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Effects of Notch signaling pathway inhibition by dibenzazepine in acute experimental toxoplasmosis

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ABSTRACT

Notch signaling pathway plays a crucial role in cellular fate across species, being important for the differentiation and development of several cell types. The aim of this study was to evaluate the effect of Notch inhibition pathway by dibenzazepine (DBZ) in histological and inflammatory alterations and, tissue parasitism in acute *Toxoplasma gondii* infection. For this, C57BL/6 mice were treated with DBZ before infection with *T. gondii*, and the small intestine, lungs and liver were analyzed. The genes related to Notch signaling pathway were assayed through qPCR in the organs, and cytokine measurement was performed in serum samples. In the small intestine, *T. gondii* infection impaired the *Hes1* and *Math1* mRNA expressions, increased the inflammation and decreased goblet and Paneth cell numbers. The DBZ-treatment was able to partially preserve these cells, however, the parasitism and inflammation were not altered. In parallel, the high IL-2, IL-6, TNF and, IFN-γ levels induced by infection were not changed with the DBZ treatment, with the IFN-γ levels even higher. In contrast, in the liver and lungs, the DBZ-treatment diminished parasitism and inflammation. Our results highlight that Notch pathway inhibition in *T.gondii* infection results in different parasitological and inflammatory outcomes depending on the organ analyzed.

1. Introduction

Differentiation and development of several cell types depend on signaling through Notch transmembrane receptors and their ligands (Fre et al., 2005; Murta et al., 2014; Huang et al., 2016; Tsao et al., 2016; Yao et al., 2018).

Notch signaling pathway begins with the interactions of Notch receptor and its corresponding ligand which initiates a series of proteolytic cleavages by the enzyme γ-secretase releasing Notch intracellular domain (NICD) from the cell membrane to the nucleus (Schroeter et al., 1998; Kopan and Ilagan, 2009). Then, NICD binds to the DNA-binding adaptor CSL (CBF1/RBPjκ) leading to the recruitment of a mastermind-like protein family (MAML 1–3) (Kopan and Ilagan, 2009) and other transcriptional coactivators of the hairy and enhancer of split (Hes) family (Jarriault et al., 1995; Kopan, 2012). Hes-1 is a repressor of the basic helix-loop-helix (bHLH) transcriptional factor Math-1 [(Atoh1 – Mouse Genome Informatics), a mammalian homolog of Drosophila atonal)] and a balance between both is suggested to be essential for cell differentiation (Jensen et al., 2000; Zheng et al., 2000). In the small intestine, enterocytes are derived from an enterocyte precursor cell that is Math-1 independent (Yang et al., 2001) and is determined by the transcription factor Hes-1 (Jensen et al., 2000), while goblet, enteroendocrine and Paneth cells are derived from a common secretory precursor that expresses Math-1 (Yang et al., 2001). Both Notch-1 and Notch-2 are expressed in intestinal epithelial cells and are responsible for governing cell fate decisions in the intestines (Schröder and Gossler, 2002; Sander and Powell, 2004; Riccio et al., 2008), being Notch-1 more prominently expressed postnatally in epithelial cells of the crypts than

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Notch-2 (Schröder and Gossler, 2002).

Several studies used pharmacological Notch signaling inhibition through γ-secretase inhibitors, resulting in the loss of proliferating crypt progenitor cells due to their conversion into post-mitotic goblet cells (Milano et al., 2004; van Es et al., 2010; VanDussen et al., 2012). *In vivo* administration of DBZ has been associated with the decrease in *Hes1* mRNA expression (van Es et al., 2010, Shinoda et al., 2010) and increase in *Math1* mRNA and protein expressions (Milano et al., 2004). The interference or inhibition of Notch signaling pathway is involved with the development of chronic intestinal inflammation (Robinson et al., 2017), chronic colitis (Obata et al., 2012) and apoptosis of crypt base stem cells in the intestine (VanDussen et al., 2012; Takada et al., 2016). Additionally, Notch pathway induction is observed in transmissible murine colonic hyperplasia (TMCH) and inflammation in *Citrobacter rodetium* infection (Ahmed et al., 2012), as well as in proliferative enteropathy (PE) in pigs induced by *Lawsonia intracellularis* infection (Huan et al., 2017).

Notch signaling is also involved in immune response. In antigen-presenting cells (APCs), the expression of Jagged or Delta-like Notch ligands regulate the differentiation of naïve T helper cells into Th2 and Th1 effector subsets, respectively (Amsen et al., 2004).

T cell–restricted Notch signaling contributes to pulmonary Th1 and Th2 immunity during *Cryptococcus neoformans* infection (Neal et al., 2017) and Notch1 and Notch2 signaling is necessary for IFN-γ secretion by Th1 cells during infection with *Leishmania major* (Auderset et al., 2012). In the liver, Notch1/Jagged1 signaling is involved in M2 polarization of macrophages that is associated with liver granuloma and fibrosis in schistosomiasis (Zheng et al., 2016). Regarding *T. gondii* infection the role of Notch signaling pathway has not been studied yet.

Susceptible C57BL/6 mice orally infected with high *T. gondii* load develop severe small intestine inflammation, resulting in necrosis of mucosal villi and mortality (Liesenfeld et al., 1996). Ileitis is caused by a Th1-type immunopathology, characterized by a $CD4^+$ T cell-mediated increase of IFN-γ, TNF, and nitric oxide (NO) (Liesenfeld et al., 1996, Heimesaat et al., 2019). *T. gondii* induces immunopathological sequelae that are not restricted to the intestines. We have, along with others researchers, shown inflammatory mediators and inflammation in extra-intestinal tissues, such as liver and lungs in acute phase of parasite infection (Heimesaat et al., 2019; Silva et al., 2010; Oliveira et al., 2020). The control of immunopathology induced by *T. gondii* infection is required to avoid necrosis of the small intestine and mortality, as well as hepatic lesions, being IL-10 a key factor in this mechanism (Suzuki et al., 2000; Gazzinelli et al., 1996).

As Notch signaling has a critical role in the maintenance of intestinal epithelia integrity and additionally contributes to T helper type immunity to different parasites, the aim of the present study was to investigate the relationship between Notch signaling pathway and *T. gondii* infection.

2. Material and methods

2.1. Animals and ethics

Female C57BL/6 and Swiss Webster mice aged 8–12 weeks were maintained at the Federal University of Uberlândia animal facilities (Rede de Biotérios de Roedores da Universidade Federal de Uberlândia, REBIR-UFU). All animal experiments were performed in accordance to the Brazilian Government's ethics and were approved by the Animal Experimental Ethics Committee (CEUA) of the Federal University of Uberlandia, under the protocol no. 088/15. All procedures were carried out in accordance with the ARRIVE guidelines and recommendations of the Guiding Principles for Biomedical Research Involving Animals of the International Council for Laboratory Animal Science (ICLAS), countersigned by the Conselho Nacional de Controle de Experimentação Animal (CONCEA; Brazilian National Consul for the Control of Animal Experimentation) and Guide for Laboratory Animal Facilities and Care (No. 8023, revised in 1965, 1968, 1972, 1978, 1985 and 1996).

2.2. Parasites

The ME 49 *T. gondii* strain was maintained in Swiss Webster mice that were inoculated with 10 cysts via intraperitoneal (i.p.) route. One month after the inoculation, the brain cysts were harvested from the Swiss mice. Cyst numbers were quantified by microscopic analysis of 10 μl aliquots of PBS-brain homogenates, in duplicates. Inoculums were adjusted to 20 cysts in 0.2 mL of PBS (pH 7.4) and used to inoculate C57BL/6 mice by oral route in this experimental work.

2.3. Experimental procedure

C57BL/6 mice were injected with 5 μM/kg/day of dibenzazepine (DBZ – Calbiochem® EMD Millipore, Burlington, MA, USA), a γ-secretase inhibitor, or with vehicle [DMSO 0.001 % in phosphate buffered saline (PBS, pH 7.4)] for 4 days by i.p. route. On the next day, mice were orally infected with 20 cysts. On day 8 post-infection (d.p.i.), mice were injected with anesthetics Ketamine (Syntec Brazil Ltda, SP, Brazil)/ Xylazine (Schering-Plough Coopers, SP, Brazil) via i.p. route. Blood samples were collected from the retro-orbital plexus and animals were euthanized by cervical dislocation. Serum samples were stored at − 80 °C for cytokine analysis. The small intestine was measured and divided into four pieces (duodenum, proximal jejunum, distal jejunum, and ileum) and processed as 'Swiss rolls' for histological analysis. The liver, lungs and small intestine were fixed in 10 % buffered formalin, and processed routinely for paraffin embedding and sectioning (4 μm of thickness). The percentage of the small intestine shortness was calculated as the ratio between the difference of the mean length of the small intestine from vehicle non-infected mice minus the length of the organ from infected mice on day 8 of infection and then multiplied by 100 over the mean length of vehicle non-infected mice small intestines (Muñoz et al., 2009).

One group of animals was monitored daily until day 30 of infection for weight loss, survival rate and morbidity score (Bartley et al., 2006).

One group of animals was DBZ-treated for 4 days and euthanized on day 1 or 8 post-treatment (dpt), being fragments of ileum collected and analyzed for Notch pathway gene expression levels by qPCR assay.

2.4. Histological and immunohistochemical analyses

For histological analysis, deparaffinized tissue sections were stained with Hematoxylin and Eosin (H&E). The inflammatory score was assayed in the entire section of the small intestine as previously described (Oliveira et al., 2020) and was represented as arbitrary units: 0–2, mild; 2.1–4, moderate; 4.1–6, severe; and above 6, very severe. The inflammatory score analyses were performed using 10x objective in a light microscope and in a blind manner. Paneth cells were counted in 100 microscopic fields per intestinal piece in H&E-stained sections, totalizing 400 fields per entire small intestine. For the quantification of goblet cells per intestinal villus, Alcian-blue (pH 2.5) staining was performed and 10 microscopic fields were counted per section of the organ with a total of 40 fields per mice. Quantification was performed under 40x objective in a light microscope and in a blinded manner.

The tissue sections of lung and liver were stained with H&E. Digital images of pulmonary tissue were acquired using a Leica DM500 microscope. Lung septal area was assessed by measuring parenchymal area using the NIH ImageJ program (https://imagej.nih.gov/ij/). A minimum of 10 representative non-overlapping fields from each lung were evaluated. The number of inflammatory foci in the liver was quantified in 40 microscopic fields per tissue section. Both analyses were performed in each group in a blinded manner.

The tissue parasitism was detected by immunohistochemistry as previously described with some modifications (Oliveira et al., 2020). Briefly, slides with deparaffinized tissue sections were placed in a

humidified chamber and incubated with 3 % hydrogen peroxide to block endogenous peroxidase activity. The antigenic unmasking was done in 0.2 M citrate buffer (pH 6.0) for a 7-min cycle in a microwave oven. To reduce nonspecific binding, sections were incubated with 0.3 % non-fat milk (Nestlé, São Paulo, SP, Brazil) in PBS at room temperature. For detection of the parasites, the slides were incubated with polyclonal anti-*T. gondii* serum (obtained from Swiss mice infected with ME49 strain) diluted in 0.01 % saponin at 4 °C overnight. Thereafter, slides were incubated with biotinylated goat anti-mouse antibody (Sigma-Aldrich, Saint Louis, MO, USA) at 37 °C for 1 h and, the assay sensitivity was improved by adding avidin-biotin-peroxidase complex (ABC kit, PK-4000; Vector Laboratories, Inc., Burlingame, CA, USA) at 37 °C for 30 min. The reaction was developed with 0.03 % H_2O_2 plus 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich). The sections were counterstained with Harris hematoxylin and examined under a light microscope using a 40x objective. The parasite quantification was performed by counting the brown-stained parasites per entire tissue section in the lung, liver and small intestine.

2.5. RNA extraction and analysis of gene expression

Frozen fragments of ileum and lung were pulverized in liquid nitrogen. The total RNA extraction was performed using TRIzol® reagent by following the manufacturer's instructions (Life Technologies, Carlsbad, CA, USA). The quantification of total RNA was measured in a GeneQuant spectrophotometer (GE Healthcare, Chicago, IL, USA) and treated with DNase I Amplification Grade (Invitrogen, Thermo Fisher Scientific, Eugene, OR, USA). The complementary DNA (cDNA) of each sample was synthesized using 1 μg of RNA with reverse transcriptase ImProm-II™ (Promega, Madison, WI, USA) on Arktik thermocycler (Thermo Fisher Scientific). Finally, the cDNA was amplified in the ABI PRISM-7500 sequence detection system (Applied Biosystems, Waltham, MA, USA) using SYBR® Green PCR Master Mix (Invitrogen) and gene-specific primers (Table 1). Samples Ct (cycle threshold) were normalized based on the expression of the reference control gene (*Gapdh*), and the relative expression of each studied gene was analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Cytokine analyses

The IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A levels in the serum samples were quantified using a cytometric bead array assay (CBA) (BD, San Jose, CA, USA) following the manufacturer's instructions. Results were recorded using a FACSCanto II Flow Cytometer (BD, Biosciences, San Jose, CA) and analyzed with FACSDiva software (BD Biosciences, San Jose, CA). The theoretical limit of detection (pg/mL) according to the kit is: IL-2, 0.1; IL-4, 0.03; IL-6, 1.4; IL-10, 16.8; IL-17A, 0.8; TNF, 0.9; IFN-γ, 0.5.

2.7. Statistical analyses

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± SEM. The survival rate was analyzed by Chi-square test; weight loss and morbidity score by two-way ANOVA, followed by Bonferroni's multiple comparison post-test. For comparing two groups, either the unpaired t-test or Mann-Whitney's test was used; for comparing multiple groups, we used one-way ANOVA followed by Bonferroni's, Sidak's or Tukey's multiple comparison test; also, Kruskal-Wallis, followed by Dunn's multiple comparisons test, was used when appropriate. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Notch pathway expression genes are altered in the small intestine in T. gondii infection, but Notch pathway is not involved in the control of tissue parasitism and inflammation

In order to evaluate whether *T. gondii* infection could interfere with Notch pathway in the small intestine, we analyzed the expression profile of genes involved in this pathway by using ileum fragments of C57BL/6 mice infected with 20 cysts of *T. gondii* (Fig. 1). *T. gondii* infection did not interfere with the intestinal *Notch1* mRNA expression (Fig. 1A). However, transcripts of *Hes1* and *Math1* decreased significantly in infected mice when compared with vehicle-treated uninfected mice (Fig. 1B, C).

As we were interested in verifying the effect of the γ-secretase inhibitor in *T. gondii* infection, a group of animals was treated with dibenzazepine (DBZ) for 4 days. After the treatment finished, mice were orally infected with 20 *T. gondii* cysts and euthanized on 8 dpi. At first, the *Notch1*, *Hes1* and *Math1* mRNA expressions were investigated in uninfected mice treated with DBZ. The treatment of animals with DBZ did not interfere significantly with *Notch1*, *Hes1* or *Math1* mRNA expression one day after the end of the treatment (Supplementary Figures 1A-C). However, animals treated with DBZ showed a diminished expression of *Notch1* and *Hes1* on day 8 after the end of treatment, showing that the effect of DBZ is progressive over the days (Supplementary Figures 1A, B).

DBZ-treated mice and *T. gondii*-infected did not have the expression of *Notch1* altered (Fig. 1A). However, *Hes1* expression was diminished in DBZ-treated infected mice in comparison with non-treated *T. gondii*-infected mice (Fig. 1B). As for *Math1* relative expression, DBZ-treated infected mice showed diminished *Math1* expression which was similar to that of infected-untreated mice (Fig. 1C). To verify whether DBZ-treatment would interfere in the intestinal cellular proliferation under *T. gondii* infection, *Ki67* mRNA expression levels were investigated in the ilea of treated mice. The treatment of uninfected mice with DBZ decreased the *Ki67* mRNA expression in the organ, mainly on 1dpt (Supplementary Figure 1D). *T. gondii* infection increased *Ki67* relative expression levels in the small intestine (Fig. 1D), and when DBZ-treated mice were infected with *T. gondii*, animals maintained elevated levels of *Ki67* expression that were similar to those of infected untreated mice (Fig. 1D). Collectively, these results showed that *T. gondii* infection downregulated mRNA expression levels of *Hes1*, *Math1* and upregulated the cellular proliferation marker, *Ki67*. When Notch pathway was blocked, mice infected with *T. gondii* showed an additional diminishing

Table 1
List of primer sequences used for qPCR gene expression assays.

Gene	Forward 5′ – 3′	Reverse 5′ – 3′
Notch1	AGCAAGAAGAAGCGGAGAGAGC	TGTCGTCCATCAGAGCACCATC
Hes1	GGAGAGGCTGCCAAGGTTTT	GCAAATTGGCCGTCAGGA
Math1	ACATCTCCCAGATCCCACAG	ACAACGATCACCACAGACCA
Defa3	CAGGCTGTGTCTGTCTCTTTTG	TCAGCGACAGCAGAGTGTGTA
Defa5	TTGTCCTCCTCTCTGCCCTTGT	ATGAAGAGCAGACCCTTCTTGG
Lyz1	GCCAAGGTCTACAATCGTTGTGAGTTG	CAGTCAGCCAGCTTGACACCACG
Ki67	CAATGTGCCTCGCAGTAAGA	GCATCTTTGGGGTTTTCTCA
*Tg*SAG1	TTTCCGAAGGCAGTGAGACG	CCATAACGCCACATCGCA
Gapdh	GAGAAACCTGCCAAGTATGATG	CAGTGTAGCCCAAGATGCCC

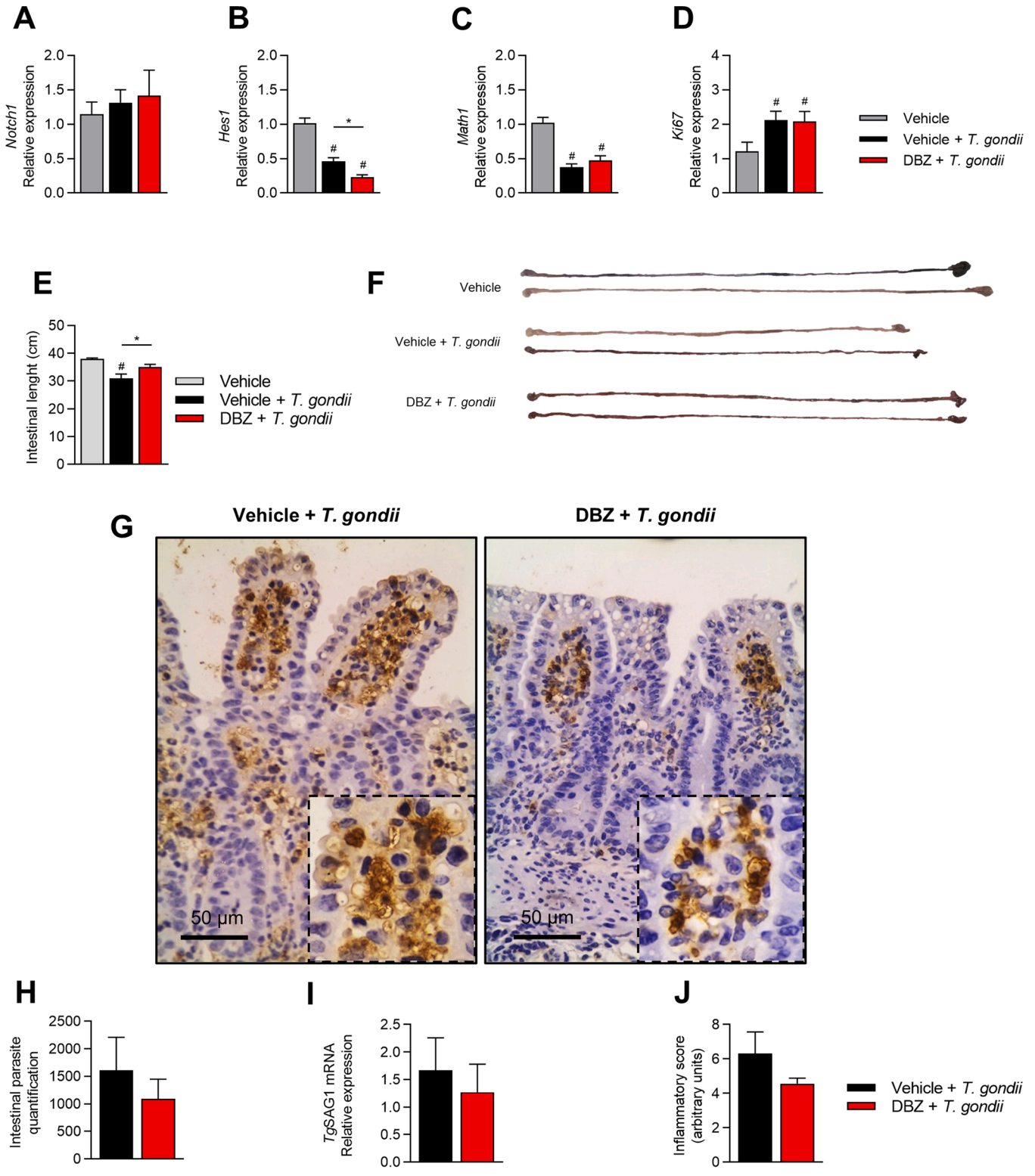

Fig. 1. Effect of Notch pathway inhibition by DBZ in the small intestine of *T. gondii* infected mice. C57BL/6 mice were treated with DBZ (5 μM/kg/day) or vehicle (0.001 % DMSO in PBS) for four days. Then, mice were infected with 20 *T. gondii* cysts, and ilea were collected on 8 dpi. The mRNA expression levels were analyzed by qPCR. The relative levels of Notch1 (A), Hes1 (B), Math1 (C) and Ki67 (D) gene expressions were calculated based on reference to the GAPDH gene in each sample, using the threshold cycle (Ct) method. The length of the organ was measured (E) and, representative macroscopic imagens of the small intestine were captured (F). The tissue parasitism was detected by immunohistochemical technique (G) and stained parasites were counted per tissue section (H). The parasitism was also investigated by qPCR to quantify the mRNA expression of a specific marker of tachyzoites, the TgSAG1 (I). Inflammatory score of the small intestine was represented as arbitrary units (J). Data are representative of two independent experiments of five mice per group and are presented as means ± SEM. One-way ANOVA followed by Sidak's (A, B, C, D) or Tukey's (E) multiple comparisons tests were used for analyses, $p < 0.05$. #Statistically different from vehicle-treated mice. *Significant difference between *T. gondii*-infected and infected DBZ-treated mice.

of *Hes1* mRNA expression.

As the intestinal epithelia are the main entrance site of *T. gondii*, we performed a macroscopic assessment of the small intestine of infected animals treated with DBZ or vehicle (Fig. 1F). The length of the small intestine was diminished in *T. gondii*-infected mice with a reduction average of 18.24 % (Fig. 1E, F). However, the small intestine of the DBZ-treated and infected showed a similar length to the vehicle-treated uninfected mice (Fig. 1E, F).

Because the small intestine length of infected DBZ-treated animals was preserved, we hypothesized that the parasitism in the small intestine would be altered. Thus, it was investigated the tissue parasitism in the organ by the immunohistochemistry technique and *Tg*SAG1 expression gene. On day 8 of infection, the number of parasites detected in the entire organ was similar between infected and DBZ-treated infected mice (Fig. 1 G, H). This result was consistent with the *Tg*SAG1 mRNA expression in the organ of infected mice, DBZ-treated or not (Fig. 1I). Additionally, the inflammatory alterations in the organ were verified. It was observed that infected animals presented inflammatory cell infiltrates in the lamina propria (LP) and submucosa with shortening and necrosis of the villi in some areas. The DBZ treatment did not significantly improve the inflammation caused by *T. gondii* infection in the small intestine (Fig. 1J).

Additionally, both experimental groups, *T. gondii*-infected and DBZ-treated and infected mice showed a similar survival rate (Supplementary Figure 2B). Regarding the morbidity score, infected animals displayed signs of illness since day 6. DBZ-treated mice infected with *T. gondii* showed smaller morbidity scores when compared with infected untreated animals until the end of the experimental period (Supplementary Figure 2C).

Thus, Notch pathway is not involved in the control of *T. gondii* proliferation and inflammation in the small intestine.

3.2. The goblet and Paneth cell populations are partially preserved in the small intestine of T. gondii-infected mice under Notch pathway inhibition

Given the significant decrease in *Hes1* mRNA expression in the DBZ-treated infected compared with vehicle-treated *T. gondii*-infected mice, we investigated the consequences of the γ-secretase inhibition on the goblet and Paneth cell numbers in the small intestine. The goblet cells were analyzed using Alcian blue staining in histological sections of the small intestine (Fig. 2B). *T. gondii* infection diminished the number of goblet cells in all of the segments of the organ (Fig. 2A–C). The DBZ-treated and infected mice showed an augmentation of the numbers of cells compared with those of vehicle-treated *T. gondii*-infected mice (Fig. 2 AC). To examine neutral mucins, tissue sections were evaluated histologically by periodic acid-Schiff (PAS) staining. PAS positive cell numbers were similar among vehicle-treated infected and DBZ-treated infected mice (Supplementary Figure 3).

Next, the primary goblet cell product, MUC2 (reviewed by Yang and Yu, 2021) (Fig. 2D), was evaluated. *T. gondii* infection diminished the *Muc2* mRNA expression in the small intestine. Despite not statistically significant, the *Muc2* mRNA expression also diminished in DBZ-treated infected mice (Fig. 2D).

Another important cell phenotype of the intestinal epithelia is the Paneth cells, producers of antimicrobial peptides (AMP) (reviewed by Bevins and Salzman, 2011). It was found that *T. gondii* infection diminished significantly the number of Paneth cells in the crypts of the small intestine. Additionally, DBZ-treated infected mice preserved partially the numbers of this cell phenotype (Fig. 2E, F). Paneth cell-associated genes *Lyz1*, *Defa3* and *Defa5* decreased significantly in *T. gondii*-infected mice regardless of whether they were treated or not with DBZ (Fig. 2G–I).

As a control of the inhibitory activity of DBZ, intestinal epithelia of uninfected mice treated with DBZ showed an increase in goblet cells and *Muc2* mRNA expression without significant alterations of the Paneth cells or PAS positive cells (Supplementary Figure 4).

Overall, secretory lineages like goblet and Paneth cells were in part recovered by the γ-secretase inhibition in the intestinal epithelia of *T. gondii*-infected mice. However, the mRNA expression of makers of each cell phenotype remained at low levels.

3.3. DBZ treatment induced an upregulation of IFN-γ systemically in mice infected with T. gondii

The cytokine profile in serum samples of DBZ-treated *T. gondii*-infected mice was evaluated (Fig. 3). The results showed that *T. gondii* infection induced an increase in IL-2, IL-6, IFN-γ and TNF (Fig. 3A–D). The treatment with DBZ in association with *T. gondii* infection increased the levels of IFN-γ in comparison to *T. gondii*-infected vehicle-treated mice (Fig. 3C). The infection with *T. gondii* did not augment the IL-4 systemic levels, however, when animals were treated with DBZ and infected with *T. gondii*, the cytokine levels were increased (Fig. 3E). Moreover, IL-10 levels were increased in infected mice irrespective of the DBZ treatment (Fig. 3F). The infection with *T. gondii* did not interfere with IL-17A levels in serum samples; nonetheless, the infection increased the cytokine levels when animals were previously treated with DBZ (Fig. 3G).

3.4. Inhibition of Notch pathway improves inflammation and parasite load in the liver and lungs of mice orally infected with T. gondii

Besides the small intestine, *T. gondii* reaches other tissues such as lungs, liver, kidney, spleen and brain (Heimesaat et al., 2019). In the acute phase of infection, C57BL/6 mice show high parasite load in the lungs that is associated with high inflammatory alterations (Silva et al., 2010). Thus, experiments were conducted to assess whether the inhibition of Notch pathway could interfere with *T. gondii* proliferation and with the inflammatory alterations in the lungs. It was shown that the infection with *T. gondii* did not alter significantly *Notch1* mRNA expression levels in the organ; however, similar to what was observed in the small intestine, the infection diminished the *Hes1* mRNA expression irrespective animals having been treated or not with DBZ (Fig. 4A, B). The infection with *T. gondii* led to an increase in septal areas indicating inflammatory cell infiltrations (Fig. 4C, D). Interestingly, DBZ-treated and infected animals improved the inflammatory changes in the lungs and showed lower parasite load compared with vehicle-treated infected mice (Fig. 4C–E). As it was previously shown that DBZ ameliorates the inflammation in the liver of rats injured by paracetamol treatment (Ahmed et al., 2021), the inflammation and parasite load were analyzed in the present study in this organ, when animals were infected and treated with DBZ. In the liver, the infection increased the number of inflammatory foci in the parenchyma; however, Notch pathway inhibition decreased these inflammatory foci (Fig. 5A, B) and decreased the tissue parasitism despite not reaching the statistical difference (Fig. 5C).

4. Discussion

Notch signaling pathway has been shown to exert important effects on several infectious diseases, such as *Ehrlichia chaffeensis, Cryptococcus neoformans*, *Leishmania major* and *Citrobacter rodentium* infections (Neal et al., 2017; Lina et al., 2016; Auderset et al., 2012; Ahmed et al., 2012). The inhibition of Notch signaling by γ-secretase inhibitor, dibenzazepine (DBZ), leads to intestinal crypt base columnar stem cells loss and differentiation of epithelial progenitors in secretory cell types (VanDussen et al., 2012) interfering with the intestinal homeostasis. As the intestinal mucosa is an important site of *T. gondii* entry, in this experimental work the relationship between Notch pathway and *T. gondii* infection in the intestinal epithelia was investigated by using DBZ. First, we evaluated the impact of *T. gondii* infection on Notch pathway genes in the small intestine of C57BL/6 susceptible mice. It was verified that *T. gondii* infection decreased the mRNA expression of Notch pathway direct target gene, *Hes1*, and its repressed transcriptional target, *Math1* (Yang et al.,

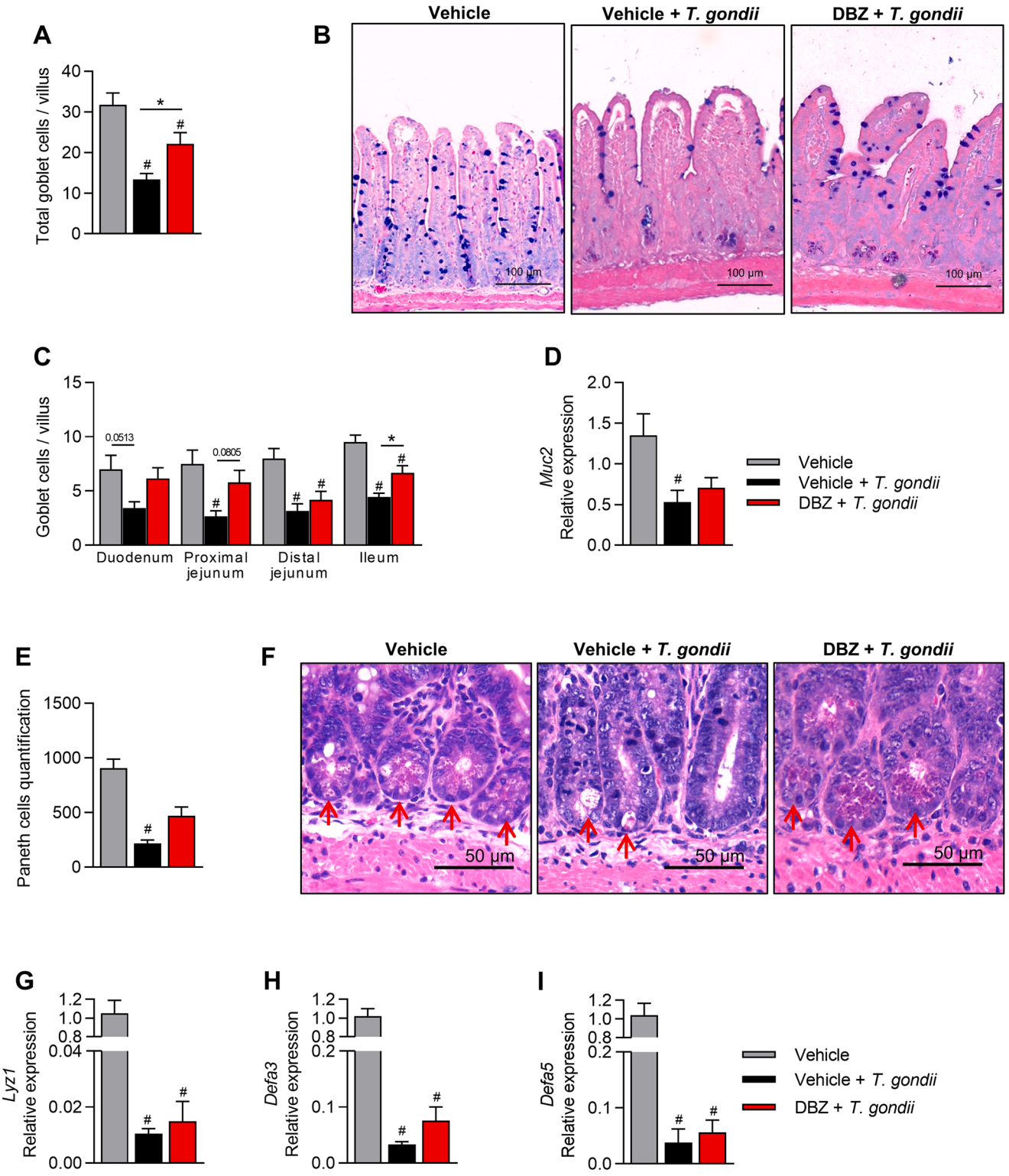

Fig. 2. Quantification of goblet and Paneth cells and, Muc2 and antimicrobial peptides mRNA expressions in the small intestine of DBZ-pre-treated *T. gondii*-infected mice. Representative photomicrographs of the small intestine stained with Alcian blue or H&E to detect goblet (B) or Paneth cells (arrows) (F), respectively. The goblet cells were counted per villus in a total of 40 microscopic fields per animal (A) or in each piece of the small intestine (C) and, Paneth cells were counted in 400 microscopic fields per tissue section of the small intestine (E). Expression levels of Muc2 (D), Lysozyme 1 (Lyz1) (G), defensin 3 (Defa3) (H) and defensin 5 (Defa5) (I) were analyzed in the ilea of mice by qPCR. Data are representative of two independent experiments of five mice per group and are presented as mean ± SEM. One-way ANOVA followed by Sidak's multiple comparisons test (A, C, E), Tukey's multiple comparisons test (D) and, Kruskal-Wallis test followed by Dunn's multiple comparisons test (G, H, I) were used for analyses. #Significant differences between vehicle-treated animals and *T. gondii*-infected mice, $p < 0.05$. *Significant differences between *T. gondii*-infected and DBZ-treated infected mice, $p < 0.05$.

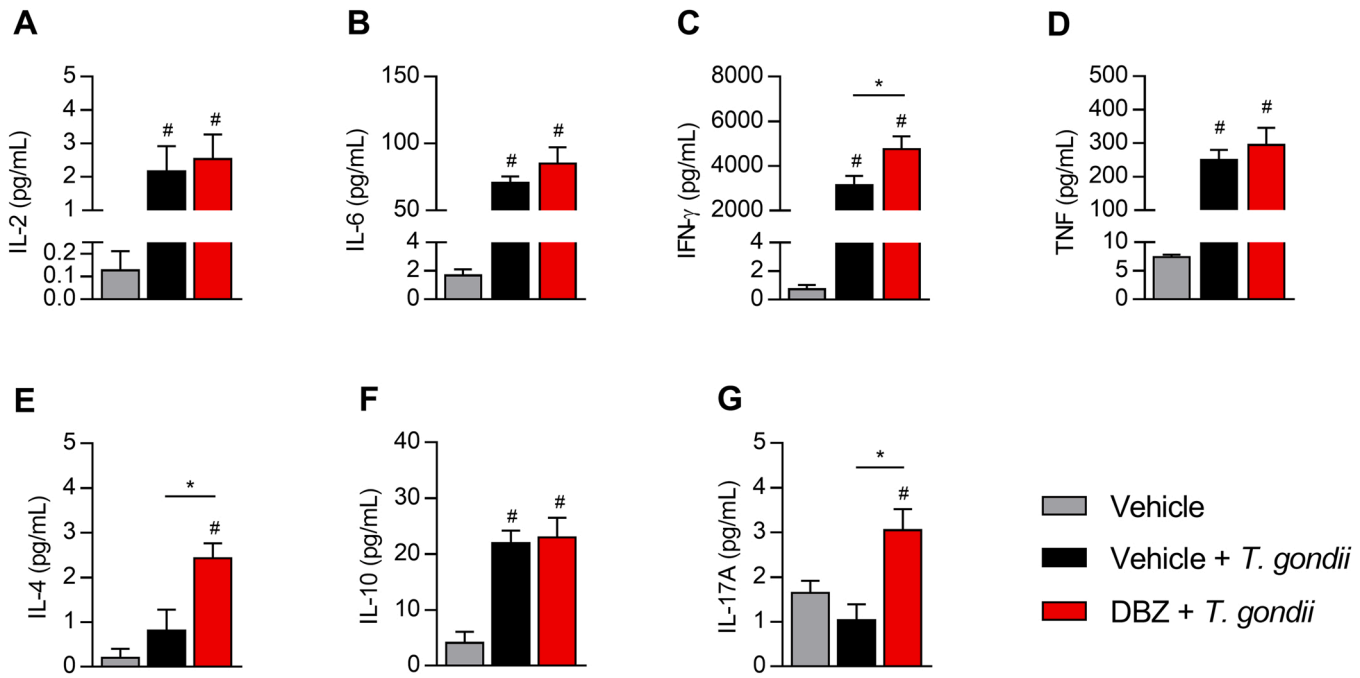

Fig. 3. Cytokine levels from C57BL/6 mice treated with γ-secretase and infected with *T. gondii.* Mice were treated with DBZ (5 μM/kg/day) or vehicle (0.001 % DMSO in PBS) for 4 days before infection with 20 cysts of *T. gondii* (ME49 strain) and euthanized after 8 days of infection. The serum was collected for measurement of IL-2 (A), IL-6 (B), IFN-γ (C), TNF (D), IL-4 (E), IL-IL-10 (F) and IL-17A (G) by CBA. Data are representative of two independent experiments of five mice per group and are presented as mean ± SEM. Differences between groups were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons test (A, E, G) or one-way ANOVA followed by Tukey's multiple comparisons test (B, C, D, F). #Significant differences between vehicle-treated group and experimental groups were represented. *Significant differences between *T. gondii*-infected and DBZ-treated infected mice. $p < 0.05$.

2001), without affecting *Notch1* mRNA expression. These data suggest that the infection with the parasite directly affects the *Hes1* and *Math1* expressions.

C57BL/6 mice infected with *Citrobacter rodentium* showed that *Hes1* and *Math1* transcripts were not altered in the cecum and colon. However, when animals were treated with DBZ an increase was observed in mortality and *C. rodentium* burden (Chan et al., 2013). Another study using NIH:Swiss mice with TMCH induced by *C. rodentium* presented increased levels of *Hes1* mRNA, which were higher on 6 and 12 days of infection, and in parallel ~~a~~ decreased levels of *Math1* mRNA expression (Ahmed et al., 2012). BALB/c mice infected with *T. cruzi* showed an augment of Notch1, NICD and Hes1 protein detection in cardiac tissues compared with non-infected animals (Guzmán-Rivera et al., 2020). Thus, depending on the infectious organism, animal species and in the case of murine experimental studies, the background of mice, the Notch target genes are differentially activated.

Several studies have shown that the inhibition of Notch pathway with different γ-secretase inhibitors has an impact on the conversion of stem cells into secretory cells in the small intestine (Milano et al., 2004; van Es et al., 2010; Droy-Drupé et al., 2012). Herein, animals were treated with DBZ with 5 μM for 4 days before infection, a dose that is known to increase goblet cell numbers (van Es et al., 2005). C57BL/6 mice DBZ-treated and infected with *T. gondii* showed similar levels of *Notch1* mRNA expression compared with *T. gondii* infected untreated mice. A further decrease was demonstrated in *Hes1* mRNA expression levels in DBZ-treated mice and a slight increase in *Math1* mRNA expression in relation to infected untreated mice, despite having remained lower when compared with uninfected mice. It is known that Notch signaling controls proliferation and differentiation of intestinal crypt base columnar stem cells (CSC), and Notch signaling inhibition by DBZ induces rapid CSC loss that is associated with decreasing Ki67 expression, a putative marker for cell proliferation (VanDussen et al., 2012). On the other hand, oral infection of C57BL/6 mice with ME-49 strain induced increased Ki67/Caspase-3 expression levels in the small and large intestines in acute phase of infection indicating proliferation/apoptosis of intestinal cells (Heimesaat et al., 2019). In accordance, in the present investigation, we showed that *T. gondii* infection induced *Ki67* mRNA expression in the small intestine, and DBZ treatment was not able to interfere with the proliferation of intestinal cells, suggesting that, the effect of *T. gondii* infection outweighs Notch pathway inhibition.

In vitro inhibition of Notch with γ-secretase inhibitor impairs IL-12-driven Th1 cell differentiation (reviewed by Amsen et al., 2015). Additionally, it was shown that Notch is involved in IL-10 production by Th1 cells (Rutz et al., 2008). In the present investigation and, in accordance with our previous studies (Oliveira et al., 2020; Araujo et al., 2021), *T. gondii* infection induced high levels of pro-inflammatory cytokines, IL-6, IFN-γ and TNF systemically, in parallel with an augmentation of anti-inflammatory IL-10. The treatment with DBZ before infection did not decrease the production of these cytokines and even increased the IFN-γ, IL-4 and IL-17 levels. It was previously shown that $CD4^+$ T cells treated with γ-secretase inhibitor before culture in Th1 condition increased slightly the IL-4 production on a 96-hour culture (Minter et al., 2005). In the present study, animals were DBZ-treated before infection and it was demonstrated that the treatment was able to increase the IL-4 systemically. Thus, with our experimental procedure, the γ-secretase inhibition before a strong Th1 stimulus, *T. gondii* infection, was not able to interfere with the exacerbated pro-inflammatory immune response provoked by the infection, despite the augmented IL-4 levels. Regarding the IL-17 levels, T cell-specific deletion of Notch receptors enhances Th17 cell differentiation in the gut and increase secretion of IL-17 (Coutaz et al., 2016). Using IL-17RA-deficient mice, reduced ileitis and prolonged survival after *T. gondii* infection was demonstrated (Guiton et al., 2010). In the present investigation, the increased levels of IL-17 in DBZ-treated mice did not worsen the ileitis induced by *T. gondii* infection, which could be related to the counterregulatory properties of the IL-4 and IL-10 increased levels. Additionally, IFN-γ itself could be interfering with direct target

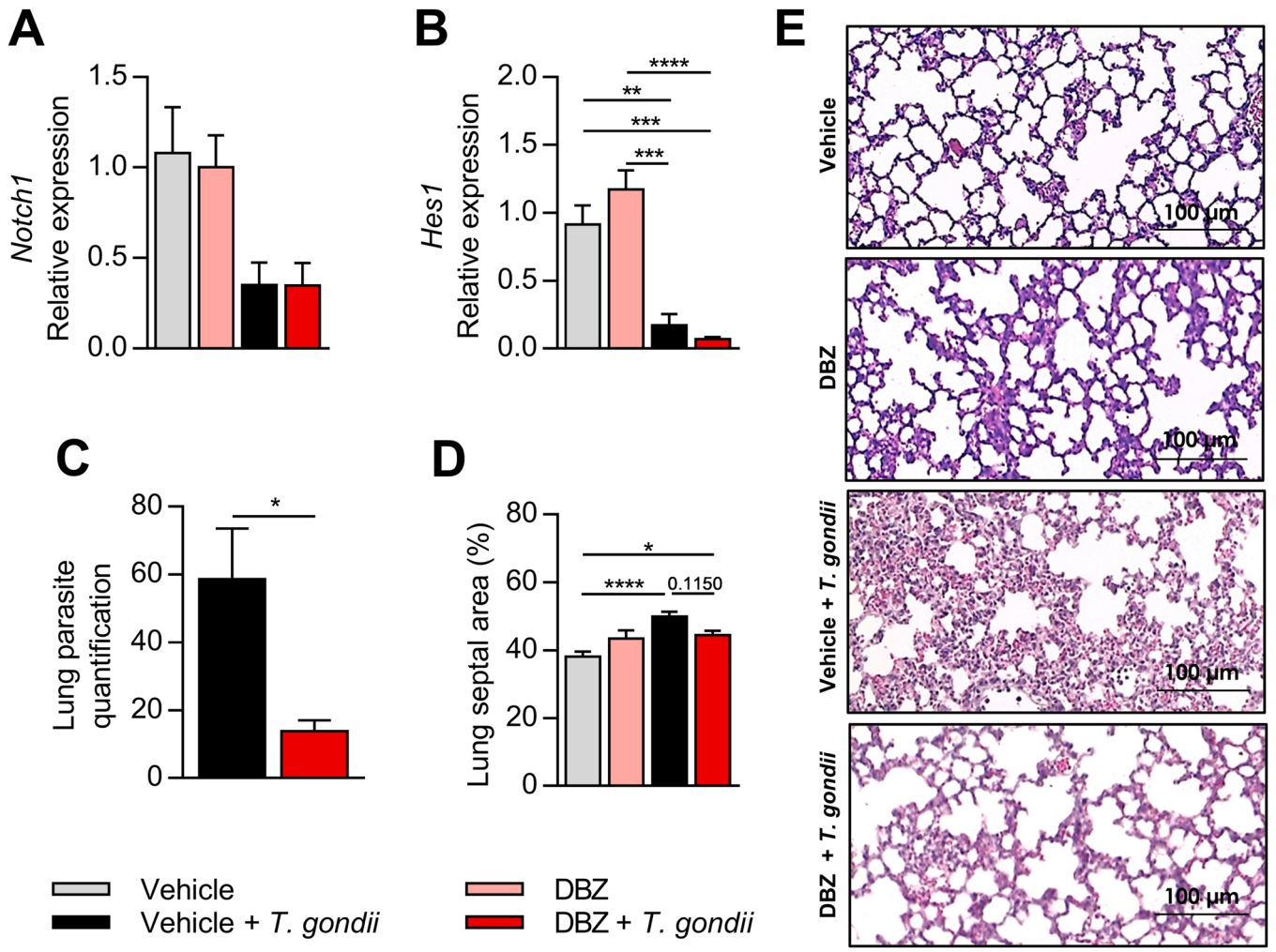

Fig. 4. *Notch1* and *Hes1* mRNA expressions and inflammatory alterations and parasite load in the lung of C57BL/6 mice DBZ-treated and infected with *T. gondii*. Mice were treated with DBZ (5 μM/kg/day) or vehicle (0.001 % DMSO in PBS) for 4 days before oral inoculation with 20 cysts of *T. gondii* and euthanized on 8 day of infection. The relative levels of *Notch1* (A) and *Hes1* (B) gene expressions were calculated by reference to the *Gapdh* gene in each sample, using the threshold cycle (Ct) method. Photomicrographs of lung tissue sections stained by H&E of vehicle- or DBZ-treated mice and infected vehicle- or DBZ-treated mice (E). Percentage of lung septal area was measured using Digital images of parenchymal area using the NIH ImageJ program. A minimum of 10 representative, nonoverlapping fields from each lung were evaluated (D). Parasite quantification in the lung (C) was performed by immunohistochemistry assay. Data are representative of two independent experiments of five mice per group and are presented as means ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test (A, B), One-way ANOVA followed by Sidak's multiple comparisons test (D) or Unpaired t test (C) $*p < 0.05$.

gene expression of Notch signaling, Hes-1, since it was previously shown that IFN-γ blocked TLR-induced activation of canonical Notch target genes by inhibiting Notch-2 signaling and downstream transcriptions (Hu et al., 2008).

Goblet cells synthesize and secrete secretory and membrane-bound mucins, predominantly mucin 2 (MUC2) in the intestine, trefoil factor 3 (TFF3) peptides, resistin-like molecule β (RELMβ), and Fc-γ binding protein (Fcgbp), which are components of the mucus that protects and lubricates the mucosa surface (reviewed by Kim and Ho, 2010). As it was previously shown by our group as well as by others (Araujo et al., 2021; Miranda et al., 2021; Pêgo et al., 2019), *T. gondii* infection decreased significantly the goblet cell numbers in the small intestine, however, the DBZ treatment was able to preserve partially the numbers of this cell phenotype. In mice, it was previously shown that TNF is involved in goblet cell depletion (Arnold et al., 1993). In human HM3 colon adenocarcinoma cells, TNF-α can upregulate the transcription of MUC2 via the PI3K/AKT/NF-κB signaling pathway, although TNF-α can also inhibit the transcription of MUC2 through the JNK pathway (Ahn et al., 2005). In the present study, we also measured the *Muc2* mRNA expression in the small intestine of DBZ-treated infected mice. Despite the increased goblet cell numbers in DBZ-treated infected mice in comparison to infected untreated, it was detected diminished *Muc2* mRNA expression, that could be related to the increased TNF levels which was not altered by DBZ-treatment.

Related to Paneth cells, this cell phenotype populates the base of the crypts of Lieberkühn in the small intestine of most mammals and is the main source of antimicrobial peptides. Paneth cells produce antimicrobials, such as proteins, α-defensins (also referred to as cryptdins (Crp) in mice), CRS, cryptdin-related sequence peptides (in mice), as well as Lysozyme C, sPLA2, secretory group IIA phospholipase A2, REG3γ, Regenerating islet-derived protein III-gamma in mice and ANG4, angiogenin 4 (reviewed by Bevins and Salzman, 2011). *In vitro*, it was previously shown that human α-defensin-5 displays a parasiticidal role against *T. gondii* (Tanaka et al., 2010) and, mouse Crp-2 and 3 were cytotoxic to *Giardia lamblia* trophozoites (Aley et al., 1994). *In vivo*, C57BL/6 mice orally infected with *T. gondii* presented Paneth cells depletion that was associated with high IFN-γ production due to an exacerbated Th1 immune response (Raetz et al., 2013; Villeret et al., 2013). Herein, it was demonstrated that the infection with the parasite increased the IFN-γ production systemically and decreased the Paneth

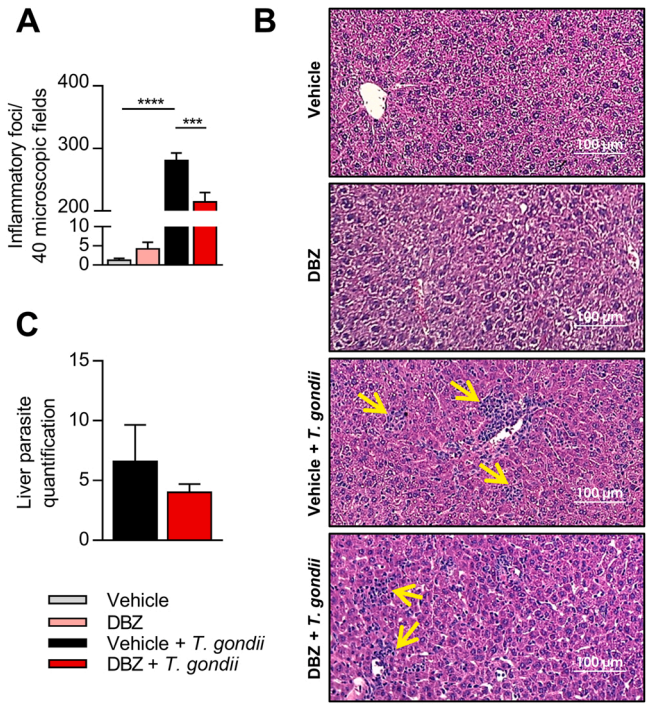

Fig. 5. Inflammatory alterations and parasite load in the liver of C57BL/6 mice DBZ-treated and infected with *T. gondii*. Mice were treated with DBZ (5 μM/kg/day) or vehicle (0.001 % DMSO in PBS) for 4 days before oral inoculation with 20 cysts of *T. gondii* and euthanized on 8 day of infection. Photomicrographs of liver tissue sections stained by H&E of vehicle- or DBZ-treated mice and infected vehicle- or DBZ-treated mice (B). The number of inflammatory foci in the liver was quantified in 40 fields per tissue section (A). Parasite quantification in the lung (C) was performed by immunohistochemistry assay. Data are representative of two independent experiments of five mice per group and are presented as mean ± SEM. One-way ANOVA followed by Tukey's multiple comparisons test (A), or Unpaired t test (C) $^{*}p < 0.05$.

cell numbers in the small intestine in association with decreased antimicrobial peptides. The DBZ treatment was able to preserve at least partially the Paneth cell numbers, however, not their antimicrobial peptides.

In the next step the survival rate, morbidity score, tissue parasitism and inflammatory alterations in the small intestine of DBZ-treated *T. gondii*-infected mice it were analyzed. Similar survival rate was observed among DBZ-treated infected and untreated infected mice, although DBZ-treated mice showed improved morbidity score compared with untreated mice. Additionally, both infected mice untreated or treated with DBZ presented similar inflammatory alterations and parasitism in the small intestine. Thus, the levels of preservation of Paneth and goblet cells by γ-secretase inhibition were not sufficient to protect mice in the present experimental work. Additionally, decreased *Muc2* and antimicrobial peptides mRNA expressions in infected mice irrespective they were treated or not with DBZ could reflect lower *Muc2* production and antimicrobial peptides that could be associated with uncontrolled tissue parasitism.

Few studies have been published using γ-secretase inhibitors or genetic deletions of Notch pathway members to test the impact on controlling parasitic infection. The treatment of mice with a specific γ-secretase inhibitor, N-[N(3,5)-difluorophenacetyl)-L-Alanyl]-S-phenylglycine t-butyl ester (DAPT), which interferes with the cellular Notch signaling pathway did not affect the *Plasmodium* growth in vivo and in vitro (Parvanova et al., 2009). On the other hand, BALB/c mice chronically infected with *Trypanosoma cruzi* showed expression and activation of Notch-1 receptor and signaling pathway in cardiac tissues and the additional induction and activation of Notch signaling by simvastatin improved the cardiac function and histopathological findings (Guzmán-Rivera et al., 2020). Specific deletion of Notch-1 and Notch-2 receptors on the T cell surface of the C57BL/6 *Leishmania major*-resistant mice induced a high susceptibility to infection showing an increase in lesion size and uncontrolled parasitism (Auderset et al., 2012). Thus, depending on the parasite, the inhibition or induction of Notch signaling pathway can improve or worsen the result of infection.

T. gondii also reaches other important organs of the host like brain, liver and lungs (Silva et al., 2010). Different from the small intestine data, our findings showed that the inhibition of Notch diminished inflammation and parasite load in the lung and liver when mice were infected with *T. gondii*. This decrease may be related to the effect of Notch inhibition directly or indirectly through changes in the small intestine and cytokine levels. A previous study using BALB/c mice infected with hepatitis B virus (HBV) demonstrated that inhibition of Notch signaling with DAPT reduced the recruitment of inflammatory cells, cytotoxic T lymphocytes and natural killer T-cells, into the liver and ameliorated local inflammation (Wei et al., 2016). Additionally, DBZ ameliorated the inflammation in the liver of rats injured by the paracetamol treatment (Ahmed et al., 2021). Few reports linking Notch pathway and pulmonary inflammation involving pathogens have been described with controversial results. C57BL/6 mice were treated during influenza infection with γ-secretase inhibitor or neutralizing antibodies specific for ligand Delta-1 that resulted in a high virus load with excessive inflammation in the lungs (Ito et al., 2011). In *Cryptococcus neoformans* infection, accumulation of inflammatory leukocytes was diminished in the lungs of mice with dominant negative mastermind-like (CCD) specifically restricted to mature $CD4^{+}$ and $CD8^{+}$ T-cells, compared with wild type mice that were associated with a diminished fungal control (Neal et al., 2017). Our results showed that Notch pathway has different behavior in different organs and that pharmacological inhibition of Notch can ameliorate inflammation and parasitism provoked by *T. gondii* in extra-intestinal organs like lungs and liver.

These results herein obtained, showed that *T. gondii* infection induced high levels of IFN-γ and TNF leading to a decrea in goblet and Paneth cells in the small intestine, which occurred at least partially dependent on Notch signaling pathway. However, Notch signaling inhibition was not efficient to revert the effectiveness of these cell phenotypes. Additionally, the infection interfered with Notch signaling direct target gene, *Hes1*, and also *Math1*, and the strong Th1 immune response induced by the parasite with high IFN-γ levels could be involved in the decreased *Hes1* expression. Thus, in the small intestine the major lesions induced by oral *T. gondii* infection are due to the strong pro-inflammatory immune response rather than the involvement of Notch signaling pathway. On the other hand, in the lung and liver, Notch signaling during *T. gondii* infection is detrimental to the host and the mechanisms involved need to be addressed.

Authors' contributions

NMS conceived the idea. MPB, YC, MPOA, NCM and ECBA performed the experiments and were involved in data acquisition. SNS and ESB contributed with technical support. MPB and NMS analyzed the data and wrote the manuscript. YC and MPOA revised the manuscript critically. All authors approved the final version of the manuscript.

CRediT author statement

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Nascimentos Santos: Investigation, Resources. **Emerson Soares Bernardes:** Investigation, Resources. **Neide Maria Silva:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tice.2022.101952.

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