disease models. Here, the infection of mice with GFP-reporter parasites indicate that PACAP displayed anti-parasitic activity, and further *in vitro* assays showed that this is an immune cell-mediated effect rather than a direct elimination of the parasites. Overall, flow cytometric and gene expression analysis showed that PACAP treatment reduced immune cell infiltration, activation, and cytokine production in the periphery and in the CNS. Additionally, a cross-talk between PACAP and neurotrophin signaling pathways was suggested to affect the immune as well as the nervous systems, and to promote neuroprotection during *T. gondii* infection.

Altogether, those findings reveal a close interaction between *T. gondii* infection at the CP and the impairment of the BCSFB function, indicating that infection-related neuroinflammation is initiated in the CP. Moreover, this study highlights the beneficial effects of PACAP application upon acute and chronic toxoplasmosis, providing new insights into the potential use of neuropeptides to counteract infection-induced inflammation.

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PUBLICATIONS

The following scientific publications were achieved during the doctoral studies of the author:

1. **FIGUEIREDO, C.A.,** Düsedau, H.P., Steffen, J., Ehrentraut, S., Dunay, M.P., Toth, G.K., Reglodi, D., Heimesaat, M.M., Dunay, I.R. (2022). *Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) alleviates T. gondii infection-induced neuroinflammation and neuronal impairment.* J Neuroinflammation 19, 274. DOI: 10.1186/s12974-022-02639-z

2. **FIGUEIREDO, C.A.,** Dunay, I.R. *IFN-γ-mediated neuronal defense mechanism targets Toxoplasma.* (2022) Trends in Parasitology. DOI: 10.1016/j.pt.2022.10.001

3. Steffen, J., Ehrentraut, S., Bank, B., Biswas, A., **FIGUEIREDO**, C.A., Düsedau, H.P., Holsken, O., Dovhan, V., Knop, L., Thode, J., Romero-Suarez, S., Infante-Duarte, C., Gigley, J., Romagnani, C., Diefenbach, A., Klose, C.S.N., Schuler, T., Dunay, I.R. (2022) *Type 1 innate lymphoid cells regulate the onset of Toxoplasma gondii-induced neuroinflammation*. Cell Rep. DOI: 10.1016/j.celrep.2022.110564

4. **FIGUEIREDO, C.A**., Steffen, J., Morton, L., Arumugam, S., Liesenfeld, O., Deli, M.A., Kroger, A., Schuler, T., and Dunay, I.R. (2022). *Immune response and pathogen invasion at the choroid plexus in the onset of cerebral toxoplasmosis*. J Neuroinflammation *19*, 17. DOI: 10.1186/s12974-021-02370-1

5. Düsedau, H.P., Steffen, J., **FIGUEIREDO, C.A**., Boehme, J.D., Schultz, K., Erck, C., Korte, M., Faber-Zuschratter, H., Smalla, K.H., Dieterich, D., et al. (2021). *Influenza A Virus (H1N1) Infection Induces Microglial Activation and Temporal Dysbalance in Glutamatergic Synaptic Transmission*. mBio 12, e0177621. DOI: 10.1128/mBio.01776-21

6. Romero-Suarez, S., Del Rio Serrato, A., Bueno, R.J., Brunotte-Strecker, D., Stehle, C., **FIGUEIREDO, C.A.**, Hertwig, L., Dunay, I.R., Romagnani, C., and Infante-Duarte, C. (2019). *The Central Nervous System Contains ILC1s That Differ From NK Cells in the Response to Inflammation.* Front Immunol *10*, 2337. DOI: 10.3389/fimmu.2019.02337

7. **FIGUEIREDO, C.A.,** Düsedau, H.P., Steffen, J., Gupta, N., Dunay, M.P., Toth, G.K., Reglodi, D., Heimesaat, M.M., and Dunay, I.R. (2019). *Immunomodulatory Effects of the Neuropeptide Pituitary Adenylate Cyclase-Activating Polypeptide in Acute Toxoplasmosis*. Front Cell Infect Microbiol *9*, 154. DOI: 10.3389/fcimb.2019.00154

8. Düsedau, H.P., Kleveman, J., **FIGUEIREDO, C.A.,** Biswas, A., Steffen, J., Kliche, S., Haak, S., Zagrebelsky, M., Korte, M., and Dunay, I.R. (2019). *p75(^{NTR}) regulates brain mononuclear cell function and neuronal structure in Toxoplasma infection-induced neuroinflammation.* Glia *67*, 193-211. DOI: 10.1002/glia.23553

INTRODUCTION

Toxoplasma gondii infection

The protozoan parasite *Toxoplasma gondii* was first described and titled in 1908 by Nicolle and Manceaux as a bow (Greek: toxon)-shaped (Greek: plasma) parasite in a gundi, a North African rodent (*Ctenodactylus gundi*), and at the same time discovered by Splendore in Brazil (Dubey, 1996). It is an obligate intracellular parasite and belong to phylum *Apicomplexa* > class *Conoidasida* > subclass *Coccidia* > order *Eucoccidiorida* > suborder *Eimeriorina* > family *Sarcocystidae* > genus *Toxoplasma*. It is the only specie of its family, although shares the Apicomplexa phylum with parasites of medical and veterinary importance e.g., *Plasmodium, Eimeria*, *Isospora, Cyclospora*, *Babesia*, *Cryptosporidium*, *Theileria*, *Sarcocystis* and *Hammondia*. The infection by *T. gondii* is determined by three parasite stages with crescent-shaped forms and approximately 5µm long and 2µm wide: a fast dividing invasive form called tachyzoite, a slowly dividing form called bradyzoite present in tissue cysts, and the sporozoite, an environmental stage protected inside an oocyst (Robert-Gangneux and Darde, 2012).

The infection caused by *T. gondii*, namely toxoplasmosis, is a worldwide zoonotic disease with approximately 30% of the world's human population chronically infected (Pappas et al., 2009). It is present with especially high seroprevalence rates in Latin America (50 to 80%) and in tropical African countries (Robert-Gangneux and Darde, 2012). The transmission incidence in the human population is mostly linked to low hygiene and culinary habits since the infection mainly occurs via ingestion of oocysts shed by felines in contaminating water, food or dust, and consumption of meat products infected with viable tissue cysts from infected intermediate hosts, including livestock animals (Jones and Dubey, 2012; VanWormer et al., 2013).

In immunocompetent humans, *T. gondii* infection usually is asymptomatic or causes only flu-like mild symptoms, rarely requiring medical intervention (Joynson and Wreghitt, 2001). Tachyzoites are rapidly suppressed by the healthy immune system, consequently converting into resistant bradyzoites and persisting within long-lived tissue cysts in muscles and the CNS (Montoya and Liesenfeld, 2004). In immunocompromised individuals, e.g., AIDS patients or immunosuppressed transplanted patients, tissue cysts can reactivate and lead to toxoplasmic encephalitis (TE), which is fatal if left untreated (Dunay et al., 2018; Luft and Remington, 1992). Still, immunocompetent adults can manifest clinical diseases, mainly ocular toxoplasmosis, one of the most frequently identified causes of uveitis (Weiss and Dubey, 2009). Studies have shown that toxoplasmosis is one of the most damaging zoonotic diseases, leading to the loss of 2-8 million disability-adjusted life years (DALYs) (Smith et al., 2021; Torgerson and Macpherson, 2011), which is a time-based measure of overall disease burden used by the World Health Organization (WHO).

Inflammation and immune response to *T. gondii* infection

The infection cycle takes place first within the intestine, after the ingestion of tissue cysts or oocysts. First, the digestion by gastric enzymes releases infecting forms of parasites in the stomach of the intermediate host, penetrating the enterocytes, and differentiating into tachyzoites. This fast-replicating form infects and replicates, potentially, inside any kind of nucleate cell (Robert-Gangneux and Darde, 2012).

After oral ingestion, parasites reach and transmigrate the intestinal epithelial barrier composed of the mucus layer, the glycocalyx of the enterocytes, and the tight junctions (TJs) between intestinal epithelial cells (Delgado Betancourt et al., 2019). The transmigration mechanisms are not fully elucidated, although studies in rodent models have proposed three different pathways: 1) paracellular transmigration, in which parasites move through intercellular junctions without disturbing barrier integrity; 2) direct infection of enterocytes, followed by intracellular replication and cellular burst; 3) Trojan horse mechanism in which immune cells are infected and molecularly hijacked by the parasite to facilitate invasion and spread of infection throughout the organism (Delgado Betancourt et al., 2019). Once established in the small intestine, parasites undergo several asexual replicative rounds inside the enterocytes. To survive intracellularly, *T. gondii* resides inside of a membranous structure called parasitophorous vacuole (PV), which is essential to create an intracellular microenvironment that allows the sequestration of host nutrients and efficient proliferation. Once disrupted, the parasite can no longer survive inside of the host cell (Kim et al., 2012), and therefore targeting the PV is the main strategy used by host cells to eliminate intracellular parasites.

While the number of parasites expands, the infection reaches the underneath lamina propria and Peyer's patches (intestinal lymphoid tissue), encountering resident immune cells such as macrophages, dendritic cells (DCs), and innate lymphoid cells (ILCs), as well as T and B cells **Figure 1** (Sasai and Yamamoto, 2019). At first, macrophages and DCs directly phagocytose opsonized parasites and apoptotic infected cells. Additionally, the direct anti-parasitic response is simultaneously followed by cellular recognition of parasite by-products (e.g., proteins and nucleic acids) and tissue damage molecules from infected cells. Accordingly, the profiling-like proteins of *T. gondii* are recognized by Toll-like receptors (TLRs)-11 and TLR12 and induce the production of interleukin (IL)-12 and IL-6 by macrophages and DCs (Koblansky et al., 2013). Of note, although critical to *T. gondii* response in mice, in humans no TLRs that recognize profilin-like proteins have been found (von Bernuth et al., 2008). For instance, human TLR7 and TLR9 have been shown to recognize the RNA and DNA from *T. gondii*, and TLR2 and TLR4 seem to recognize parasitic glycosylphosphatidylinositols (GPI) and induce the production of tumor necrosis factor (TNF) by macrophages (Andrade et al., 2013; Debierre-Grockiego et al., 2007). Currently, it is suggested that in humans multiple other pathways are involved in the recognition of *T. gondii* and initial cytokine production.

Further on, this recognition of local infection triggers an amplified immune cell activation, which includes high production of chemokines (e.g., C-C motif chemokine ligand 2 (CCL2) and C-X-C motif chemokine 2 (CXCL2)) and further recruitment of $Ly6C^{hi}$ inflammatory monocytes, $Ly6G^{+}$ neutrophils, granulocytes, NKs and DCs to the infected villi of the small intestine (Dunay et al., 2008; Muller and Randolph, 1999). Together, both resident and recruited cells produce a pool of local proinflammatory cytokines (TNF, IL-1β, IL-6, IL-12, and interferon-gamma (IFN-γ)), orchestrating parasite clearance and the subsequent adaptive immune response by B and T cells. During the initial infection, the production of IL-12 has been shown to be crucial to the anti-parasitic response (Gazzinelli et al., 1994; Khan et al., 1994). Mainly secreted by macrophages and DCs, IL-12 induces the proliferation of NK cells and T cells ($CD4^{+}$ and $CD8^{+}$), activation of their cytotoxic activity, and large production of IFN-γ. IFN-γ is the ultimate mediator of host resistance to *T. gondii* (Gazzinelli et al., 1993; Suzuki et al., 1988). During infection, important initial IFN-γ producers are T cells, NK cells, type 1 ILCs (ILC1s), and neutrophils (Biswas et al., 2017; Lopez-Yglesias et al., 2021; Steffen et al., 2022).

The key role of IFN-γ in the control of *T. gondii* infection relies on its downstream molecular signaling involving the stimulation of hundreds of genes that orchestrate the proliferation and differentiation of immune cells, and the overall cell-autonomous immune response (Pittman and Knoll, 2015). In macrophages, IFN-γ promotes the intensive production of antimicrobial reactive oxygen species (ROS), and nitric oxide (NO) via the inducible NO synthase (iNOS) which is also expressed by $Ly6C^{hi}$ inflammatory monocytes (Green et al., 1990). The intensive and continuous production of ROS and NO eliminate parasites, but eventually cause massive tissue destruction (Dupont et al., 2012).

Moreover, IFN-γ crucial importance is the stimulation of genes involved in cell-autonomous immune response, a cell-intrinsic defense system present in hematopoietic and non-hematopoietic cells. Two group members of this system are particularly important for the control of intracellular parasite replication: the immunity-related GTPases (p47GTPases, also called IRGs) and the guanylate binding proteins (p65GTPases, also called GBPs). In the group of IRGs, immunity-related GTPase family M member-1 (IRGM1) and IRGM3 (also called IFN-γ-induced GTPase (IGTP)) coordinate the active disruption of the PV in infected cells, and their genetic ablation in mice caused the high inability to control *T. gondii* infection (Henry et al., 2009). Furthermore, GBP1,2,3,5 and 7 have also been shown to be involved in PV disruption, as genetic ablation of the entire cluster of GBPs resulted in intact PVs and increased parasite burden (Yamamoto et al., 2012). Nonetheless, *T. gondii* has evolved to partially escape cell-autonomous responses by secreting proteins e.g., rhoptry bulb protein (ROP)-5, -18 to interfere with PV targeting and destruction by IRGs and GBPs **Figure 1** (Delgado Betancourt et al., 2019).

While infection further expands locally, the adaptive immune response mediated by $CD4^{+}$ and $CD8^{+}$ T cells is initiated. These cells can directly recognize different parasite secretory antigens and will encounter innate immune cells locally or upon traffic to lymphoid tissues, to perform antigen presentation and clonal expansion (Sturge and Yarovinsky, 2014; Tsitsiklis et al., 2019). After the acute phase of infection, $CD4^{+}$ and $CD8^{+}$ T cells become the main producers of IFN-γ. Of note, in the C57BL/6 mouse

model of infection, IFN-γ is predominantly released by $CD4^+$ rather than $CD8^+$ T cells (Sturge and Yarovinsky, 2014). Although crucial for parasite control, high local levels of IFN-γ, TNF and NO cause an intense inflammatory damage of the intestine, which characterizes a distinct *T. gondii* immunopathology in mice, and has established *T. gondii* as an important model pathogen for the study of Th1 immunity (Burger et al., 2018). Moreover, apart from IFN-γ production, $CD8^+$ T cells exert cytotoxic activity to specifically kill infected cells via MHC-I recognition, and this process is highly dependent on the genetic background of the MHC-I haplotype of the host (Suzuki et al., 1994; Suzuki et al., 2010).

Finally, an intensive immune response to eliminate *T. gondii* leads to an overall reduction of parasite burden but eventually imposes parasitic mechanisms of escape and survival. One of them is the hijacking of migratory immune cells present at the site of infection. It has been shown that these infected cells develop a hypermigratory behavior contributing to parasite spread into immunologically restricted regions, such as the CNS (Lambert et al., 2006; Ueno et al., 2015). Additionally, fast-replicating tachyzoites convert to bradyzoites, the resistant parasite form which persists in tissue cysts and characterizes the establishment of chronic infection. At this point, the parasite can already be found invading the CNS.

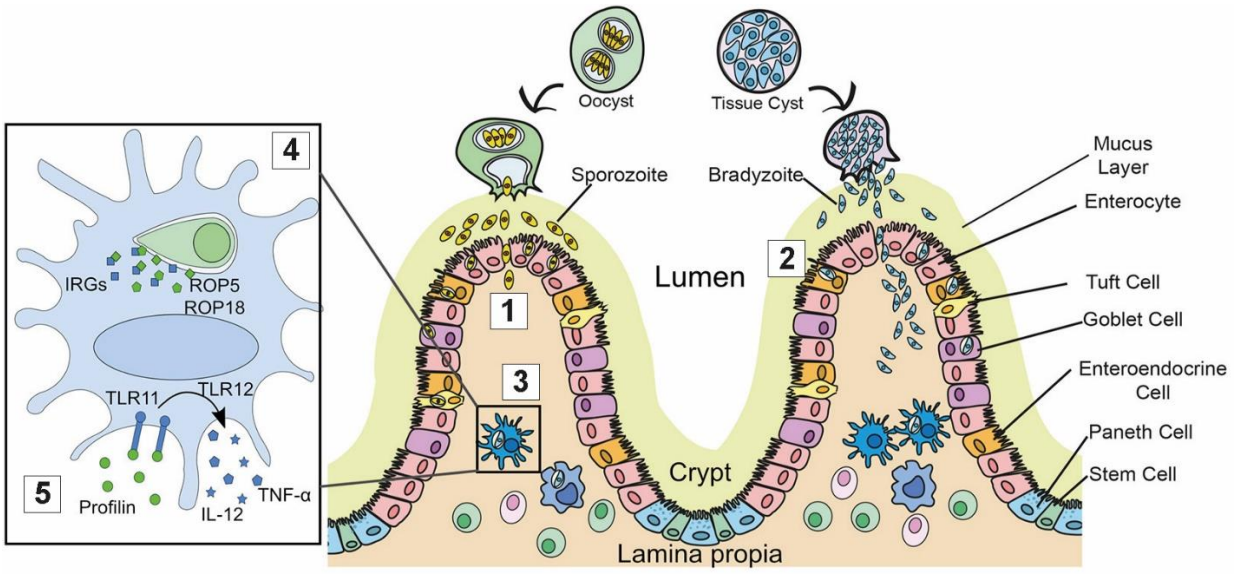

Figure 1. Intestinal *T. gondii* invasion and initial immune response.
Parasites reach the small intestine and transpose the intestinal epithelium to reach the lamina propria (**1**) via paracellular transmigration, (**2**) by infection of enterocytes and cell lysis, and (**3**) by hijacking immune cells. Once in the lamina propria, parasites can further bolster their spread by hijacking immune cells via (**4**) intracellular secretion of ROP5/18 and consequent inhibition of cell-autonomous defense. Still, parasitic secreted antigens such as profilin (**5**) can be detected via TLR11/12 inducing the release of IL-12 and TNF, for example, by macrophages. Adapted from Delgado Betancourt et al., 2019

Cerebral infection and neuroinflammation

The acute phase is followed by the latent chronic stage, where the encysted parasites hide from the immune system in immune-privileged sites such as the CNS. The cysts remain during the lifetime of the host within infected neurons, potentially leading to altered neuronal function that contributes to behavioral and neuropsychiatric diseases. Importantly, in immunocompromised patients, the latent parasite cysts are the cause of reactivated disease that progresses to uncontrolled parasite replication, immune cell infiltration in the brain, and lethal TE when left untreated (Montoya and Liesenfeld, 2004; Munoz et al., 2011; Parlog et al., 2015). TE or *T. gondii*-infection-induced neuroinflammation has been studied in C57BL/6 mouse genetic background, which have a different allele in the MHC-I locus ($H\text{-}2^b$ haplotype) (Suzuki et al., 1994). For this reason, those animals develop a lethal chronic progressive neuroinflammation, with high parasite loads and extensive inflammation (Brown and McLeod, 1990; Schluter and Barragan, 2019).

Once parasites reach the brain by crossing the CNS barriers, they potentially invade all cell types encountered. In this context, *T. gondii* has been shown to readily invade murine cerebral endothelial cells, astrocytes, and microglial cells in *in vivo* and *in vitro* setups (Forrester et al., 2018). However, neurons are the preferred host cells in the CNS, as they show extensive interaction with parasites, and moreover shown to harbor tissue cysts (Cabral et al., 2016). This parasite predilection for neurons still remains poorly understood, but several studies suggest that *T. gondii* persists in neurons because these cells are unable to clear intracellular parasites. One of the studies supporting this hypothesis observed a lack of MHC-I expression by infected neurons, which were not recognized by cytotoxic $CD8^+$ T cells (Salvioni et al., 2019). In contrast, a recent study showed that neurons are able to elicit cell-autonomous defense upon IFN-γ exposure, a process considered inexistent before in those cells, allowing partial intracellular elimination of *T. gondii* (see **Figure 2**) (Chandrasekaran et al., 2022; Figueiredo and Dunay, 2022).

The infection of hematopoietic and non-hematopoietic cells of the CNS triggers a local pro-inflammatory immune response with the release of TNF, IL-1β, IL-6, and NO by activated resident microglia and astrocytes (Blanchard et al., 2015; Fischer et al., 1997). Early activation and accumulation of resident leukocytes, i.e., ILC1s, in the brain contributes to the initial production and release of IFN-γ (Steffen et al., 2022). Additionally, a rapidly invaded neutrophil subset has shown to be another early source of IFN-γ in the brain (Biswas et al., 2017). Consequently, IFN-γ signaling activates cell-autonomous mechanisms of intracellular parasite elimination and stimulates intense overall production of chemokines such as CXCL9 and CXCL10 (Bhandage et al., 2019). These chemokines, in addition to astrocyte-produced CCL2, orchestrate the recruitment of peripheral $Ly6G^+$ neutrophils, $Ly6C^{hi}$ inflammatory monocytes, NK cells, DCs, $CD4^+$ and $CD8^+$ T cells to the brain (Matta et al., 2021). Particularly, $Ly6C^{hi}$ inflammatory monocytes have been shown to be crucial to the control of cerebral toxoplasmosis, as they produce IL-1α, IL-1β, IL-6, TNF, and anti-parasitic ROS and NO (Biswas et al., 2015). Their recruitment into the brain was shown to be dependent on the expression of adhesion molecules, particularly P-selectin glycoprotein ligand 1 (PSGL-1), and on the expression of the C-C motif chemokine receptor type 2 (CCR2) which recognizes brain levels of CCL2 (Benevides et al., 2008; Biswas et al., 2015). Moreover, the spatiotemporal

analysis revealed that monocytes accumulated within the vascular lumen before entering the parenchyma, and were found next to *T. gondii* clusters with a consistent pattern of infiltration (Schneider et al., 2019). Importantly, the beneficial contribution of monocytes to cerebral infection was further observed when ILC1s and Ly6G$^+$ neutrophils were absent, resulting in impaired monocyte recruitment and increased parasite burden (Steffen et al., 2022).

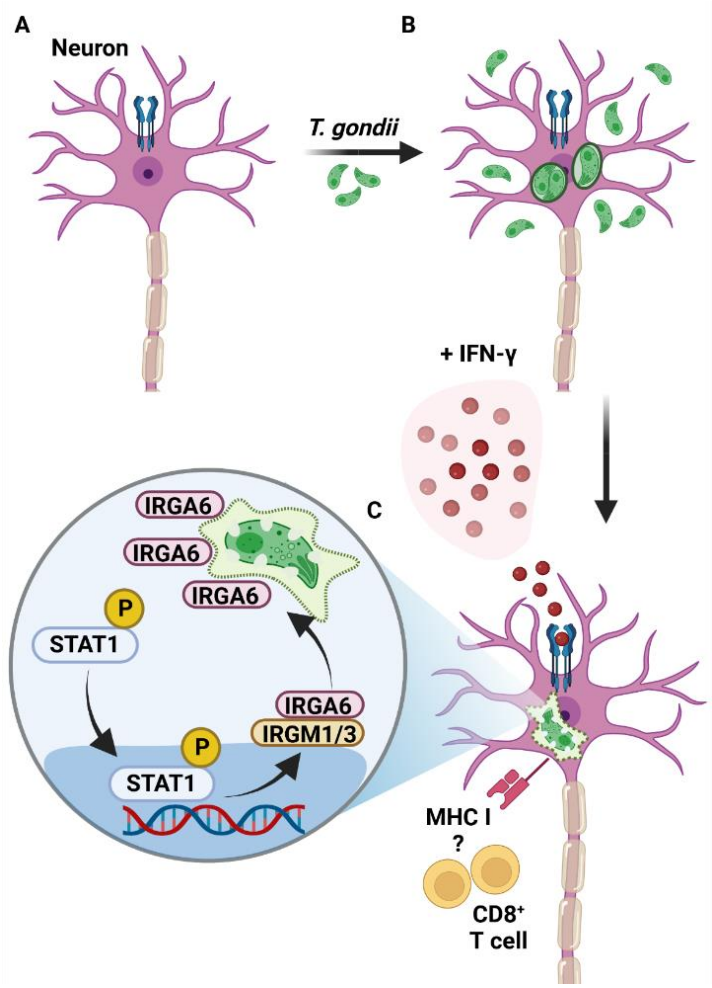

Figure 2. Neuronal cell autonomous immune response targets *T. gondii*.

(**A**) Steady-state neurons expressing IFN-γ receptor; (**B**) *T. gondii* infection in neurons allow parasite intracellular development, especially in case of a parasite strain resistant to murine IRG-dependent mechanisms; (**C**) The presence of IFN-γ promotes elevated expression of total and phosphorylated STAT1 levels, which induce expression of components from the IRG system (e.g., IRGA6, IRGM1/3) that accumulate at the parasite vacuole for its elimination. The direct involvement of MHC I expression and recognition by CD8$^+$ T cells still needs to be clarified. Schematic picture created with BioRender.com, from *Figueiredo and Dunay, 2022*

The early recruitment of initial immune cells is complemented by a subsequent infiltration of CD4$^+$ and CD8$^+$ T cells. Those cells become the main mediators of parasite control by sustained production of IFN-γ, and the cytotoxic perforin-dependent activity of CD8$^+$ T cells. This latter parasite-specific cytotoxic mechanism has shown to be dispensable for the control of acute infection but critical for long-lasting parasite control in the CNS (Salvioni et al., 2019). In resistant murine models of *T. gondii* infection (mice of NMRI, BALB/c, B6.C H-2^d background), this feature is intact, and animals develop a chronic latent

infection with low inflammation. In a susceptible C57BL/6 mouse model, this cytotoxic mechanism is impaired, as differences in the MHC-I locus do not allow recognition of certain secreted *T. gondii* antigens, and the animals will not further control parasite replication, eventually succumbing with high levels of inflammation (Schluter and Barragan, 2019).

Many aspects of the anti-parasitic immune response to *T. gondii* are equivalent in the CNS and the intestine, including high levels of inflammatory damage and pathology (Dupont et al., 2012; Suzuki, 2020). However, in the brain, persistent infection establishes an ongoing neuroinflammation that can remain lifelong and is able to modify neuronal function and synapses. In this case, experimental infection models have shown that *T. gondii* neuroinflammation and chronic infection can alter the levels of several neurotransmitters like dopamine, gamma-aminobutyric acid (GABA), glutamate, and their corresponding receptor signaling pathways. Simultaneously, these changes were found to be followed by loss of synapses, reduction of neuronal dendritic spines, decreased synapse density, and an overall altered synaptic protein composition (e.g., postsynaptic scaffolding protein Shank3 and postsynaptic density protein 95 (PSD-95)) (Blanchard et al., 2015; Carrillo et al., 2020; French et al., 2019; Lang et al., 2018; Parlog et al., 2015). In addition, albeit essential for parasite control, the distinct and constant high levels of IFN-γ in the brain can contribute to modifying CNS function, as IFN-γ is involved in dendritic remodeling (Monteiro et al., 2016), synaptic stripping by activation of microglia (Di Liberto et al., 2018), and proliferation of neuronal precursors (Sun et al., 2010), therefore potentially disrupting neurogenesis and promoting neurodegeneration (Monteiro et al., 2017). Consequently, neurological and behavior alterations upon *T. gondii* infection have been found in rodents and humans (Castano Barrios et al., 2021; Flegr, 2007; Kannan and Pletnikov, 2012; Xiao et al., 2009). The seroprevalence for the parasite was associated with altered cognitive functions and several neuropsychiatric conditions, including depression, suicidal and bipolar behavior, and schizophrenia (Arling et al., 2009; Berrett et al., 2017; Sutterland et al., 2015; Torrey et al., 2012; Zhu, 2009). Regardless of countless scientific evidence, the relationship between CNS *T. gondii* infection and its influence on human neuropsychiatric conditions is still not completely clear.

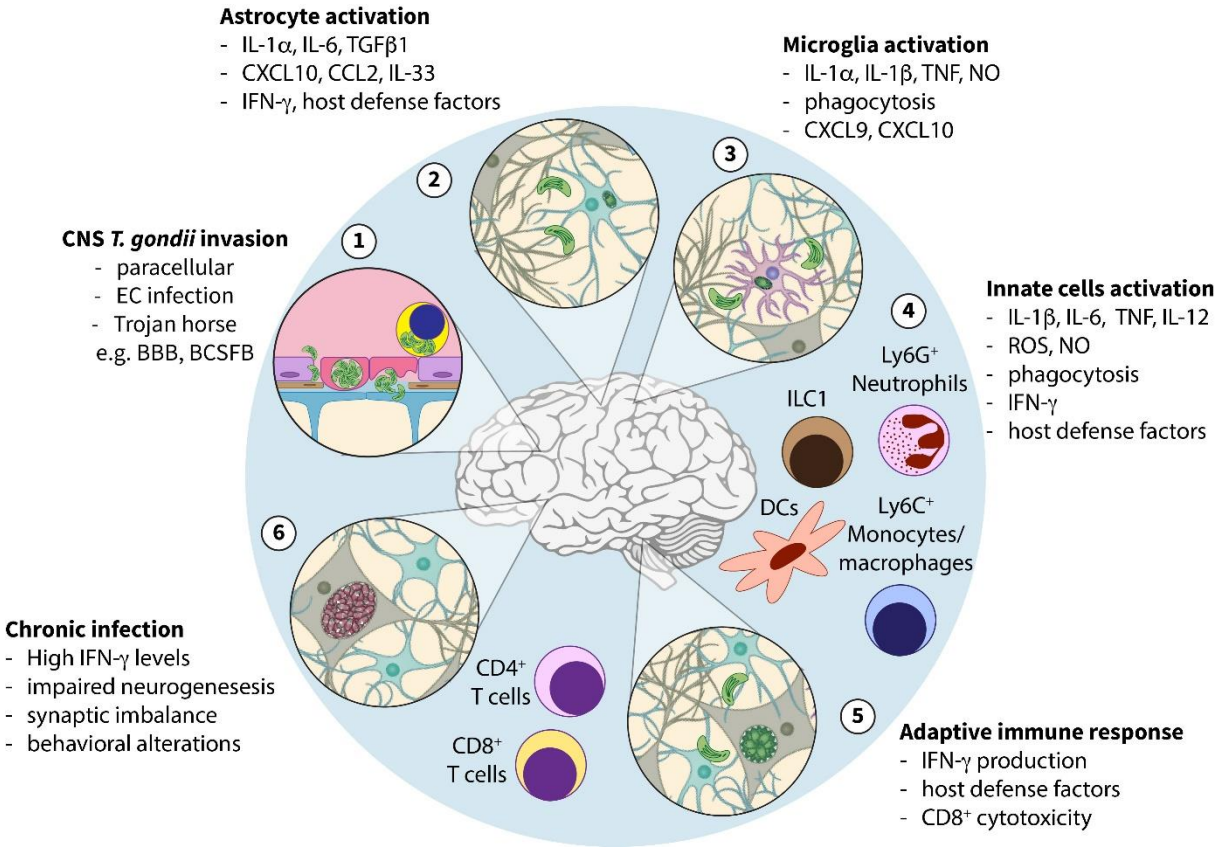

Figure 3. CNS infection by *T. gondii* and neuroinflammation

Schematic overview of the *T. gondii*-induced neuroinflammatory response. (**1**) *T. gondii* parasites invade the CNS borders to infect brain parenchyma. The infection causes activation, cytokine and chemokine production by (**2**) astrocytes and (**3**) microglia, triggering the accumulation of (**4**) activated innate cells as ILC1s, Ly6G$^+$ neutrophils, Ly6C$^+$ inflammatory monocytes, macrophages, and DCs. Subsequently, (**5**) adaptive immune response by CD4$^+$ and CD8$^+$ T cells intensifies IFN-γ production and parasite elimination by cell-autonomous host defense factors and cytotoxic activity. To evade immune recognition, *T. gondii* forms cysts within neurons, a hallmark of (**6**) chronic infection, which is associated with impaired neurogenesis, synaptic imbalance and behavioral alterations. *Adapted from Matta et al., 2021.*

CHAPTER 1 – IMMUNE RESPONSE TO *T. GONDII* AT THE CHOROID PLEXUS

Central nervous system barriers

The biological barriers of the CNS encase the brain and the spinal cord, serving regulatory and protective mechanisms to guarantee a homeostatic and fully functional microenvironment for neuronal cells. They regulate the movement of immune cells and molecules derived from the dynamic milieu of the bloodstream, assuring to shield the CNS from infections and detrimental inflammation (Engelhardt et al., 2017; Matta et al., 2021). Although the main focus of this chapter is on the CP and the BCSFB, it is fundamental to understand the other CNS barriers due to their interdependence and simultaneous involvement in neuroinflammation (see **Figure 3**).

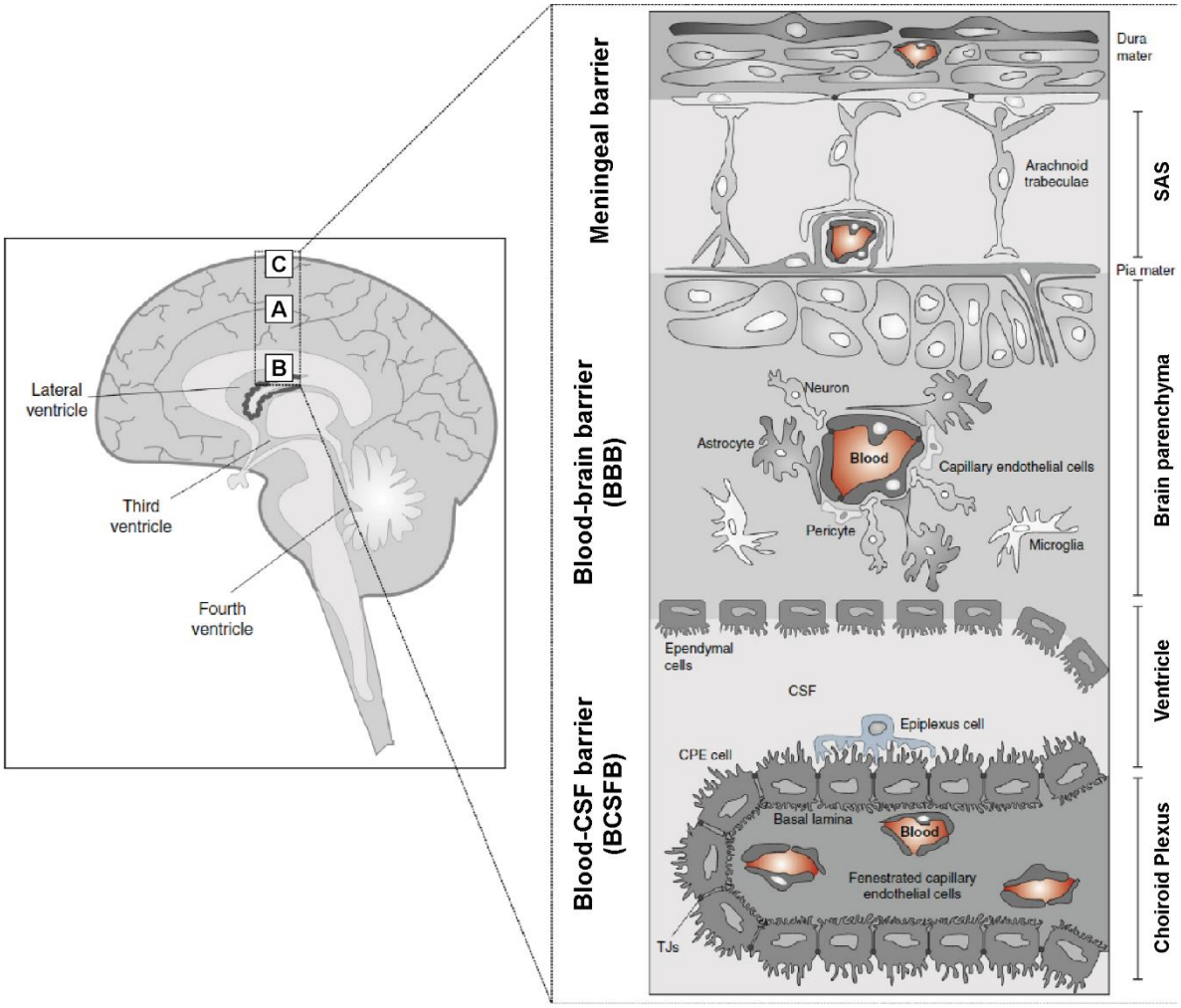

Figure 4. The main CNS barriers

(**A**) The BBB with non-fenestrated endothelial cells expressing TJs, pericytes, astrocytic endfeet, and cellular projections of glia cells and neurons. (**B**) The BCSFB, mainly composed of the choroid plexus with highly secretory epithelial cells in contact with the CSF, the fenestrated capillaries, and local immune cells e.g., epiplexus cells. (**C**) The meningeal barrier with dura mater, arachnoid and pia mater, creating the sub-arachnoid space. Adapted from *Demeestere et al., 2015.*

Blood-brain barrier

The BBB is the most studied biological barrier between the peripheral blood and the CNS parenchyma. Over the years, the understanding of the BBB has evolved from being a simple physical barrier to the functional combination of physiological properties possessed by the endothelial cells that limit the permeability of blood vessels (Profaci et al., 2020). The BBB is mainly comprised of non-fenestrated endothelial cells that express tight junction proteins (TJs) and transport-carrier-systems, lying in direct contact with the bloodstream. The endothelial cell layer is additionally sealed by pericytes, astrocytic endfeet, and cellular projections of glial cells and neurons. Collectively, these cells form the neurovascular unit, the block-builders of the BBB. Although providing an interface between the brain and the blood, the BBB does not have the properties of an immunological niche compared to the meningeal and the CP interface (Croese et al., 2021). Still, upon signals of tissue stress and inflammation, circulating leukocytes adhere to activated endothelial cells and transpose the BBB, a process mediated by selectins like E- and P-selectin and their corresponding ligands on leukocytes (Feustel et al., 2012).

Loss of BBB function and its properties have been reported in neuroinflammatory diseases, such as multiple sclerosis (MS), epilepsy, stroke, and CNS infections. The disruption of the BBB causes ion dysregulation, edema, and enhanced neuroinflammation, resulting in neuronal dysfunction and neuronal degeneration. Although the term "breakdown" implies physical damage, any changes in the physiological properties of the BBB and its transport systems can significantly shape the CNS environment (Profaci et al., 2020). The BBB is also responsible for shielding the CNS from pathogen invasion, although some of them developed mechanisms to overcome this protection, transpose it, and cause cerebral infection.

Meningeal barrier

Three meningeal membranes ensheath the brain and the spinal cord. Anatomically, the most outer membrane is the dura mater, which possesses fenestrated capillaries that lack a barrier function hence allowing an unrestricted entry of blood-derived components within the dura mater, e.g., immune cells, cytokines, and pathogens (Engelhardt et al., 2017; Rua and McGavern, 2018). Next, the arachnoid mater is closely attached to the inner surface of the dura mater and the external surface of the underneath layer, the pia mater, creating the subarachnoid space (SAS) through which the CSF flows. Being the most internal meningeal layer, the pia mater lies directly on top of the glia limitans, constituted by a thin layer of extracellular-matrix (ECM) components and cell-protrusion endings at the surface of the CNS parenchyma (Rua and McGavern, 2018).

Advanced studies of the meningeal barriers have shown that based on their unique immunological landscape, they participate in a variety of brain functions, such as neuronal development (Siegenthaler and Pleasure, 2011), neuronal firing (Alves de Lima et al., 2020), neuronal connectivity (Filiano et al., 2016), and homeostatic behavior of mice (Siegenthaler et al., 2009). Indeed, meningeal layers harbor different immune cells, such as monocytes, neutrophils, DCs, B and T cells, NKs, mast cells, eosinophils, ILCs, and macrophages (Van Hove et al., 2019). Of note, some of these cells are suggested to be originated not from

the bloodstream but from the bone marrow present in the skull (Brioschi et al., 2021; Cugurra et al., 2021). Moreover, meningeal barriers are exploited as an early gateway for CNS pathogens e.g., HIV, Zika virus, and *Trypanosoma brucei*, and can support acute and chronic inflammatory responses associated with significant neurological damage (Rebejac et al., 2022; Rua and McGavern, 2018). In this regard, local meningeal macrophages (MM) have been recently considered the "guardians" of brain integrity. First, they reacted to peripheral challenges such as lipopolysaccharide (LPS), and lymphocytic choriomeningitis virus (LCMV), leading to a transient infection and activation of the meninges in the case of LCMV. Second, specific genetic ablation of components of the interferon type I response (e.g., STAT1 and interferon (alpha and beta) receptor (IFNAR)) on MM resulted in extensive viral spread into the CNS, with infection of several meningeal areas, that led to fatal meningitis (Rebejac et al., 2022).

Choroid plexus and the Blood-cerebrospinal fluid barrier

Located within the four brain ventricles, the CP is a highly vascularized secretory tissue responsible for the production of the CSF. The CP is a villous and selective organ formed by adjacent epithelial cells anchored to a basal lamina, and its inner core contains fibroblast-like stromal cells and resident immune cells surrounding a dense vascular network formed by fenestrated endothelial cells. Choroidal epithelial cells are tightly interconnected by intercellular tight junctions (TJs), which form at the most apical part of the lateral space between neighboring cells. They are formed by a combination of transmembrane proteins, such as claudins and occludins that are connected to actin filaments of the cytoskeleton through scaffold cytoplasmic proteins, such as zona occludens 1 (ZO-1). Besides assuring cell polarity, those TJs are actively involved in the formation of a paracellular barrier that restrains free diffusion of ions, molecules, and cells at cell-cell junctions. In fact, claudins are the main ones responsible for controlling paracellular permeability and are classified into two main groups. Barrier-forming claudins are responsible for sealing the paracellular space, while the channel-forming claudins allow the selective passage of electrolytes and small solutes, and therefore, they determine the level of permeability of the BCSFB to components derived from the blood (Castro Dias et al., 2019). The specific repertoire of expressed claudins at the BCSFB and the way these proteins dimerize with each other between adjacent CP epithelial cells is what determines the specific and intrinsic barrier property of the BCSFB (Ghersi-Egea et al., 2018). Moreover, CP epithelial cells display specific transport systems, for example, the efflux ATP-binding cassette (ABC) transporters and solute carrier (SLC) transporters. Other additional regulatory functions of the CP include the modulation of sex hormones (Marques et al., 2017) and catabolite detoxification (Strazielle et al., 2004). Together, TJs and transporter systems control the molecular and cellular composition of the CSF (Castro Dias et al., 2019; Ghersi-Egea et al., 2018).

In addition, the CP is a unique neuro-immune interface that has been shown to actively integrate signals between the brain parenchyma and the periphery to regulate CNS immunity (Baruch, M Schwartz 2014). Alterations or even disruption of the CP epithelium in response to stressful events, such as CNS infection, trauma, and inflammation, have detrimental effects on barrier permeability compromising the

BCSFB functions (Engelhardt and Sorokin, 2009). BCSFB breakdown has been implicated in neurodegenerative diseases, such as Alzheimer's disease (AD) (Brkic et al., 2015; Demeestere et al., 2015; Rosenberg, 2009; Vandenbroucke, 2016; Vandenbroucke et al., 2012) and during infection-induced inflammation (Gorle et al., 2018; Vandenbroucke et al., 2012). Often, BCSFB breakdown indicates the involvement of the matrix metalloproteinases (MMPs) in the modification of ECM proteins and TJs at the CP. Moreover, unique changes on the CP have been examined in mitochondrial disorders (e.g., Kearns-Sayre syndrome) (Spector and Johanson, 2010), MS and experimental autoimmune encephalomyelitis (EAE) (Rodriguez-Lorenzo et al., 2020), schizophrenia (Kim et al., 2016), and sepsis (Marques et al., 2009). Moreover, recent studies have described that the BCSFB serves as a hotspot for direct pathogen infiltration into CNS, such as bacteria, viruses, fungi, and parasites (Lauer et al., 2018).

T. gondii invasion of CNS: BBB and Choroid plexus involvement

Despite the CNS being shielded from peripheral infections and inflammation, *T. gondii* parasites are able to overcome these protective barriers and invade the CNS. Previous studies have proposed different mechanisms of parasite invasion, including (1) active paracellular migration of free parasites, (2) transmigration of hypermotile infected leukocytes, defined as the "Trojan horse" mechanism, and (3) infection and replication of parasites within brain endothelial cells (Barragan and Sibley, 2002; Bhandage et al., 2020; Coombes et al., 2013; Courret et al., 2006; Konradt et al., 2016; Lachenmaier et al., 2011; Olafsson and Barragan, 2020; Ueno et al., 2015). Still, more recent studies have shown contrasting findings that kept the mechanisms of parasite invasion under debate. One of them described that parasitized immune cells, despite their enhanced motility, do not transpose endothelial layers efficiently (Drewry et al., 2019). In contrast, a more complex study using intravital 2-photon microscopy revealed that infected immune cells shuttle *T. gondii* within brain blood vessels and within the brain (Schneider et al., 2022). When compared to extracellular parasites on their own, infected cells showed enhanced dissemination of infection within the brain. In addition, the proposed hypermotility induced by parasite hijacking did not occur in the brain, as *T. gondii*-infected cells traveled more slowly inside brain blood vessels than non-infected cells (Schneider et al., 2022).

Besides the decisive involvement of the BBB in the parasite invasion, another barrier and potential interface for pathogen interaction within the CNS is the CP and the BCSFB itself. Data concerning the contribution of the CP to *T. gondii* invasion and subsequent neuroinflammation are controversial and incomplete. For example, the analysis of postmortem samples from immunodeficient patients with cerebral toxoplasmosis identified the CP as a site of infection (Falangola and Petito, 1993). On the contrary, models of reactivated TE indicate no evidence for the involvement of CP in systemic parasitic dissemination (Dellacasa-Lindberg et al., 2007; Schluter and Barragan, 2019). Moreover, after studies unveiled that the CP shapes brain function and plasticity via immunological control of the CNS (Schwartz and Baruch, 2014), no further studies were conducted to elucidate the contribution of the CP to *T. gondii* infection.

Aim of this Study

The focus of the study was to investigate the actual involvement of the CP in the invasion and immune response during the onset of cerebral infection caused by *T. gondii*. Alterations in the BCSFB are often associated with neurological diseases and neurodegeneration, which are also associated with cerebral *T. gondii* infection. Due to the fundamental role of the CP to shape CSF composition, brain function, and immunological control of the CNS, the interaction of *T. gondii* with the BCSFB and its consequences on infection-induced neuroinflammation were further evaluated.

Results – Publication 1

The data indicate that in the onset of infection, endothelial cells in the CP were initially infected by *T. gondii* and became activated prior to BBB endothelial cells recognized by MHC-II upregulation. Additionally, the CP elicited an early local immune response with upregulation of IFN-γ, TNF, IL-6, and host-defense factors, as well as swift expression of CXCL9 chemokine, compared to the brain microvasculature. Consequently, distinct TJ disturbances were found in the CP of infected mice. These effects were associated with the upregulation of MMP-8 and MMP-13 in infected CP *in vivo*, which was further confirmed by *in vitro* infection of primary CP epithelial cells. Notably, early barrier damage and functional loss were detected by increased BCSFB permeability to FITC-dextran *in vivo*, which was extended over the course of infection. Together, these data reveal that *T. gondii* infection at the CP is detrimental to BCSFB function and integrity, indicating the initiation of neuroinflammation upon cerebral toxoplasmosis.

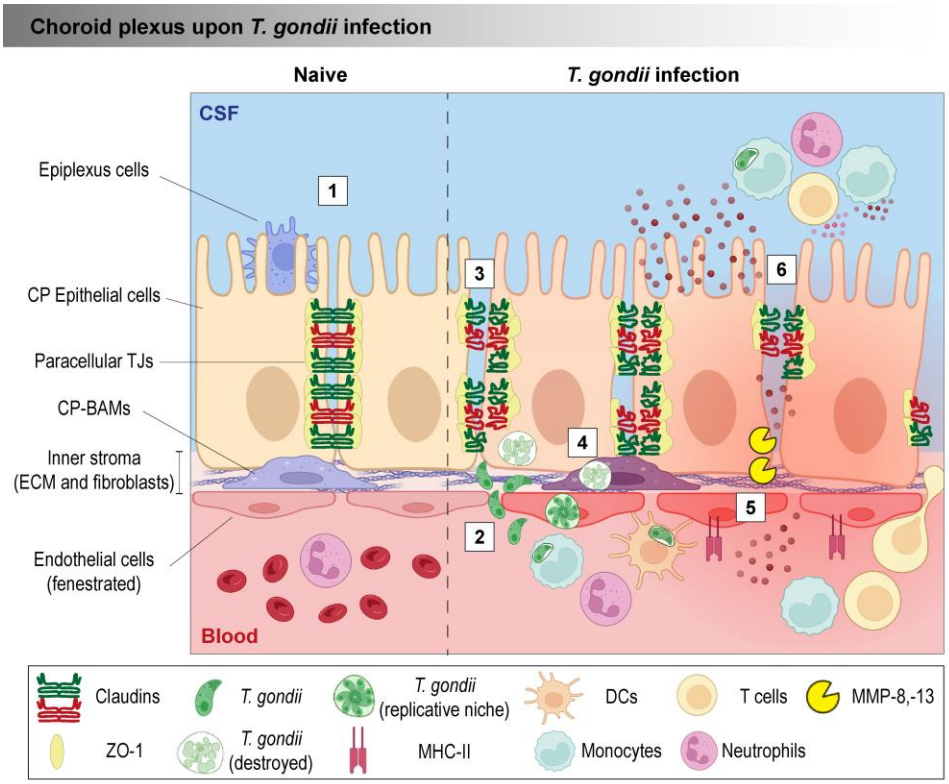

Figure 5. Graphical abstract - Publication 1.

Immune response and pathogen invasion at the choroid plexus in the onset of cerebral toxoplasmosis. **(1)** Naive CP shows epithelial cells interconnected with TJs of the claudin family and scaffold ZO-1 proteins to established a functional paracellular barrier. **(2)** Upon *T. gondii* infection, parasites reach the CP from the vasculature, and infect endothelial cells and resident leukocytes. **(3)** Presence of *T. gondii* in the CP stroma causes disturbance of TJ structure. **(4)** Parasites establish a replicative niche in endothelial cells, and are believed to not survive inside of CP epithelial cells and resident CP-BAMs due to cell autonomous host defense factors. **(5)** Compared to the BBB, CP endothelial cells show early activation by MHC-II expression, signaling to recruitment of peripheral immune cells via CXCL9. Local immune response at the CP involves production of IFN-γ, TNF, IL-6, the chemokine CCL2, and the proteinases MMP-8 and MMP-13. **(6)** Consequently, paracellular TJs are disturbed, and barrier function is compromised, allowing inflammatory cytokines, parasites and immune cells to traffic between blood and CSF. Created with Biorender.com

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Immune response and pathogen invasion at the choroid plexus in the onset of cerebral toxoplasmosis

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Abstract

Background: *Toxoplasma gondii* (*T. gondii*) is a highly successful parasite being able to cross all biological barriers of the body, finally reaching the central nervous system (CNS). Previous studies have highlighted the critical involvement of the blood–brain barrier (BBB) during *T. gondii* invasion and development of subsequent neuroinflammation. Still, the potential contribution of the choroid plexus (CP), the main structure forming the blood–cerebrospinal fluid (CSF) barrier (BCSFB) have not been addressed.

Methods: To investigate *T. gondii* invasion at the onset of neuroinflammation, the CP and brain microvessels (BMV) were isolated and analyzed for parasite burden. Additionally, immuno-stained brain sections and three-dimensional whole mount preparations were evaluated for parasite localization and morphological alterations. Activation of choroidal and brain endothelial cells were characterized by flow cytometry. To evaluate the impact of early immune responses on CP and BMV, expression levels of inflammatory mediators, tight junctions (TJ) and matrix metalloproteinases (MMPs) were quantified. Additionally, FITC-dextran was applied to determine infection-related changes in BCSFB permeability. Finally, the response of primary CP epithelial cells to *T. gondii* parasites was tested in vitro.

Results: Here we revealed that endothelial cells in the CP are initially infected by *T. gondii*, and become activated prior to BBB endothelial cells indicated by MHCII upregulation. Additionally, CP elicited early local immune response with upregulation of IFN-γ, TNF, IL-6, host-defence factors as well as swift expression of CXCL9 chemokine, when compared to the BMV. Consequently, we uncovered distinct TJ disturbances of claudins, associated with upregulation of MMP-8 and MMP-13 expression in infected CP in vivo, which was confirmed by in vitro infection of primary CP epithelial cells. Notably, we detected early barrier damage and functional loss by increased BCSFB permeability to FITC-dextran in vivo, which was extended over the infection course.

Conclusions: Altogether, our data reveal a close interaction between *T. gondii* infection at the CP and the impairment of the BCSFB function indicating that infection-related neuroinflammation is initiated in the CP.

Keywords: *Toxoplasma gondii*, Neuroinflammation, Choroid plexus, Blood–CSF barrier, Blood–brain barrier, Tight junctions, Matrix metalloproteinases

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Background

Toxoplasmosis is a foodborne parasitic disease caused by the obligate intracellular protozoan *Toxoplasma gondii* (*T. gondii*). It is estimated that more than one-third of the world's human population is infected with *T. gondii* and

its seroprevalence increases gradually with age [1–3]. Following uptake via oral ingestion of contaminated food or water, the parasites proliferate within a variety of nucleated cells, infect circulating leukocytes, successfully cross all the barriers of the body and spread throughout host tissues [4]. Despite of the central nervous system (CNS) being an immune privileged site shielded from peripheral infections and inflammation, *T. gondii* parasites are able to invade the CNS. Previous studies have proposed different mechanisms of parasite invasion, including: (1) active paracellular migration of free parasites; (2) transmigration of hypermotile infected leukocytes, defined as "Trojan horse" mechanism, and (3) infection and replication of parasites within brain endothelial cells [5–12]. Once *T. gondii* crosses the brain biological barriers, like the blood–brain barrier (BBB), they invade brain-resident cells and persist in cysts lifelong [13–15]. The cyst formation within neurons develops a stress-mediated response followed by ongoing basal neuroinflammation, which leads to altered neuronal function, and potentially to behavioral alterations and neuropsychiatric diseases [16–19]. In immunodeficient individuals, the infection can lead to disruption of tissue cysts and uncontrolled parasite proliferation, resulting in toxoplasma encephalitis (TE) [2, 20, 21].

Several studies have demonstrated the decisive involvement of the BBB in the invasion of *T. gondii* and the ensuing development of neuroinflammation [22, 23]. In fact, the choroid plexus (CP) is another barrier and potential interface for pathogen invasion into CNS. The CP is the main structure forming the blood–CSF barrier (BCSFB), and is crucial for CNS homeostasis and cerebrospinal fluid (CSF) secretion. Located within the four brain ventricles, the CP is a villous and selective organ formed by adjacent epithelial cells anchored to a basal lamina and an inner core of resident immune cells surrounding a dense vascular network of fenestrated endothelial cells. Choroidal epithelial cells are tightly interconnected by tight junction proteins (TJ), and control the molecular and cellular composition of the CSF [24, 25]. The ability of this unique neuro-immune interface to actively integrate signals between brain and periphery is fundamental to CNS immunity [26], in which CP regulation of immune cell trafficking is considered a central point in the initiation of inflammatory brain responses [27]. Alterations or even disruption of the CP epithelium in response to stressful events have detrimental effects on barrier permeability compromising the BCSFB functions [28]. Indeed, BCSFB breakdown has been implicated in neurodegenerative diseases [29–33] and during infection-induced inflammation [31, 34], often indicating the involvement of matrix metalloproteinases (MMPs). Moreover, recent studies have described that the BCSFB serves as a hotspot for direct pathogen infiltration into the CNS [35]. However, data concerning the contribution of the CP to *T. gondii* invasion, and subsequent neuroinflammation are controversial and incomplete. For example, the analysis of post-mortem samples from immunodeficient patients with cerebral toxoplasmosis identified the CP as a site of infection [36]. On the contrary, models of reactivated TE indicate no evidence for the involvement of CP in systemic parasitic dissemination [37, 38].

Here, we demonstrate that, prior to the activation of endothelial cells in the BBB, parasites invading the CNS rapidly seize choroidal endothelial cells. As a result, a prompt immune response is initiated in the CP as shown by the upregulation of cytokines, chemokines, and host defense factors, followed by the expression of MMPs, TJ disturbance of CP epithelial cells, and subsequent increased barrier permeability. Together, our results show that the infection takes place in the CP, and indicate sudden functional impairment of the BCSFB upon the onset of the CNS invasion by *T. gondii*.

Methods

Mice and infections in vivo

Experiments were conducted with female C57BL/6 J mice (8–14 weeks old, purchased from Janvier, Cedex, France). All animals were group-housed in a 12-h day/night cycle at 22 °C with free access to food and water under specific pathogen-free conditions and according to institutional guidelines approved by the Animal Studies Committee of Saxony-Anhalt. In order to investigate early *T. gondii* infection, mice were infected by intraperitoneal (*i.p.*) injection of either cysts or tachyzoites. For cyst infection, 2 cysts of the type II ME49 strain harvested from the brains of female NMRI mice infected *i.p.* with *T. gondii* cysts 6–12 months earlier were used as previously described [39]. For tachyzoites infection, type II *T. gondii* reporter parasites of the PTG-GFPS65T strain were grown in monolayers of human foreskin fibroblast (HFF) with DMEM medium (FG0435, Biochrom, Germany), supplemented with 10% fetal bovine serum (FBS) (ThermoFisher, Germany), 1% penicillin/streptomycin (Pen/Strep; Sigma, USA) and 1% non-essential amino acids (NEEA) (ThermoFisher, Germany) as previously described [40]. Freshly egressed parasites were filtered through a 5 µm Millex-SV syringe filter (Millipore, Germany), and the number of living tachyzoites determined by counting under a bright-field microscope using Trypan Blue 0.4%. Mice were infected *i.p.* with 1×10^5 reporter parasites in 200 µl PBS.

Organ isolation

Mice were deeply anaesthetized by isoflurane inhalation (Baxter), the CSF was collected and thereafter animals

were transcardially perfused with 60 ml PBS. For immunofluorescence samples, perfusion was additionally done with 20 ml of 4% paraformaldehyde (PFA) in PBS. Brain, spleen and spinal cord were removed and stored in sterile ice-cold PBS or RNAlater (Sigma) for further processing. Samples stored in RNAlater were kept at 4 °C overnight and afterwards transferred to − 80 °C.

Cerebrospinal fluid collection

CSF was collected by the *cisterna magna* puncture technique as described elsewhere [41]. In short, deeply anaesthetized animals were immobilized in a prone position with the head forming a 135° angle with the body, and a sagittal incision of the skin was made inferior to the occiput. Using a stereomicroscope (Stemi 305; ZEISS), the subcutaneous tissue and muscles were dissected, and a glass capillary tube was introduced into the *cisterna magna* through the dura matter, lateral to the arteria *dorsalis spinalis*. An average of 15 µl of CSF per animal were collected, and kept on ice until further processing. Samples were macroscopically assessed for blood contamination, and discarded when contamination was detected.

Choroid plexus and brain tissue isolation

Isolated brains were placed under a stereomicroscope in dissection buffer containing HBSS (ROTI®Cell, Roth), and 10 mM HEPES (Gibco, ThermoFisher). CPs were isolated from the lateral, third and fourth brain ventricles, and were either processed for total RNA/DNA isolation, or placed in digestion buffer for further cell isolation. Cerebellum, olfactory bulbs, and adjacent brain meninges were removed and discarded. The remaining brain tissue was used for cell isolation followed by flow cytometric analysis, or further utilized for brain microvessels isolation.

Brain microvessels isolation

Brain microvessels were isolated as previously described [42] with a few modifications, and used for immunofluorescence, or total RNA/DNA isolation. Briefly, brain hemispheres were minced with a scalpel, and homogenized in digestion buffer (HBSS, with 6.75 g/l glucose, 20 mM HEPES) containing 1 mg/ml DNAse I. After incubation (10 min, 37 °C), homogenate was washed in FACS buffer (PBS w/o Ca/Mg, 2 mM EDTA, 2% v/v FBS, 10 mM HEPES). The resultant pellet was resuspended and separated by successive centrifugations in 20% (w/v) bovine serum albumin (BSA)-DMEM/F12 solution. To remove remaining myelin debris, the pellet containing microvessels was resuspended in PBS, fractioned on 22% (v/v) Percoll® (Sigma, #GE17089101) gradient solution and centrifuged for 10 min, 600 × g, w/o brake. The microvessels pellet was recovered and extensively washed in PBS/HEPES.

Cell isolation

Isolated CP, the remaining brain tissue, liver and lymph nodes (inguinal and mesenteric) were further processed in order to obtain single cell suspensions. CP samples were incubated (20 min, 37 °C) in digestion buffer (PBS w/ Ca/Mg, 2% v/v FBS, 10 mM HEPES) containing 1 mg/ml DNAse I (Sigma, #DN25) and 1 mg/ml Collagenase/Dispase (Sigma, #11097113001). Digested tissues were mechanically dissociated using syringes connected to 22- and 26G needles, then cells were washed with FACS buffer, and used for further analysis. For the isolation of brain cells, liver and lymph nodes, tissues were minced with a scalpel, and homogenized in digestion buffer (HBSS, with 6.75 g/l glucose, 20 mM HEPES) containing DNAse I and Collagenase/Dispase as previously mentioned. Homogenate was incubated (40 min, 37 °C, 200 rpm), and filtered through a 70-µm cell strainer (Falcon®, #352350). The cell suspension was centrifuged (400 × g, 10 min, 4 °C), and the cell pellet separated in a 25–70% discontinuous Percoll® gradient for 20 min without brake. Cells were recovered from the gradient interface, washed with FACS buffer, and used for further analysis.

Flow-cytometric analysis

Cells were resuspended in FACS buffer, and stained as previously described [39, 40]. In short, cells were incubated with an anti-mouse CD16/32 unconjugated antibody (clone 93, BioLegend) and stained with fixable viability dye Zombie NIR (BioLegend), for 20 min at 4 °C. Subsequently, cells were stained (30 min, 4 °C) using fluorochrome-conjugated antibodies. CD11b (PerCP-Cy5.5, clone M1/70) purchased from eBioscience, CD45 (BV510, clone 30-F11), MHCII (BV711, clone M5/114.15.2), VE-cadherin (BV421, clone BV13), and gp38 (AF488, clone PMab-1) purchased from BioLegend. CD31 (APC, clone MEC13.3) purchased from BD Biosciences. Cells were washed (400 × g, 5 min 4 °C) with FACS buffer, fixed in 4% PFA for 15 min at 4 °C and re-suspended in FACS buffer. For CP, cells were additionally permeabilized with eBioscience™ Permeabilization buffer (Invitrogen), incubated with antibody rabbit-anti-TTR (Abcam) for 40 min, and stained with secondary antibody anti-rabbit AF488 (ThermoFisher). Cells were acquired using Attune NxT Flow Cytometer (ThermoFischer). Data were analyzed using FlowJo software (version 10.5.3, FlowJo LLC, OR, USA).

FITC-dextran permeability assay

BCSFB and BBB permeability were measured as previously described [34] with modifications. In short, 4 kDa FITC-dextran (Sigma, #46944) was diluted in PBS, and administered intravenously (*i.v.*) at 75 mg/kg body weight mice, 30 min before CSF and brain collection. CSF was collected through the *cisterna magna* as previously described, diluted 100-fold in PBS, and spun down (1000 × g, 5 min). The resulting supernatant was further used for analysis. After perfusion, isolated brains were weighted, minced with a scalpel, homogenized in formamide (Roth) at 0.8 ml per 100 mg tissue, and incubated overnight (37 °C, 200 rpm). Homogenates were spun down (12,000 × g, 5 min), and supernatants were collected and diluted twofold in PBS. All samples were measured in triplicates at λex/λem = 485/520 nm using SpectraMax M5e (Molecular Devices LLC).

Choroid plexus epithelial cells and *T. gondii* in vitro infection

Primary CP epithelial cell culture was obtained as previously described [43, 44] with modification. Isolated CP from 9 to 14 mice, one-week-old, were used in each preparation. Tissues were pooled in digestion buffer (PBS w/ Ca/Mg, 2% v/v FBS, 10 mM HEPES) containing 1 mg/ml DNAse I and 2 mg/ml Collagenase/Dispase, and incubated (20 min, 37 °C). Digested tissues were mechanically dissociated and cells were washed (400 × g 5 min) with FACS buffer (PBS w/o Ca/Mg, 2 mM EDTA, 2% v/v FBS, 10 mM HEPES). Cell pellet was resuspended, and cultivated with complete medium (DMEM/F12, supplemented with 10% FBS, 1% penicillin/streptomycin, 1% ITS (insulin–transferrin–selenite), 40 mg/ml human-EGF (epidermal growth factor, PeproTech, Germany, #AF-100–15). Cells were grown until confluence, for approximately 7 to 10 days, in 12 well plates previously coated with poly-L-lysine (PLL), or on top of PLL-coated coverslips. Once confluent, cells were infected at multiplicity of infection (MOI) = 5 with type II PTG-GFPS65T tachyzoites, and incubated at 37 °C. After 6 h, culture medium was removed, cells were washed with PBS, and further used for total RNA/DNA isolation, flow cytometric analysis (validation) or immunofluorescence. Naïve controls were treated with pre-warmed fresh medium.

Immunofluorescence

Immunofluorescence staining was performed for CP whole mount, BMV mount, brain sections and cell culture coverslips. For brain sections, isolated brains were post-fixed (4 h, at 4 °C), soaked in 30% sucrose in PBS (2 days, at 4 °C), and frozen in cryo media (OCT Compound, Tissue Tek). Coronal sections (20 µm) were obtained (Thermo Scientific CryoStar NX50) and only sections containing CP were stained. BMV mount, brain sections and coverslips were stained directly on glass slides. CP whole mount staining were performed utilizing a free-floating approach. All samples from different origins were stained and mounted with the same protocol unless otherwise stated. In short, samples were fixed (4% PFA in PBS, 20 min, 4 °C), washed twice with washing solution (PBS 0.1% (v/v) Triton X-100), and blocked/permeabilized with PBS 0.3% (v/v) Triton X-100 5% normal-goat-serum (NGS) and unconjugated F(ab')2-goat anti-mouse IgG (H + L) antibody (1:500, Thermo Scientific), for 2 h at 4 °C. Next, samples were incubated with antibody solution (PBS 0.1% (v/v) Triton X-100 2% NGS) containing the primary antibodies of interest: anti-SAG1 (1:50, clone D61S, ThermoFisher), anti-GFP (1:1000, #132004, SYSY), anti-E-cadherin (1:500, #PA5-85088, ThermoFisher), anti-PDGFRβ (1:200, clone R.140.4, ThermoFisher), anti-ZO-1(1:50, clone R26.4C, ThermoFisher), anti-IBA-1(1:500, #234004, SYSY), anti-CD31 (1:50, clone MEC13.3, BioLegend), anti-claudin-2 (1:500, clone MH44, ThermoFisher), VE-cadherin (1:40, clone BV13, ThermoFisher). Primary antibodies were incubated overnight at 4 °C, then samples were washed twice, and incubated (30 min, RT) in antibody solution with secondary antibodies (1:1000) tagged with AF488, AF555, AF594 and AF647 according to each antibody host. Next, samples were washed twice, and mounted with ProLong™ Gold Antifade Mountant with DAPI (ThermoFisher). In some samples, anti-CD45 (clone 30-F11) conjugated antibody was used, and an additional final staining step was added. For brain sections, antigen retrieval (10 mM citrate buffer, pH 6.0, 0.1% Tween-20) was performed at 96 °C for 30 min, before the blocking and permeabilization step. Images were generated using a Leica TCS SP8 microscope, and analyzed using the ImageJ software (ImageJ 1.52p).

DNA and RNA isolation

Total DNA and RNA were isolated from CP, BMV, spleen, spinal cord, brain and CP epithelial cell culture. Samples from CP, BMV and CP epithelial cell culture were isolated with Quick-DNA/RNA Miniprep kit (Zymo Research, Germany) according to the manufacturer's instructions. Brain, spinal cord, and spleen samples were first homogenized with TriFast (Peqlab, 30-2010) using tubes containing Zirconium oxide beads (Precellys, P000926-LYSK0-A) in a BeadBug 6 homogenizer (Biozym). DNA was isolated from the homogenate according to the manufacturer's instructions. RNA was isolated from the homogenate by isopropanol precipitation or using peqGOLD total RNA kit (Peqlab) following the manufacturer's instructions. The concentration and purity of DNA and RNA samples were determined using

NanoDrop 2000 spectrophotometer (ThermoFisher; Germany), and samples were stored at − 80 °C until further use.

qPCR

Parasite burden was assessed in triplicates using 40 ng of isolated DNA, FastStart Essential DNA Green Master and LightCycler® 96 System (both Roche, Germany), as previously described [40]. Thermal-cycling parameters were set as follows: initial activation (95 °C, 10 min), 55 amplification cycles consisting of denaturation (95 °C, 15 s), annealing (60 °C, 15 s) and elongation (72 °C, 15 s). The DNA target was the published sequence of the highly conserved 35-fold-repetitive B1 gene of *T. gondii* [45, 46]. Murine argininosuccinate lyase (*Asl*) was used as reference gene for normalization and relative DNA levels were determined by the ratio *gene of interest/reference gene* and subsequently normalized to mean values of control group [47]. Primers were synthetized by Tib MolBiol (Germany) and used at 200 nM final concentration. Primer sequences are described elsewhere [see Additional file 1].

RT-qPCR

Gene expression levels of cytokines, inflammatory mediators, host-defense factors, tight junctions and MMPs were assessed in triplicates using 20 ng total RNA, TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems, Germany) and LightCycler® 96 (Roche, Germany) as previously described [40]. Thermal-cycling parameters were set as follows: reverse transcription (48 °C, 30 min), inactivation (95 °C, 10 min) followed by 55 cycles of denaturation (95 °C, 15 s) and annealing/extension (60 °C, 1 min). Utilized TaqMan® Gene Expression Assays (Applied Biosystems, Germany) are listed elsewhere [see Additional file 1]. *Hprt* was chosen as *reference gene* and relative mRNA levels were determined by the ratio *gene of interest/reference gene* and subsequently normalized to mean values of control group.

For the genes analyzed using SYBR Green technology, Power SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, Germany) was used. Samples were analyzed in triplicates (20 ng of isolated mRNA per reaction) using LightCycler® 96 with the following parameters: reverse transcription (48 °C, 30 min), inactivation (95 °C, 10 min) followed by 55 cycles of denaturation (95 °C, 15 s) and annealing/extension (60 °C, 1 min) and melting curve analysis. The primer sequences are listed elsewhere [see Additional file 1] and were synthetized by Tib MolBiol and used at 100 nM final concentration. *Hprt* was chosen as reference gene and relative mRNA levels were determined by the ratio *gene of interest/reference gene* and subsequently normalized to mean values of control group.

Statistical analysis

Results were statistically analyzed using GraphPad Prism 7 (GraphPad Software Inc., USA), *post-test* corrections were applied according to software recommendations. Statistical significance was set to $p \leq 0.05$. All data are presented as arithmetic mean and standard error of the mean (SEM) and are representative of at least two independent experiments. For parasite burden analysis, normalized data were analyzed by multiple *t*-test, with Holm–Šidák correction for multiple comparisons. For flow cytometric analyses, normalized data were analyzed by one-way ANOVA, with Tukey's correction. For RT-qPCR, data were analyzed by one-way ANOVA followed by Dunnett's correction. BCSFB permeability assay was analyzed by multiple *t*-test to compare between BCSFB and BBB, and one-way ANOVA followed by Dunnett's correction was used to compare differences between day 0 and the time-point analyzed from the same barrier type. RT-qPCR data from primary cell culture were analyzed by Student's *t*-test.

Results

Choroid plexus is infected by *T. gondii* upon the invasion

BCSFB is a gateway to the CNS for different pathogens as well as immune cells under certain inflammatory conditions [26, 35, 48–50]. Assuming that the CP is involved in the parasite entry and early immune response upon the invasion of the brain by *T. gondii*, we investigated the presence of parasites in the CNS through the course of infection. As BBB endothelial cells were shown previously to be directly infected by *T. gondii* [7], we isolated brain microvessels (BMV) that contain endothelial cells, and compared them to isolated CP (Fig. 1A) for parasite burden over time by PCR. Our results revealed the early presence of parasites on CP at 3 dpi, which was increased by day 5 and reduced by day 7 (Fig. 1B). In the BMV, the parasite burden only strongly increased by day 7 (Fig. 1B). Next, CP and BMV of infected animals were isolated and immuno-stained as a three-dimensional whole mount preparation. Within the CP at 5 dpi, we detected parasites ($SAG1^+$) co-localized with immune cells ($CD45^+$) (Pearson's coefficient, $r=0.57$), and also extracellular parasites present in the paracellular and stromal regions between the CP epithelial cells (E-$cadherin^+$) without co-localization with E-cadherin (Pearson's coefficient, $r=0.013$) (Fig. 1C). In the BMV, parasites were found to be associated to the microvasculature, although no co-localization were detected for PDGFRβ $^+$pericytes (Pearson's coefficient, $r=0.099$) and only a few for $ZO{-}1^+$ endothelial cells (Pearson's coefficient, $r=0.112$) (Fig. 1D), which constitute the main components of cerebral microvasculature forming the BBB [51]. Overall, parasites were found in the CP, and apparently more frequently in initial

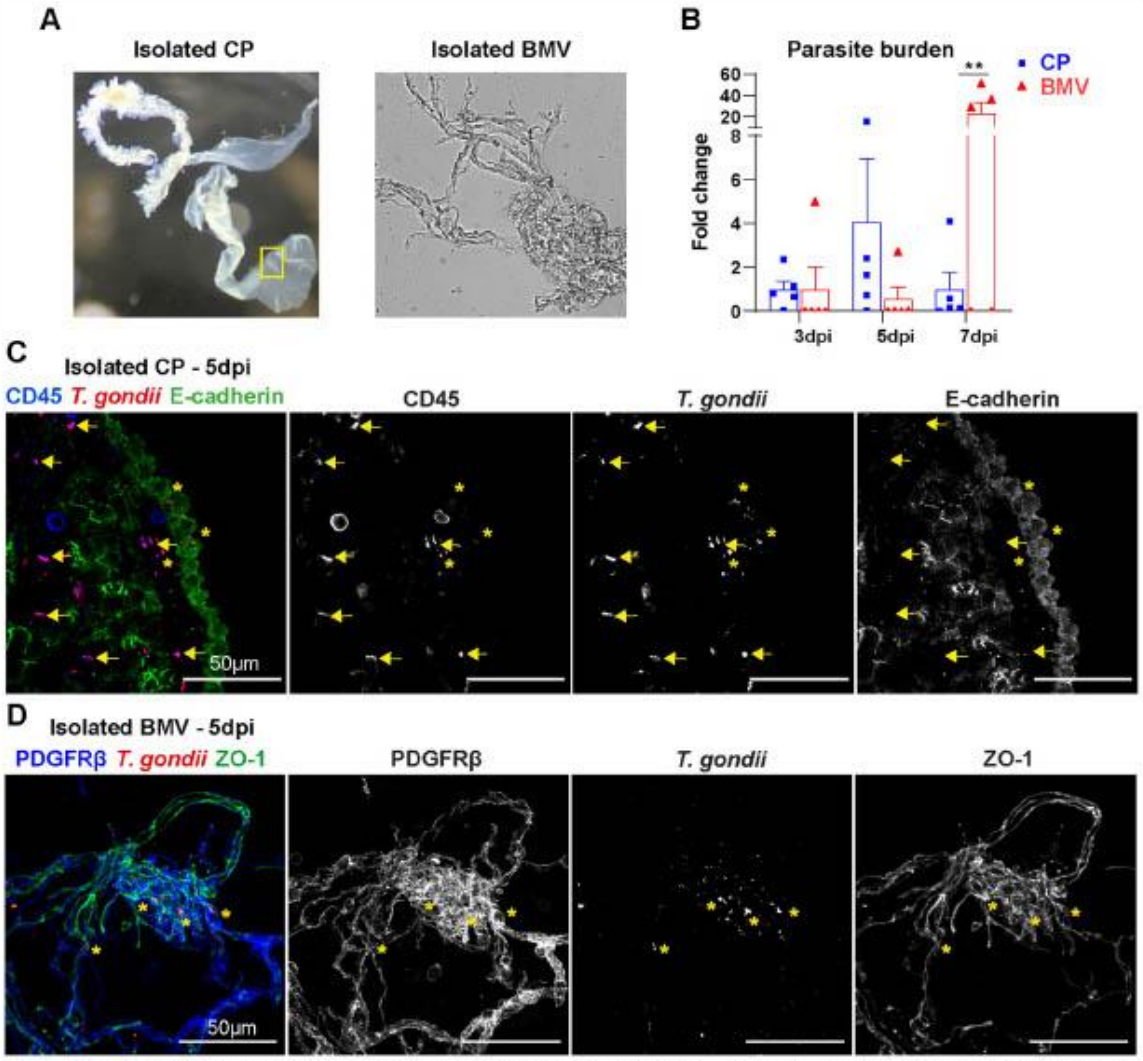

Fig. 1 Detection of *T. gondii* in the CP. Mice were infected *i.p.* with 2 cysts of *T. gondii* type II ME49 and samples were collected at 5dpi. From brains, CPs were isolated under stereomicroscope, and the remaining brain tissue was processed for isolation of BMVs. **A** Representative image of freshly isolated CP and a phase-contrast image of isolated BMV. **B** Parasite burden qPCR analysis of isolated CPs (blue) and BMVs (red) from the correlated animals, which were infected at the same time, and analyzed at 3, 5 and 7dpi. The analysis was performed based on the presence of B1 gene of *T. gondii* (*TgB1*) normalized to the murine gene *Asl*. Data were normalized to the mean values of 3 dpi, and bar charts show individual values of a representative experiment, and mean + SEM. $n = 5$ per group. Statistical analysis was performed by multiple *t*-test, with Holm–Šidak correction for multiple comparisons. $^{**}p < 0.01$. **C** CP whole mount staining to identify immune cells (CD45, blue), *T. gondii* (SAG1, red) and epithelial cells (E-cadherin, green). Scale bars = 50 µm **D** BMVs immune-staining to identify pericytes (PDGFRβ, blue), *T. gondii* (SAG1, red), and the tight junction ZO-1 (green). Yellow arrows indicate the co-localization of parasites with CD45$^+$ immune cells, and yellow asterisks *T. gondii* signal alone. Scale bars = 50 µm

infection at the BCSFB interface than the BBB. Immunostaining of isolated BMV confirmed the presence of *T. gondii* tachyzoites in BMV at 7 dpi, as detected previously by PCR [see Additional file 2]. Spleens were used as controls to monitor the parasite burden levels in the periphery, indicating the usual spread of the parasites throughout tissues [see Additional file 2]. Parasite burden was just detected starting at 7 dpi in both brain parenchyma (after CP removal) and spinal cord, evidencing the

early infection through the CP and the microvasculature [see Additional file 2].

Choroid plexus endothelial and immune cells are targeted by *T. gondii*

Given the early presence of *T. gondii* in the CP, we sought to determine the target cell types in this compartment. Therefore, mice were infected *i.p.* with 10×10^5 tachyzoites of the *T. gondii* strain expressing the fluorescent protein GFP (PTG-GFPS65T). First, we analyzed coronal brain sections showing CP from lateral ventricles and found parasites within the choroidal endothelial cells at 7 dpi (Fig. 2A) [and see Additional file 3]. Indeed, at 7 dpi parasites were additionally found in the cortex and the adjacent brain areas [see Additional file 2], simultaneously to the CP [see Additional file 3]. To identify infected immune cells in the BCSFB, isolated CP whole mounts were co-stained for CD45 and CD31. At 3 dpi, we observed *T. gondii* parasites associated to immune cells and endothelial cells in the CP (Fig. 2B). Of note, recruited amoeboid-shape CD45*hi* leukocytes did not appear to be associated to parasites, rather a resident stellate CD45*int* CP macrophages (referred as CP-BAMs (border-associated-macrophages) or epiplexus cells) were co-localized with parasites at this time-point [see Additional file 3]. CP-resident macrophages were also identified by IBA-1 staining and morphology [see Additional file 4]. CP endothelial cells were infected with intracellular parasites at 3 and 5 dpi, but at 7 dpi infected cells were less abundant. Additionally, parasites were also found in the inner core of the CP parenchyma, suggesting the infection of mesenchymal derived stromal cells. Altogether, our results demonstrate the dynamic infiltration of parasites into endothelial and immune cells of the CP in the early phase of infection, confirming our initial data on parasite burden.

Early immune response at the choroid plexus prior to the BBB

Pathogens infecting the CP elicit local immune responses which are associated with the activation of endothelial, epithelial and tissue-resident immune cells [35]. To characterize the early immune response to *T. gondii* in the CP in more details, we first compared the MHCII expression on endothelial cells from CP and remaining brain tissue (Fig. 3A–H). In agreement with the spatiotemporal differences in pathogen distribution (Fig. 1), MHCII upregulation on endothelial cells was first evidenced in the CP followed by later activation of brain endothelial cells (Fig. 3A–H). The definition of endothelial cells as $CD45^-$ $CD31^+$was sufficient to identify endothelial cells from both tissues, and nor the additional endothelial marker VE-cadherin neither the fibroblastic marker GP38 (podoplanin) improved the recognition of endothelial cells [see Additional file 5]. The kinetics of MHCII expression by microglia was comparable to brain endothelial cells and reached maximum levels at 7 dpi [see Additional file 6]. Moreover, MHCII expression was analyzed on CP epithelial cells even though less than 10% of the cells express this marker, a significant increase was observed [see Additional file 6]. Of note, total mRNA expression of MHCII (*H2-Aa*) in the CP was also increased during infection [see Additional file 7]. Taken together, choroidal endothelial cells responded earlier to *T. gondii* than brain endothelial cells and microglia, indicating that the CP is a critical interface for early parasite recognition.

To further compare the local inflammatory response in the BCSFB versus the BBB, we analyzed the mRNA expression of pro-inflammatory cytokines which play a central role in host defense upon *T. gondii* infection. Therefore, CP and the BMV were isolated from infected brains and expression of IFN-γ, TNF, and IL-6 were measured (Fig. 3I). Compared to the BMV, CP expression of IFN-γ was higher and earlier elevated. BMV only reached comparable IFN-γ levels at 7 dpi. TNF was significantly higher in the CP at both 5 and 7 dpi and IL-6 levels peaked at 3 dpi and was higher in the CP at 7 dpi (Fig. 3I). We also evaluated the expression of type I interferon-β (IFN-β, *Ifnb1*), which were highly expressed in the CP [see Additional file 7]. Furthermore, the cell-autonomous immune response against intracellular *T. gondii* infection is mediated by IFN-γ-inducible GTPases, designated as host-defense factors (*Irgm1, Igtp, Gbp2b*). Following the elevated IFN-γ levels, we detected higher expression of those genes on the CP compared to the BMV (Fig. 3J). Previous studies have shown specific immune cell

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Fig. 2 *Toxoplasma gondii* infection of endothelial and immune cells in the CP. **A** Animals were infected *i.p.* with 1×10^5 *T. gondii* type II PTG-GFP tachyzoites. The brains were isolated at 7 dpi, and coronal sections were immune-stained with anti-GFP (green), anti-CD31 (magenta) and DAPI (cyan). First row indicate the location of the CP (magenta rectangle) in the lateral ventricle (VL) according to the mouse brain atlas. Second row magnify the CP area above. Yellow arrowheads indicate the detection of parasites co-localized with endothelial cells (CD31). Scale bars = 50 µm **B** Isolated CPs from animals infected *i.p.* with 1×10^5 PTG-GFP tachyzoites were immune-stained as whole-tissue mount with anti-CD45 (blue), anti-SAG1 (red), and anti-CD31 (green). Animals were infected at the same time, but tissues were analyzed at 3, 5 and 7 dpi. Yellow arrows indicate CD45/SAG1 co-localization, showing immune cells carrying parasites through the BCSFB. Arrowheads indicate co-localization of CD31/SAG1, and asterisks represent SAG1 signal alone. White square indicates magnified area of co-localization signal and orthogonal views of a z-stack analysis. GFP signal plus SAG1 staining were detected in the same channel and colored in red for better visualization. Scale bars = 50 µm. *CTX* cortex, *Cc* corpus callosum, *CdP* caudoputamen, *LS* lateral septal, *VL* lateral ventricle

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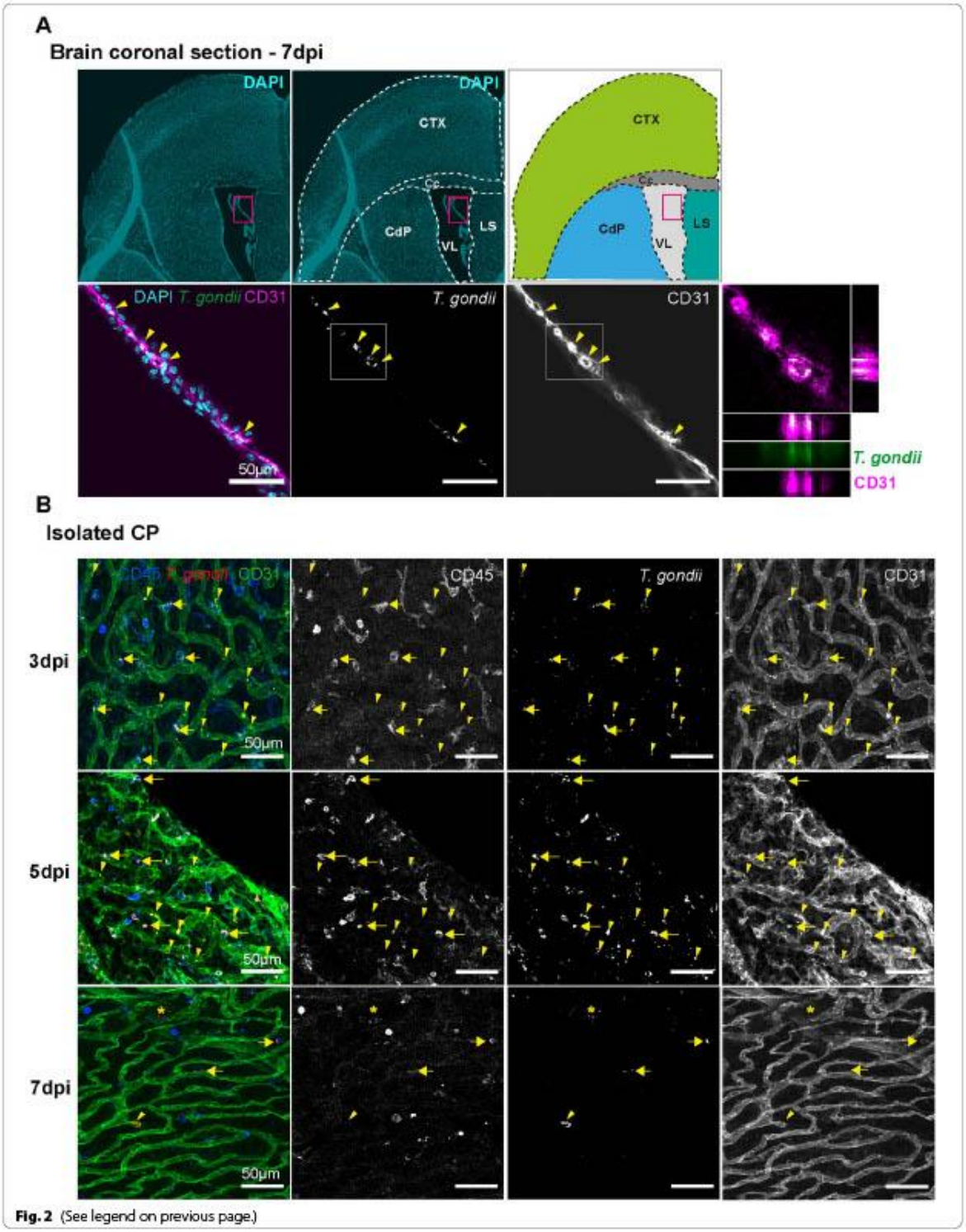

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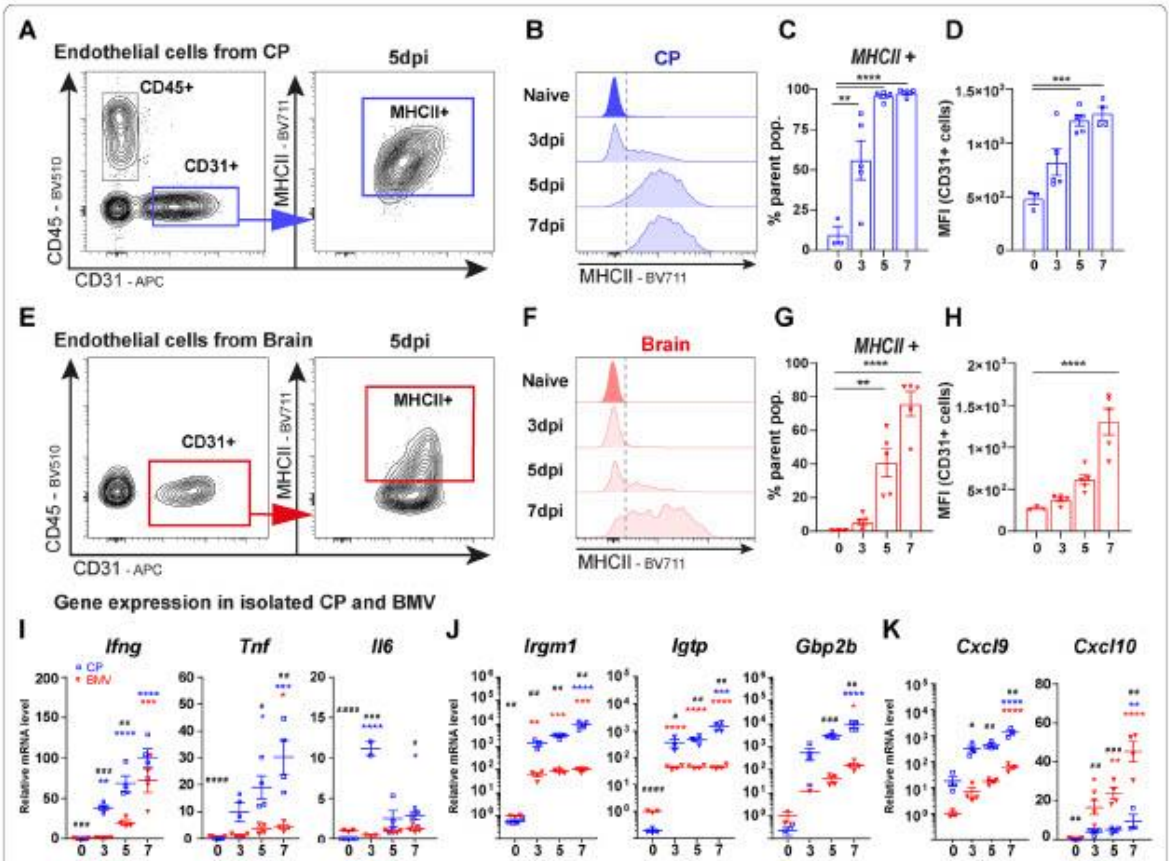


Fig. 3 Initial immune response in the CP and BBB upon *T. gondii* infection. Single live cells from (**A–D**) isolated CP and (**E–H**) brain were analyzed by flow cytometry, and the expression of MHCII was evaluated on endothelial cells ($CD31^+CD45^-$) from both tissues. Dot plots (**A, E**) represent the gating strategy based on FMO controls. Histograms (**B, F**) provide a visual representation of the temporal MHCII expression levels by endothelial cells over *T. gondii* early phase of infection. Bar charts (**C, G**) show the frequency in % of cells derived from parent population ($CD31^+CD45^-$). Bar charts (**D, H**) show MFI values of MHCII expression on the endothelial cells. RT-PCR of total RNA isolated from CP and BMV was performed for (**I**) cytokine expression, (**J**) IFN-γ-regulated host-defense factors, and (**K**) chemokines. Data from **C, D, G, H** show individual values and mean ± SEM, $n = 5$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (one-way ANOVA, with Tukey's correction). Data from **I, J, K** were normalized to naïve (day 0) BMV mean, and individual values as mean ± SEM are shown, $n = 2–4$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$, (one-way ANOVA, with Dunnett's correction). # indicates significant difference between CP and BMV from the same time-point, and * indicates significant difference between day 0 and the time-point being analyzed from the same tissue

trafficking molecules to be upregulated on CP epithelial cells in the presence of IFN-γ and TNF [50]. Accordingly, CXCL9 expression was higher in CP throughout the observation period. On the contrary, CXCL10 was more pronounced in the BMV, and both chemokines were progressively increased during the infection (Fig. 3K). At last, M-CSF (macrophage colony-stimulating factor, *Csf1*) and CX3CL1 (fractalkine), which are chemokines involved in monocyte trafficking across the BCSFB [52] were also found upregulated in the CP at 7 dpi, while no alteration was detected for M-CSF on BMV [see Additional file 7]. Of note, ICAM-1 involved in the general trafficking of immune cells was also upregulated at the CP [see Additional file 7]. Overall, *T. gondii* elicited an early and local immune response at the BCSFB, with the expression of pro-inflammatory cytokines, IFN-γ-inducible GTPases, and leukocyte trafficking molecules.

Loss of TJ integrity affects BCSFB function in early infection

TJ molecules constitute the main components of the paracellular barrier established by CP epithelial cells, therefore they assume an essential role determining cellular and molecular compounds delivered from blood into CNS [25]. To investigate the outcome of *T. gondii*

infection at the BCSFB, we analyzed the expression of TJs in isolated CP tissue over the course of infection. Claudins form the majority group of the integral membrane TJ proteins, which establish complex interactions with intracellular linker proteins like zonula occludens proteins (ZO, *Tjp*) [53]. Upon *T. gondii* infection at the CP, we detected lower expression levels of Claudin-2, -3, -5, and -11 compared to naïve group, suggesting a TJ alteration (Fig. 4A). Moreover, the breakdown of epithelial barriers has been associated with increased activity of matrix metalloproteinases (MMPs), which were indicated to be detrimental for BCSFB integrity during neuroinflammation [33]. Here, infected CP tissue displayed a robust upregulation of MMP-8 and MMP-13 (Fig. 4B), pointing towards a potential involvement of those endopeptidases in the epithelial barrier perturbation upon parasite invasion of the BCSFB. Taken together, the expression levels of claudins and MMPs in the infected CP suggested a barrier disruption. Indeed, immunostaining for Claudin-2 on brain sections showed ruffled-shape irregularities on the contour of CP epithelial cells in infected mice, instead of the smooth and continuous staining pattern depicted in naïve CP (Fig. 4C) [see Additional file 8]. In addition, ZO-1 staining indicates a grade of erasure in the infected CP, interestingly depicted in the same area of Claudin-2 morphological alteration. Finally, we determined whether altered TJ and MMP expression patterns affected CP barrier function. For this purpose, a FITC-dextran permeability assay was performed to assess barrier function in vivo. The extravasation of the fluorochrome-conjugated polysaccharide was quantified in the CSF for CP integrity, and in the remaining brain homogenate for BBB integrity (Fig. 4D). In contrast to the BBB, BCSFB permeability increased over time. A significant difference between both compartments was first visible at 7 dpi and further increased from 10 to 23 dpi. BBB vascular alterations were not depicted [see Additional file 9]. Thus, our results reveal a continuous increase in BCSFB permeability, suggesting long-standing detrimental effects of *T. gondii* infection on CP barrier integrity. Altogether, *T. gondii* infection results in TJs disturbances in the CP, with possible involvement of MMP-8 and MMP-13, culminating in functional loss and increased permeability of the BCSFB.

Response of choroid plexus epithelial cells to *T. gondii* in vitro

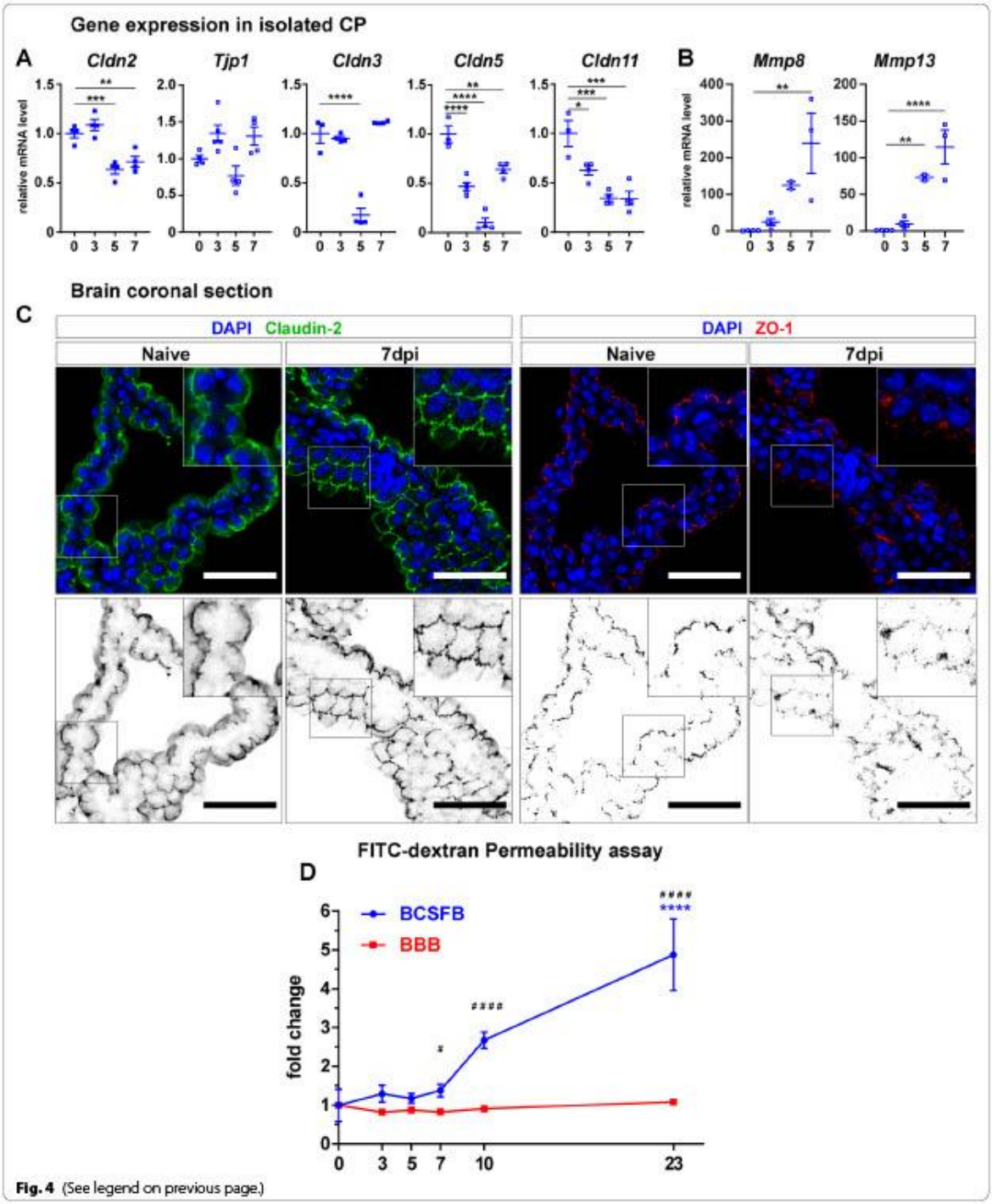
For a more detailed characterization of *T. gondii* infection effects in the CP epithelium, we developed an in vitro culture model for murine primary CP epithelial cells. First, we confirmed the ability of the cultivated cells to establish adhesion junctions and express ZO-1 (honeycomb shape) (Fig. 5A). In addition, cell cultures were largely devoid of IBA-1$^+$myeloid cells and contained mainly CD45$^-$ CD31$^-$ E-cadherin$^+$epithelial cells (Fig. 5B). Subsequently, CP epithelial cells were cultured in the presence or absence of GFP$^+$ *T. gondii* and the expression of the TJ proteins ZO-1 and Claudin-2 were quantified (Fig. 6). ZO-1 (Fig. 6A) and Claudin-2 (Fig. 6B) showed a poor and non-continuous distribution, characterized by strand breaks and puncta upon infection, compared to naïve controls. Additionally, the gene expression analysis of the cultivated epithelial cells showed no expression of IFN-γ, but increased expression of IFN-γ-receptor (*Ifngr2*) upon infection. The tissue damage-associated cytokines TNF and IL-6 were also upregulated, and the chemokine CCL2, responsible for myeloid cell recruitment was about 60-fold higher on infected cultures (Fig. 6C). We also detected a discrete reduction of ZO-1 (encoded by *Tjp1*) expression upon infection, without change and in the levels of Claudin-2 and Claudin-11 (Fig. 6D). In agreement with (Fig. 4B), MMP-8 and MMP-13 showed a robust upregulation upon infection, suggesting that CP epithelial cells are relevant source of MMPs (Fig. 6E). Overall, these results demonstrate that epithelial cells from CP directly respond to *T. gondii* infection, and contribute to the local inflammatory response and BCSFB damage.

Discussion

The pivotal role of the CP in the orchestration of neuroinflammation became evident in the recent years [49, 54–56], and distinct pathogens have been described exploiting the CP as gateway into CNS [35]. These microbes cross CNS barriers via paracellular entry, transcellular penetration, or via infected immune cells ("Trojan horse") [57]. The same mechanisms have been proposed for *T. gondii* invasion of the BBB [23], but only

(See figure on next page.)

Fig. 4 Dysregulated tight junctions in the CP affect BCSFB function during early infection. **A** Tight junctions and **B** MMP-8, and MMP-13 expression analysis (RT-PCR) of total RNA isolated from CP at 3, 5 and 7 dpi. Data show individual values and mean ± SEM, $n = 2–5$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (one-way ANOVA, with Dunnett's correction). **C** Immunofluorescence of coronal brain sections stained for identification of Claudin-2 (green) and ZO-1 (red) tight junctions from naïve and 7 dpi mice. White squares identify the regions of interest shown in higher magnification in the white upper squares. Lower panels indicate the respective inverted separated channels for each staining without DAPI to visualize the protein disturbances. Scale bars = 50 µm. **D** Functional FITC-dextran permeability assay comparing the leakage of the BCSFB (blue) versus BBB (red) throughout the course of infection. Data show mean ± SEM ($n = 5$) for each time-point analyzed. $^{****}p < 0.0001$, $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$. # indicates significant difference between BCSFB and BBB from the same time-point (multiple *t*-test), and * indicates significant differences between day 0 and the time-point being analyzed from the same barrier type (one-way ANOVA, with Dunnett's correction)



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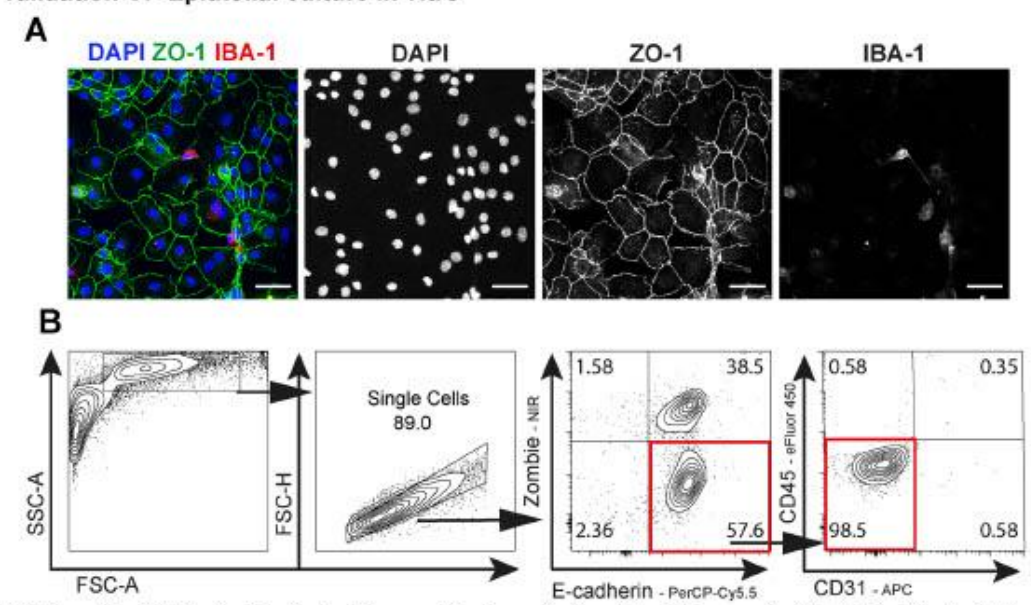

Fig. 5 Validation of CP epithelial cell culture in vitro. **A** Representative image of cultured choroid plexus epithelial cells stained for the tight junction ZO-1. The myeloid cell marker IBA-1 was utilized as control for immune cell contamination. **B** Flow-cytometric validation of cultured epithelial cells, identified as E-cadherin$^+$CD45$^-$CD31$^-$. Numbers represent the frequency from parent population

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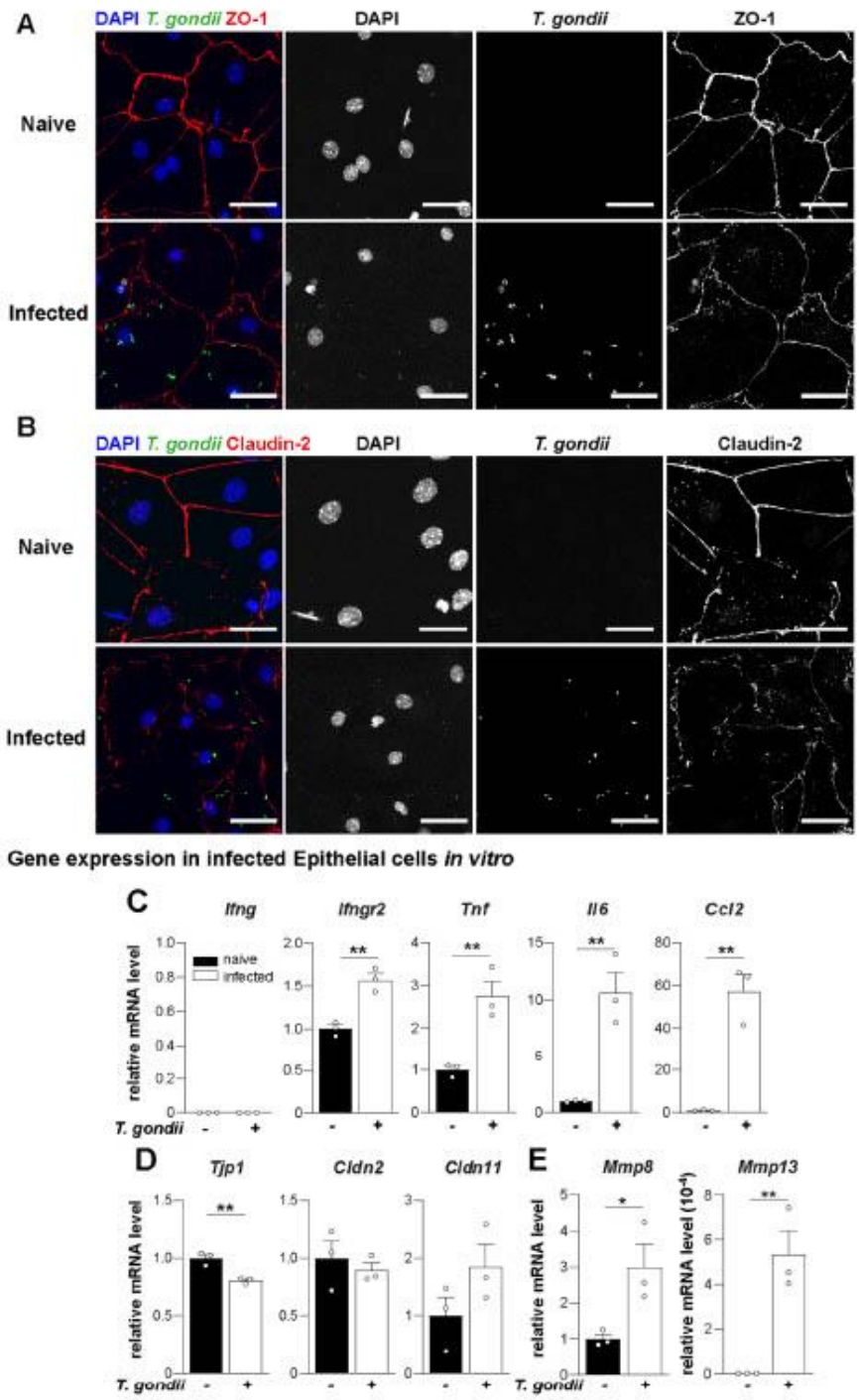
Fig. 6 Epithelial CP cells response against *T. gondii* in vitro. **A**, **B** Primary cultures of CP epithelial cells were infected for 6 h with reporter *T. gondii* type II PRU-GFP tachyzoites at MOI = 5, and controls remained non-infected. Cultivated cells were immune-stained for detection of (**A**) ZO-1 (red) and (**B**) Claudin-2 (red). DAPI stained the nucleus (blue). GFP-reporter *T. gondii* (green). Scale bars = 50 µm. **C**–**E** Naïve and infected cultures were analyzed for gene expression of (**C**) cytokines and *Ifngr2*, (**D**) ZO-1 (*Tjp1*) and tight junctions, and (**E**) MMPs. Bar charts show individual mean values of triplicates from a representative experiment, and the mean + SEM ($n = 3$) for each time-point analyzed. Data were normalized by naïve means, besides *Mmp13* which was not detectable on naïve samples $^*p < 0.05$, $^{**}p < 0.01$ (Student's *t*-test)

few studies have explored the CP as a gateway for *T. gondii* [36].

Here we demonstrate that *T. gondii* was found in the CP at the onset of infection. In a model of reactivated TE, there was no evidence for the involvement of the CP in *T. gondii* dissemination [37, 38]. Whether parasites are able to translocate from blood to the CSF, via CP, and further infect CSF-inundated areas (e.g., brain, spinal cord) is still not fully understood. In fact, we have previously described the extent of spinal cord pathology in a model of experimental chronic toxoplasmosis, suggesting that parasites translocate into CSF while the infection progresses [58]. Currently, the lack of tools to specifically block parasite entry into one or another barrier presents a major challenge to unveil their respective contributions to the parasite invasion. Here, we propose that CP infection is the initial step to the development of the neuroinflammation, resulting in the disruption of the BCSFB. Evidences show that the collapse of this barrier and its regulatory mechanisms allow immune cells to enter the CNS and initiate neuroinflammatory diseases [27]. As the disease evolves, the parasite spread increases, *e.g.*, through circulation of infected monocytes which simultaneously can cross the CNS via transposition of BBB and CP. It is likely that the higher parasite burden on BMV at 7 dpi indicate parasite shuttling to small CNS capillaries due to infected embolized monocytes, as suggested by previous studies [59].

Our findings suggest that the activation of the CP vasculature is involved in the orchestration of the anti-parasitic immune responses at early time points of infection. We observed that choroidal endothelial cells upregulated MHCII expression in response to *T. gondii* earlier than brain endothelial cells or microglia. Of note, brain endothelial cells have been described to serve as a replicative niche for *T. gondii* invasion of the CNS [7], but



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whether activated endothelial cells can control parasite replication in vivo is still unknown. Though, early MHCII presentation by CP endothelial cells suggests that they may play a role in antigen-presentation and interaction with T cells during *T. gondii* infection. They likely initiate a prompt recruitment of immune cells to the CP, orchestrating the onset of infection-induced neuroinflammation. In line with our findings, CP has been considered previously as a niche for T-cell proliferation and stimulation within the CNS [60]. Vascular and epithelial alterations at the CP, followed by other alterations like reduced choroidal synthesis, transport capacity, and CSF secretion were shown during aging, and were described to be intensified in Alzheimer's disease [61]. Therefore, our data confirm the CP as an important interface to early pathogen interaction with endothelial cells, likely promoting damage recognition, antigen-presentation and immune cell recruitment.

Infections at the BCSFB has proven to promote CNS inflammation, inducing cytokines and chemokine secretion locally, and in the CSF, mediating immune cell activation and recruitment [62]. The CP senses CNS injury and rapidly responds to inflammation, upregulating the expression of adhesion molecules and chemokines receptors essential for leukocyte trafficking [50]. Upon the establishment of infection at the CP, we have found that *T. gondii* elicited early and local immune response at the BCSFB, with expression of pro-inflammatory cytokines, IFN-γ-inducible GTPases, and leukocyte trafficking molecules. In face of early detection of $CD45^+$ cells associated to *T. gondii* on the CP, we hypothesize that a transient burden of extracellular parasites in the blood likely reach the CP interface, infecting the choroidal endothelial cells and challenging the resident stromal macrophages and epithelial cells. Additionally, IFN-γ, TNF and IL-6 upregulation in the CP of infected mice might be due to the combined action of immune and epithelial cells, which showed elevated levels of gene expression after in vitro culture with *T. gondii*. This suggests a direct anti-parasitic, pro-inflammatory response of the CP epithelial cells. However, IFN-γ gene expression was only detectable in the CP in vivo, but not in CP epithelial cells co-cultured with *T. gondii*. This indicates that IFN-γ expression is restricted to immune cells. Whether they are CP-resident and/or recruited immune cells from the periphery remains to be clarified. IFN-γ-signaling through CP epithelium have been shown to tightly regulate cell recruitment into CNS [50], and probably will determine the immune cell dynamics through the CP upon *T. gondii* infection. Besides, we detected upregulation of IFN-γ-receptor (*Ifngr2*) on in vitro-infected CP epithelial cells, likely resulting in the increased expression of leukocyte trafficking genes CSF-1, CX3CL1, ICAM-1, CXCL9 and CXCL10, as previous described [50]. Moreover, we found CXCL9 more expressed in CP rather than in BMV through the first week of infection. Indeed, CXCL9 is known to induce T-cell activation and recruitment into the brain during cerebral toxoplasmosis [63]. Hereby, CXCL9 could be a differential trafficking molecule expressed by the CP to specific modulated the control of *T. gondii* infection.

Dysfunctional BCSFB is part of the pathophysiology leading to increased neuroinflammation [32]. At the BCSFB, the permeability is determined by TJ, specifically the Claudin-family proteins. Claudin-1, -2, -3 are highly expressed in the CP, and have been shown to be sensitive to inflammation [64–67]. In our analysis, Claudin-2 was downregulated and structurally disorganized within CP epithelial cells, displaying a ruffled shape upon infection. TJ ruffling frequently correlates with increased paracellular permeability caused by altered anchoring into actin filaments [68–70]. This anchoring is dependent on the scaffold protein ZO-1, which here displayed a grade of erasure in the infected CP. Accordingly, alterations of Claudins and ZO-1 in the CP have also been described in experimental autoimmune encephalomyelitis (EAE) and LPS-sepsis models [71, 72]. Of note, the infection of BCSFB by *Trypanosoma brucei* has indicated direct parasite interactions with Claudins of the CP, although the mechanisms of how they induce TJs opening is still not known [73, 74]. Still, the disruption of the TJ has been associated to the activity of MMPs enzymes implicated on BCSFB breakdown in neurodegenerative diseases and bacterial meningitis [29, 75]. We detected increased expression levels of the MMP-8 and MMP-13 on the infected CP, with both upregulated on in vitro CP epithelial cultures after *T. gondii* infection. Indeed, previous studies have described MMP-8 contribution to BCSFB leakage, and MMP-13 association with TJ dysregulation [31, 76].

Importantly, here we discovered that the CP damage extended to the chronic phase of experimental murine toxoplasmosis, presented by increased BCSFB permeability, which correlates to our previous findings on neuronal impairment during chronic toxoplasmosis [18, 20, 39, 77]. Therefore, we propose that the substantial change on the CP permeability has detrimental implications for CSF composition and impairment of the CNS drainage. Both aspects may contribute to the increased neuroinflammation and subsequent neuronal damage during toxoplasmosis.

Conclusion

In summary, we detected that the CP is initially infected early after the onset of *T. gondii* infection, where next to CP endothelial cells also immune cells are targeted, determining a rapid inflammatory response and loss of BCSFB integrity and functionality. These processes are likely driven by TJ disturbance of Claudins within CP epithelial cells and by MMP activity. Thus, dysfunctional BCSFB may early enhance and further contribute to *T. gondii*-induced neuroinflammation, therefore emerging as a crucial target for new therapeutic approaches in cerebral toxoplasmosis.

Abbreviations

AF488: Alexa Fluor 488; AF555: Alexa Fluor 555; AF594: Alexa Fluor 594; AF647: Alexa Fluor 647; ANOVA: Analysis of variance; BBB: Blood–brain barrier; BCSFB: Blood–cerebrospinal fluid barrier; BMV: Brain microvessels; CCL2: Chemokine (C–C motif) ligand 2; CD: Cluster of differentiation; CNS: Central nervous system; CP: Choroid plexus; CP-BAMS: Choroid plexus border-associated macrophages; CSF: Cerebrospinal fluid; CX3CL1: Chemokine (C-X3-C motif) ligand 1; CXCL10: Chemokine (C-X-C motif) ligand 10; CXCL9: Chemokine (C-X-C motif) ligand 9; DAAD: Deutscher Akademischer Austauschdienst; DAPI: 4′,6-Diamidino-2-phenylindole; DFG: Deutsche Forschungsgemeinschaft; DMEM: Dulbecco's modified Eagle medium; DNA: Deoxyribonucleic acid; DNAse I: Deoxyribonuclease type I; dpi: Days post-infection; EDTA: Ethylenediamine tetraacetic acid; EGF: Epidermal growth factor; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; FITC: Fluorescein isothiocyanate; Gbp2b: Guanylate binding protein 1; GFP: Green fluorescent protein; HBSS: Hank's balanced salt solution; HEPES: 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid; HFF: Human foreskin fibroblasts; IBA-1: Ionized calcium binding adaptor molecule 1; ICAM-1: Intercellular adhesion molecule 1; Ifngr2: IFN-γ-receptor; IFN-β: Type I interferon-beta; IFN-γ: Interferon gamma; IgG: Immunoglobulin G; Igtp: Interferon gamma-induced GTPase; IL-6: Interleukin 6; Irgm1: Immunity-related GTPase family M protein 1; ITS: Insulin–transferrin–selenite; LPS: Lipopolysaccharides; M-CSF: Macrophage colony-stimulating factor; MHCII: Major histocompatibility complex type II; MMPs: Metalloproteinases; MOI: Multiplicity of infection; NEEA: Non-essential amino acids; NGS: Normal goat serum; OCT: Optimal cutting temperature; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; PDGFRβ: Platelet derived growth factor receptor β; PFA: Paraformaldehyde; PLL: Poly-L-lysine; RNA: Ribonucleic acid; RT: Room temperature; RT-qPCR: Quantitative reverse transcription PCR; SAG1: Surface antigen 1; SEM: Standard error of the mean; *T. gondii*: *Toxoplasma gondii*; TE: Toxoplasma encephalitis; Tjp1: Tight junction protein-1; TJs: Tight junctions; TNF: Tumor necrosis factor; TTR: Transthyretin; ZO-1: Zona occludens-1.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-021-02370-1.

Additional file 1: Table S1. Oligonucleotide primers used for qPCR and RT-qPCR.

Additional file 2. Detection of *T. gondii* on BMV, brain with removed CP and Spinal cord. Animals were infected *i.p.* with 2 cysts of *T. gondii* type II ME49. CPs were removed and the remaining brain tissue was processed for isolation of BMVs. (A) BMVs from 7 dpi were stained to identify pericytes (PDGFRβ), *T. gondii* (SAG1), and the tight junction ZO-1. White square area is shown in higher magnification on the right image, and the yellow arrow points to the disseminated signal for *T. gondii*. (B) Parasite burden in spleens of infected mice at 3, 5 and 7 dpi. The analysis was performed based on the presence of B1 gene of *T. gondii* (TgB1) normalized to the murine gene *Asl*. Data were normalized to the mean values of 3 dpi, and bar charts show individual values of a representative experiment, and mean + SEM, $n = 4$. *$p < 0.05$ (multiple t-test, with Holm-Sidak correction). (C) Brains were isolated, CP removed, and remaining total brain homogenate was processed for parasite detection. (D) Spinal cords from infected mice were also used to quantify parasite burden. The analysis was performed based on expression of *Sag1* gene normalized to *Hprt* expression. Bar charts show individual values of a representative experiment, and mean + SEM, $n = 4$ (multiple t-test, with Holm-Sidak correction).

Additional file 3. Detection of *T. gondii* in the CP and brain. Animals were infected *i.p.* with 2 cysts of *T. gondii* type II ME49. The brains were isolated, and coronal sections or CP whole mount were immune-stained with anti-SAG1 (light blue), anti-CD31 (red), anti-Ecadherin (green) and DAPI (dark blue). (A) Confocal image of parasite detection on endothelial cells at 7 dpi. (B) Magnified region of interested previously identified by white square. (C) Parasites identified in the brain cortex, and white square region is magnified in (D). (E) Parasites on CP and adjacent brain ventricular areas at 7 dpi. (F) CP whole tissue mount from animals infected *i.p.* with 1×10^5 *T. gondii* type II PRU-tdTomato tachyzoites, showing detection of parasites inside immune and endothelial cells at 3 dpi.

Additional file 4. IBA-1 staining and morphological identification of CP resident macrophages. Brain coronal sections of naïve animals were stained for identification of CP resident macrophages (elongated cells, red) indicated by yellow arrow. White squares indicate magnified region of interested. Scale bars = 50 µm.

Additional file 5. Alternative surface marker for identification of endothelial cells. Single cells suspensions obtained from CP (A-D), brain (E–H), liver (I-K) and lymph nodes (L-M) were digested and processed under the same conditions, and additional surface markers gp38 and VE-cadherin were used to verify identification of endothelial cells. Dot plots and numbers are from a representative sample. Bar charts represent the frequency in % of cells from parent population. Data represent individual values and mean ± SEM, $n = 3$.

Additional file 6. MHCII expression by microglia and CP epithelial cells. Single cells suspensions were discriminated based on FSC-SSC parameters, singlets, and viable cells (Zombie NIR negative). (A) Brain cells were gated and identified as microglia ($CD11b^+CD45^{int}$), $CD45^+$ immune cells, and double negative (DN) cells. (B) Choroid plexus cells were first divided in two main populations based on FSC-SSC. Bigger, viable cells were first defined as $CD45^-CD31^-$ then positive for the CP epithelial cell marker TTR (transthyretin). Smaller, viable cells were gated as $CD45^+CD31^-$ immune cells, and $CD45^-CD31^+$ endothelial cells. (C) Representative contour plots showing microglia and (D) CP epithelial cells MHCII expression at 7 dpi. Bar charts represent the frequency in % of cells from parent population, and MFI values of MHCII expression levels. Data represent individual values and mean ± SEM, $n = 5$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (one-way ANOVA, with Tukey's correction).

Additional file 7. Complementary gene expression analysis of CP and BMVs. RT-PCR of total RNA from isolated tissue, for (A) MHCII expression on CP, (B, C) expression of interferon-beta-1 (*Ifnb1*) and macrophage colony-stimulating factor (*Csf1*), respectively, in isolated CP and BMV. (D, E) Expression of fractalkine (*Cx3cl1*) and intercellular-adhesion-molecule-1 (*Icam1*) on CP. Data show individual values and mean + SEM, $n = 3$–5, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ (A, D, E, one-way ANOVA with Tukey's correction; B, C, one-way ANOVA with Dunnett's correction).

Additional file 8. Complementary staining of Claudin-2 in the CP. Brain coronal sections of naïve and 7 dpi animals were stained for identification of morphological alterations of Claudin-2 (green) in CP epithelium. Scale bars = 50 µm.

Additional file 9. Complementary BBB evaluation of VE-cadherin and ZO-1 upon infection. Animals were infected *i.p.* with 1×10^5 *T. gondii* type II PTG-GFP tachyzoites (green). The brains were isolated at 7 dpi, and coronal sections were immune-stained for identification of VE-cadherin and ZO-1 tight junctions (red). Cortical and hippocampal areas were imaged. White squares identify the regions of interest shown in higher magnification.

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Authors' contributions
CAF and SA performed experiments and analyzed data. JS, LM, AK, OL, MAD, TD critically discussed experimental design, provided material and co-edited the manuscript. CAF and IRD conceived experimental design, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All animal experiments were approved by the respective authorities (Landesverwaltungsamt Halle, Sachsen-Anhalt, Germany) in accordance with German and European legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Publisher's Note

CHAPTER 2 – THE NEUROPEPTIDE PACAP UPON *T. GONDII* INFECTION

The neuropeptide PACAP

PACAP (Pituitary adenylate cyclase-activating polypeptide) was discovered together with many other hypothalamic hormones in the 1980s from the extract of ovine hypothalamic tissue, and currently it accumulates over 30 years of scientific studies (Denes et al., 2019). PACAP remained highly conserved during its evolution, being structurally identical in many species from tunicates to mammals. Together with secretin and vasoactive intestinal peptide (VIP), PACAP belongs to the glucagon superfamily (Sherwood et al., 2000). In nature, PACAP exists in two biologically active forms, the full-length PACAP1-38 representing about 90% of total natural frequency, and the form PACAP1-27 with shorter C-terminal (Kovacs et al., 2021). PACAP1-38 (or PACAP38, for simplicity, referred to as PACAP from now on) is a 38-amino acid of amphipathic structure, with a preponderance of arginine (R) and lysine (K) basic residues (see **Figure 5**). This high polycationic nature and its helical conformation set PACAP as a cell-penetrating peptide, and also suggest potential antimicrobial effects (Zasloff, 2021).

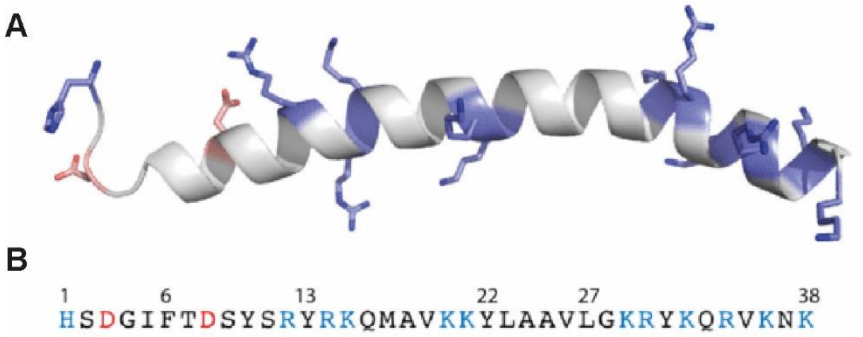

Figure 6. The neuropeptide PACAP38
(**A**) Molecular structure of PACAP38 (PDBID: 2D2P). (**B**) Amino acid sequence of PACAP38, with basic residues in blue and acidic residues in red. Adapted from (Liao et al., 2019).

PACAP has been described to be widely expressed in the CNS of mammals, and therefore it is classified as a neuropeptide (Hirabayashi et al., 2018; Szabo et al., 2021). According to recent annotations in the Human Protein Atlas program database, single-cell RNA-sequencing analyzes have shown expression of PACAP (encoded by *Adcyap1* gene) mostly by granulocytes and excitatory neurons, and to a lesser extent (10 fold) by specific epithelial cells and fibroblasts, T cells, microglia, and plasma cells (database, 2022; Karlsson et al., 2021). PACAP employs its biological activity through binding to three widely distributed G protein-coupled receptors (GPCRs) i.e., PAC1R, VPAC1R, and VPAC2R (Soles-Tarres et al., 2020), which triggers a series of intracellular signaling pathways, culminating in the production of cyclic adenosine monophosphate (cAMP) (Langer, 2012; Vaudry et al., 2009). Of note, PACAP receptors are present not only on the plasma membrane but also within the nucleus and mitochondria. The higher intracellular abundance of some PACAP receptors is believed to explain why PACAP shows a "bell shape" curve of actions, where a concentration threshold is needed to activate intracellular receptors (Yu et al.,

2013). Because PACAP has a small size, it can be artificially synthesized by solid phase strategy and then explored in many different experimental setups *in vivo* and *in vitro*. Notably, PACAP penetrates the BBB entering the CNS via specific peptide transporter system-6 (PTS-6), showing the highest influx ratio from blood to CNS among other peptides of the glucagon superfamily (Dogrukol-Ak et al., 2004). In fact, PACAP has an intracerebral transfer rate 6 times faster than morphine (Banks et al., 1993). However, its application as a drug still faces many challenges common to other natural peptides, such as poor metabolic stability and rapid blood clearance (Bourgault et al., 2011). Still, many promising strategies have been applied to improve PACAP properties and obtain a clinical therapeutic drug (Erak et al., 2018).

The dual and ambiguous role of PACAP

Immunomodulatory properties

PACAP has been described to strongly modify inflammation and clinical symptomatology in different rodent models of inflammatory diseases (Abad et al., 2006; Toth et al., 2020). PACAP receptors have different affinities for PACAP, with PAC1R possessing the highest affinity (Pozo et al., 1997). In addition, the receptors are differently expressed among immune cells; for example, PAC1R and VPAC1R are constitutively expressed by monocytes, peritoneal macrophages, and microglia, while T cells do not express PAC1R. VPAC2R is rarely detected under steady-state, but it is induced in T cells, monocytes and macrophages upon inflammation. Notably, microglia do not express VPACR2 in steady-state, nor upon inflammatory stimulation (Delgado et al., 2004; Delgado et al., 1996b). In addition, the expression of VPACR1 and VPACR2 was found in type 2 ILC (ILC2s) (Nussbaum et al., 2013), and were implicated in the resolution of inflammation (Delgado, 2016; Talbot et al., 2015).

As an immunomodulator, PACAP exerts a dual role in regulating innate immunity depending on the activation status of the cells and their environment. Several studies reported that PACAP is a potent mediator for both innate and adaptive immunity, primarily assuming an anti-inflammatory role. Exposure to PACAP inhibits the pro-inflammatory response of macrophages, such as the production of TNF (Delgado et al., 1999) and IL-6 (Martinez et al., 1998a), as well as the chemokines CCL2, CCL3, and CCL5 (Delgado et al., 2003). Additionally, PACAP treatment leads to the polarization of T helper cells to a type 2 (Th2) phenotype (Delgado, 2003). It also promotes the development of tolerogenic DCs and favors the generation of regulatory T cells (Tregs), suppressors of immune responses (Delgado et al., 2005). In contrast, PACAP was able to stimulate the phagocytic activity, adhesion, and mobility of resting macrophages as well as the release of free radicals and IL-6 (Delgado et al., 1996a; Garrido et al., 1996; Martinez et al., 1998b), altogether highlighting PACAP-dependent immunomodulation as a crucial mechanism for pathogen elimination.

Overall, the immunomodulatory properties of PACAP are considered ambiguous, transiting from inhibition to stimulation, pro-inflammatory and anti-inflammatory, depending on the experimental conditions and cell types (Abad and Tan, 2018), and therefore they request further investigation.

Neuroprotective properties

PACAP has shown neuroprotective potential in several neurological disorders. In experimental models of Parkinson's disease (PD), PACAP exerted anti-apoptotic and anti-autophagic effects in dopaminergic neurons, and prevented behavioral alterations (Lamine-Ajili et al., 2016; Maasz et al., 2017). In AD models, administration of PACAP also showed an anti-apoptotic effect, associated with increased expression of Aβ-degradation enzymes and improved cognitive function of the treated transgenic animals (Rat et al., 2011). In Huntington's disease (HD) model, PACAP reduced the formation of mutant huntingtin aggregates and promoted hippocampal expression of brain-derived neurotrophic factor (BDNF) (Cabezas-Llobet et al., 2018). Finally, upon traumatic brain injury, PACAP exerted antioxidative effects, prevented apoptosis, increased BDNF levels, and stimulated axonal growth (Shioda and Nakamachi, 2015). Despite these beneficial effects observed in animal models, clinical studies targeting PACAP signaling mainly addressed nociception (i.e., pain) upon different conditions, such as nephrotic syndrome, cluster headache, migraine, and major depression (Denes et al., 2019; Dominguez-Moreno et al., 2022).

As previously mentioned, the neuromodulatory properties of PACAP were shown to be involved with the signaling of the neurotrophin BDNF, promoting neuronal survival and synaptic plasticity (Frechilla et al., 2001). The family of neurotrophins comprises of four secreted proteins characterized by their ability to modulate survival, differentiation, and apoptosis of neurons (Bothwell, 2016). BDNF, nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) exert their functions by binding to tropomyosin-regulated kinase (Trk) receptors (TrkA, TrkB, TrkC), and the p75 neurotrophin receptor ($p75^{NTR}$). Interestingly, neurotrophin receptors were shown to be transactivated by PACAP in human monocytes, triggering their activation (El Zein et al., 2008; El Zein et al., 2007; El Zein et al., 2006; El Zein et al., 2010). Together, a growing body of evidence proposes an intertwined interaction among PACAP, neurotrophins, and immune cells. In this context, a recent report highlighted the effect of neurotrophin signaling via $p75^{NTR}$, which consisted in the alteration of innate immune cell activation and modification of the structural plasticity of neurons (Dusedau et al., 2019). Therefore, the modulatory effect of PACAP on immune cells via neurotrophins added another complicated and unexplored path in the interface of the nervous and immune system, which requires further exploration.

PACAP and infectious diseases

Associated with the ability of PACAP to modulate innate immune cells, it was proposed that PACAP could also directly affect pathogens. Anti-parasitic effects of PACAP were first described against the protozoa *Trypanosoma brucei* (*T. brucei*), which is the causative agent of African sleeping sickness. The study showed a membrane-lytic effect in parasites, attributed to the amphipathic and cationic structure of PACAP, establishing a close association with autophagy and apoptosis-like cell death (Delgado et al., 2009). Based on these findings but also considering the structural features of PACAP, other studies proposed that it could act as a broad spectrum antimicrobial peptide and that increased expression of PACAP during the course of a viral or bacterial infection suggested a strong relation with immune response (Debbabi et al., 2018; Lugo et al., 2019; Starr et al., 2018). More recently, a study used bioinformatics tools to scan a peptide database for the probability of inducing deformation in bacterial membranes and found PACAP among the most promising candidates. Experimentally, the study confirmed the antimicrobial activity of PACAP against gram-positive and gram-negative bacterial species and against *Candida albicans* (Lee et al., 2021).

Besides the protozoan *T. brucei*, PACAP was investigated as a therapeutic approach for experimental intestinal inflammation caused by *T. gondii*. Upon exogenous administration of synthetic PACAP, the acute inflammation and the extra-intestinal sequelae were diminished, although no effects on parasite burden were detected (Bereswill et al., 2019; Heimesaat et al., 2014). So far, those studies have used *T. gondii* infection to obtain a model for intestinal inflammation, restricting their spectrum of analysis regarding the overall infection itself. Accordingly, many other implications of PACAP treatment during *T. gondii* infection were still left unexplored. Moreover, *T. gondii* infection in the brain establishes an ongoing neuroinflammation and modifies neuronal function and synapse composition (Blanchard et al., 2015; French et al., 2019; Lang et al., 2018; Parlog et al., 2014). As no current therapy is able to completely eliminate the parasites from the CNS, new therapeutic strategies are necessary and must combine the reduction of infection-induced neuroinflammation and prevention of neuronal death and synaptic dysfunction (Dunay et al., 2018).

Aim of the Study

This part of the study set to elucidate the impact of exogenous PACAP administration throughout the infection with *T. gondii*. First, the effects of PACAP on the innate immune response and parasite elimination were explored during peripheral acute infection [**Publication 2**]. Next, the beneficial impact of PACAP and its modulation of neuroinflammation was evaluated during chronic cerebral infection [**Publication 3**].

Results

Publication 2

Upon acute peripheral *T. gondii* infection, exogenous administration of PACAP resulted in the reduction of inflammatory monocyte and neutrophils recruitment to the peritoneal cavity and increased the expression of MHC-II by peritoneal DCs. By infecting mice with GFP-reporter parasites, PACAP-treated animals showed a reduction of infected cells among the peritoneal myeloid populations. In these cells, increased transcriptional levels of the cell-autonomous host defense factors IRGM1 and IRGM3 were detected, indicating enhanced anti-parasitic activity of PACAP. This effect was further examined *in vitro*, where an immune cell-mediated effect of PACAP was uncovered, rather than direct damage to the parasites. Next, utilizing an *in vitro* phagocytosis assay, PACAP enhanced the phagocytic capacity of macrophages while increasing their surface expression of F4/80. Adding to this anti-parasitic effect, the neuropeptide attenuated the transcriptional levels of pro-inflammatory mediators *in vivo*, such as IFN-γ, TNF, IL-6, CCL2, and iNOS, while upregulating the gene expression of its own receptors VPAC1R and VPAC2R. Furthermore, PACAP-treated animals displayed increased transcriptional levels of BDNF in peritoneal cells and diminished expression of the $p75^{NTR}$ by $Ly6C^{hi}$ inflammatory monocytes, pointing towards the fundamental contribution of PACAP to neurotrophin signaling in immune cells.

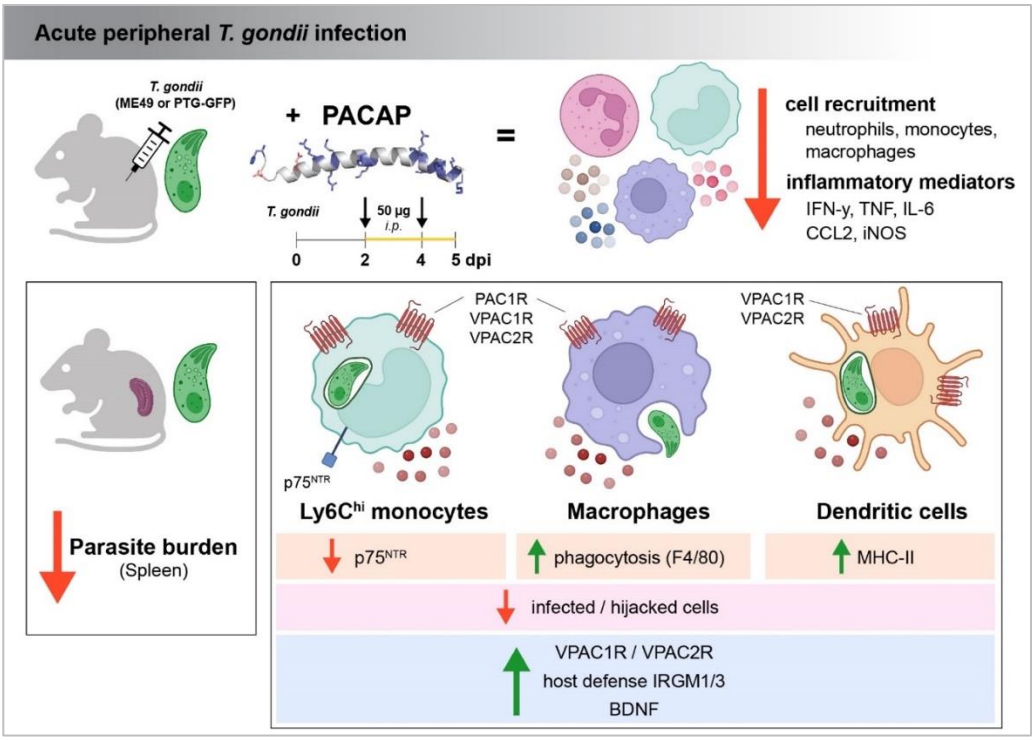

Figure 7. Graphical abstract - Publication 2

Immunomodulatory effects of PACAP upon acute *T. gondii* infection. PACAP treatment upon experimental acute peripheral infection resulted in reduced immune cell recruitment to the peritoneal cavity and reduced expression of pro-inflammatory mediators. PACAP reduced the parasite burden in treated mice and modulate the different aspects of monocytes, macrophages and dendritic cells, pointing out the immune cell-mediated anti-parasitic activity. This effect was associated with increased phagocytic capacity, upregulation of cell-intrinsic host defense factors, and modulation of neurotrophin signaling on immune cells. Created with Biorender.com

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Immunomodulatory Effects of the Neuropeptide Pituitary Adenylate Cyclase-Activating Polypeptide in Acute *Toxoplasmosis*

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Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP) is an endogenous neuropeptide with distinct functions including the regulation of inflammatory processes. PACAP is able to modify the immune response by directly regulating macrophages and monocytes inhibiting the production of inflammatory cytokines, chemokines and free radicals. Here, we analyzed the effect of exogenous PACAP on peripheral immune cell subsets upon acute infection with the parasite *Toxoplasma gondii (T. gondii)*. PACAP administration was followed by diminished innate immune cell recruitment to the peritoneal cavity of *T. gondii*-infected mice. PACAP did not directly interfere with parasite replication, instead, indirectly reduced parasite burden in mononuclear cell populations by enhancing their phagocytic capacity. Although proinflammatory cytokine levels were attenuated in the periphery upon PACAP treatment, interleukin (IL)-10 and Transforming growth factor beta (TGF-β) remained stable. While PACAP modulated VPAC1 and VPAC2 receptors in immune cells upon binding, it also increased their expression of brain-derived neurotrophic factor (BDNF). In addition, the expression of p75 neurotrophin receptor ($p75^{NTR}$) on $Ly6C^{hi}$ inflammatory monocytes was diminished upon PACAP administration. Our findings highlight the immunomodulatory effect of PACAP on peripheral immune cell subsets during acute *Toxoplasmosis*, providing new insights about host-pathogen interaction and the effects of neuropeptides during inflammation.

Keywords: pituitary adenylate cyclase-activating polypeptide (PACAP), *Toxoplasma gondii*, acute infection, monocytes, macrophages, innate immunity, neurotrophins

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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP) is a 38-amino-acid neuropeptide in the glucagon superfamily together with secretin and vasoactive intestinal peptide (VIP) (Sherwood et al., 2000). PACAP is widely expressed in the peripheral and central nervous systems (CNS) and functions as a neurotransmitter, neuromodulator and neurotrophic factor (Waschek, 2002; Zhou et al., 2002; Dejda et al., 2005; Botia et al., 2007; Armstrong et al., 2008; Abad and Tan, 2018). The nervous and immune systems participate in a complex bidirectional cross-talk through neuropeptides such as PACAP/VIP in multiple organs (Abad and Tan, 2018). Correspondingly, PACAP strongly improves the outcome of inflammatory disorders, such as rheumatoid arthritis, septic shock, inflammatory bowel disease, and multiple sclerosis in rodent models (Abad et al., 2001, 2003; Martinez et al., 2002; Gonzalez-Rey et al., 2006; Tan et al., 2009).

PACAP acts by binding to three specific membrane receptors from the G protein-coupled receptors (GPCR) family: PAC1, VPAC1, and VPAC2. The PAC1 receptor is highly expressed in the nervous system and possesses the highest affinity for PACAP, while the receptors VPAC1 and VPAC2 have the same lower affinity for PACAP, and are expressed among different cell types (Pozo et al., 1997). The majority of immune cells express one or more PACAP receptors. For example, PAC1 is expressed on peritoneal macrophages, microglia and pulmonary dendritic cells (DCs) (Delgado et al., 2004a). VPAC1 is constitutively expressed in T cells, macrophages, monocytes and DCs (Delgado et al., 2004a). VPAC2 is rarely expressed in these cells during a resting state, but its expression is induced following lipopolysaccharide (LPS) stimulation *in vitro* (Delgado et al., 1996b). Recently, the expression of VPAC1 and VPAC2 was found in innate lymphoid cells (ILC) 2 (Nussbaum et al., 2013), and has been implicated in the resolution of inflammation (Talbot et al., 2015).

Many studies have demonstrated that PACAP acts as a neuronal growth factor during development and regeneration (Waschek, 2002; Deguil et al., 2007; Watanabe et al., 2007). The neuromodulatory properties of PACAP were shown to be involved with the neurotrophin signaling of brain-derived neurotrophic factor (BDNF), promoting neuronal survival and synaptic plasticity (Frechilla et al., 2001). The family of neurotrophins is comprised of four secreted proteins, characterized by their ability to modulate survival, differentiation, and apoptosis of neurons (Bothwell, 2016). BDNF, nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4 (NT-4) exert their functions via interaction with tropomyosin receptor kinases (Trk) TrkA, TrkB, TrkC, and the p75 neurotrophin receptor ($p75^{NTR}$). The effect of PACAP on neurotrophin signaling was presented in human monocytes, where the exposure to the neuropeptide resulted in pro-inflammatory cell activation with increased Ca^{2+} mobilization (El Zein et al., 2006, 2007, 2008, 2010).

As an immunomodulator, PACAP exerts a dual role in regulating innate immunity depending on the activation status of cells and their environment. Several studies reported that PACAP is a potent immunomediator for both innate and adaptive immunity, primarily assuming an anti-inflammatory role. Exposure to PACAP inhibits the pro-inflammatory response of macrophages, such as the production of tumor necrosis factor (TNF) (Delgado et al., 1999b) and interleukin 6 (IL-6) (Martinez et al., 1998a), as well as the chemokines MCP-1 (CCL2), MIP1-α (CCL3) and RANTES (Delgado et al., 2003). Additionally, PACAP treatment leads to polarization of T helper cells to a Type 2 (Th2) phenotype (Delgado, 2003). It also promotes the development of tolerogenic DCs and favors the generation of regulatory T cells (Tregs), suppressors of immune responses (Delgado et al., 2005). In contrast, PACAP was able to stimulate the phagocytic activity, adhesion and mobility of resting macrophages as well as release of free radicals and IL-6 (Delgado et al., 1996a; Garrido et al., 1996; Martinez et al., 1998b), associating PACAP with a crucial mechanism to pathogen elimination.

Antiparasitic effects of PACAP were first described against the protozoa *Trypanosoma brucei*, showing a membranelytic effect, closely associated with autophagy and apoptosis-like cell death (Delgado et al., 2009). More recently, we showed that administration of PACAP ameliorated acute small intestinal inflammation and extra-intestinal sequelae caused by *Toxoplasma gondii* (*T. gondii*) infection (Heimesaat et al., 2014; Bereswill et al., 2019). *Toxoplasma gondii* is an obligate intracellular parasite acquired by oral ingestion of contaminated food or water. The parasite infects the small intestine and then differentiates to its rapidly replicating stage (tachyzoite), which is able to infect all nucleated cells through active penetration (Dobrowolski and Sibley, 1996). After crossing the intestinal barrier, the parasites encounter both resident and recruited immune cells, resulting in parasite elimination, antigen presentation and cytokine production (Buzoni-Gatel et al., 2006).

The successful dissemination of *T. gondii* within the host is highly dependent on invading migratory immune cells and the ability of these immune cells to phagocyte the parasite. Elimination of *T. gondii* involves a complex recruitment of immunity-related GTPases (IRGs) and guanylate-binding proteins (GBPs) (Zhao et al., 2009; Fentress et al., 2010). After infection, these host defense factors are known to accumulate in the membrane of the parasitophorous vacuole (PV) culminating with its disruption and parasite elimination (Macmicking, 2012). Moreover, a particular preference for myeloid cells has been explored as a key mechanism for parasite dissemination into the CNS (Weidner and Barragan, 2014; Blanchard et al., 2015).

Our group has previously demonstrated the critical importance of myeloid cells controlling *T. gondii* infection in the periphery as well as in the CNS (Dunay et al., 2008, 2010; Biswas et al., 2015; Mohle et al., 2016). Besides, we recently showed the innate immune response and the influence of neurotrophin signaling upon *T. gondii*-induced neuroinflammation. Particularly, neurotrophin signaling via $p75^{NTR}$ altered innate immune cell behavior and changed the structural plasticity of neurons (Dusedau et al., 2019).

Here, we set out to evaluate the immunomodulatory effects of PACAP on the innate immune response during *T. gondii* acute infection. We show that PACAP is able to reduce immune cell recruitment and enhance phagocytic capacity of mononuclear cells, promoting parasite elimination. At the same

time, the neuropeptide attenuated pro-inflammatory mediators while upregulating its own receptors. Interestingly, we detected altered expression of BDNF and $p75^{NTR}$ in peritoneal cells, pointing toward the contribution of PACAP to the parasite elimination and neurotrophin signaling in immune cells upon acute *Toxoplasmosis*.

MATERIALS AND METHODS

Animals

Experiments were conducted with female C57BL/6JRj mice (8 weeks old, purchased from Janvier, Cedex, France). All animals were group-housed in a 12 h day/night cycle at 22°C with free access to food and water under specific-pathogen-free conditions, according to institutional guidelines approved by the Animal Studies Committee of Saxony-Anhalt.

T. gondii in vitro Culture

Tachyzoites of ME49 and PTG-GFP type II strain of *T. gondii* were grown in monolayers of human foreskin fibroblast (HFF) cells cultured in DMEM medium (FG0435, Biochrom, Germany), supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher, Germany), 1% Penicillin/Streptomycin (Pen/Strep; Sigma, USA) and 1% non-essential amino acids (NEEA) (Thermo Fisher, Germany) (Morisaki et al., 1995). HFF cells and tachyzoites were scrapped from culture flasks, spinned down at 500 × g for 10 min and passed through 20 and 22G needles to liberate intracellular parasites. To obtain a host cell-free parasite suspension, the solution was filtered through a 5 µm Millex-SV syringe filter (Millipore, Germany). The parasite suspension was pelleted at 800 × g for 20 min, resuspended in 1 ml sterile phosphate-buffered saline (PBS) and the number of living tachyzoites was determined by counting under a light microscope using Trypan Blue 0.4%. Subsequently, the freshly egressed parasite suspension was used for infections and plaque assay experiments.

Experimental Acute *T. gondii* Infection and PACAP Administration

In order to investigate acute *T. gondii* infection in the peritoneal cavity, all mice were infected by intraperitoneal (i.p.) injection of 1×10^4 ME49 or PTG-GFP tachyzoites, freshly harvested from HFF cultures, in a final volume of 200 µl with PBS. For PACAP treatment, 50 µg (11 nmol/mouse) of the synthesized neuropeptide PACAP38 dissolved in 200 µl of PBS was administrated i.p. on days 2 and 4 post infection. Non-treated control mice received PBS only. At day 5 post infection (dpi) peritoneal exudate cells were collected by peritoneal lavage (Fentress and Sibley, 2011) for further analysis. Spleens were collected and stored in Allprotect Tissue Reagent (Qiagen, Germany) at −80°C until further processing.

Flow Cytometric Analysis

Single cell suspensions were first incubated with ZOMBIE NIR™ fixable dye (BioLegend, San Diego, CA) or 7-AAD Viability Staining Solution (BioLegend) for live/dead discrimination. To prevent unspecific binding of antibodies, anti-FcγIII/II receptor antibody (clone 93) was applied to cells before staining with fluorochrome-conjugated antibodies against cell surface markers in FACS buffer (PBS, supplemented with 2% FBS and 0.1% sodium azide). CD11b (M1/70), Ly6C (HK1.4), MHCII I-A/I-E (M5/114.15.2), and F4/80 (BM8) were all purchased from eBioscience (San Diego, USA). Ly6G (1A8) and CD11c (N418) were purchased from BioLegend and $p75^{NTR}$ (MLR2) from Abcam (Germany). Cells were incubated for 30 min at 4°C, washed and subsequently analyzed. Fluorescence Minus One (FMO) controls were used to determine the level of autofluorescent signals for each conjugated antibody. Data was acquired using a BD FACS Canto II (BD Biosciences, USA) or Attune NxT flow cytometer (Thermo Fisher, Germany) and analyzed using FlowJo (v10, FlowJo Inc., USA). A minimum of 2×10^5 cells per samples were acquired.

Plaque Assay

All experiments were conducted using fresh syringe-released extracellular tachyzoites. As previously described (Arroyo-Olarte et al., 2015), 200 parasites per well were used to infect HFF monolayers in six-well plates at different concentrations of PACAP. Briefly, parasitized cells were incubated for 7 days, fixed with cold methanol, and then stained with crystal violet. Plaques were imaged and scored for their sizes and numbers using the ImageJ software (NIH, US).

Generation of Bone Marrow-Derived Macrophages

For generation of bone marrow-derived macrophages (BMDMs), femurs and tibias of 8 to 12 weeks old C57BL/6JRj mice were collected. Bones were flushed with a syringe filled with DMEM (FG0435, Biochrom, Germany) containing 10% FBS and 1% Pen/Strep to extrude bone marrow onto a 40 µm cell strainer. The obtained cell suspension was then spinned down for 10 min at 400 × g, 4°C and the cells were seeded into 6 well/plate using DMEM supplemented with 10% FBS, 1% Pen/Strep, 10 ng/ml recombinant murine GM-CSF (315-03, PeproTech, USA) and incubated at 37°C, 5% CO_2. After 10 days, cells were primed to M1 macrophage phenotype with 150 Units/ml recombinant murine IFN-γ (315-05, Peprotech, USA) for 10 h, and then with 20 ng/ml of LPS (L2630, Sigma-Aldrich, USA) for another 12 h. Following stimulation, cells were washed and subsequently used for phagocytosis assay.

In vitro Phagocytosis Assay

Phagocytosis assay was performed with M1 macrophages generated as described above and assessed in triplicates by incubation of cells with carboxylated, yellow-green fluorescent FluoSpheres™ (F8823, Fisher Scientific, Germany) in serum-free DMEM (with 1% Pen/Strep) in the presence of PACAP (0.1, 1, and 10 µM) for 4 h at 37°C, 5% CO_2. Negative controls were established by keeping cells at 4°C throughout the experiment. After incubation, cells were washed twice with PBS to remove remaining microspheres before detachment with Accutase® solution (423201, BioLegend). Finally, detached cells were washed with PBS and directly stained with fluorochrome-conjugated antibodies for flow cytometric analysis. *In vitro* phagocytosis of microspheres was assessed by the median

fluorescence intensity (MFI) of the positive population in the FITC-fluorescence channel. Further, percentages of cells in the FITC-positive fraction where divided according to the amount of microspheres internalized.

DNA and RNA Isolation

DNA and RNA samples were isolated from peritoneal exudate cells and spleens of acutely infected mice. Spleen samples were homogenized in lysis buffer using BashingBeads Lysis tubes (Zymo Research, Germany) and isolated using AllPrep DNA/RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. For peritoneal exudate cells, parts of the cell suspensions were pelleted down, resuspended in lysis buffer and processed as describe above. The concentration and purity of DNA and RNA samples was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher; Germany).

qPCR

Parasite burden was assessed in triplicates using 30 ng of isolated DNA, FastStart Essential DNA Green Master and LightCycler® 96 System (both Roche, Germany), as described previously (Biswas et al., 2017). Thermal-cycling parameters were set as follows: initial activation (95°C, 10 min), 45 amplification cycles consisting of denaturation (95°C, 15 s), annealing (60°C, 15 s) and elongation (72°C, 15 s). The DNA target was the published sequence of the highly conserved 35-fold-repetitive B1 gene of *T. gondii* (Burg et al., 1989; Lin et al., 2000). Murine argininosuccinate lyase (*Asl*) was used as reference gene for normalization and relative DNA levels were determined by the ratio *gene of interest / reference gene* and subsequently normalized to mean values of control group (Butcher et al., 2011). Primers were synthetized by Tib MolBiol (Germany) and used at 300 nM final concentration.

RT-qPCR

Expression levels of cytokines, inflammatory mediators, host-defense factors, neurotrophins, and neurotrophin receptors were assessed in triplicates using 30 ng isolated RNA, TaqMan® RNA-to-C_T™ *1-Step* Kit (Applied Biosystems, Germany) and LightCycler® 96 (Roche, Germany) as previously described (Mohle et al., 2016). Thermal-cycling parameters were set as follows: reverse transcription (48°C, 15 min), inactivation (95°C, 10 min) followed by 45 cycles of denaturation (95°C, 15 s) and annealing/extension (60°C, 1 min). Utilized TaqMan® Gene Expression Assays (Applied Biosystems, Germany) are listed in **Supplementary Table 1**. *Hprt* was chosen as a reference gene and relative mRNA levels were determined by the ratio *gene of interest/reference gene* and subsequently normalized to mean values of control group.

The expression of PACAP receptors were evaluated using *Power* SYBR® Green RNA-to-CT™ 1-Step Kit (Applied Biosystems, Germany). Samples were analyzed in triplicates (30 ng of isolated mRNA per reaction) using LightCycler® 96 and the following parameters: reverse transcription (48°C, 30 min), inactivation (95°C, 10 min) followed by 55 cycles of denaturation (95°C, 15 s) and annealing/extension (60°C, 1 min) and melting curve analysis. The primer sequences are listed in **Supplementary Table 1** and were synthetized by Tib MolBiol and used at 100 nM final concentration. Expression of *Hprt* was chosen as reference gene and relative mRNA levels were determined by the ratio *gene of interest / reference gene* and subsequently normalized to mean values of control group.

Statistical Analysis

Results were statistically analyzed using GraphPad Prism 7 (STATCON, Germany) and two-tailed unpaired *t*-test was used on flow cytometry, qPCR and RT-qPCR data, and considered significant for $p \leq 0.05$. Statistical analysis of phagocytosis assay data was carried out by applying one-way ANOVA with *post-hoc* Holm-Sidak test. For plaque assay, data was analyzed by one-way ANOVA followed by *post-hoc* Bonferroni test. All data are presented as arithmetic mean ± standard error of the mean (SEM) and are representative of two to three independent experiments.

RESULTS

Immune Cell Recruitment Is Reduced Following PACAP Administration

Upon acute infection with *T. gondii*, neutrophil granulocytes, inflammatory monocytes and DCs are recruited to the site of infection (Robben et al., 2005; Dunay et al., 2008; Dunay and Sibley, 2010). To assess the effect of PACAP on cell recruitment and activation upon acute infection, mice were infected with tachyzoites, followed by administration of PACAP or PBS (control). The peritoneal exudate cells were collected and characterized by flow cytometry (**Figure 1A**). As our previous studies demonstrated the critical importance of myeloid cells in the control of *T. gondii* infection (Dunay et al., 2010; Biswas et al., 2015; Mohle et al., 2016), we analyzed the mononuclear compartment based on the expression of CD11b and Ly6G. While the $CD11b^+Ly6G^+$ subset defined neutrophil granulocytes, the fraction of $CD11b^+Ly6G^-$ cells was further discriminated into $CD11c^{hi}MHCII^{hi}$ DCs as previously described (Dupont et al., 2014). Subsequently, remaining immune cells were then defined according to Ly6C expression as $Ly6C^{hi}$ inflammatory monocytes and $Ly6C^-$ peritoneal macrophages.

In general, the PACAP-treated group presented less recruited cells in the peritoneal cavity (control: $1.67 \times 10^5 \pm 0.10 \times 10^5$ vs. PACAP: $0.95 \times 10^5 \pm 0.07 \times 10^5$; $p = 0.0012$) (**Figure 1B**). When compared to the controls, administration of PACAP significantly reduced the recruitment of all analyzed myeloid cell subsets. $Ly6C^{hi}$ inflammatory monocytes appeared to be the most affected cell population (control: $4.34 \times 10^4 \pm 0.10 \times 10^3$ vs. PACAP: $2.99 \times 10^4 \pm 2.41 \times 10^3$; $p = 0.00003$), followed by $Ly6C^-$ (control: $3.19 \times 10^4 \pm 2.75 \times 10^3$ vs. PACAP: $2.08 \times 10^4 \pm 2.15 \times 10^3$; $p = 0.0003$) and neutrophils (control: $1.62 \times 10^4 \pm 2.09 \times 10^3$ vs. PACAP: $7.96 \times 10^3 \pm 7.7 \times 10^2$; $p = 0.0043$). No evident difference was found for DCs (control: $4.02 \times 10^3 \pm 6.10 \times 10^2$ vs. PACAP: $1.92 \times 10^3 \pm 2.29 \times 10^2$; $p = 0.4301$) (**Figure 1C**). As the ability to present antigens is dependent on MHCII expression, we evaluated whether this was modulated by PACAP treatment upon acute *T. gondii* infection. Our data indicated that PACAP

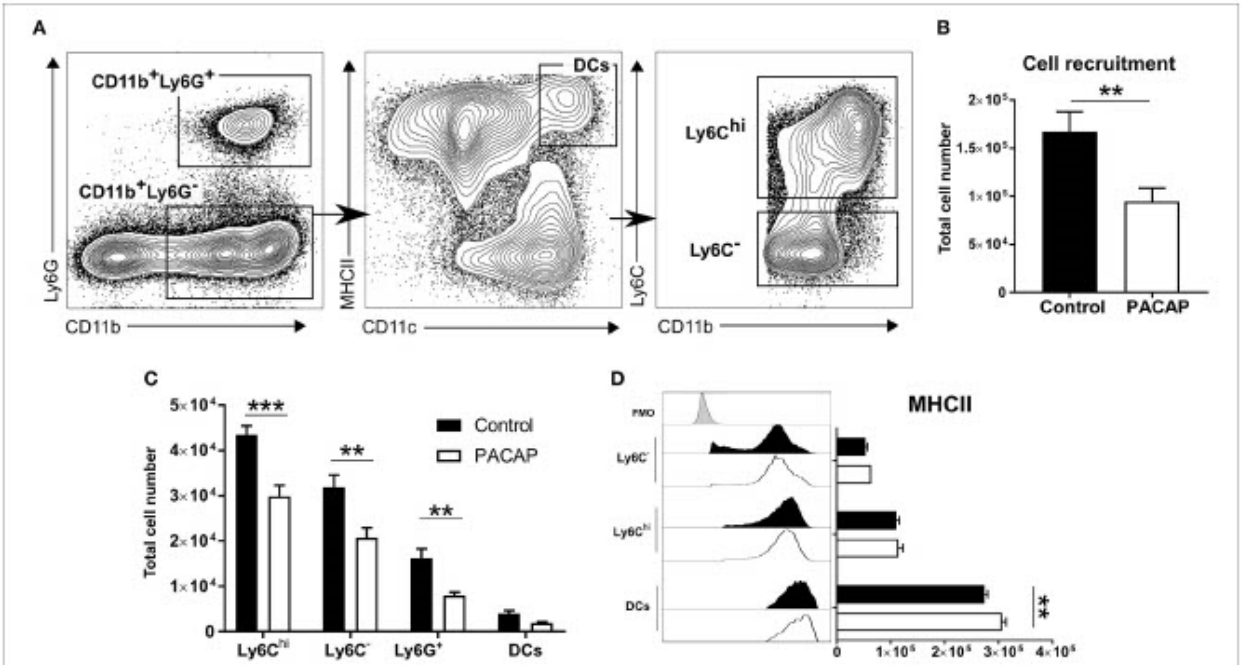

FIGURE 1 | Immune cell recruitment and activation upon PACAP administration. Peritoneal cells of acutely-infected mice were analyzed by flow cytometry. Cells were selected based on the forward-scatter/side-scatter plot (FSS/SSC) and living, single cells were chosen for further analysis (not shown). **(A)** Gating strategy used to discriminate CD11b+Ly6G+ neutrophils and CD11b+Ly6G− monocyte-derived cells. CD11b+Ly6G− monocyte-derived cells were further divided into $CD11c^{hi}MHCII^{hi}$ DCs and then differentiated according to Ly6C expression: $Ly6C^{hi}$ inflammatory monocytes and $Ly6C^{-}$ peritoneal macrophages. **(B)** Shows the total recruitment of living single cells. **(C)** Bar charts show cell recruitment of the identified cell populations in peritoneal cavity exudate of control and PACAP-treated groups (control: black bars; PACAP-treated: white bars). **(D)** Histograms and bar charts show expression of the activation marker MHCII on peritoneal cell subsets according to median fluorescence intensity (MFI). Control (black bars/histogram) and PACAP-treated (white bars/histogram); FMO (gray histogram) data are expressed as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed unpaired *t*-test).

was able to increase the expression of MHCII on peritoneal DCs (control: $2.74 \times 10^5 \pm 5.58 \times 10^3$ vs. PACAP: $3.06 \times 10^5 \pm 8.25 \times 10^3$; $p = 0.0012$) (**Figure 1D**) but not on the other myeloid subsets ($Ly6C^{-}$, control: $5.36 \times 10^4 \pm 3.23 \times 10^3$ vs. PACAP: $6.35 \times 10^4 \pm 1.92 \times 10^3$; $p = 0.2448$; $Ly6C^{hi}$, control: $1.11 \times 10^5 \pm 4.91 \times 10^3$ vs. PACAP: $1.15 \times 10^5 \pm 8.21 \times 10^3$; $p = 0.6767$). Thus, PACAP reduced the recruitment of mononuclear cells to the peritoneal cavity upon *T. gondii* infection and increased MHCII expression on peritoneal DCs.

Antiparasitic Effect of PACAP Is Immune Cell-Mediated

Infected migratory immune cells, such as DCs, monocytes and macrophages are responsible for the parasite dissemination throughout host tissues, including lung, spleen, and also the peritoneal cavity (Ueno et al., 2014). As previous reports demonstrated the anti-parasitic effect of PACAP, we set out to analyze whether PACAP administration is able to affect the presence of *T. gondii* in myeloid-derived cell subsets. By infecting mice with a GFP-fluorescent reporter parasite, we were able to elucidate *T. gondii* occurrence in cell subsets isolated from the peritoneal cavity via flow cytometry. Here, the experimental group receiving PACAP showed a marked reduction of infected cells in all myeloid populations (**Figures 2A′–C′**). Alongside with $Ly6G^{+}$ neutrophil granulocytes, $Ly6C^{hi}$ monocytes represented the cell subset with the strongest reduction of parasitic GFP signal, while DCs and $Ly6C^{-}$ macrophages were less affected (neutrophils, control: 32 ± 5.6% vs. PACAP: 6.2 ± 0.34%; $p = 0.027$; $Ly6C^{hi}$, control: 26 ± 3% vs. PACAP: 5.5 ± 0.3%; $p = 0.0004$; $Ly6C^{-}$, control: 15 ± 1.5% vs. PACAP: 3.4 ± 0.19%; $p = 0.0003$; DCs, control: 21 ± 2.5% vs. PACAP: 3.6 ± 0.65%; $p = 0.0006$) (**Figures 2A–C**). In order to address if this was mediated by the anti-parasitic effect of PACAP, we first performed an *in vitro* plaque assay using HFF culture infected with *T. gondii* in the presence of different PACAP concentrations (0.1, 1, and 10 μM) (**Figure 2D**). In contrast to our *in vivo* data in the peritoneal cavity, these results revealed that PACAP did not directly affect the size (**Figure 2D′**) or the number of plaques (**Figure 2D″**). Thus, PACAP has no direct effect on parasite elimination and/or impairment of the parasite development.

As infections with intracellular pathogens promote the expression of host defense factors such as IRGs and GBPs in myeloid cells, we hypothesized that these pathways were modulated by PACAP possibly explaining the reduced parasite burden *in vivo*. To this end, expression of IRGs (IRGM1, IRGM3) and GBP2b on peritoneal cells isolated from acutely

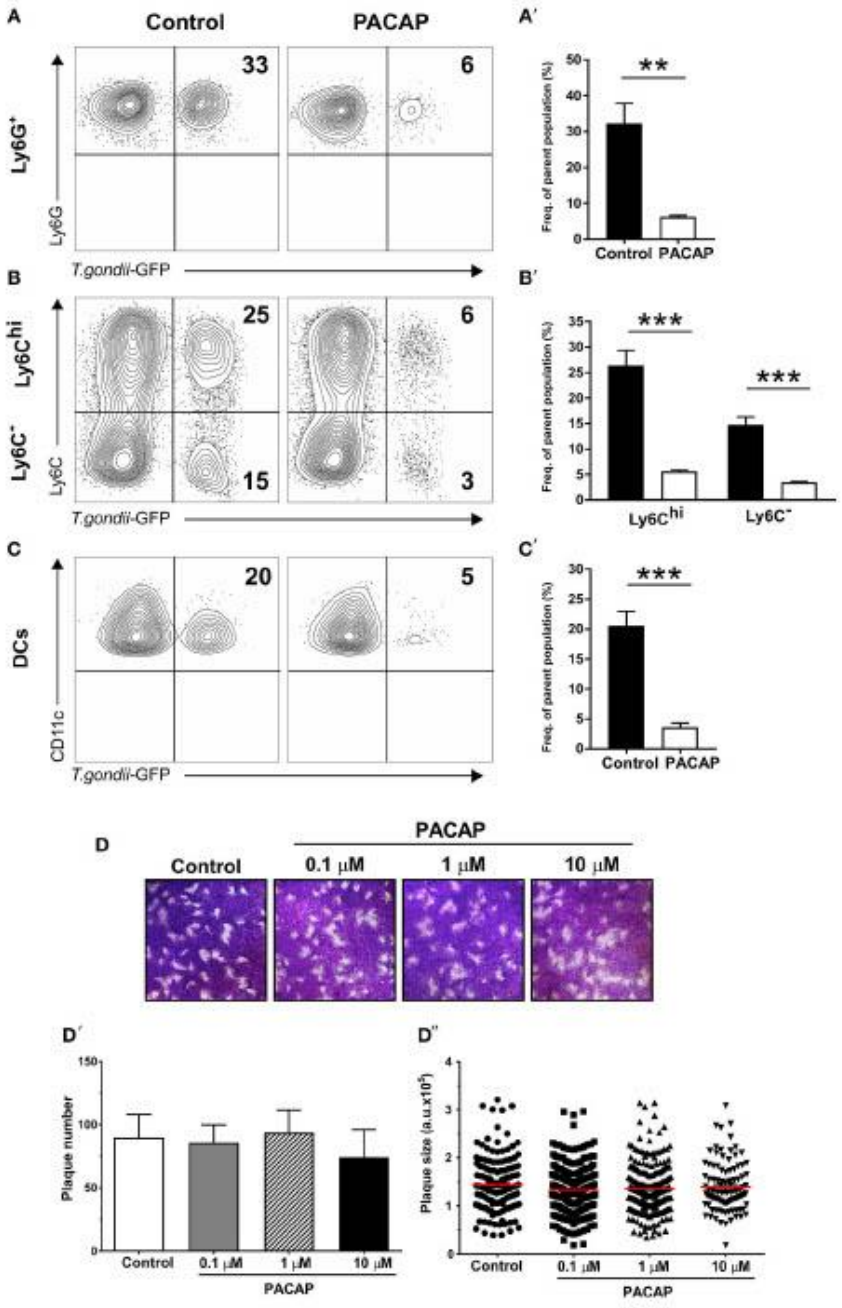

FIGURE 2 | Anti-parasitic effect of myeloid peritoneal immune cells upon PACAP-treatment. Peritoneal cells of acutely-infected mice were isolated and analyzed by flow cytometry. A GFP-fluorescent *T. gondii* reporter was used to track the presence of the parasite in myeloid peritoneal subsets selected as described above. **(A–C)** Contour plots show the presence of GFP$^+$ cells for each cell subset in control (left column) and PACAP-treated (right column) group. Numbers represent the mean percentage of parent population for each group from a representative experiment ($n = 4$). **(A′–C′)** Bar charts compare the frequency of GFP$^+$ cells. Control (black bars) and PACAP-treated (white bars). **(D–D″)** Representative images show *in vitro* replication of *T. gondii* by plaque assays in the presence of PACAP. Plaques from three independent experiments were scored for size and numbers using ImageJ. **(D)** Images of the formed plaques initially infected with 200 tachyzoites/well; numbers indicate different PACAP concentration. **(D′)** Scored number of plaques under different concentrations of PACAP. **(D″)** Scored plaque sizes shown as arbitrary units (a.u.) in the presence of different PACAP concentrations. Data are expressed as mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed unpaired *t*-test).

infected mice was assessed by RT-qPCR (**Figure 3A**). Both IRGs were upregulated in the group that received PACAP (IRGM1: $p = 0.0329$; IRGM3: $p = 0.0220$), suggesting that the observed decrease in parasite burden might be associated with an improved phagocytic ability, also reported to be modulated by PACAP (Delgado et al., 1996a). Therefore, we performed, a phagocytosis assay with bone marrow-derived macrophages (BMDMs) primed to a classically activated M1 phenotype and stimulated with different concentrations of PACAP (**Figure 3B**). When compared to the non-treated group all PACAP-treated groups showed an increased phagocytosis as displayed by the MFI, with the highest effect observed at 1 μM concentration (control vs. 0.1 μM PACAP: $p < 0.0001$; control vs. 1 μM PACAP: $p < 0.0001$; control vs. 10 μM PACAP: $p < 0.0001$). With the signal of FITC-fluorescent microspheres being well distinguishable by flow cytometry, we were further able to subdivide the composition of each experimental group based on the number of beads being phagocyted (**Figure 3B′**). In line with the MFI data, all PACAP-treated groups showed an increased number of internalized microspheres when compared to the non-treated control. Moreover, treatment with 1 μM PACAP resulted in the largest fraction of BMDMs with more than 3 beads engulfed when compared to other groups (control: 68.3 ± 1.007 %; 0.1 μM PACAP: 79.33 ± 0.9939; 1 μM PACAP: 88.9 ± 1.0044 %; 10 μM PACAP: 81.3 ± 0 %). These findings were further supported by analysis of BMDMs with respect to their expression of the macrophage marker F4/80 (**Figure 3C**). Also here, PACAP treatment resulted in upregulation of F4/80 that was most prominent in the group treated with 1 μM PACAP (control vs. 0.1 μM PACAP: $p < 0.0045$; control vs. 1 μM PACAP: $p < 0.0004$; control vs. 10 μM PACAP: $p < 0.004$). Altogether, these results demonstrate that the neuropeptide PACAP was able to reduce the presence of *T. gondii* in peritoneal myeloid cells not by a direct anti-parasitic effect but by modulating their phagocytic capabilities.

Decreased Parasite Burden and Expression of Inflammatory Mediators

Previous studies have shown that PACAP inhibits the production of pro-inflammatory cytokines (Martínez et al., 1998a; Delgado et al., 1999a,b). In order to evaluate how PACAP affects the acute inflammation caused by *T. gondii*, we assessed the parasite burden and the gene expression of inflammatory mediators (**Figure 4**). The results show a reduced parasite load ($p = 0.0171$) in the PACAP-treated group and reduced expression of IFN-γ ($p = 0.0022$), TNF ($p = 0.0069$), IL-6 ($p = 0.0009$), CCL-2 ($p = 0.0009$), and iNOS ($p = 0.0451$). Expression levels of IL-12, IFN-β, IL-10, and TGF-β did not differ between PACAP and the control group. In summary, PACAP was able to reduce parasite burden while diminishing the robust Th1 response characteristic for *T. gondii* infection without affecting anti-inflammatory mediators.

Immune Cells Upregulate PACAP Receptors and Neurotrophin Expression

PACAP-mediated effects on immune cells are elicited by binding of the neuropeptide to its receptors PAC1, VPAC1, and VPAC2, whereby the main anti-inflammatory effect is primarily exerted through VPAC1 resulting in activation of the cAMP/PKA pathway (Delgado et al., 2004a). This signaling pathway regulates the activity of a range of transcription factors critical for expression of the most inflammatory mediators (Delgado et al., 1998, 1999a; Delgado and Ganea, 1999, 2001). Therefore, we evaluated whether PACAP modulates the expression of its intrinsic receptors on peritoneal immune cells upon *T. gondii* infection (**Figure 5A**). Our results show that the neuropeptide was able to increase the expression of VPAC1 and VPAC2 ~3 fold when compared to the control group (VPAC1: $p = 0.00001$; VPAC2: $p = 0.035$). Thus, while PACAP binds to its own receptor, it also regulates their expression on innate immune cells.

Previous studies have shown the involvement of VPAC1 in myeloid cells and the PACAP-mediated signal transduction into neurotrophin signaling (El Zein et al., 2007). In general, neurotrophins are associated with the growth, development and survival of neuronal cells in the CNS (Mitre et al., 2017). However, besides neuronal tissue, neurotrophin receptors of the Trk superfamily and the p75 neurotrophin receptor ($p75^{NTR}$) were detected in a variety of immune cells (Frossard et al., 2004; Fischer et al., 2008; Minnone et al., 2017), thus emphasizing the interdependency of the neuronal and immune system. In accordance with that, our previous work indicates that neurotrophin signaling via the $p75^{NTR}$ affects innate immune cell behavior upon *T. gondii*-induced neuroinflammation (Dusedau et al., 2019). Therefore, to further explore the influence of PACAP-mediated signaling on the neurotrophin signaling pathway as another modulator of the innate immune response, we analyzed the expression of BDNF and neurotrophin receptors ($p75^{NTR}$, TrkA, TrkB, and TrkC) in peritoneal exudate cells upon acute infection (**Figure 5B**). Surprisingly, BDNF expression level was found to be elevated ~6 fold in PACAP-treated animals when compared to the control group (BDNF, $p = 0.0129$; $p75^{NTR}$, $p = 0.0584$; TrkA, $p = 0.9990$; TrkB, $p = 0.9992$; TrkC, $p = 0.9674$). Altogether, our results indicate an upregulation of BDNF expression upon PACAP treatment, suggesting a modulation of neurotrophin pathways in innate immune cells upon acute *T. gondii* infection.

PACAP Reduce $p75^{NTR}$ Expression on $Ly6C^{hi}$ Monocytes

We have recently highlighted the upregulation of $p75^{NTR}$ on innate immune cells in the blood and brain of infected animals in *T. gondii*-induced neuroinflammation (Dusedau et al., 2019). In the periphery, the infection increased $p75^{NTR}$ expression on myeloid cell subsets, which are essential for control of toxoplasmosis (Dunay et al., 2010; Biswas et al., 2015). To this end, we analyzed expression of $p75^{NTR}$ on $Ly6C^{hi}$ inflammatory monocytes and $Ly6C^{-}$ resident macrophages isolated from the peritoneal cavity upon acute infection. In the PACAP-treated group, the $p75^{NTR}$ expression on $Ly6C^{hi}$ monocytes was significantly reduced ($Ly6C^{hi}$, $p < 0.00001$; $Ly6C^{-}$, $p = 0.29$) (**Figure 5C**) with no changes in frequency of $p75^{NTR+}$ cells for $Ly6C^{hi}$ ($p = 0.276$) or $Ly6C^{-}$ ($p = 0.5181$) (**Figure 5D**). Taken together, our results point toward the anti-inflammatory effect of

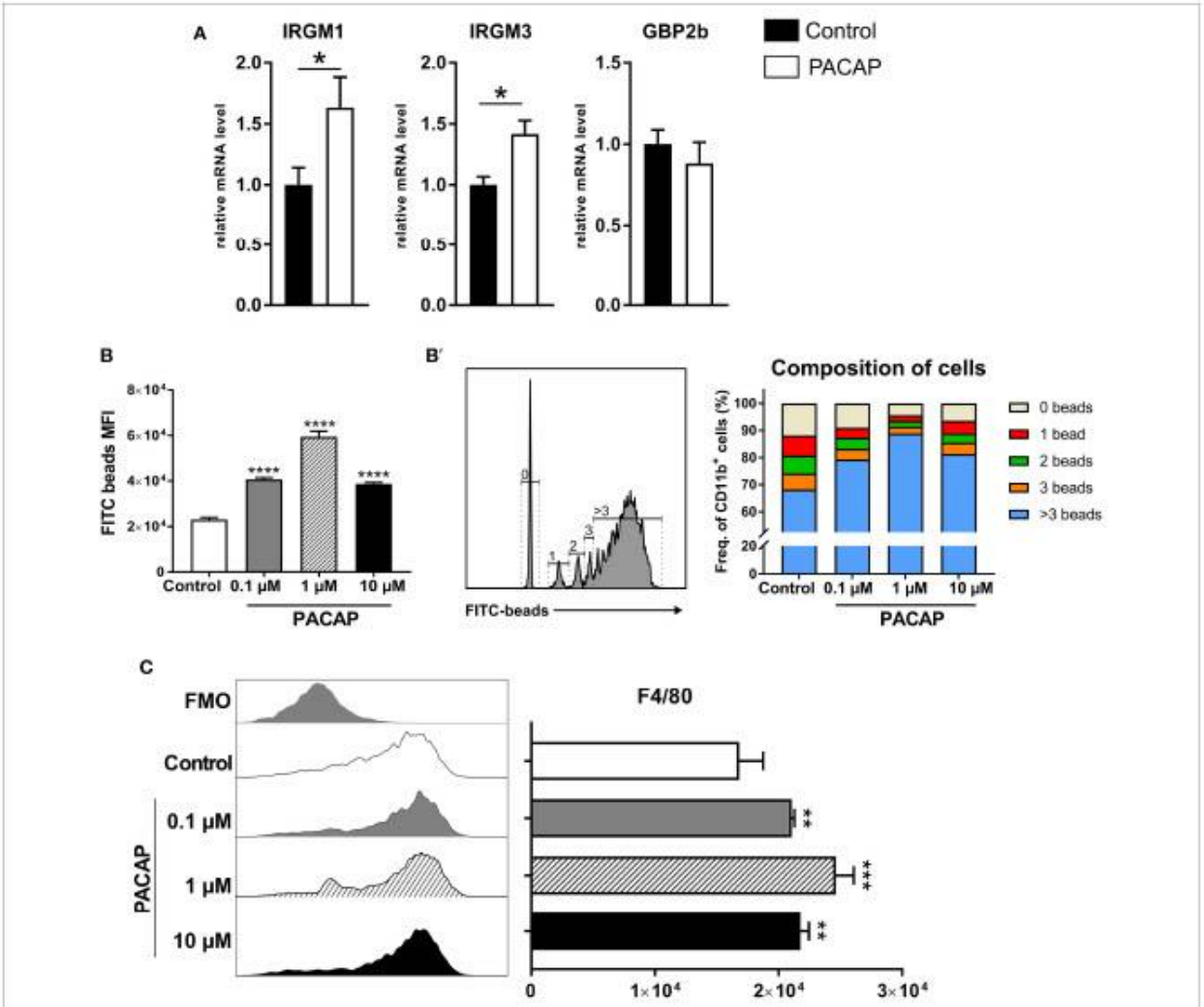

FIGURE 3 | PACAP treatment increases expression of host-defense factors and phagocytosis. **(A)** Bar charts show gene expression of IRGs and GBP2b associated with parasite elimination in peritoneal exudate cells assessed by RT-qPCR. The gene expression levels of host defense factors were determined by RT-qPCR using *Hprt* as reference gene. Data were further normalized to mean values of control group; Control (black bars) and PACAP-treated (white bars). Bar charts present results as mean ± SEM, $n = 4$; $^*p < 0.05$ (two-tailed unpaired *t*-test). **(B)** Phagocytosis of BMDMs primed to M1 phenotype was assessed by incubation with FITC-fluorescent microspheres in the presence of different PACAP concentrations (0.1, 1, and 10 µM); phagocytic capability was evaluated by the MFI of the positive populations. **(B')** Histograms and bar charts show frequency cells fractioned according to the amount of microspheres internalized. **(C)** Histograms and bar charts show activation marker F4/80 expressed on BMDMS in the phagocytosis assay. Data are expressed as mean ± SEM (one-way ANOVA with *post-hoc* Holm-Sidak test).

PACAP on Ly6C^{hi} monocytes, and suggest the involvement of p75^{NTR} in the acute inflammatory response against *T. gondii*.

DISCUSSION

We hypothesized that application of the neuropeptide PACAP might modulate the behavior of myeloid-derived mononuclear cells and potentially contribute to the resolution of the infection and parasite elimination. Accordingly, we investigated the immunomodulatory effect of PACAP on innate immune cells isolated from the peritoneal cavity during experimental acute *T. gondii* infection. We detected an interaction of PACAP and neurotrophin signaling that suggests a contribution to the resolution of acute *Toxoplasmosis*.

Previously, we have reported that PACAP administration ameliorates acute small intestinal inflammation and extra-intestinal sequelae during acute ileitis caused by *T. gondii* infection (Heimesaat et al., 2014; Bereswill et al., 2019). Besides, we demonstrated the critical importance of myeloid cells to control *T. gondii* infection in the periphery as well

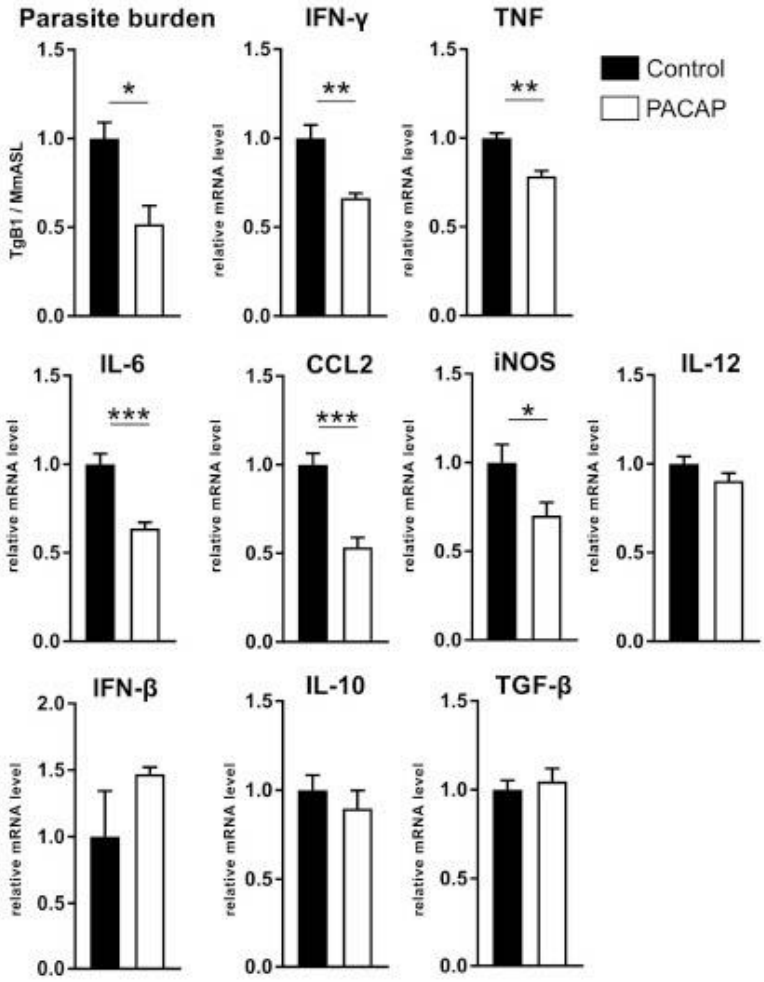

FIGURE 4 | PACAP alters parasite burden and inflammatory mediators. DNA and RNA were isolated from spleens of acutely infected mice and analyzed by qPCR and RT-qPCR. The parasite burden was determined based on the presence of B1 gene of *T. gondii* (*TgB1*) normalized to the murine gene *Asl*. The gene expression levels of inflammatory mediators were determined by RT-qPCR using *Hprt* as reference gene. Data were further normalized to mean values of control group; Control (black bars) and PACAP-treated (white bars). Bar charts present results as mean ± SEM, $n = 4$. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (two-tailed unpaired *t*-test).

as in the CNS (Dunay et al., 2008, 2010; Biswas et al., 2015; Mohle et al., 2016). Furthermore, our recent studies have revealed the emerging role of neurotrophin signaling via $p75^{NTR}$ that affects innate immune cell behavior and influences structural plasticity of neurons upon *T. gondii*-induced neuroinflammation (Dusedau et al., 2019). Therefore, we analyzed the effects of PACAP on the course of acute *T. gondii* infection in mice.

Initially, our data revealed that in the peritoneal cavity, the number of recruited myeloid cells, especially $Ly6C^{hi}$ inflammatory monocytes, was reduced by the administration of exogenous PACAP. PACAP has been shown to modulate chemokines produced by activated macrophages and adhesion molecules expressed by granulocytes, thereby affecting the recruitment of different immune cell subsets (Ganea and Delgado, 2002; El Zein et al., 2008). Under steady-state conditions, two types of macrophages are found in the peritoneal cavity: large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs) (Ghosn et al., 2010). During inflammation, the peritoneal cell composition dramatically changes, with a massive recruitment of $Ly6C^{hi}$ monocytes that give rise to new SPMs, while LPMs leave the peritoneal cavity and migrate to the omentum for antigen presentation (Cassado Ados et al., 2015). Upon *in vitro* LPS stimulation, SPMs developed a pro-inflammatory profile as indicated by TNF, CCL3 and RANTES production (Cain et al., 2013). These findings align with our previous results where $Ly6C^{hi}$ monocytes contributed to *T. gondii* removal via production of TNF, iNOS

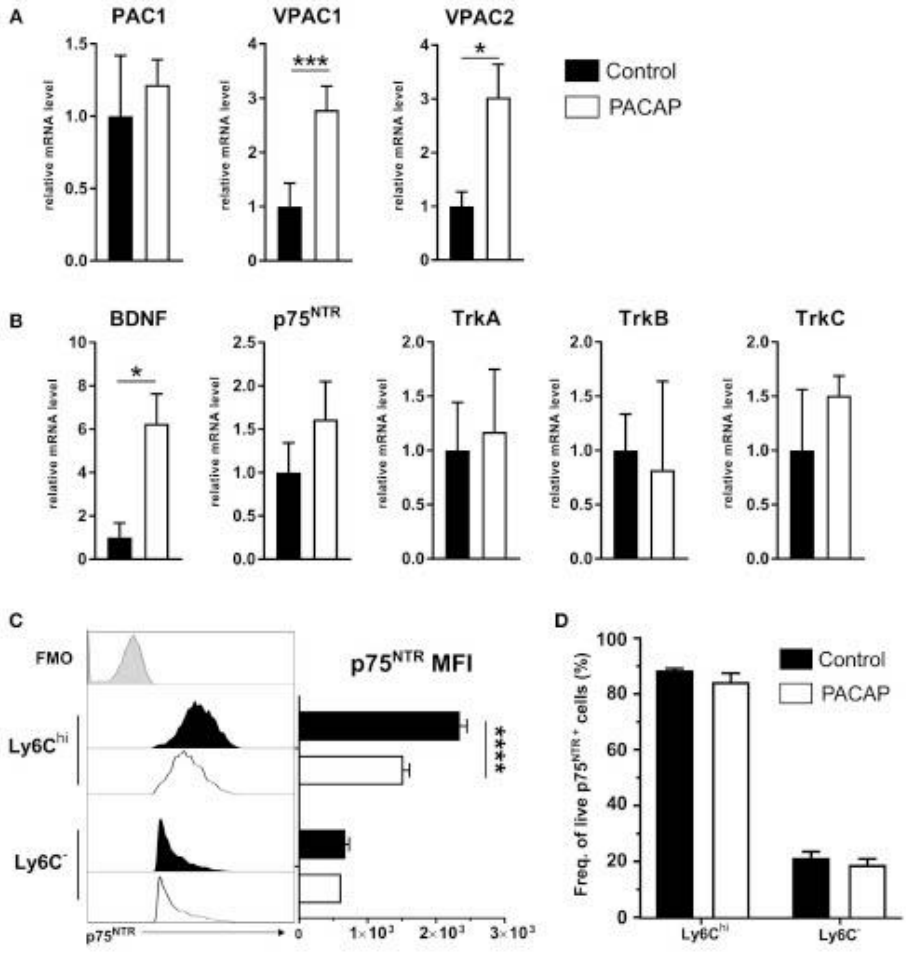

FIGURE 5 | Expression of PACAP receptors, BDNF and neurotrophin receptors on peritoneal cells. Gene expression of **(A)** PACAP receptors and **(B)** BDNF and neurotrophin receptors was assessed by RT-qPCR. Control (black bars) and PACAP-treated (white bars); data are expressed as mean ± SEM, $^{*}p < 0.05$, $^{***}p < 0.001$ (two-tailed unpaired *t*-test). **(C)** Flow cytometric analysis of $p75^{NTR}$ expression on immune cell subsets; the histograms show the FMO control (light gray-tinted), control group (black-tinted) and PACAP group (black non-tinted); bar charts present the values of MFI for $p75^{NTR}$ expression. **(D)** Frequency of cells from parent population of living, single $p75^{NTR+}$ cells. Data are presented as mean ± SEM, $n = 4$. $^{****}p < 0.0001$ (two-tailed unpaired *t*-test).

and reactive oxygen species (ROS) (Dunay et al., 2008, 2010; Dunay and Sibley, 2010; Karlmark et al., 2012). Analyzing the gene expression of selected chemokines, cytokines and inflammatory mediators, we detected that exposure to PACAP diminished the levels of TNF, IL-6, and iNOS similarly to our previous observations in the intestine (Heimesaat et al., 2014). Moreover, the expression of the chemokine CCL2, important for the recruitment of $Ly6C^{hi}$ monocytes (Biswas et al., 2015), was also significantly downregulated further supporting our observations of reduced myeloid cell recruitment. Levels of the anti-inflammatory cytokine IL-10 were not elevated, but remained balanced upon PACAP administration in our experimental setup. These data are in line with a previous finding in a study with experimental autoimmune encephalomyelitis (EAE), where PACAP was able to reduce IFN-γ levels but had no effect on production of IL-10 by spleenocytes (Kato et al., 2004).

Interestingly, we observed a decreased systemic parasite burden despite IFN-γ being negatively affected by the administration of PACAP. Generally, downregulation of IFN-γ, the main driving force against *T. gondii* infection, would result in an uncontrolled parasite replication (Suzuki et al., 1988). Contrary to previous reports with *Trypanosoma* (Delgado et al., 2009), we did not detect direct antiparasitic effects of PACAP on the *T. gondii* replication, which was assessed by monitoring changes in the size or the number of plaques. Instead, IFN-β was found to be upregulated by tendency after exposure to PACAP. The expression of the inflammatory cytokine IFN-β is upregulated following *T. gondii* infection (Mahmoud et al., 2015). Anti-parasitic effects of type I IFNs in

myeloid cells are independent from iNOS and IFN-γ-induced effects. IFN-β acts via induction of IRGM1 that accumulates on the parasitophorous vacuole (PV), in order to disrupt it (Mahmoud et al., 2015). Therefore, we further analyzed the expression of IRGs (IRGM1, IRGM3, GBP2b) and other host defense factors associated with PV disruption. Here, we observed upregulated expression of IRGM1/IRGM3, which implies that PACAP modulates immune cell-mediated parasite elimination rather than direct anti-parasitic effects. Our results align with previous studies (Delgado et al., 1996a), where macrophages exhibit an increased F4/80 expression and enhanced phagocytic capacity.

In contrast to Ly6C^{hi} monocytes and Ly6G^{+} neutrophils, CD11c^{+} DC recruitment was not affected by PACAP. However, the expression of MHCII in the PACAP-treated group was increased, pointing toward a promotion of antigen recognition and subsequent activation of lymphocytes. In line with previous studies, PACAP-mediated effects on DCs have shown to be mainly induced by signaling through VPAC1, which is the same receptor-mediated pathway utilized for macrophages (Delgado et al., 2004b). Indeed, PACAP administration upregulated the expression of VPAC1 and VPAC2 but not PAC1 by peritoneal exudate cells from acutely infected mice. All three receptors result in the activation of cyclic adenosine monophosphate (cAMP) and the subsequent activation of protein kinase A (PKA) (Delgado et al., 2004a). Specific studies using agonists and antagonists for PACAP receptors have established VPAC1 as the major mediator of the immunomodulatory effects from PACAP, both *in vitro* and *in vivo*, with moderate involvement of VPAC2, and minimal or none from PAC1 (Delgado et al., 2004a). Interestingly, in human neutrophils and monocytes, PACAP interaction with VPAC1 and the NGF receptor TrkA resulted in calcium mobilization and subsequent pro-inflammatory activation (El Zein et al., 2006, 2007). Even though PACAP exposure had no effect on the gene expression of Trk receptors, we detected an upregulation of BDNF expression by immune cells upon PACAP administration.

It was previously reported that PACAP upregulated BDNF expression in primary neuronal cultures from rat cerebral cortex, as well as in human neuroblastoma cells upon injury (Frechilla et al., 2001; Shintani et al., 2005; Brown et al., 2013). BDNF is able to influence the immune system via modulation of cytokine expression in peripheral blood mononuclear cells (Vega et al., 2003). BDNF was shown to also be produced by immune cells (Kruse et al., 2007), to modulate monocyte chemotaxis, participate in tissue-healing mechanisms (Samah et al., 2008), and enhance macrophage phagocytic activity (Hashimoto et al., 2005). Recently, we have described a specific role of neurotrophins and their receptors during neuroinflammation. We described that the BDNF receptor p75^{NTR} has a functional impact on the activation status of innate immune cells during *T. gondii*-induced neuroinflammation (Dusedau et al., 2019).

Here, PACAP was able to reduce the overall recruitment of myeloid-derived mononuclear cells to the peritoneal cavity; in particular, Ly6C^{hi} monocytes. Interestingly, the same cell subset presented higher p75^{NTR} expression than Ly6C^{-} peritoneal macrophages, and the administration of PACAP exclusively reduced p75^{NTR} expression on Ly6C^{hi} monocytes. In previous studies (Lee et al., 2016), the use of an antagonist blocker of p75^{NTR} reduced the recruitment of inflammatory monocytes to the CNS, further suggesting that neurotrophin signaling is involved in immune cell migration. Moreover, in a model of in EAE model, the induction of the inflammation resulted in expression of p75^{NTR} in endothelial cells. Although they focus on endothelial p75^{NTR} expression, the study reports a differential immune cell recruitment to the CNS of p75^{NTR} knockout mice, with reduced numbers of cells from the monocyte-macrophage lineage (Kust et al., 2006). In other studies, p75^{NTR} expression by immune cells was reported to increase by a factor of 10 in response to injury (Ralainirina et al., 2010). Our previous work also showed p75^{NTR} upregulation on resident microglia cells and myeloid-derived mononuclear cell subsets in *T. gondii*-infected brains (Dusedau et al., 2019). These studies supported our findings, where immune cell recruitment and the expression of p75^{NTR} upon inflammation were reduced upon PACAP treatment.

Alongside BDNF, p75^{NTR} signaling in immune cells can be modulated by levels of neurotrophin precursors (pro-neurotrophins). proBDNF has been shown to negatively affect neuronal plasticity and cell death, reinforced by elevated levels of proBDNF detected in peripheral macrophages (Wong et al., 2010; Luo et al., 2016; Dusedau et al., 2019). Here we observed a downregulation of p75^{NTR}, implying a reduced influence of pro-neurotrophins on Ly6C^{hi} monocytes and thus having a beneficial effect on the resolution of inflammation. The increase of BDNF gene expression in response to PACAP treatment and the involvement of neurotrophins/receptors with VPAC1, especially on neutrophils and monocytes, suggest a possible interaction between p75^{NTR}, BDNF and PACAP within myeloid cells during an inflammatory response. However, future experiments should investigate whether the downregulation of p75^{NTR} is directly PACAP-mediated or a result of an overall reduced inflammation.

In our experiments, the effect of PACAP on parasite elimination may correlate with the synergic, indirect effect of elevated BDNF expression by peritoneal immune cells. However, the role of p75^{NTR} signaling in relation to the immune system remains poorly understood due to the complex interplay of mature vs. pro-neurotrophins, and heterodimeric interactions with Trk receptors (Meeker and Williams, 2014). In summary, our results indicate different routes of PACAP-mediated regulation of the innate response during acute *T. gondii* infection. As a potent immunomodulator, PACAP has been shown to contribute to the resolution of acute inflammation and parasite elimination by innate immune cells. Furthermore, our findings point toward a potential connection between PACAP and neurotrophin-mediated signaling in Ly6C^{hi} inflammatory monocytes. Taken together, these results contribute to the understanding of

the interaction between the nervous and immune systems through neuropeptides.

AUTHOR CONTRIBUTIONS

CF and HD performed experiments and analyzed data. JS, NG, MD, GT, DR, and MH critically discussed experimental design, provided material, and co-edited the manuscript. ID conceived experimental design. CF and ID wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2019.00154/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publication 3

Upon chronic *T. gondii* cerebral infection, application of PACAP resulted in ameliorated brain inflammation and pathology, indicated by a reduced number of inflammatory foci and apoptotic cells. Importantly, suppression of brain inflammation did not trigger uncontrolled parasite burden. More specifically, this effect was followed by reduced recruitment of peripheral myeloid cells to the brain, such as Ly6C^{hi} inflammatory monocytes. PACAP-treated animals also displayed reduced microglia activation that was measured by MHC-II expression, and diminished production of iNOS, TNF and IL-1β by microglial cells. Accordingly, PACAP administration resulted in reduced transcriptional levels of pro-inflammatory mediators, and the chemokines CCL2, CXCL9 and CXCL10 in infected brains. In contrast to the effect on myeloid cells, PACAP did not affect the recruitment of T cells to the brain, but it diminished IFN-γ production by CD4^{+} T cells. In addition, PACAP administration simultaneously improved neuronal health, as transcriptional levels of BDNF were increased while levels of p75^{NTR} were downregulated. Notably, PACAP altered the expression of transporters involved in the brain synaptic composition, favoring a less dysfunctional neuronal network of glutamatergic and GABAergic signaling, which is specifically affected during *T. gondii*-induced neuroinflammation.

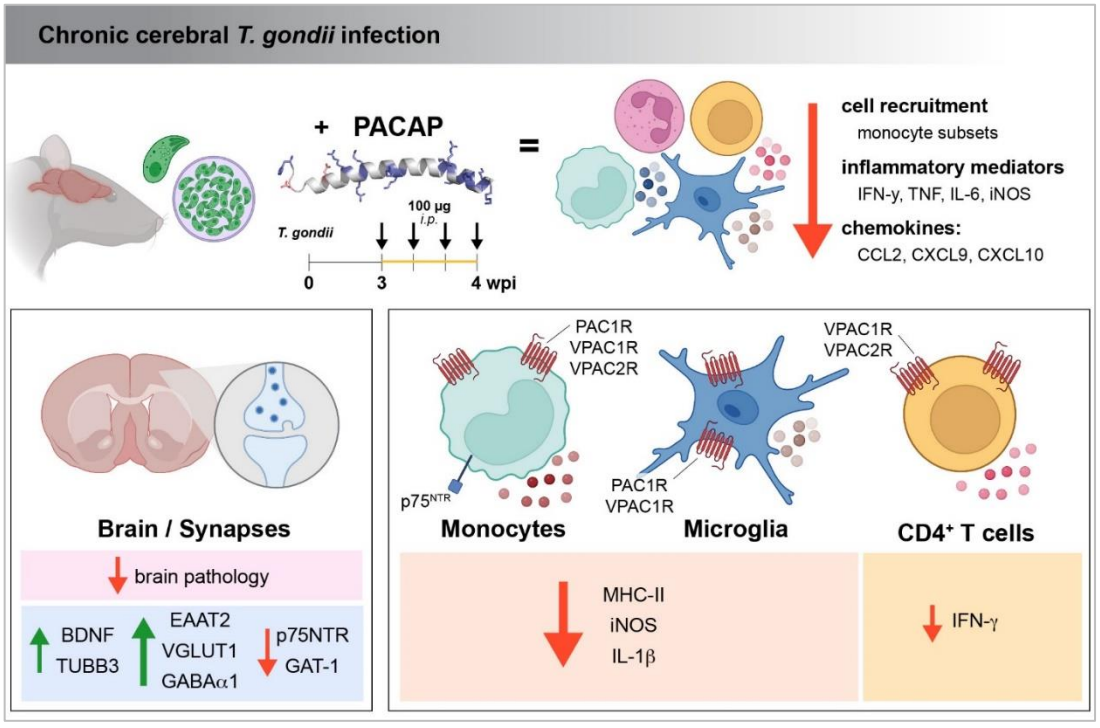

Figure 8. Graphical abstract - Publication 3

The neuropeptide PACAP alleviates *T. gondii* infection-induced neuroinflammation. PACAP treatment of chronically infected mice reduced infiltration of peripheral myeloid cells, and their inflammatory mediators between cytokines and chemokines. Specifically, PACAP affect monocytes and microglia expression of MHC-II, and their production of iNOS and IL-1β. While cerebral T cells numbers were not altered, PACAP reduced IFN-γ production by CD4+ T cells. Adding to the immunomodulatory effects, PACAP promoted neuronal health, as indicated by upregulation of BDNF and TUBB3, and the altered expression of synaptic transporters from glutamatergic and GABAergic signaling that were affected upon cerebral toxoplasmosis. Created with Biorender.com

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RESEARCH Open Access

The neuropeptide PACAP alleviates *T. gondii* infection-induced neuroinflammation and neuronal impairment

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Abstract

Background: Cerebral infection with the protozoan *Toxoplasma gondii* (*T. gondii*) is responsible for inflammation of the central nervous system (CNS) contributing to subtle neuronal alterations. Albeit essential for brain parasite control, continuous microglia activation and recruitment of peripheral immune cells entail distinct neuronal impairment upon infection-induced neuroinflammation. PACAP is an endogenous neuropeptide known to inhibit inflammation and promote neuronal survival. Since PACAP is actively transported into the CNS, we aimed to assess the impact of PACAP on the *T. gondii*-induced neuroinflammation and subsequent effects on neuronal homeostasis.

Methods: Exogenous PACAP was administered intraperitoneally in the chronic stage of *T. gondii* infection, and brains were isolated for histopathological analysis and determination of pathogen levels. Immune cells from the brain, blood, and spleen were analyzed by flow cytometry, and the further production of inflammatory mediators was investigated by intracellular protein staining as well as expression levels by RT-*q*PCR. Neuronal and synaptic alterations were assessed on the transcriptional and protein level, focusing on neurotrophins, neurotrophin-receptors and signature synaptic markers.

Results: Here, we reveal that PACAP administration reduced the inflammatory foci and the number of apoptotic cells in the brain parenchyma and restrained the activation of microglia and recruitment of monocytes. The neuropeptide reduced the expression of inflammatory mediators such as IFN-γ, IL-6, iNOS, and IL-1β. Moreover, PACAP diminished IFN-γ production by recruited $CD4^+$T cells in the CNS. Importantly, PACAP promoted neuronal health via increased expression of the neurotrophin BDNF and reduction of $p75^{NTR}$, a receptor related to neuronal cell death. In addition, PACAP administration was associated with increased expression of transporters involved in glutamatergic and GABAergic signaling that are particularly affected during cerebral toxoplasmosis.

Conclusions: Together, our findings unravel the beneficial effects of exogenous PACAP treatment upon infection-induced neuroinflammation, highlighting the potential implication of neuropeptides to promote neuronal survival and minimize synaptic prejudice.

Keywords: PACAP, Neuroinflammation, *Toxoplasma gondii*, Cerebral toxoplasmosis, Immunomodulation, Neuroprotection

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Background

Neurological disorders are mainly associated with the development of neuroinflammation in response to autoimmune recognition, neurodegenerative accumulation

of toxic metabolites, or due to infectious diseases [1, 2]. Most frequent infections of the central nervous system (CNS) are caused by viral, bacterial and fungal pathogens, although neurotropic protozoan parasites such as *Toxoplasma gondii* (*T. gondii*) can cause cerebral toxoplasmosis, a persistent subclinical to severe neuroinflammatory disease [3, 4]. It is estimated that one-third of the world's human population is infected with *T. gondii* and the seroprevalence is even higher in other mammals [5–7]. Once invading neuronal tissue, the parasites evoke an efficient pro-inflammatory immune response with release of tumor necrosis factor (TNF), interleukin (IL)-6, IL-1β, and nitric oxide (NO) by activated resident microglia and astrocytes [8, 9]. The local immune response is intensified by the recruitment of peripheral lymphoid and myeloid cells to the brain, which are crucial for parasite control mainly via interferon-gamma (IFN-γ) production [10–13]. The persistent infection in the brain establishes an ongoing neuroinflammation for the host lifetime, and is able to modify neuronal function and synapse composition [9, 14–16]. Indeed, neurological and behavior alterations upon *T. gondii* infection have been highlighted in rodent and human studies [17–20], and associated to altered cognitive function [21] and several neuropsychiatric conditions including depression, suicidal behavior, and schizophrenia [22–24]. As no current therapy is able to completely eliminate the parasites from the CNS, new therapeutic strategies are necessary to reduce infection-induced neuroinflammation, preventing neuronal death and minimizing synaptic prejudice [25].

The neuropeptide PACAP (Pituitary Adenylate Cyclase-Activating Polypeptide) has been described to strongly modify inflammation and clinical symptomatology in different rodent models of inflammatory diseases [26, 27]. The neuropeptide also acts as a neurotransmitter, neuromodulator, and neurotrophic factor [28, 29]. The full-length biologically active form of PACAP (PACAP1-38) is a 38-amino-acid neuropeptide commonly described as being widely expressed in the CNS of mammals [28, 30]. According to recent annotations in the Human Protein Atlas program database, single-cell RNA analyses have shown expression of PACAP (codified by *Adcyap1* gene) mostly by granulocytes and excitatory neurons, and in lesser manner (10 fold) by specific epithelial cells and fibroblasts, T cells, microglia, and plasma cells [31, 32]. PACAP employs its biological activity through binding to three widely distributed G protein-coupled receptors (GPCRs) named PAC1R, VPAC1R and VPAC2R [33], which triggers a series of intracellular signaling pathways, mainly involving production of cyclic adenosine monophosphate (cAMP) [34, 35]. PACAP receptors have different affinities for PACAP, with PAC1R possessing the highest affinity [36]. In addition, the receptors are differently expressed among immune cells, for example PAC1R and VPAC1R are constitutively expressed by monocytes, peritoneal macrophages and microglia, while T cells do not express PAC1R. VPAC2R is rarely detected under steady-state, but its expression is induced upon inflammation [37–39]. Despite neuroprotective potential in several neurological disorders such as Parkinson's [40, 41], Alzheimer's [42], and Huntington's disease [43], as well as traumatic brain injury [44], clinical studies targeting PACAP signaling have been conducted addressing nociception upon nephrotic syndrome, cluster headache, migraine, and major depression [45, 46]. In fact, immuno-modulatory properties of PACAP were found to be ambiguous, transiting from inhibition to stimulation depending on the experimental conditions and cell types [47], and therefore they request further investigation.

Our previous studies applying the acute infection-induced inflammation model have demonstrated that the resolution of intestinal inflammation and enhanced phagocytic capacity of mononuclear cells was achieved by exogenous administration of PACAP [39, 48, 49]. As PACAP has shown beneficial effects in neurological and inflammatory diseases and given its active transport into the brain across the blood–brain barrier (BBB) [50], we set out to investigate the effects of exogenous PACAP administration during cerebral toxoplasmosis, a *T. gondii*-induced neuroinflammation model. Our results point toward amelioration of brain inflammation, with reduced recruitment of myeloid cells and marked reduction of microglia activation and cytokine production, without triggering uncontrolled parasite burden. In addition, our results provide evidence that PACAP promotes neuronal health via BDNF/$p75^{NTR}$ axis modulation, favoring a less dysfunctional neuronal network of glutamatergic and GABAergic signaling, which is specifically affected during *T. gondii*-induced neuroinflammation.

Methods

Mice and *T. gondii* infection-induced neuroinflammation model

Experiments were conducted with female C57BL/6J mice (8–14 weeks old, purchased from Janvier, Cedex, France). All animals were group-housed in a 12-h day/night cycle at 22 °C with free access to food and water under specific pathogen-free conditions and according to institutional guidelines approved by the Animal Studies Committee of Saxony-Anhalt. In order to investigate *T. gondii*-induced neuroinflammation, mice were infected by intraperitoneal (i.p.) injection of cysts of the type II ME49 strain, previously harvested from brains of female NMRI mice infected i.p. with *T. gondii* cysts 6–12 months before, as described elsewhere [51]. Briefly, brains isolated from

NMRI mice were mechanically homogenized in 1 ml sterile phosphate-buffered saline (PBS), and the number of cysts was determined in ≥ 80 µl homogenate using a bright field microscope. The homogenate was diluted in sterile PBS, and animals were infected i.p with two cysts. In our model, cerebral toxoplasmosis is established after 3 weeks post-infection, as described elsewhere [11, 15, 16, 52].

PACAP treatment and organ collection

For all experiments, PACAP1-38 (the full-length 38-amino acid peptide, simply referred here as PACAP) was synthesized using solid phase strategy combined with the *Fmoc* chemistry methodology at the Department of Medical Chemistry, University of Szeged (Hungary) as previously described [53]. To investigate the effect during *T. gondii*-induced neuroinflammation, 100 µg of PACAP in phosphate-buffered saline (PBS; 5 mg per kg body weight, 22 nM per mouse) or PBS (for non-treated controls) were administered i.p. on alternating days for one week, from the third to fourth week post-infection (4 administrations in total). One day after the last treatment, animals were killed, blood samples obtained by puncture of the vena cava inferior and diluted in ice-cold PBS for flow cytometric analysis. Next, animals were transcardially perfused with 60 ml sterile ice-cold PBS, and brain, lung, and spleen were collected and stored in sterile ice-cold PBS for flow cytometric analysis, or stored in RNAlater® (Sigma, Germany) for nucleic acid isolation and subsequent *q*PCR or RT-*q*PCR analysis. Samples stored in RNAlater® were kept at 4 °C overnight and then stored at −80 °C.

Cell isolation

Isolation of immune cells from brain was performed as previously described [52, 54]. Briefly, brain hemispheres were mechanically homogenized in dissection buffer (HBSS, Gibco™, Germany), supplemented with 50 mM glucose (Roth, Germany) and 13 mM HEPES (pH 7.3, Sigma), using a glass potter, and then filtered through a 70-µm cell strainer (Falcon®, Corning, Germany). The cell suspension was washed (400 g, 10 min, 4 °C) with PBS and fractionated on a discontinuous 30%–70% isotonic Percoll® gradient (GE Healthcare, Germany) (800 g, 30 min without brake, 4 °C). After the removal of myelin debris, cells in the interphase comprising mononuclear cells were isolated and washed with FACS buffer (PBS w/o Ca^{2+}/Mg^{2+}, 2% v/v fetal bovine serum (FBS), 10 mM HEPES, 0.1% sodium azide), centrifuged (400 g, 10 min, 4 °C) and immediately used for flow cytometric analysis. To isolate immune cells from spleens, organs were homogenized and sieved through a 40-µm cell strainer (Falcon®, Corning). Isolated cells from spleen and blood samples were treated with erythrocyte lysis buffer (eBioscience, Germany), and washed twice with FACS buffer (400 g, 10 min, 4 °C) before staining and flow cytometric analysis.

Flow cytometric analysis

For surface staining, single-cell suspensions were first incubated with ZOMBIE NIR™ fixable dye (BioLegend, San Diego, CA) for live/dead discrimination. To prevent unspecific binding of antibodies, anti-mouse CD16/32 antibody (clone 93, BioLegend) was applied to cells before staining with fluorochrome-conjugated antibodies against cell surface markers in FACS buffer. CD11b (clone M1/70), Ly6C (clone HK1.4), CD4 (clone GK1.5) and CD8a (clone 53–6.7) were all purchased from eBioscience. CD45 (30-F11), Ly6G (1A8), CD3 (17A2), MHCII I-A/I-E (clone M5/114.15.2) were purchased from BioLegend. Cells were incubated for 30 min at 4 °C, washed (400 g, 10 min, 4 °C) and subsequently analyzed. Fluorescence Minus One (FMO) controls were used to determine the positive signals for each conjugated antibody.

Flow cytometric analysis of intracellular cytokines was performed as described elsewhere [52]. Briefly, isolated immune cells from the brain were re-stimulated with Toxoplasma Lysate Antigen (TLA) at 20 µg/ml for 2 h [55]. Then, Brefeldin A (10 µg/ml, GolgiPlug, BD Biosciences) and Monensin (10 µg/ml GolgiStop, BD Biosciences) were added and the cells were further incubated for additional 4 h. After one washing step with FACS buffer (400 g, 10 min, 4 °C), cells were incubated with ZOMBIE NIR™ and anti-mouse CD16/32 antibody prior to surface staining described above. Afterwards, cells were fixed in 4% paraformaldehyde (PFA) for 15 min and subsequently permeabilized with Permeabilization Buffer (BioLegend). Intracellular proteins were stained with fluorochrome-conjugated antibodies against IFN-γ (XMG1.2; eBiosciences), iNOS (clone 6, BD Biosciences), IL-1β (clone NJTEN, eBiosciences), and TNF (clone MP6-XT22, BioLegend) in Permeabilization Buffer. Matching isotype controls were used to assess the level of unspecific binding. Data were acquired using the Attune™ NxT flow cytometer (Thermo Fisher; Germany) and analyzed using FlowJo™ (v10, LLC, BD Life Sciences, USA). A minimum of 2×10^5 cells per sample was acquired and analyzed.

DNA and RNA isolation

For DNA and RNA isolation, brains, lungs, and spleens were removed from RNAlater® and homogenized in Tri-Fast™ (Peqlab, VWR, LLC, Germany) or lysis buffer using BashingBeads Lysis tubes (Zymo Research Europe, Germany) and BeadBug 6 homogenizer (Biozym, Germany). DNA and RNA were isolated from the homogenate by

Table 1 Oligonucleotide primers used for qPCR and RT-qPCR based on SYBR Green

Protein	Gene		Sequence (5′–3′)
ASL	*Asl*	Fw	TCTTCGTTAGCTGGCAACTCACCT
		Rv	ATGACCCAGCAGCTAAGCAGATCA
BAG1*	*Bag1*	Fw	GACGTGGAGTTCGACAGCAAA
		Rv	ATGGCTCCGTTGTCGACTTCT
GAPDH	*Gapdh*	Fw	TTGTCAAGCTCATTTCCTGGTATG
		Rv	TGGTCCAGGGTTTCTTACTCCTT
SAG1*	*Sag1*	Fw	ATCGCCTGAGAAGCATCACTG
		Rv	CGAAAATGGAAACGTGACTGG
B1*	*B1*	Fw	GCATTGCCCGTCCAAACT
		Rv	AGACTGTACGGAATGGAGACGAA
GAD65	*Gad2*	Fw	GGAATCTTTTCTCCTGGTGGC
		Rv	CACTCACCAGGAAAGGAACAAA
GAD67	*Gad1*	Fw	CTGAACCGAGCCTGTTCCTG
		Rv	TCATACGTTGTAGGGCGCAG
GAT-1	*Slc6a1*	Fw	GTTGGACTGGAAAGGTGGTCT
		Rv	AGCTTTCGGAAGTTGGGTGT
GAT-2	*Slc6a13*	Fw	GCCTCGGGAACAACCAGTAAT
		Rv	GACAGGGATGCCACAGGTAAA
GAT-3	*Slc6a11*	Fw	CGGCTGGGTATATGGAAGCA
		Rv	GCCCCAAGCAGGATATGTGT
VGAT	*Slc32a1*	Fw	CACTGCGACGATCTCGACTT
		Rv	CACGAACATGCCCTGAATGG
PAC1R	*Adcyap1r1*	Fw	GGCTGTGCTGAGGCTCTACTTTG
		Rv	AGGATGATGATGATGCCGATGA
VPAC1R	*Vipr1*	Fw	GATGTGGGACAACCTCACCTG
		Rv	TAGCCGTGAATGGGGGAAAAC
VPAC2R	*Vipr2*	Fw	GCGGTGTCTGGGACAACATC
		Rv	CTGTGACATTTTCCCCAACGT

**T. gondii*

isopropanol precipitation using Total RNA Kit peqGOLD® (Peqlab, VWR) or isolated using AllPrep DNA/RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Concentration and purity of DNA and RNA was determined using NanoDrop 2000 spectrophotometer (Thermo Fisher) and samples stored at −80 °C until further use.

*q*PCR

Parasite burden was assessed in triplicates using 30 ng of isolated DNA, FastStart Essential DNA Green Master (Roche, Germany) and LightCycler® 96 System (Roche) as described elsewhere [39]. Thermal-cycling parameters were set as follows: initial activation (95 °C, 10 min), 45 amplification cycles consisting of denaturation (95 °C, 15 s), annealing (60 °C, 15 s), and elongation (72 °C, 15 s). The DNA target was the published sequence of the highly conserved 35-fold-repetitive B1 gene of *T. gondii* (*TgB1*) [56, 57], and murine argininosuccinate lyase (*Asl*) was used for normalization [58]. Primers listed in Table1 were synthetized by Tib MolBiol (Germany) and used at 300 nM final concentration.

RT-*q*PCR

Transcription levels of inflammatory mediators, host-defense factors, neurotrophins, neurotrophin receptors, and synaptic proteins were assessed in triplicates using 30 ng isolated RNA, TaqMan® RNA-to-CT™ 1-Step Kit (Applied Biosystems, Germany), and LightCycler® 96 (Roche, Germany) as described elsewhere [39]. TaqMan® Gene Expression Assays (Thermo Fisher) utilized are listed in Table 2. *Hprt* was chosen as reference gene and relative mRNA levels were determined by the ratio *gene of interest/reference gene* and subsequently normalized to mean values of control group. To determine parasite life-stage conversion, the expression of

Table 2 Oligonucleotide primers used for RT-qPCR based on TaqMan®

Protein	Gene	Gene expression assay
BDNF	*Bdnf*	Mm04230607_s1
GABAα1	*Gabra1*	Mm00439046_m1
GBP-1	*Gbp2b*	Mm00657086_m1
HPRT	*Hprt*	Mm01545399_m1
IFN-γ	*Ifng*	Mm00801778_m1
Irgm3	*Igtp*	Mm00497611_m1
IL-1β	*Il1b*	Mm00434228_m1
IL-6	*Il6*	Mm00446190_m1
Irgm1	*Irgm1*	Mm00492596_m1
NGF	*Ngf*	Mm00443039_m1
$P75^{NTR}$	*Ngfr*	Mm01309638_m1
iNOS	*Nos2*	Mm00440485_m1
Nt-3	*Ntf3*	Mm01182924_m1
TrkA	*Ntrk1*	Mm01219406_m1
TrkB	*Ntrk2*	Mm00435422_m1
VGLUT1	*Slc17a7*	Mm00812886_m1
EAAT2	*Slc1a2*	Mm01275814_m1
TNF	*Tnf*	Mm00443258_m1

stage-specific genes for tachyzoites (*Sag1*) and bradyzoites (*Bag1*) [59] was evaluated using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Thermo Fisher). Samples were analyzed in triplicates (50 ng of isolated mRNA per reaction) using LightCycler® 96 (Roche) and the following parameters: reverse transcription (48 °C, 30 min), inactivation (95 °C, 10 min) followed by 55 cycles of denaturation (95 °C, 15 s), annealing/extension (60 °C, 1 min), and melting curve analysis. The primers used for *Sag1* (SAG1), *Bag1* (BAG1) and murine *Gapdh* are listed in Table 1, and were synthetized by Tib MolBiol and used at 100 nM final concentration. Expression of *Gapdh* was chosen as reference gene and relative mRNA levels were determined by the ratio *gene of interest/reference gene* and subsequently normalized to mean values of control group. Similarly, transcriptional levels of glutamate decarboxylase enzymes, GABA transporters, and PACAP receptors were determined as described above, using Power SYBR® Green RNA-to-CT™ 1-Step Kit (Thermo Fisher) with 30 ng mRNA per reaction and primers listed in Table 1 used at 200 nM final concentration.

Histopathology and immunohistochemistry

For histopathological analysis, brains were fixed with 4% paraformaldehyde (PFA) at 4 °C immediately after removal, and embedded in paraffin. Sagittal sections were prepared at 5 µm. To evaluate the general pathological changes and the number of inflammatory foci sections were stained using hematoxylin and eosin (H&E). Brain sections were further analyzed by in situ immunohistochemistry for quantification of $F4/80^+$ macrophages and apoptotic cells. Primary antibodies against F4/80 (1:50, clone BM8, eBioscience) and cleaved Caspase-3 (1:200, Cell Signaling, USA) were used. A minimum of 2–4 sagittal sections were analyzed per animal. Axiovert 200 bright field microscope equipped with an AxioCam ERc 3 digital camera and ZEN software (ZEISS, Germany) were used. The number of positively stained cells was assessed in 10 randomly chosen images (40× objective) within brain cortical regions. The total number of inflammatory foci was assessed using a 10× objective across the brain sections. Slides were analyzed in an independent and blinded manner.

Western blot analysis

Proteins were isolated from whole brain homogenates previously prepared with TriFast™ (Peqlab, VWR, LLC, Germany) using an optimized method described elsewhere [60]. Protein concentrations were determined using Bradford reagent (Bio-Rad Protein Assay, Bio-Rad, Germany) and read at $\lambda = 595$ nm using SpectraMax M5e (Molecular Devices LLC). Total of 15 µg of protein from each sample were separated by SDS-PAGE 10%, and transferred to nitrocellulose membrane (Amersham™ Protran™, GE Healthcare, Germany). Membranes were blocked for 2 h in ROTI®Block solution diluted in Tris-Buffered Saline (TBS) containing 0.1% (v/v) Tween-20 (Roth, Germany) (TBS-T), and subsequently incubated overnight at 4 °C with primary antibodies in the same blocking solution. Accordingly, GAD65 (Synaptic System, SYSY, Germany, #198,104, 1:1000), GAD67 (SYSY, #198,211, 1:500), TrkB (CST, #4603, 1:1000), beta-III-Tubulin (Sigma, #T8660, 1:1000) and beta-actin (CST, #13E5, 1:1000) were used. In the following day, membranes were washed three times (10 min each) in TBS-T and then incubated for 1 h room temperature with secondary HRP-conjugated antibodies diluted in TBS-T at 1:10,000. Anti-guinea pig (Invitrogen, #A18769), anti-rabbit (Jackson ImmunoResearch, #111–035-144) and anti-mouse (Jackson ImmunoResearch, #115–035-068) were used. Membranes were revealed using SuperSignal™ West kit (Thermo Fisher, #34,078 and #34,095) and densitometric analysis of the blots were performed using ImageJ/Fiji.

Statistical analysis

Results were statistically analyzed using GraphPad Prism 7 (GraphPad Software Inc., USA). Data were considered statistically significant with $p \leq 0.05$. All data are presented as arithmetic mean with standard error of the

mean (SEM), collected from at least two independent experiments. For histopathological data, *q*PCR and RT-*q*PCR, data were analyzed by non-parametric Mann–Whitney *U*-test. For flow cytometric and western blot analyses, data were analyzed by two-tailed unpaired *t*-test.

Results

PACAP alleviates brain pathology upon cerebral *T. gondii* infection

T. gondii neuroinfection is characterized by activation of glial cells and recruitment of immune cells to the CNS. Albeit promotion of pathogen clearance, the inflammatory responses also result in severe brain lesions and destruction of neuronal tissue [61]. To assess the effects of exogenous PACAP administration upon cerebral toxoplasmosis, we utilized a murine model susceptible to chronic progressive infection [9]. Therefore, C57BL/6 J animals were infected with two cysts of type II ME49 parasite strain. After the initial acute infection, 100 µg of PACAP or vehicle only (for non-treated controls) were administered i.p. starting three weeks post-infection (Fig. 1A). One day after the last treatment, brain tissue was collected for histopathological analyses (Fig. 1B–G). In non-treated control- and PACAP-treated animals, we detected parenchymal hemorrhage and inflammatory foci of cellular infiltrates. In contrast, the number of inflammatory foci was strongly reduced in PACAP-treated mice (Fig. 1B–C). Additionally, PACAP treatment resulted in reduced numbers of F4/80$^+$ cells (Fig. 1D, E), which was paralleled by lower number of brain apoptotic cells (Casp3$^+$) (Fig. 1F, G). Hence, these results point towards an anti-inflammatory and neuroprotective effect of PACAP. In order to assess whether this amelioration of inflammation negatively affected parasite control, we determined *T. gondii* burden in the brain of mice. Our results revealed an unaltered parasite burden, and no alteration of parasite life-stage conversion upon PACAP treatment (Fig. 1H). Similarly, parasite burden in peripheral organs was also not affected (Fig. 1I). Thus, PACAP treatment of chronically infected mice suggested amelioration of neuroinflammation without hampering parasite control. Accordingly, we hypothesize that PACAP effects are likely due to modulation of immune cell recruitment to the CNS and their activation status.

PACAP restricted myeloid cell recruitment and reduced microglia and monocyte activation

In previous studies, we demonstrated that neutrophils and myeloid cells continuously infiltrate the CNS during cerebral toxoplasmosis [11, 12]. As suggested by the histopathological analysis, we hypothesize that PACAP ameliorates neuroinflammation and reduces cellular death by restraining immune cell infiltration to the brain. For further investigation, we isolated immune cells from the brain of *T. gondii*-infected animals, and assessed the differences between PACAP-treated animals and infected non-treated controls regarding immune cell recruitment and activation. CD45 and CD11b were used as markers to identify lymphocytes (CD45$^+$CD11b^{low}, upper left gate), myeloid cells (CD45$^+$CD11b^{high}, upper right gate) and activated microglia (CD45^{int} CD11b^{int}, lower right gate) (Fig. 2A). Myeloid cells were further discriminated by Ly6G expression, providing differentiation between neutrophil granulocytes (Ly6G$^+$CD11b$^+$, upper gate) and mononuclear cells (Ly6G$^-$ CD11b$^+$, lower gate). Then, mononuclear cells were classified according to Ly6C expression, and subdivided in CD11b$^+$Ly6C^{hi} inflammatory monocytes (upper gate), CD11b$^+$Ly6C^{int} monocytes-derived dendritic cells (DCs) (middle gate) and CD11b$^+$Ly6C^{lo} monocyte-derived macrophages (lower gate) as previously described by our group [11]. When compared to control, PACAP-treated mice showed a reduced number of recruited myeloid cell subsets (Fig. 2B). Also, the recruitment of Ly6G$^+$ neutrophil granulocytes was reduced without reaching significance ($p=0.065$) in PACAP group (Fig. 2C). Next, we hypothesized that the reduction of cell recruitment to the brain of PACAP-treated mice might simply be a reflection of diminished peripheral immune response. Thus, we investigated the immune cell compartment in blood (Fig. 2D-–F) and spleen (Fig. 2G–I), and no changes in myeloid cell frequency were detected between control and PACAP group. Further on, we assessed whether PACAP would modulate the activation status of monocytes subsets in terms of MHCII expression, which is crucial for a potent inflammatory adaptive immune response [62]. We detected reduced MHCII expression by Ly6C^{hi} inflammatory monocytes in the brain (Fig. 3A). In addition, we assessed the production of NO, a key role pro-inflammatory mediator measured via expression of inducible NO synthase (iNOS). Levels of iNOS were reduced on Ly6C^{int} and Ly6C^{lo} monocyte subsets upon PACAP treatment (Fig. 3B). Moreover, the production of pro-inflammatory cytokines TNF and IL-1β was reduced in Ly6C^{int} and Ly6C^{lo} subsets, respectively (Fig. 3C, D). Next to the altered activation of infiltrating myeloid cells, we found that the activation status of resident microglia was changed upon PACAP, indicated by reduced expression of MHCII, and intracellular production of iNOS, TNF, and IL1-β (Fig. 3E–H). At last, we assessed the overall impact of PACAP in the transcriptional level of inflammatory mediators and chemokines in the entire brain tissue, and we detected

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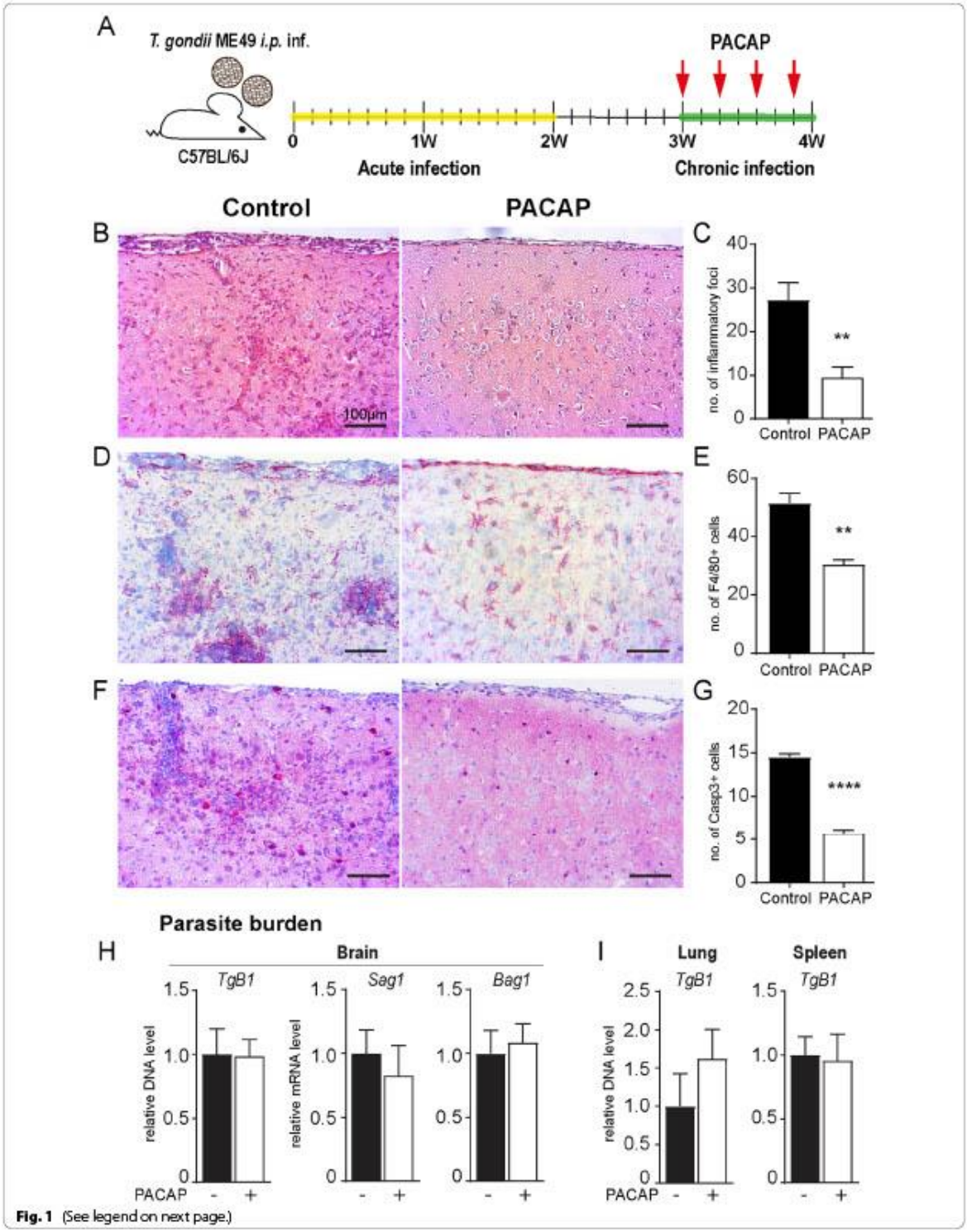

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Fig. 1 PACAP administration alleviates severe brain pathology upon *T. gondii* encephalitis. **A** Experimental design and PACAP administration schedule. Mice were infected with 2 cysts of *T. gondii* (type II strain ME49) and treated with 100 µg PACAP i.p. on alternating days starting at 3 weeks post-infection; yellow indicates acute infection, followed by an intermediated phase (2 to 3 weeks post-infection, not indicated), and green indicates chronic infection. **B**, **D**, **F** Histological brain sections from infected control animals (left column) and PACAP-treated animals (right column). **B** H&E staining and **C** bar charts show number of inflammatory foci quantified across 10 consecutive brain sections per mouse. **D** F4/80 staining and **E** bar charts show the mean of F4/80$^+$cells across 10 random cortical areas per mouse. **F** Caspase-3 (Casp3) staining and **G** bar charts show the mean of Casp3$^+$cells across 10 random cortical areas per mouse. Data show mean + SEM, n = 4, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (unpaired two-tailed t-test). Scale bars = 100 µm. (H) Parasite burden was determined by *q*PCR analysis of TgB1 abundance, and RT-qPCR of *Sag1* and *Bag1* gene expression in brain homogenate. (I) Parasite burden in peripheral organs determined by TgB1 abundance. Bar charts present results normalized to mean values of control group as mean + SEM obtained in two independent experiments and analyzed together, $n = 4–5$ (Mann–Whitney *U*-test); Control (black bars) and PACAP-treated (white bars)

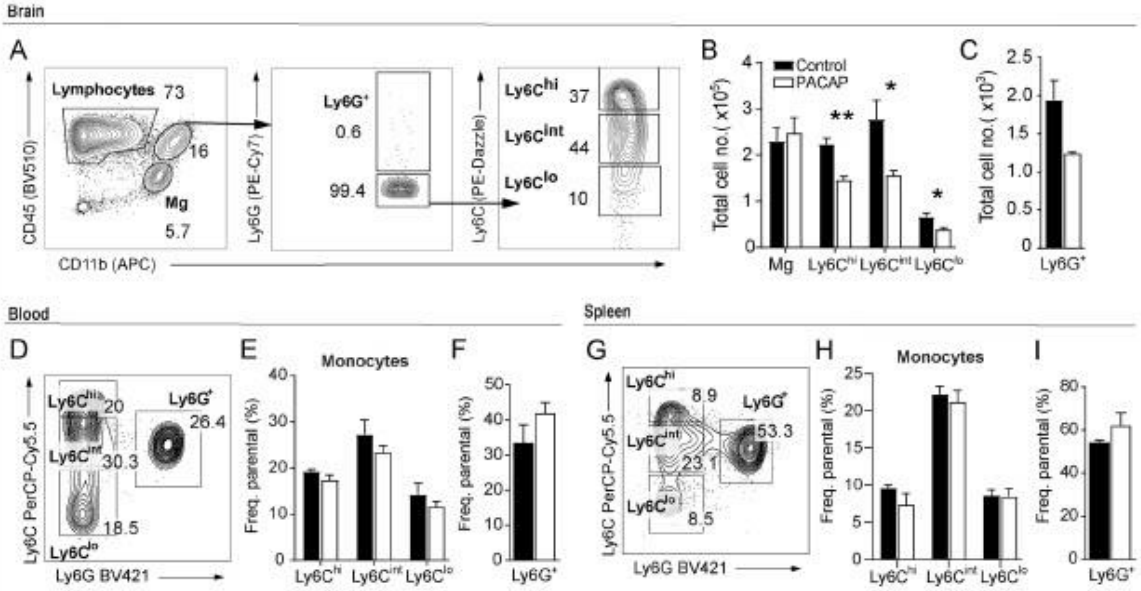

Fig. 2 Analysis of microglia and myeloid immune cell subsets upon PACAP treatment. Immune cells were isolated from brain, blood and spleen, and analyzed by flow cytometry. Only single, live immune cells were considered for analysis. **A** Representative gating strategy applied for discrimination of CD45^{hi}CD11b$^-$lymphocytes and CD45^{int}CD11b^{int} microglia (Mg). CD45^{hi}CD11b^N myeloid cells (upper right gate) were further discriminated into Ly6G$^+$ neutrophils, and Ly6G$^-$ mononuclear cells were differentiated according to Ly6C expression in Ly6C^{hi} inflammatory monocytes, Ly6C^{int} monocyte-derived DCs and Ly6C^{lo} monocyte-derived macrophages. **B**, **C** Bar charts show total cell numbers of microglia and myeloid immune cell subsets in the brain. **D**, **G** Myeloid cells from blood and spleen were first identified as CD11b$^+$ (not shown). Contour plots show the classification of selected cells into monocyte subsets (according to Ly6C expression) and Ly6G$^+$ neutrophils. Bar charts compare the cell frequency in the blood (**E**, **F**) and spleen samples (**H**, **I**) as percentages from parent population. Data show individual values as mean + SEM, $n = 4–5$, $^*p < 0.05$, $^{**}p < 0.01$ (unpaired two-tailed t-test) from a representative experiment; Control (black bars) and PACAP-treated (white bars)

reduced expression of IFN-γ, IL-6, iNOS (*Nos2*), IL-1β, CCL2, CXCL9 and CXCL10 (Fig. 3I). Regarding modulation of PACAP receptors levels in overall brain, all receptors presented higher expression upon infection, and only PAC1R was reduced upon PACAP treatment (Additional file 1A). In sum, PACAP impacted the recruitment of myeloid cells to brain, reduced the expression of inflammatory mediators and chemokines in the brain parenchyma.

PACAP diminished IFN-γ production by recruited CD4$^+$T cells in infected brains

Next, we analyzed the impact of PACAP on the lymphoid T cells recruited to the brain upon *T. gondii* infection. Upon chronic toxoplasmosis, T cell-derived IFN-γ production represents the major driving force to parasite control, and it is mainly mediated by CD4$^+$and CD8$^+$T cells [63–65]. Although no difference in parasite burden was previously detected in PACAP-treated brains,

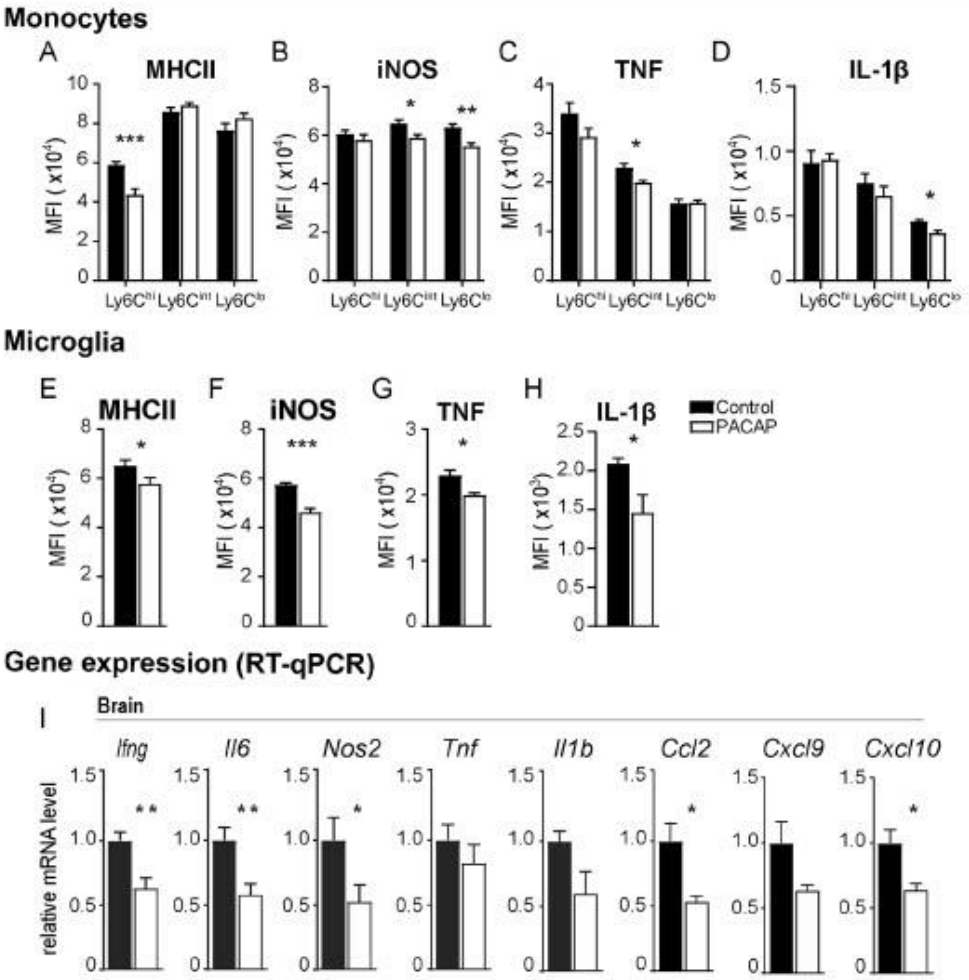

Fig. 3 Monocyte and microglia activation and inflammatory mediators. Previously identified brain monocytes subsets and microglia were analyzed by flow cytometry for surface expression of MHCII (**A**, **E**), and for intracellular production of iNOS, TNF and IL-1β (**B–D**, **F–H**). Bar charts show values of median fluorescence intensity (MFI) previously defined for each population and marker, based on their respective fluorescence-minus-one (FMO) controls (not shown). Data represent mean values + SEM, $n = 4–5$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (unpaired two-tailed *t*-test); I gene expression of inflammatory mediators and chemokines was assessed in whole brain homogenate. Bar charts represent mean values + SEM obtained in two independent experiments and analyzed together, $n = 4–5$ per experiment $^{*}p < 0.05$, $^{**}p < 0.01$ (Mann–Whitney *U*-test); Control (black bars) and PACAP-treated (white bars)

IFN-γ transcripts were reduced, as well as the expression of CXCL9 and CXCL10 chemokines, particularly important to T cell recruitment into the brain upon cerebral toxoplasmosis [66] (Fig. 3I). For this reason, we further investigated whether PACAP would affect T cell recruitment and their IFN-γ production. No difference was found in the total cell number of $CD4^+$ and $CD8^+$ T cells between the treated and control group (Fig. 4A, B). In contrast, the intracellular IFN-γ production was significantly decreased in $CD4^+$ T cells upon PACAP treatment, whereas it remained unaltered in $CD8^+$ T cells (Fig. 4C, D). As IFN-γ signaling regulates cell-intrinsic host defense factors against intracellular parasites, we analyzed the expression of *Igtp*, *Gbp2b* and *Irgm1* in the brain tissue, and despite the reduction of IFN-γ, only *Igtp* showed reduced expression (Fig. 4E). Thus, PACAP treatment did not alter recruitment of T cells, but reduced IFN-γ production by $CD4^+$ T cells

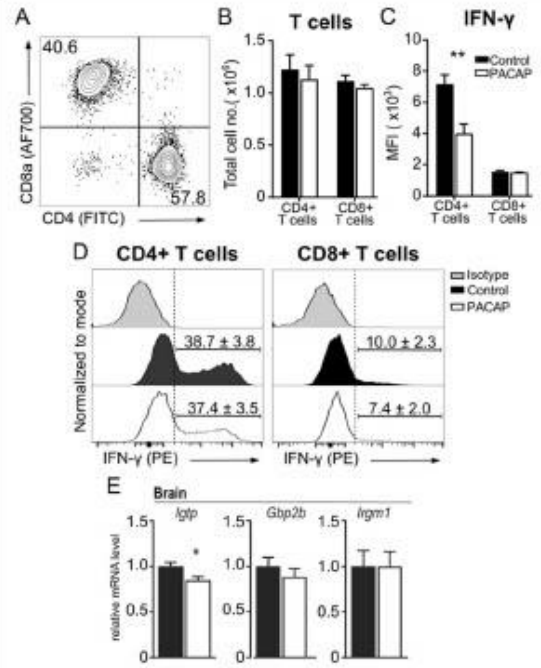

Fig. 4 Lymphocyte infiltration into brain, IFN-γ production and host-defense factors. Immune cells were isolated from brain, and identified as single, live, $CD45^{hi}CD11b^{-}CD3^{+}$ lymphocytes as shown above. **A** Representative gating illustrates discrimination of $CD4^{+}$ and $CD8^{+}$ T cells, and values in quadrant gates indicate cell frequency from parent population. **B** Total cell number of recruited $CD4^{+}$ and $CD8^{+}$ lymphocytes to the brain. **C–D** Intracellular analysis of IFN-γ production. Bar charts present MFI values as mean + SEM, $n = 4$, $^{**}p < 0.01$ (unpaired two-tailed *t*-test). Histograms show the representative IFN-γ production by $CD4^{+}$ and $CD8^{+}$ T cells in comparison to isotype-control. Bars and numbers above histograms indicate mean percentage of positively stained cells + SEM. **E** Gene expression levels of host-defense factors in the whole brain homogenate were assessed using RT-qPCR. Bar charts represent mean values + SEM obtained in two independent experiments and analyzed together, $n = 4$–5 per experiment, $^{*}p < 0.05$ (Mann–Whitney *U*-test). Control (black bars) and PACAP-treated (white bars)

PACAP modulates BDNF and $p75^{NTR}$ levels and modifies infection-induced synaptic imbalance

As the inflammatory response during cerebral toxoplasmosis is known to modify neuronal function and synapse composition [9, 14–16], we next set out to investigate whether the beneficial anti-inflammatory outcome of PACAP extended to neuronal survival and improved synaptic plasticity in the brain. In previous studies, PACAP has been associated with neurotrophins and their respective receptors [67], which are crucial for growth, development, and survival of neuronal tissue [68]. Therefore, we analyzed the transcriptional levels of the neurotrophins BDNF, NGF, NT-3, and the neurotrophin receptors $p75^{NTR}$, TrkA, and TrkB in the brain of PACAP-treated animals (Fig. 5A-B). When compared to control group, PACAP treatment increased BDNF transcriptional levels (Fig. 5A), a crucial factor for neuronal function [69], and simultaneously reduced expression of $p75^{NTR}$ (Fig. 5B), a receptor associated with neuronal cell death and reduced synaptic function [70, 71], but not the other neurotrophin receptors (Fig. 5B). TrkB, the main receptor for BDNF also showed no difference at protein levels (Additional file 2). Those findings prompted us to evaluate whether PACAP could compensate the reduction on synaptic alterations caused by cerebral immune response against *T. gondii*, particularly in the glutamatergic neurotransmission pathways previously described [16]. We detected increased transcriptional levels of the glutamate transporter EAAT2 and vesicular glutamate transporter VGLUT1, both implicated in neuronal protection and avoidance of excessive excitatory neuronal stimuli [72, 73] (Fig. 5C). Furthermore, we detected increased transcriptional levels of the α1 subunit of the GABA-a post-synaptic receptor (GABAAα1), an important component of the inhibitory GABAergic signaling, suggesting that PACAP probably try to counter-balance exacerbated excitatory neuronal signaling (Fig. 5C). Those results prompted us to extend our analysis to the glutamate–GABA axis. Accordingly, PACAP had no evident effect on glutamate decarboxylases GAD65 and GAD67, responsible for the most GABA amount synthesized from glutamate in the mouse brain [74] (Fig. 5D, F). Further on, we found a reduced transcriptional level of GAT-1 (Fig. 5E), a critical transporter implicate in the availability of GABA in the synaptic cleft and associated functions [75, 76]. No difference was detected for the other investigated transporters (GAT-2, GAT-3, VGAT). Of note, GAT-1 and GAT-2 were found more expressed in the brain upon *T. gondii* infection, while GAT-3 and VGAT were less expressed Additional file 1, as previous depicted for other neuronal markers upon infection [15, 16]. At last, we found increased levels of the specific neuronal component β-III tubulin (TUBB3) in the PACAP-treated brains, which in addition to transcriptional VGLUT1 levels indicates a reduced neurological damage, as previously described for chronic *T. gondii* infection [77]. Together, our results suggest that PACAP promoted neuronal viability likely via BDNF/$p75^{NTR}$ modulation, and can modify synaptic excitatory/inhibitory neuronal imbalance present upon *T. gondii*-induced neuroinflammation.

Discussion

Cerebral infection with *T. gondii* causes chronic inflammation leading to neuronal alterations and behavioral changes of the host. The elicited immune response is

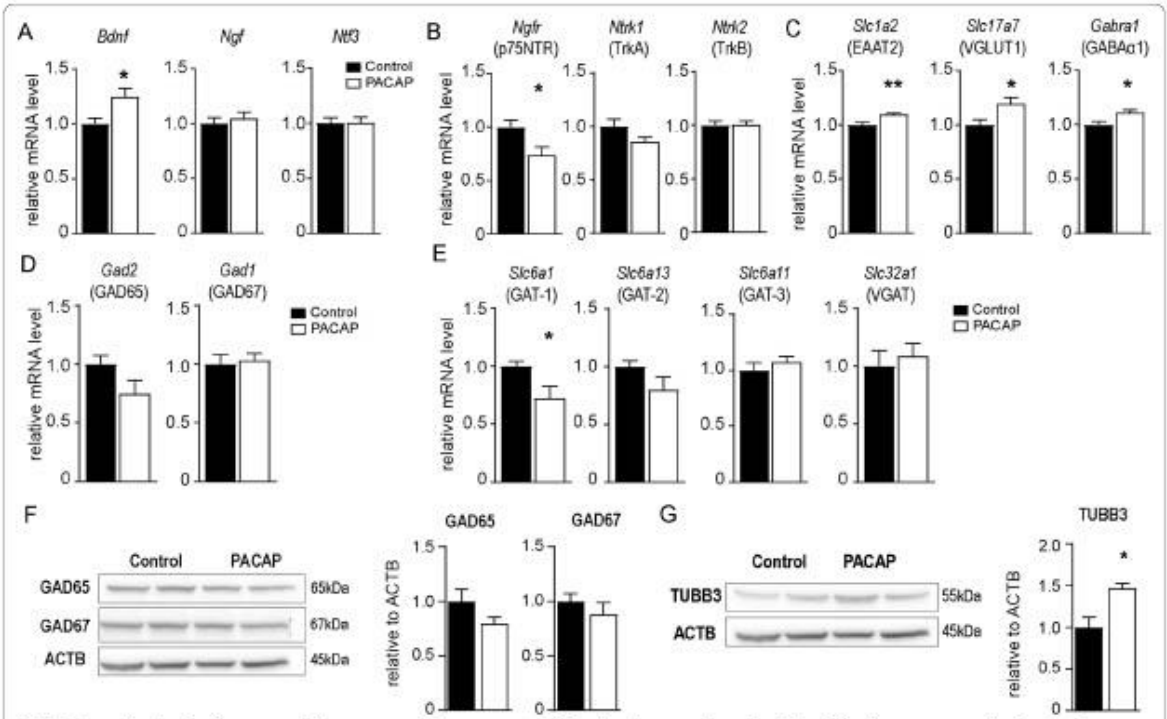

Fig. 5 Expression levels of neurotrophins, neurotrophin-receptors and functional neuronal markers. Overall brain gene expression levels of **A** neurotrophins and **B** neurotrophin-receptors; **C** gene expression levels of glutamate transporters (EAAT2, VGLUT1) and GABAα1 subunit; **D** expression of glutamate decarboxylases GAD65 and GAD67 and **F** western blot analysis; **E** expression levels of GABA transporters (GAT-1, GAT-2, GAT-3, VGAT); **G** protein levels of beta-III-tubulin (TUBB3). Bar charts represent mean values + SEM obtained in two independent experiments and analyzed together, $n = 4–5$ per experiment, $^{*}p < 0.05$, $^{**}p < 0.01$ (Mann–Whitney *U*-test); Control (black bars) and PACAP-treated (white bars). Western blot membranes show two representative samples of each group, and bar charts represent mean values + SEM, $n = 4$

essential for parasite control in the brain, although simultaneously entails damage to the neuronal tissue. Here, we demonstrate the beneficial effects of the neuropeptide PACAP on infection-induced neuroinflammation in the murine model of cerebral toxoplasmosis. PACAP administration reduced brain pathology, microglia activation, and infiltration of monocytes, diminished production of pro-inflammatory cytokines, and bolstered neuronal protection. In previous studies, PACAP was able to modify clinical symptomatology of certain neurological disorders for example Parkinson's [40, 41], Alzheimer's [42], Huntington's disease [43], and traumatic brain injury [44]. Additionally, administration of PACAP has been shown to modulate inflammation in murine models of septic shock, rheumatoid arthritis, Crohn's disease, and multiple sclerosis [78–84]. In fact, although exerting a prominent impairment of inflammation, the immunomodulatory ability of PACAP was found to be ambiguous, alternating from inhibitory to stimulatory effects [47]. Moreover, as modulator of microbial infections, PACAP has exhibited direct antimicrobial activity against bacteria and fungi [85]. In support, our previous studies indicate as well a reduction of *T. gondii* parasite burden in the acute phase of the infection in the periphery, through an indirect immunomodulatory anti-parasitic effect [39].

In our experimental model of cerebral toxoplasmosis, PACAP reduced the number of apoptotic caspase-3^{+} cells in the brain parenchyma. According to previous studies, the beneficial effects of PACAP on neurological diseases are mainly attributed to inhibition of caspase-3 activation mediated by receptor PAC1R [86], which is expressed by inhibitory neurons, astrocytes, and microglia [87]. Therefore, we suggest that PACAP treatment may act via PAC1R to reduce brain cell death upon *T. gondii* cerebral infection. Moreover, our histopathological examination and flow cytometric analysis indicates reduction of inflammatory foci and infiltration of immune cells in the infected brains following PACAP treatment. Likely, this immunomodulation is a secondary branch-effect of PACAP, also mediated by binding to PAC1R or other receptors in brain parenchymal cells. Previously, we and others have described the important contribution

of recruited peripheral immune cells to control parasite burden in the brain in the cerebral toxoplasma model [11, 12, 88]. Surprisingly, we observed that restraining immune cell infiltration into the brain did not affect parasite control and stage conversion. Parasite levels in the periphery remained constant in the PACAP-treated group as well. In the acute toxoplasma model, we have previously detected reduced parasite burden by PACAP immunomodulation of mononuclear cells, enhancing their phagocytic capacity, although PACAP did not interfere directly with parasite replication in vitro [39]. During acute infection, the fast replicating tachyzoites are the predominant life stage of *T. gondii*, and are susceptible to immune recognition and elimination, as well as to known therapeutic drugs [25]. In contrast, during chronic infection, tachyzoites that escaped the initial immune response convert into bradyzoites, which form cysts within a variety of tissues including brain and muscles [10]. *T. gondii* cysts are shielded from therapeutic drugs and evade host immune responses [89], which suggests they are also protected from the immune response of cells modulated by PACAP treatment, and therefore could explain no difference in parasite burden. Altogether, PACAP provided neuroprotection and markedly modulated immune cell behavior without compromising parasite control.

In addition to the reduced infiltration of myeloid cells into the brain, PACAP modified the activation status and cytokine production of brain immune cells, pointing towards an anti-inflammatory effect. Markedly, Ly6C^{hi} inflammatory monocytes were reduced in number and activation status, exhibiting reduced MHCII expression. Additionally, PACAP slightly diminished levels of pro-inflammatory mediator iNOS and TNF by Ly6C^{int} monocytes-derived DCs, while iNOS and IL-1β levels were reduced by Ly6C^{lo} monocyte-derived macrophages. In our previous studies, we demonstrated the critical importance of Ly6C^{hi} monocytes upon cerebral toxoplasmosis [11]. In fact, antibody-mediated ablation of CCR2^{+} Ly6C^{hi} monocytes increased parasite burden and decreased survival of infected mice, also ablating Ly6C^{hi} monocytes in the blood [11]. In contrast, PACAP administration did not alter the presence of monocytes in the periphery, but still restricted their infiltration into the brain, reducing inflammation without compromising parasite control. Therefore, we hypothesize that PACAP exerted a specific immunomodulatory effect on the infiltrating monocytes to prevent CNS immunopathology. We have previously demonstrated that PACAP restricted the recruitment of monocytes and neutrophils, reduced their expression of pro-inflammatory cytokines, and enhanced their phagocytic capabilities upon *T. gondii* acute infection [39]. Here we additionally detected that PACAP treatment diminished CCL2 levels in the brain, which can further reduce myeloid cell infiltration. We suggest that anti-inflammatory effects of PACAP in the brain involve microglia modulation, and overall shift of the local brain inflammatory environment. In our dataset, PACAP reduced microglial MHCII expression and their production of pro-inflammatory mediators iNOS, TNF, and IL-1β. Similarly, in vitro studies have reported anti-inflammatory effects of PACAP on microglial cells, which was demonstrated by reduction of iNOS and IL-1β production in an LPS-sepsis model [90].

Further investigation on the inflammatory brain environment upon *T. gondii* infection interestingly showed that exogenous PACAP did not interfere with T cell infiltration into the brain, although it reduced IFN-γ intracellular production by CD4^{+}T cells, and the overall brain expression of CXCL9 and CXCL10. During cerebral toxoplasmosis, CD4^{+}and CD8^{+}T cells are the major source of IFN-γ in the brain, directly controlling parasite replication [91, 92]. Pointed out by previous studies, CD4^{+}T cells are targets for PACAP regulation, indicating inhibition of Th1 and favoring of Th2 differentiation and response [93–95]. Moreover, in vitro experiments indicated that macrophages and DCs treated with PACAP induce Th2 cytokines and inhibit IFN-γ in primed CD4^{+}T cells [94, 96]. Besides, our analysis showed reduction of the IFN-stimulated host-defense factor *Igtp*, even though the parasite burden was not altered. In our dataset, the absence of the modulation by PACAP on the recruitment of T cell can be explained by the late administration of the neuropeptide at the third week post-infection, when the pathology is already established in the CNS. This hypothesis is supported by previous studies, where peripheral depletion of CD4^{+}and CD8^{+}T cells did not show any effect on intracerebral T cell number, indicating that this recruitment was negligible after acute infection [65]. The reduction of IFN-γ production and *Igtp* expression suggest that PACAP may not abolish the strong Th1 response induced by *T. gondii*, but can restrain brain immunopathology.

Overall, the neuroprotective and immunomodulatory effects of PACAP are mostly attributed to the complex and wide distribution of its receptors in a variety of cell types, including neurons and immune cells. Upon inflammation, most of inhibitory immuno-modulation of PACAP targets pro-inflammatory signaling cascades including NF-κB and MAPK pathways, consequently downregulating an array of cytokines and chemokines produced by innate immune cells [47]. Our previous data have shown that PACAP administration increased expression levels of its own receptors VPAC1R and VPAC2R in immune cells, which were followed by reduced infiltration of monocytes and neutrophils, and

reduced pro-inflammatory cytokine expression during acute toxoplasmosis [39]. Additionally, several studies indicate that the anti-inflammatory activity of PACAP on macrophages, monocytes, and DCs are primarily exerted through VPAC1R [97–100]. While VPAC1R is constitutively expressed in those cells, VPAC2R is induced in lymphocytes, monocytes, and macrophages after inflammatory stimulation, and has been linked to increased Th2 response [93–95]. Aligned with our finding that PACAP reduced IFN-γ production by $CD4^+$T cells in the brain, studies with transgenic mice overexpressing the receptor VPAC2R in $CD4^+$T cells developed increased Th2 responses, while the Th1 response prevailed in VPAC2R-deficient mice [95, 101]. Of note, VPAC1R and VPAC2R were also found expressed by the newly discovered innate lymphoid cells 2 (ILC2), the innate-correlated cells of the Th2 adaptive immune response, showing to be important for immune response [102]. In microglia, PAC1R is the main receptor mediating anti-inflammatory effects of PACAP, inhibiting NO production during ischemia, reducing pro-inflammatory cytokine release, and inducing a microglial phenotype transformation associated to regenerative growth, tissue repair and clearance of local cellular debris [45, 103]. Taken together, we suggest that the beneficial effects of PACAP upon cerebral toxoplasmosis rely partially on the differential expression of PACAP receptors on the immune cells directly involved in the disease control.

Further on, we demonstrate that PACAP exerted not only beneficial anti-inflammatory effects on cerebral toxoplasmosis, but also showed a neuroprotective outcome in the infected brain parenchyma. Interestingly, we found higher transcriptional levels of the neurotrophin BDNF in the brain of PACAP-treated mice, and no differences were detected for NGF or NT-3. In fact, PACAP-mediated neuroprotection have been attributed to enhanced expression of neurotrophins and related receptors via PAC1R signaling [33]. Of note, PAC1R is considered the main PACAP receptor in the brain, and the one with the highest affinity for PACAP [104]. In terms of neurotrophins, BDNF is crucial for neuronal survival, and its expression was also found reduced upon neurodegeneration [105] and cerebral toxoplasmosis [16]. Indeed, in vitro studies on primary neuronal cultures revealed that PACAP stimulates and recovers BDNF expression upon injury conditions, mainly via PAC1R [67, 106]. Additionally, reduced hippocampal BDNF was also detected in PAC1R knockout mice [107]. Overall, enhancement of BDNF expression is proposed as a key mechanism in neuroprotection and rescue of cognitive impairment via PAC1R [33]. Since PACAP affected BDNF transcriptional levels, we sought to further investigate the modulation of neurotrophin-related receptors. BDNF mainly binds to TrkB (*Ntrk2*), NGF to TrkA (*Ntrk1*), and the binding of neurotrophins to $p75^{NTR}$ (*Ngfr*) is more promiscuous and complex, and it is thought to define the affinity of the neurotrophins and their precursors to other receptors [68]. In fact, $p75^{NTR}$ has been nominated as death-receptor, and binding of the BDNF precursor to $p75^{NTR}$ has shown to induce cell death and reduce synaptic function [108]. Surprisingly, we found that PACAP reduced expression of $p75^{NTR}$ in the brain, and did not alter TrkB levels, suggesting an additional contribution of PACAP to neuronal survival via BDNF–$p75^{NTR}$ axis modulation. Other studies in stroke models have shown that PACAP reduced $p75^{NTR}$ levels, regulated the activity of TrkB by increased phosphorylation, and consequently increased affinity for BDNF [109, 110]. We have previously demonstrated that $p75^{NTR}$ signaling not only shaped brain neuronal architecture, but also altered the behavior of innate immune cells during neuroinflammation [52]. Moreover, we have previously found a beneficial association of $p75^{NTR}$ and immune cells upon PACAP treatment of acute *T. gondii* infection, in which reduced expression of $p75^{NTR}$ on $Ly6C^{hi}$ inflammatory monocytes and increased overall BDNF gene expression was associated with reduction on pro-inflammatory mediators and enhanced parasite elimination [39]. Therefore, we suggest that PACAP-mediated neuroprotective effects and immunomodulatory properties provide a beneficial overlap via modulation of BDNF/$p75^{NTR}$ axis to counteract *T. gondii* neuroinflammation. In addition, PACAP increased the levels of TUBB3, a specific cytoskeleton component of neurons shown to be modified upon cerebral *T. gondii* infection [77], reinforcing PACAP contribution to neuronal survival. Still, future studies are necessary to address the involvement of PACAP, BDNF precursors and $p75^{NTR}$ function in immune cells within the brain.

Besides neurotrophins, PACAP-treated mice showed an increase in the expression levels of synaptic markers EAAT2 and VGLUT1 from neuronal glutamatergic pathway, and GABAAα1 from inhibitory GABAergic pathway. As recently shown in our previous studies, the expression of these synaptic markers was significantly decreased upon cerebral toxoplasmosis, suggesting that *T. gondii* neuroinflammation shifted the synaptic excitation–inhibition balance towards excitotoxicity at the transcriptional [16] and protein level [15]. Detrimental alterations in the glutamatergic and GABAergic signaling upon *T. gondii* infection have also been further characterized, and suggest a neurodegenerative state of the infected CNS [77, 111]. Here, PACAP's overall reduction of brain inflammation without dysregulation of parasite control points towards a less dysfunctional neuronal network. The explanation relies on the reduced IFN-γ production, MHCII expression, NO

production and other pro-inflammatory cytokines in the brain, which have detrimental effects on neurons [112]. Antibody-mediated depletion of IFN-γ during cerebral toxoplasmosis partially recovered synaptic alterations, although it triggered an uncontrolled parasite burden in the brain [16]. Therefore, PACAP exerts amelioration of brain immunopathology and consequently reduces synaptic dysfunction.

Finally, it is important to point out the limitations of our study. Exogenous administration of PACAP is sought to recover and/or enhance the beneficial effects of the endogenous presence of the neuropeptide, which are diminished or lost during neuroinflammation [45]. We applied PACAP via i.p. injections, and despite the relatively short half-life in the organism, we achieved neuroprotective results. In recent studies, intranasal administration of PACAP has proven to deliver more efficiently the neuropeptide to the brain, showing rapid absorption via nasal mucosa and high uptake in the occipital cortex and striatum [113, 114]. Still, PACAP is transported directly across the BBB in a very rapid transport rate [50, 115], and its uptake was found particularly high in the hypothalamus and hippocampus [116]. Besides the administration route and brain-region-specific penetration, another aspect not explored in this study is the effect of PACAP specific on astrocytes, which are known to contribute to recover the neuronal homeostasis during cerebral toxoplasmosis. We believe that PACAP restriction of myeloid immune cell infiltration and the shifted brain inflammatory environment, together, can be partially mediated by the effect of PACAP on astrocytes, once they also express PACAP receptors. Moreover, a comprehensive characterization of the PACAP receptors and neurotrophin receptors on Ly6C$^+$ monocytes and on the recently described ILCs are necessary for the understanding of the neuro-immune mechanism involved in neuroinflammation and neurodegeneration.

Conclusions

In summary, exogenous administration of PACAP ameliorated *T. gondii* infection-induced brain pathology, restrained the recruitment of peripheral myeloid cells to the brain, and reduced the activation status and production of pro-inflammatory mediators by microglia and monocytes, resulting in neuroprotection. This immunomodulation restricted the detrimental effects of neuroinflammation. Moreover, PACAP promoted neuronal health likely via BDNF/p75^{NTR} axis modulation, resulting in diminished dysregulation of the neuronal network with implications on glutamatergic and GABAergic signaling inflicted by cerebral toxoplasmosis. Together, our findings unravel that exogenous PACAP administration provides beneficial cumulative effects of neuroprotection and immunomodulation to overcome infection-induced neuroinflammation.

Abbreviations

AC: Adenylate cyclase; AKT: Protein kinase B; ANOVA: Analysis of variance; BBB: Blood–brain barrier; BDNF: Brain-derived neurotrophic factor; cAMP: Cyclic adenosine monophosphate; Casp3: Cleaved caspase-3; CCL2: Chemokine (C–C motif) ligand 2; CD: Cluster of differentiation; CNS: Central nervous system; DCs: Dendritic cells; DNA: Deoxyribonucleic acid; EAAT2: Excitatory amino acid transporter 2; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; FMO: Fluorescence minus one; GABA: Gamma-aminobutyric acid; Gbp2b: Guanylate binding protein 1; GPCRs: G protein-coupled receptors; H&E: Hematoxylin and eosin; HBSS: Hanks' balanced salt solution; HEPES: 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; IFN-γ: Interferon gamma; Igtp: Interferon gamma-induced GTPase; IL-6: Interleukin 6; iNOS: Inducible nitric oxide synthase; IP3: Phosphatidylinositol 3 kinase; Irgm1: Immunity-related GTPase family M protein 1; MHCII: Major histocompatibility complex type II; LPS: Lipopolysaccharides; NGF: Nerve growth factor; NO: Nitric oxide; NT-3: Neurotrophin-3; p75NTR: P75 neurotrophin receptor; PACAP: Pituitary Adenylate Cyclase-Activating Polypeptide; PBS: Phosphate-buffered saline; PCR: Polymerase chain reaction; PFA: Paraformaldehyde; PKA: Protein kinase A; PKC: Protein kinase C; PLC: Phospholipase C; RNA: Ribonucleic acid; RT-qPCR: Quantitative reverse transcription PCR; SEM: Standard error of the mean; TLA: Toxoplasma lysate antigen; TNF: Tumor necrosis factor; TrkA: Tropomyosin receptor kinase A; TrkB: Tropomyosin receptor kinase B; VGLUT1: Vesicular glutamate transporter 1.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-022-02639-z.

Additional file 1. Complementary transcriptional levels of PACAP receptors and neuronal markers. Overall brain gene expression levels for (A) PACAP receptors, (B) glutamate decarboxylases GAD65 and GAD67, and (C) GABA receptors. Additional naïve dataset was introduced as comparison to previously unknown transcriptional levels in the brain of the analyzed genes, but the values were normalized by the control group (infected) as shown in the previous figures for appropriate comparison. Bar charts represent mean values + SEM obtained in two independent experiments and were analyzed together, n = 4-5 per experiment, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 (ANOVA with Tukey correction); Naïve (gray bars), control (black bars) and PACAP-treated (white bars).

Additional file 2. Complementary protein levels of neurotrophin receptor TrkB. Overall brain expression levels of TrkB in control vs PACAP-treated animals. Western blot membrane shows two representative samples of each group, and bar charts represent mean values + SEM, n = 4.

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Author contributions

CAF performed experiments and analyzed data. HPD, JS, SE, MPD, GT, DR, MMH critically discussed experimental design, provided material and co-edited the manuscript. IRD conceived experimental design and supervised the project. CAF and IRD wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All animal experiments were approved by the respective authorities (Landesverwaltungsamt Halle, Sachsen-Anhalt, Germany) in accordance with German and European legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Publisher's Note

FINAL DISCUSSION

Choroid Plexus and *T. gondii* infection

Parasite invasion at the CP

The pivotal role of the CP in the orchestration of neuroinflammation became evident in recent years (Baruch et al., 2013; Kunis et al., 2015; Schwartz and Baruch, 2014; Shechter et al., 2013), and distinct pathogens have been described exploiting the CP as a novel gateway into the CNS (Lauer et al., 2018). Although, as a gateway for *T. gondii*, only a few studies have explored the CP rather than the BBB (Falangola and Petito, 1993). Here, **Publication 1** demonstrated that *T. gondii* was found in the CP at the onset of infection, opposing previous studies on reactivated TE models, where no evidence for the involvement of the CP in *T. gondii* dissemination was detected (Dellacasa-Lindberg et al., 2007; Schluter and Barragan, 2019). Whether parasites are able to translocate from blood to the CSF via CP and further infect CSF-inundated areas (e.g., brain, spinal cord) is still not fully disclosed. In fact, a previous study described the extent of infection within the spinal cord during chronic toxoplasmosis, suggesting that parasites translocate into the CSF while the infection progresses (Mohle et al., 2014). Currently, the lack of tools to selective block one or another barrier (i.e., BCSFB or BBB) presents a major challenge to unveiling their respective contributions to the parasite invasion. In the present study, described in details in **Publication 1**, CP infection resulted in structural alterations of the CP epithelial TJs and increased the permeability of the BCSFB. Previous evidence indicates that alterations in this barrier allow immune cells and inflammatory mediators to enter the CNS and enhance neuroinflammatory diseases (Fleischer et al., 2021). Accordingly, the detrimental effects of *T. gondii* infection on the BCSFB integrity is probably one of the initial steps in the development of neuroinflammation. From this point on, parasitic spread increases while the disease evolves, for example, through the circulation of infected leukocytes that can transpose the BBB and CP. In this regard, at 7dpi time point, a higher parasite burden was detected in the brain microvessels (BMV) rather than the CP, which likely indicates parasite shuttling to the small CNS capillaries, and confirms findings recently published by others (Drewry et al., 2019; Schneider et al., 2022). The reduced parasite burden in the CP at this time point could probably be explained by the development of the CP's local immune response.

CP local immune response

The activation of the CP vasculature presented in the **Publication 1** may contribute to the orchestration of the anti-parasitic immune response at early time points of the infection. The choroidal endothelial cells upregulated the expression of MHCII prior than brain endothelial cells and microglia, suggesting that the CP vasculature plays a prompt role in antigen presentation and interaction with DCs and T cells. Of note, BBB endothelial cells have been described to serve as a replicative niche for *T. gondii* invasion of the CNS (Konradt et al., 2016), but whether activated endothelial cells (from CP or BBB) can control intracellular parasite replication *in vivo* is still unknown. Further on, the increased expression of the

intercellular adhesion molecule 1 (ICAM-1) detected in the infected CP suggests local recruitment of immune cells.

In the face of early detection of $CD45^+$ cells associated with *T. gondii* in the CP, **Publication 1** hypothesizes that a transient burden of extracellular parasites in the blood likely reach the CP interface, infecting the choroidal endothelial cells and challenging the resident stromal macrophages and epithelial cells. Accordingly, infection at the CP elicited early and local immune response at the BCSFB, with expression of pro-inflammatory cytokines, cell-intrinsic host defense factors, and leukocyte trafficking molecules. IFN-γ, TNF, and IL-6 were upregulated in the CP of infected mice, suggesting not only an immune cell-derived response but also implying the involvement of choroidal epithelial cells in this response, as *in vitro* culture of those cells responded to *T. gondii* in the absence of immune cells. This indicates a direct anti-parasitic, pro-inflammatory response of the CP epithelial cells. It is still unknown whether endothelial and epithelial cells of the CP have different responses to *T. gondii* infection and whether this could explain preferences for parasite targeting (epithelial *vs.* endothelial cells) at the BCSFB. Moreover, whether immune cells found in the CP are resident and/or recruited from the peripheral circulation remains to be clarified. *In vivo*, the fact that in **Publication 1** CXCL9 gene expression was more elevated in the CP rather than in BMV could, additionally, suggest the recruitment of specific immune cells into the brain via the CP during cerebral infection (Ochiai et al., 2015). For example, a previous study has shown that the CP served as an educative gateway for the recruitment of inflammatory immune cells, which in the CSF acquired an inflammation-resolving phenotype before infiltrating the CNS parenchyma (Shechter et al., 2013). Thus, the CP emerged as a crucial interface for early pathogen interaction within CNS borders, likely promoting damage recognition, antigen presentation and immune cell recruitment.

BCSFB dysfunction

Dysfunctional BCSFB is part of the pathophysiological process leading to increased neuroinflammation (Demeestere et al., 2015). BCSFB permeability is mainly determined by the paracellular barrier established by TJs between the epithelial cells of the CP, specifically the TJs containing claudins anchored by ZO-1 scaffold protein to the actin cytoskeleton. In **Publication 1**, claudin-2 was found to be downregulated and overall structurally disorganized between CP epithelial cells, and together with ZO-1 alterations, could explain the increased BCSFB permeability upon infection. Accordingly, those alterations in the CP have also been described in EAE and LPS-induced sepsis model (Marques et al., 2009; Shrestha et al., 2014). Interestingly, infection of the BCSFB by *Trypanosoma brucei* has indicated direct parasite interactions with claudins between CP epithelial cells, although the mechanisms of how they induce TJ opening is still not known (Mogk et al., 2014a; Mogk et al., 2014b). Whether there is a direct and active interaction of *T. gondii* with TJ of the CP is still to be defined. Likely, MMPs are involved in TJ disruption, as increased transcriptional levels of the MMP-8 and MMP-13 were found *in vivo* in isolated infected CP and *in vitro* in CP epithelial cultures after *T. gondii* infection. Previously, these proteinases have been implicated in BCSFB breakdown in neurodegenerative diseases and bacterial meningitis (Leppert et al.,

2000; Rosenberg, 2009), and have been associated with TJ dysregulation (Vandenbroucke et al., 2013; Vandenbroucke et al., 2012).

Moreover, the alterations found in the CP and the increased BCSFB permeability likely relate to neuronal alterations, as the CSF composition drastically changes. For example, besides the abundant entrance of inflammatory cytokines and immune cells present in the blood, CP and CSF modifications of composition (e.g., hormones, neurotransmitters, neurotrophins, and metabolic waste) have been associated with neuropsychiatric and neurodegenerative disorders such as dementia, AD, schizophrenia and HD (Stopa et al., 2018). Previous reports indicate that neuronal alterations are present during chronic toxoplasmosis (Dusedau et al., 2019; French et al., 2019; Lang et al., 2018; Parlog et al., 2015). The detrimental modifications in CP and the BCSFB depicted in **Publication 1** might contribute to progressive neuronal impairment, as increased BCSFB permeability to FITC-dextran was extended throughout the chronic phase of infection. Thus, substantial changes in the CP architecture and permeability have detrimental implications for the molecular and cellular composition of the CSF and may contribute to increased neuroinflammation and subsequent neuronal damage during toxoplasmosis.

PACAP treatment and *T. gondii* infection

PACAP: Inflammation and anti-parasitic effects

The work presented here hypothesized that the application of the neuropeptide PACAP might modulate the behavior of myeloid-derived mononuclear cells and potentially contribute to the resolution of the infection and parasite elimination. In line with previous reports, the results from **Publication 2** and **Publication 3** reinforced the previously described anti-inflammatory effect of PACAP, such as the reduction of pro-inflammatory cytokines and chemokines (Abad et al., 2003; Abad et al., 2001; Abad et al., 2002; Azuma et al., 2008; Delgado et al., 2001; Martinez et al., 2002; Tan et al., 2009). In fact, the recruitment of immune cells and their production of pro-inflammatory mediators are essential for parasite elimination, regardless of tissue damage and functional loss. In this present study, administration of exogenous PACAP showed to reduce levels of IFN-γ, which is crucial for *T. gondii* control and intracellular parasite elimination via cell-autonomous host defense factors. Although this effect would suggest a negative outcome on systemic parasite control in treated animals, the opposite was observed. In **Publication 2**, PACAP reduced systemic parasite burden and increased the transcriptional levels of host defense factors IRGM1 and IRMG3 directly in isolated immune cells from infected animals. The same cells subsets also displayed increased transcriptional levels of VPAC1R and VPAC2R, suggesting that these PACAP receptors are involved in the anti-parasitic response mediated by PACAP treatment and maybe a positive feedback loop. To confirm that PACAP induced elimination of intracellular *T. gondii*, animals were infected with GFP-reporter parasites and treated with PACAP the same way as before. Similarly, treated animals showed a reduced percentage of infected myeloid immune cells. Moreover, *in vitro* experiments with PACAP from **Publication 2** indicated an enhanced phagocytic capacity of macrophages. At last, in the face of previous studies suggesting antimicrobial effects induced by features of PACAP's structure, a

possible direct anti-parasitic effect of PACAP was assessed. No direct effect of PACAP on *T. gondii* parasites was detected *in vitro*. In conclusion, the anti-parasitic effect of PACAP was mediated by immune cells, likely via VPAC1R and VPAC2R binding that enhanced the expression of host defense factors and their phagocytic capacity. Therefore, PACAP was able to counteract excessive detrimental inflammation and promote parasite elimination.

In **Publication 3**, the experimental approach of PACAP administration was focused on the effects of the neuropeptide in the brain during chronic cerebral *T. gondii* infection. As depicted before, PACAP exerted a potent anti-inflammatory effect and reduced the recruitment and activation of myeloid cells in the brain. Additionally, PACAP decreased microglia production of iNOS, TNF, and IL-1β and further resulted in diminished IFN-γ production by $CD4^+$ T cells. Together, this immunomodulation of PACAP has raised concerns as previously depicted in **Publication 2**: from one side, PACAP reduced local brain pathology, but on the other, it could trigger uncontrolled parasite replication. Surprisingly, parasite burden and stage conversion in the brain, but also in other peripheral organs, remained unaltered upon PACAP treatment, despite reduced levels of IFN-γ. Together, in **Publication 2**, the parasite burden was reduced, and in **Publication 3** parasite burden was not altered; one explanation for divergent results would rely on the ability of *T. gondii* to circumvent immune recognition. Upon chronic infection, tachyzoites might escape the immune response by converting into bradyzoites, forming cysts within the brain. The persistency of intracellular parasites is likely linked to their ability to impair the action of the IRGs host defense factors, e.g., IRGM1 and IRGM3. Possibly, the stimulation of these factors by PACAP treatment, as seen in **Publication 2** is no longer effective for the infected cells in which the parasites have already successfully interfered with these IRGs during cerebral infection. To cause this interference, one strategy used by *T. gondii* is to inoculate rhoptry bulb proteins (ROPs) in the host cell. For example, ROP5, ROP17, and ROP18 cause phosphorylation and degradation of the IRGs, avoiding their accumulation in the PV and parasite elimination (Sasai and Yamamoto, 2019). Altogether, *T. gondii* elimination by PACAP is likely mediated by cell-autonomous defense factors and will be dependent on the ability of parasites to counteract those defense factors and avoid intracellular elimination. How the anti-parasitic effects of PACAP and the parasite evasion mechanisms are interconnected is still not understood. Therefore, future studies using genetically ablated models for IRGs or specific blocking agents for host defense factors would help to clarify this finding.

Moreover, the overall restraining effect of PACAP treatment on microglia, myeloid and T cells in the infected brain suggested that an excessive immune response is present, as parasite control and parasite stage conversion remained unaffected. This excessive immune response, markedly of a pro-inflammatory profile, probably is a compensatory mechanism to the inability of this mouse model (C57BL/6) to effectively suppress brain inflammation. The genetic background of these animals has differences in the MHC-I locus, which impairs the recognition of the secreted *T. gondii* antigenic protein GRA6. As a consequence, the cytotoxic activity of $CD8^+$ T cells is impaired, and animals will eventually succumb to high levels of brain inflammation (Schluter and Barragan, 2019). Therefore, PACAP treatment mediated the beneficial

reduction of severe brain pathology caused by the excessive immune response, ameliorating CNS tissue damage.

PACAP and BDNF/p75^{NTR} in immune cells

The results presented here indicate that PACAP receptors would participate in neurotrophin signaling, specifically regarding BDNF and the neurotrophin receptor p75^{NTR} in immune cells. In this regard, BDNF can be produced by different immune cells and even modulate cytokine production, chemotaxis of monocytes and enhance their phagocytic activity (Hashimoto et al., 2005; Kruse et al., 2007; Samah et al., 2008; Vega et al., 2003). The p75^{NTR} is a receptor that binds to all neurotrophins, and mediates cell survival and migration. It can act as a co-receptor for the neurotrophins Trk receptors, enhancing their specificity and selectivity of them for the correlated neurotrophins. In fact, p75^{NTR} has high affinity by neurotrophin precursors, which can antagonize the effect of mature neurotrophins (Chao, 2003). Because PACAP receptors are GPCRs, after PACAP binding their signaling can cause transactivation of Trk receptors (Chao, 2003). Specifically, TrkA and TrkB are the main receptors for NGF and BDNF, respectively, and they were found to be activated by GPCR ligands in the absence of those neurotrophins (Rajagopal et al., 2004). Considering that VPAC1R and VPAC2R were found upregulated upon *T. gondii* infection and that PACAP significantly influenced the behavior of myeloid cells, a possible explanation may be found in the transactivation of neurotrophin receptors by PACAP.

In **Publication 2** and **Publication 3**, no alterations were detected in transcriptional levels of TrkA and TrkB. In contrast, in **Publication 2**, PACAP treatment increased levels of BDNF in immune cells, and the receptor p75^{NTR} showed reduced surface expression on Ly6C^{hi} inflammatory monocytes. In **Publication 3**, PACAP treatment resulted in an observation: increased BDNF and reduced p75^{NTR} transcriptional levels in total brain homogenates. Previous report highlights that p75^{NTR} is upregulated in myeloid cells and microglia but not on lymphocytes during cerebral *T. gondii* infection (Dusedau et al., 2019). From one side, PACAP can bind to its receptors on myeloid cells and increase their expression of BDNF. In addition, the binding to VPAC1R and/or VPAC2R can transactivate the Trk receptors, associated or not with p75^{NTR}, and trigger their intracellular signaling linked to BDNF, reinforcing it. On another side, PACAP-mediated reduction of p75^{NTR} on monocytes may alter their interaction with neurotrophins. Studies using antagonists of p75^{NTR} have observed reduced recruitment of inflammatory monocytes after traumatic brain injury, further strengthening the link of neurotrophins and immune cell migration (Lee et al., 2016). Moreover, previous data indicated the accumulation of pro-BDNF in the brain upon *T. gondii* infection (Dusedau et al., 2019). Probably a reduced expression of p75^{NTR} suggests the reduced influence of pro-neurotrophins on Ly6C^{hi} monocytes with a beneficial effect on the resolution of inflammation. In fact, both suggested mechanisms are possibly contributing to the immunomodulation of myeloid cells. Yet, the remaining questions are: 1) Is this receptor transactivation occurring upon PACAP treatment? 2) How does p75^{NTR} modulation take place and may alter the behavior of myeloid cells and their interaction with neurotrophins? 3) How much of this interaction is influenced by pro-neurotrophins that are also expressed upon

inflammation, triggering antagonistic signaling pathways? Further studies of myeloid cells upon PACAP treatment shall consider the phosphorylation status of Trk receptors and the use of agonists/antagonists for PACAP and neurotrophins to better understand those interactions. Finally, another relevant aspect of being considered is the influence of BDNF signaling in the evasion of apoptosis by immune cells. It was previously described that BDNF binding to TrkB-expressing T cells leads to their protection from apoptosis upon EAE (Frota et al., 2009). The constant presence of activated immune cells in the brain parenchyma is especially important during chronic neuroinflammation, as depicted in diseases such as MS (Wang and Tian, 2021), but also in chronic cerebral toxoplasmosis. To what degree this interaction of PACAP, immune cells and neurotrophins promotes neuronal protection *vs* persistence of inflammation still needs to be evaluated. Taken together, the data gathered here indicate that PACAP treatment is able to modulate the behavior of myeloid cells upon *T. gondii* infection, possibly with the involvement of BDNF and $p75^{NTR}$.

PACAP, neuroinflammation and synaptic dysfunction

Synaptic alterations in the brain have an intimate relation with neuronal health, in particular upon chronic neuroinflammation. During the onset of brain infection, local neuroinflammation is fundamental for pathogen recognition and elimination. However, it also entails damage to neuronal tissue in short- and long-term (Farmen et al., 2021). Prolonged activation and recruitment of immune cells into the CNS induces the release of cytotoxic ROS and NO, which ultimately trigger apoptosis of neurons promoting neuronal degeneration (Herrup and Yang, 2007; Lyman et al., 2014).

The main, but not unique, drivers of this cytotoxic response are microglia, macrophages, and recruited immune cells (Becher et al., 2017). In **Publication 3**, the administration of PACAP resulted in an overall anti-inflammatory effect with a reduced number of inflammatory cuffs. Specifically, the reduced number of $F4/80^+$ cells in the cortex revealed by histology supports the depicted reduction of $CD11b^+Ly6C^{lo}$ monocyte-derived macrophages detected by flow cytometry. Of note, the remaining $F4/80^+$ cells apparently increased F4/80 expression in the cortex and additionally on the meninges (pia mater), suggesting an increased phagocytic activity after PACAP administration. In **Publication 2**, *in vitro* PACAP treatment increased F4/80 expression and phagocytic capacity of macrophages, supporting those findings upon cerebral infection. Moreover, the meningeal barriers can serve as a gateway for CNS pathogens, and the meningeal macrophages (MM) have been shown to be crucial for the control of CNS viral infections (Rua and McGavern, 2018). Thus, further studies might explore not only the modulation of MM by PACAP but also explore their contribution to *T. gondii* neuroinflammation.

Further on, in **Publication 3**, PACAP treatment induced less infiltration of inflammatory monocytes and reduced microglial production of iNOS, TNF and IL-1β. Consequently, reduced neuroinflammation showed to impact parenchymal cell death, as PACAP further leads to a diminished number of apoptotic cells in infected brains. Indeed, PACAP increased levels of β-III-tubulin (TUBB3), a cytoskeleton component of neurons. As chronic *T. gondii* infection causes a loss of structural complexity of axons and dendrites (Parlog et al., 2014), the effects of PACAP point towards neuronal protection. Often,

the use of anti-inflammatory drugs describes the attenuation of neuronal degeneration and neurological symptoms in the context of bacterial meningitis (de Gans et al., 2002). However, upon cerebral toxoplasmosis, this approach can be critical for parasite control, leading to unresolved TE (Dunay et al., 2018). Here, PACAP showed to reduce *T. gondii* neuroinflammation without affecting parasite control. In fact, PACAP has shown a potent anti-apoptotic effect, mainly via PAC1R binding, directly inhibiting the caspase-dependent mitochondrial apoptotic pathway (Ohtaki et al., 2006).

Ultimately, neuronal health implies the preservation of a functional synaptic network. In previous studies, the inflammatory milieu induced by chronic *T. gondii* infection was detrimental to synaptic protein composition, mainly downregulating components of the glutamatergic neurotransmission pathway (Lang et al., 2018). In **Publication 3**, PACAP increased transcriptional levels of the excitatory amino acid transporter 2 (EAAT2) and vesicular glutamate transporter 1 (VGLUT1) both implicated in glutamate transport. Particularly, glutamate is a potent excitatory neurotransmitter, and its excessive accumulation in the CNS induces neuronal apoptosis cascade, characterizing a noxious effect called excitotoxicity (Verma et al., 2022). This neurotoxicity is reported among many models of neurodegenerative diseases, e.g., MS, amyotrophic lateral sclerosis (ALS), and epilepsy. It is highly suggested to occur upon cerebral toxoplasmosis, as the accumulation of extracellular glutamate in *T. gondii*-infected brains was detected (David et al., 2016). Modulation of the glutamatergic pathway suggests that PACAP can counteract glutamate excitotoxicity. Still, different cell types can be involved in this regulation, as the expression of PAC1R, the main PACAP receptor, is quite ubiquitous in the CNS. Moreover, the reduction of inflammation, and specifically, the use of neutralizing antibodies to reduce IFN-γ levels has been shown to ameliorate glutamatergic synaptic alterations, despite of detrimental consequences for parasite control (French et al., 2019).

Collectively, PACAP has shown a pleiotropic neuroprotective effect, ranging from: 1) reduction of inflammation, 2) reduction of apoptosis, 3) modulation of neurotrophin signaling, and 4) modulation of synaptic composition. Therefore, PACAP demonstrated beneficial effects upon *T. gondii*-induced neuroinflammation. Further studies shall focus on the modulatory mechanisms of the neuropeptide PACAP on specific cell types - glial, immune cells and neurons -, and how these modulations would contribute to ameliorate neuronal impairment and promote neuroprotection upon cerebral infection.

FINAL CONCLUSION

This present work demonstrated the involvement of the CP, and the beneficial immunomodulatory impact of the neuropeptide PACAP on *T. gondii* infection. The CP showed to be initially infected early in the onset of the infection. Alongside with CP endothelial cells, immune cells were also targeted by the parasite, inducing a rapid inflammatory response with loss of BCSFB integrity and functionality. This response was likely driven by TJ disturbances within CP epithelial cells and by the activity of MMPs. Thus, the dysfunctional BCSFB may initially enhance and further contribute to *T. gondii*-induced neuroinflammation.

Concerning the neuropeptide PACAP, this study indicates that the PACAP application ameliorated overall inflammation in the periphery and in the CNS by reducing immune cell infiltration, activation, and cytokine production, particularly by myeloid cells. PACAP also showed an immune cell-mediated anti-parasitic activity, with enhanced phagocytic capacity. In addition, the results demonstrate the involvement of PACAP in neurotrophin signaling through the modulation of the immune response. Simultaneously, PACAP reduced neuroinflammation and modified neuronal dysregulation, promoting neuroprotection during cerebral *T. gondii* infection.

Altogether, the CP has emerged as an important CNS barrier actively involved in *T. gondii* infection, which opens new perspectives for diagnostic and therapeutic approaches. Notably, PACAP unveiled an exclusive role in neuro-immune interactions through providing neuroprotection and beneficial immunomodulation overcoming *T. gondii* infection.

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AD	Alzheimer's disease
AIDS	Acquired immunodeficiency syndrome
ALS	Amyotrophic lateral sclerosis
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCSFB	Blood-cerebrospinal fluid barrier
BDNF	Brain-derived neurotrophic factor
BMV	Brain microvessels
cAMP	Cyclic adenosine monophosphate
Casp3	Caspase 3
CCL2	C-C motif chemokine ligand 2
CCL3	C-C motif chemokine ligand 3
CCL5	C-C motif chemokine ligand 5
CCR2	C-C motif chemokine receptor type 2
CD	Cluster of differentiation
CNS	Central nervous systems
CP	Choroid plexus
CP-BAMs	Choroid plexus border-associated macrophages
CSF	Cerebrospinal fluid
CXCL10	C-X-C motif chemokine 10
CXCL2	C-X-C motif chemokine 2
CXCL9	C-X-C motif chemokine 9
DALYs	Disability-adjusted life years
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
EAAT2	Excitatory amino acid transporter 2
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cells
ECM	Extracellular matrix
FITC	Fluorescein isothiocyanate
GABA	Gamma-aminobutyric acid
GBPs	Guanylate binding proteins
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPCRs	G protein-coupled receptors
GPI	Glycosylphosphatidylinositols
GTP	Guanosine triphosphate
HD	Huntington’s disease
HIV	Human immunodeficiency virus
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
IFNAR	Interferon (alpha and beta) receptor
IGTP	Interferon gamma-induced gtpase
IL	Interleukin

ILCs	Innate lymphoid cells
iNOS	Inducible NO synthase
IRGM1	Immunity-related gtpase family M member-1
IRGM3	Immunity-related gtpase family M member-3
IRGs	Immunity-related gtpases
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MM	Meningeal macrophages
MMPs	Matrix metalloproteinases
MS	Multiple sclerosis
NGF	Nerve growth factor
NKs	Natural killer cells
NO	Nitric oxide
NT-3	Neurotrophin-3
NT-4	Neurotrophin-4
$p75^{NTR}$	P75 neurotrophin receptor
PACAP	Pituitary adenylate cyclase-activating polypeptide
PD	Parkinson's disease
PRRs	Pattern recognition receptors
PSGL-1	P-selectin glycoprotein ligand 1
PTS-6	Peptide transporter system-6
PV	Parasitophorous vacuole
RNA	Ribonucleic acid
ROP	Rhoptry bulb protein
ROS	Reactive oxygen species
SAS	Subarachnoid space
Shank3	SH3 and multiple ankyrin repeat domains
SLC	Solute carriers
STAT1	Signal transducer and activator of transcription 1
TCR	T cell receptor
TE	Toxoplasmic encephalitis
Th2	T helper type 2
TJs	Tight junctions
TLR	Toll-like receptors
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
Trk	Tropomyosin regulated kinase
TUBB3	Beta-III-tubulin
VGLUT1	Vesicular glutamate transporter 1
VIP	Vasoactive intestinal peptide
WHO	World health organization
ZO-1	Zonula occludens-1

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