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Resiniferatoxin lowers TNF-α, NO and PGE_2 in the intestinal phase and the parasite burden in the muscular phase of *Trichinella spiralis* infection

J. L. Muñoz-Carrillo[1] | J. J. Muñoz-Escobedo[2] | C. H. Maldonado-Tapia[1] | F. Chávez-Ruvalcaba[1] | M. A. Moreno-García[1]

[1]Laboratory of Cell Biology and Microbiology, Academic Unit of Biological Sciences, Autonomous University of Zacatecas, Zacatecas, México

[2]Academic Unit of Odontology, Autonomous University of Zacatecas, Zacatecas, México

Correspondence
José Luis Muñoz-Carrillo, Laboratory of Cell Biology and Microbiology, Academic Unit of Biological Sciences, Autonomous University of Zacatecas. Zacatecas, México.
Email: mcbjlmc@gmail.com

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Summary

During the course of infection with *Trichinella spiralis*, an inflammatory response is triggered at the intestinal level in the host, playing a crucial role in the expulsion and elimination of the parasite. However, several studies have demonstrated that this inflammatory response is harmful to the host; hence, the importance of studying molecules with therapeutic potential like resiniferatoxin, which is known to have an anti-inflammatory effect both in vitro and in vivo. In this article, we evaluated the anti-inflammatory activity of resiniferatoxin during the intestinal phase of *T. spiralis* infection by quantitatively determining the levels of TNF-α, NO and PGE_2 as well as the percentage of eosinophils in the blood and intestinal pathology. In addition, parasite burden was determined during the muscle infection. Our results show that resiniferatoxin lowered the serum levels of TNF-α, NO and PGE_2, as well as the percentage of eosinophils in the blood and intestinal pathology during the intestinal infection. Moreover, resiniferatoxin also lowered the parasite burden in muscle, resulting in a reduction of the humoral response (IgG) associated to treatment with resiniferatoxin. These findings suggest a potential therapeutic use of the anti-inflammatory effect of resiniferatoxin, which also contributes to host defence against the challenge of *T. spiralis* infection.

KEYWORDS

eosinophilia, inflammation, resiniferatoxin, *Trichinella spiralis*

1 | INTRODUCTION

Currently, almost 4 million people are infected with helminth parasites worldwide, which represent a serious global health and economic problem.[1] Nematode parasites belonging to the genus *Trichinella* show a cosmopolitan distribution. To date, 12 different species are known to cause trichinellosis,[2] a zoonotic parasitic disease characterized by being able to infect a wide variety of hosts, including humans.[3,4]

The life cycle of the *Trichinella spiralis* begins in a new host when meat containing infective larvae (L1) is ingested. L1 digested from meat in the stomach pass to the intestine where they invade intestinal epithelial cells. The parasites undergo development in the intestine from L1 to adults (AD).[5,6] AD live in intestinal epithelial cells for several weeks, where they reproduce and female worms release newborn larvae (NBL) into tissue. These NBL become distributed throughout the body, where they penetrate skeletal muscle cells and develop to L1, which are infective to the next host.[5,7]

Host reacts to the parasite entry activating the immune system with different mechanisms which characterize the intestinal and muscular phases of infection. The immune response against *T. spiralis* at the intestinal level depends on the $TCD4^+$ cells,[8] which can both suppress or promote the inflammatory response through the

synthesis of diverse cytokines.[9] During the intestinal phase the immune response is mixed (Th1/Th2) with the initial predominance of the Th1 type of response and the subsequent domination of Th2 response, protective and responsible for the parasite expulsion,[10] characterized by secretion of cytokines such as interleukin (IL) -4, -5, -10 and -13, as well as immunoglobulin E (IgE) and the mobilization of eosinophils and mast cells.[8,11] The activity of IL-4 and IL-13 is also required for the release of tumour necrosis factor (TNF-α) through the activation of intestinal mucosal mast cells,[12,13] thus promoting local inflammation.[14] One of the effects of TNF-α is the induction of inducible nitric oxide synthase (iNOS), resulting in the production of nitric oxide (NO),[15,16] which finally acts mainly as an effector molecule against both extracellular and intracellular parasites.[17] However, several studies have demonstrated that the inflammatory response driven by TNF-α and NO is deleterious to the host, since they also favour the development of enteropathy, while leaving unmodified the expulsion of *T. spiralis*.[18,19] In this context, the treatment of the inflammatory response during trichinellosis involves the use of anti-inflammatory steroids[20,21]; however, their side effects limits their therapeutic use since it is known that they significantly increase the parasite burden of *T. spiralis* in the muscle tissue of the host[22,23]. Hence, the necessity to study molecules with therapeutic potential as is the case of resiniferatoxin (RTX). RTX is a vanilloid derived from a cactus-like plant named *Euphorbia resinifera*. Most of the biological actions of RTX are mediated by the transient receptor potential vanilloid 1 (TRPV1), by desensitizing and blocking nociception, thus having an analgesic effect.[24-26] Besides the multiple actions mediated by the TRPV1, it has been demonstrated that RTX is a potential immunomodulator, since it has a potent anti-inflammatory effect by reducing the expression of nuclear factor κB (NF-κB),[27] cyclooxygenase-2 (COX-2) and iNOS, thus inhibiting the synthesis of both prostaglandine-E_2 (PGE_2) and NO.[28]

For these reasons, the aim of this study was to evaluate if RTX is capable of lowering the levels of pro-inflammatory mediators, TNF-α, NO and PGE_2 of the inflammatory response during the intestinal phase of infection by *T. spiralis*. Furthermore, we have assessed the effect of administering RTX during the intestinal phase on the development of the muscular phase of infection. Based on these considerations, our goal was to test the potential therapeutic use of RTX in the treatment of the inflammatory response triggered during trichinellosis, while preventing the side effects of the anti-inflammatory steroids, which are known to suppress the immune response of the host, thus favouring the implantation of the parasite in muscle tissue.

2 | MATERIAL AND METHODS

Female Sprague-Dawley rats with a body weight between 250 and 300 g were used. Groups of six rats were formed in the following fashion: a healthy control group (HC), a healthy control group treated with RTX (HC-RTX), three control groups infected with *T. spiralis* ($CITsp_1$, $CITsp_2$ and $CITsp_3$) sacrificed on days 7, 14 and 28 post infection (p.i.) respectively; two control groups treated with dexamethasone (DX) on day 1 p.i. ($Tsp\text{-}DXD1_1$ and $Tsp\text{-}DXD1_2$), sacrificed on day 7 and 28 p.i. respectively; two control groups infected with *T. spiralis* treated with DX on day 7 p.i., ($Tsp\text{-}DXD7_1$ and $Tsp\text{-}DXD7_2$), sacrificed on day 14 and 28 p.i. respectively; two groups infected with *T. spiralis* treated with RTX on day 1 p.i., ($Tsp\text{-}RTXD1_1$ and $Tsp\text{-}RTXD1_2$), sacrificed on day 7 and 28 p.i. respectively; and two groups infected with *T. spiralis* treated with RTX on day 7 p.i. ($Tsp\text{-}RTXD7_1$ and $Tsp\text{-}RTXD7_2$), sacrificed on day 14 and 28 p.i. respectively. This study was reviewed and approved by the ethical committee and the academic council of the Academic Unit of Biological Sciences of the Autonomous University of Zacatecas (UAZ), in accordance with the Mexican Official Norm (NOM-062-ZOO-1999), published by the Secretariat of Agriculture, Livestock, Rural Development, Fisheries and Food (SAGARPA) in the Official Gazette of the Federation (México) on June 28, 2001.

2.1 | Experimental infection

Rats, obtained from the animal facility of the Academic Unit of Biological Sciences of the Autonomous University of Zacatecas, were infected orally using 500 L1 of *T. spiralis*. The parasite (Mexican strain) was identified with Edoardo Pozio PhD, in the *Istituto Superiore di Sanita* in Rome, Italy, and has been maintained by serial passage in mice and rats since 1986 at the Laboratory of Cell Biology and Microbiology at the Academic Unit of Biological Sciences from the Autonomous University of Zacatecas. Zacatecas, México. All of the animals were maintained in temperature-controlled rooms and fed with rodent balanced food.

2.2 | Anti-inflammatory treatment

Control groups Tsp-DXD1 and TspDXD7 were treated with commercial dexamethasone sodium phosphate (dose:1 mg/kg) administered intraperitoneally.[29]

Experimental groups Tsp-RTXD1 and Ts-RTXD7 were treated with resiniferatoxin (Sigma-Aldrich, St. Louis, MO, USA dose: 20 μg/kg), dissolved in vehicle (physiological solution 0.9% NaCl) administered intraperitoneally.[30]

2.3 | Determination of serum PGE_2 and TNF-α

The concentrations of serum PGE_2 and TNF-α in rats were quantitatively determined in the CITsp control groups, and 90 minutes after treatment administration in both the groups treated with DX and RTX, on days 1 and 7 p.i., using ELISA kits (R&D systems, Minneapolis, MN, USA).

2.4 | Determination of serum NO

The concentration of serum NO in the rats was quantitatively determined in the CITsp control group, and 90 minutes after treatment administration in both the groups treated with DX and RTX, on days 1 and 7 p.i., using a Total Nitric Oxide and Nitrate/Nitrite Parameter Assay Kit (R&D systems).

2.5 | Determination of the percentage of eosinophils in the blood

A blood smear was performed on days 1, 7, 14, 28 and 4 months after infection for the CITsp group and on days 1, 7, 14 and 28 for the groups treated with DX and RTX. After the blood smears, the Wright's stain method (Golden Bell Reactive 82300) was used to determine the percentage of eosinophils in the peripheral blood. Eosinophils in the sample were observed with an optical light microscope (Carl Zeiss Primo Star, model 3708) using a 100× immersion objective. The average number of eosinophils per 100 white cells was determined from three different counts (300 white cells in total).

2.6 | Evaluation of intestinal pathology

The duodenum, jejunum and ileum portions of the small intestine were obtained from *T. spiralis* infected control groups, as well as those treated with DX and RTX sacrificed on day 7 and 14 p.i. The portions were fixed in 10% formol solution and embedded in paraffin wax. Then, sections were cut and stained with haematoxylin and eosin on a glass microscope slide.[6,31,32] The integrity and damage of the intestinal tissue were evaluated by measuring the lengths (in micrometers, μm) of the ten crypts and villi [18] for each rat, using the ZEN (Blue Edition Carl Zeiss Microscopy GmgH 37081 Gottingen, Germany) imaging software (Carl Zeiss Primo Star microscope Carl Zeiss Microscopy GmgH 37081 Gottingen, Germany, model 3708) using a 10× objective.

2.7 | Determination of implanted *Trichinella spiralis* infective larvae in muscle tissue

Samples of muscle tissue such as masseter, tongue, leg and diaphragm,[33,34] were obtained from both the CITsp groups and the groups that were treated with DX and RTX and sacrificed on day 28 p.i. The samples were placed between two compression plates composed of two microscope slides and were observed after compression under a light microscope (Carl Zeiss Primo Star microscope, model 3708) using 4×, 10× and 40× objectives in order to assess the presence of nurse cells. The average number of implanted *T. spiralis* L1 in muscle tissue [3,32] was determined for each sample by averaging the total count of L1 from three fields observed using a 4× objective. All the samples of muscle (masseter, tongue, leg and diaphragm) were stained with haematoxylin and eosin.[31]

2.8 | Determination of the parasite burden

Muscle tissue was obtained from the groups that were sacrificed on day 28 p.i., and was thoroughly grinded (using an Oster processor model 3212). Portions of 15 g of grinded muscle tissue were taken and placed in a tulle sleeve bag inside a separation funnel containing artificial digestive solution of 0.3% pepsin (1:10 000), 7% HCl (37%, 0.2 mol/L) and 90% distilled water. Samples were incubated at 37°C for 24 hours in an incubator (Thelco, Model 4, Precision Scientific Co Chicago, USA.) Larvae packages, that resulted of digestion separated and settled at the bottom of the separation funnel were retrieved in conical tubes. After three PBS washes (pH 7.3), the parasite burden in samples of 15 g was determined.[35]

2.9 | Obtaining for *Trichinella spiralis* total soluble (TS) antigen

The L1 larvae isolated from the muscle after artificial digestion from an infected rat were washed three times in PBS (pH 7.3). The L1 larvae were placed in a mortar in a frozen environment where they were lysed and homogenized in liquid nitrogen for 10 minutes at -20°C using a protease inhibitor (SIGMAFAST Protease Inhibitor Tablets; Sigma-Aldrich). Subsequently, the suspension of homogenized larvae was centrifuged in Eppendorf tubes at 1, 100 g units for 90 minutes at 4°C (Spectrafuge 24D, Labnet, International Inc. Edison, NJ, USA, Model C2400-B). Finally, the supernatant containing the TS antigen was separated and stored in Eppendorf tubes until further use.[32] The concentration of the *T. spiralis* TS antigen was determined using the Bradford method. A bovine serum albumin standard curve (BSAC) was obtained and the absorbance at 595 nm was measured in a microplate reader (iMark Microplate Reader BIO-RAD, model 10465).[36]

2.10 | Detection of serum IgM and IgG

The IgM and IgG were detected through the dot enzyme-linked immunosorbent assay (Dot-ELISA). The ST antigen (10 μL/dot with a protein concentration of 10 μg) was spotted onto nitrocellulose paper (NC; Sigma). The NC paper spotted with TS antigen was air-dried between 8 and 12 hours at room temperature and then blocked with skimmed milk at 3% in PBS for 1 hour. After blocking, two PBS-Tween washes (0.5% Tween 20 in PBS) were performed with constant stirring for 10 minutes. A third PBS wash with stirring was carried out for 10 minutes. Then, the NC paper was incubated with diluted rat sera (1:10 in blocking solution) and incubated for 1 hour at room temperature with gently shaking. The NC paper was then washed with PBS-Tween (0.5% Tween 20 in PBS) and incubated with diluted (1:2000 in PBS) rabbit anti-rat IgG and IgM (peroxidase-conjugated; Sigma-Aldrich) for 1 hour at room temperature. After a final washing with PBS-Tween the NC paper was exposed to 3,3-diaminoobenzidine-tetrahydrochloride solution (DAB) composed of 20 mL of PBS (pH 7.2), 20 μL of 3% hydrogen peroxide, and 10 mg of DAB (Sigma-Aldrich). The reaction was stopped after 5 minutes by washing with deionized water.[37]

2.11 | Statistical analysis

Results are presented as mean ± standard deviation (SD). Significance was determined by a one-way analysis of variance (ANOVA) to test for overall differences between group means. Student's *t*-test for paired samples was used to compare means of paired samples. A P value<.05 was considered statistically significant. Statistical analyses were performed in Graphpad PRISM for Windows version 6 (Graphpad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | Induction of pro-inflammatory mediators TNF-α, NO and PGE_2 during *Trichinella spiralis* infection

Rats infected orally with 500 L1 of *T. spiralis* showed a significant increase (compared to the HC group) in the levels of both serum TNF-α (73.7 ± 5.49 pg/mL, *P*<.001) and NO (369.2 ± 68.55 µmol/L, *P*<.05) 24 hours p.i., at the beginning of the initial intestinal phase. On day 7 p.i., levels of serum TNF-α and NO remained significantly increased (65.4 ± 9.10 pg/mL and 329.9 ± 17.4 µmol/L respectively, *P*<.05). Similarly, levels of serum PGE_2 increased both on day 1 and 7 p.i., (15 438 ± 2119 pg/mL y 13 990 ± 647 pg/mL respectively), although these differences were not statistically significant (*P*>.05) when compared to the HC group. On day 14 p.i., (between phases enteral and parenteral), a decrease in the levels of TNF-α, NO and PGE_2 was observed. However, although TNF-α level decreased with respect to day 7 (*P*<.05), it was significantly higher (31.4 ± 1.71 pg/mL) than HC group. On the other hand, NO decreased below the NO baseline levels of the HC group (36.1 ± 8.33 µmol/L), although this decrement was not statistically significant. Level of PGE_2 also decreased significantly (8, 589 ± 1, 275 pg/mL, *P*<.05) when compared to the HC group. On day 21 p.i., (muscular phase), the levels of serum TNF-α and PGE_2 increased significantly (67.1 ± 11.89 pg/mL and 16, 871 ± 266 pg/mL respectively, *P*<.05) with respect to the HC group. In addition, although the level of NO also increased, it was lower than the baseline level (89.4 ± 10.65 µmol/L). Finally, on day 28 p.i., basal levels of TNF-α, NO and PGE_2 were observed (21.1 ± 4.03 pg/mL, 47.4 ± 5.51 µmol/L and 10, 394 ± 1, 806 pg/mL respectively) as compared to the HC group (Figure 1).

3.2 | Resinifertoxine lowers the production of pro-inflammatory mediators during the intestinal phase of *Trichinella spiralis* infection

On day 1 p.i., RTX was administered to the TSp-$RTXD1_1$ group. As a result, levels of serum PGE_2, TNF-α and NO decreased significantly to 6219 ± 1325 pg/mL (*P*<.001), 53.6 ± 1.40 pg/mL (*P*<.01) and 178.5 ± 1.44 µmol/L (*P*<.01) respectively, when compared to the CITsp group. A similar decrease was observed on the levels of serum PGE_2, TNF-α and NO (3902 ± 785 pg/mL, 27.7 ± 6.19 pg/

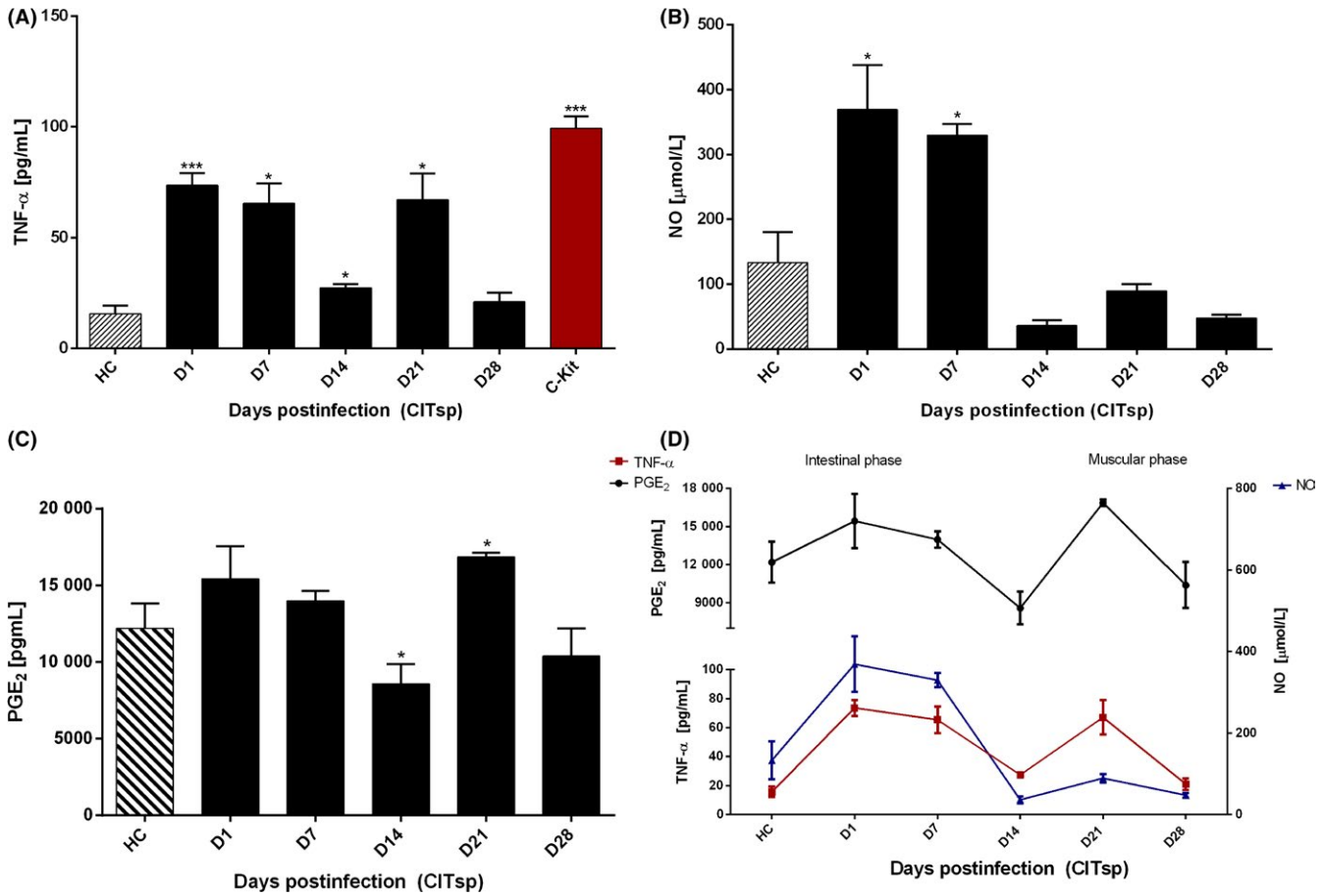

FIGURE 1 Production of pro-inflammatory mediators TNF-α, NO and PGE_2 during *Trichinella spiralis* infection. Levels of (A) TNF-α, (B) NO and (C) PGE_2 in serum of rats on days 1, 7, 14, 21 and 28 of *T. spiralis* infection were measured by ELISA. CITsp group (black bars), HC group (striped bars), kit control TNF-α (red bar). (D) The course of the release of TNF-α (red line), NO (blue line) and PGE_2 (black line) during phases of *T. spiralis* infection. Values are presented as group means ± SD, indicating the level of significance (**P*<.05, ***P*<.01 and ****P*<.001)

mL and 21.4 ± 6.13 µmol/L respectively, P<.001) in the Tsp-DXD1$_1$ group, which was treated with DX on day 1 p.i. (Figure 2A-C). Levels of serum PGE_2, TNF-α and NO of the group treated with RTX on day 7 p.i., decreased significantly to 2455 ± 503 pg/mL, 33.9 ± 2.0 pg/mL and 119.2 ± 8.49 µmol/L respectively (P<.001) when compared to the levels of the CITsp group (Figure 2D-F). The same significant decrease was observed on the levels of serum PGE_2 (3088 ± 1625 pg/mL, P<.001), TNF-α (19.7 ± 1.45 pg/mL, P<.001) and NO (290.2 ± 16.25 µmol/L, P<.05) of the Tsp-DXD7$_1$ group, treated with DX on day 7 p.i. (Figure 2D-F).

3.3 | Resiniferatoxin lowers the percentage of eosinophils in the blood during the intestinal phase of *Trichinella spiralis* infection

A significant increase in the percentage of peripheral eosinophils (9 ± 3.5%, P<.05) during *T. spiralis* infection in rats was observed 24 hours p.i., when compared to that of the HC group (4 ± 0.4%). The count of eosinophils remained increasing significantly at days 7 and 14 p.i., (21 ± 3.5% and 25 ± 3.8% respectively, P<.001). However, the percentage of eosinophils decreased on days 21 and 28 to 13 ± 5.4% and 8 ± 1.7% respectively. Finally, four months after infection, a significant increase in the percentage of eosinophils was observed (7 ± 0.7%, P<.001). Group HC-RTX also showed a significant decrease in the percentage of eosinophils (3 ± 0.5%, P<.01) compared to the HC group (Figure 3A).

At day 1 p.i., RTX and DX were administered to the groups Tsp-RTXD1 and Tsp-DXD1 respectively. A significant decrease in the percentage of peripheral eosinophils was observed in both groups (4 ± 0.4% and 5 ± 0.8% respectively, P<.01) when compared to the CITsp group. Similarly, on day 7 p.i., both groups showed a further decrease in the percentage of eosinophils (2 ± 1% and 2 ± 0.8% respectively, P<.001). In contrast, at day 14 p.i., we observed an increase in the percentage of peripheral eosinophils to 5 ± 1.1% and 11 ± 1.6% in the Tsp-RTXD1 and Tsp-DXD1 groups respectively, but both percentages were still significantly lower than the of the CITsp group on day 14 p.i. Finally, on day 28 p.i., both groups presented a similar percentage of eosinophils (8 ± 2.6% and 8 ± 1% respectively) as that of the CITsp group (Figure 3B). Similarly, the groups Tsp-RTXD7 and Tsp-DXD7, treated with RTX and DX respectively at day 7 p.i., a decreased percentage of eosinophils was observed (15 ± 0.8% and 13 ± 2.7% respectively, P<.001), with respect to the CITsp group. Similarly, on day 14 p.i., both treatments (RTX and DX) significantly lowered the percentage of eosinophils (7 ± 1.9% and 12 ± 1% respectively) when compared to the CITsp group. Finally, on day 28 p.i., the Tsp-RTXD7 and Tsp-DXD7 groups presented a percentage of eosinophils similar to that of the CITsp group (10 ± 1.2% and 7 ± 1.1% respectively, Figure 3C).

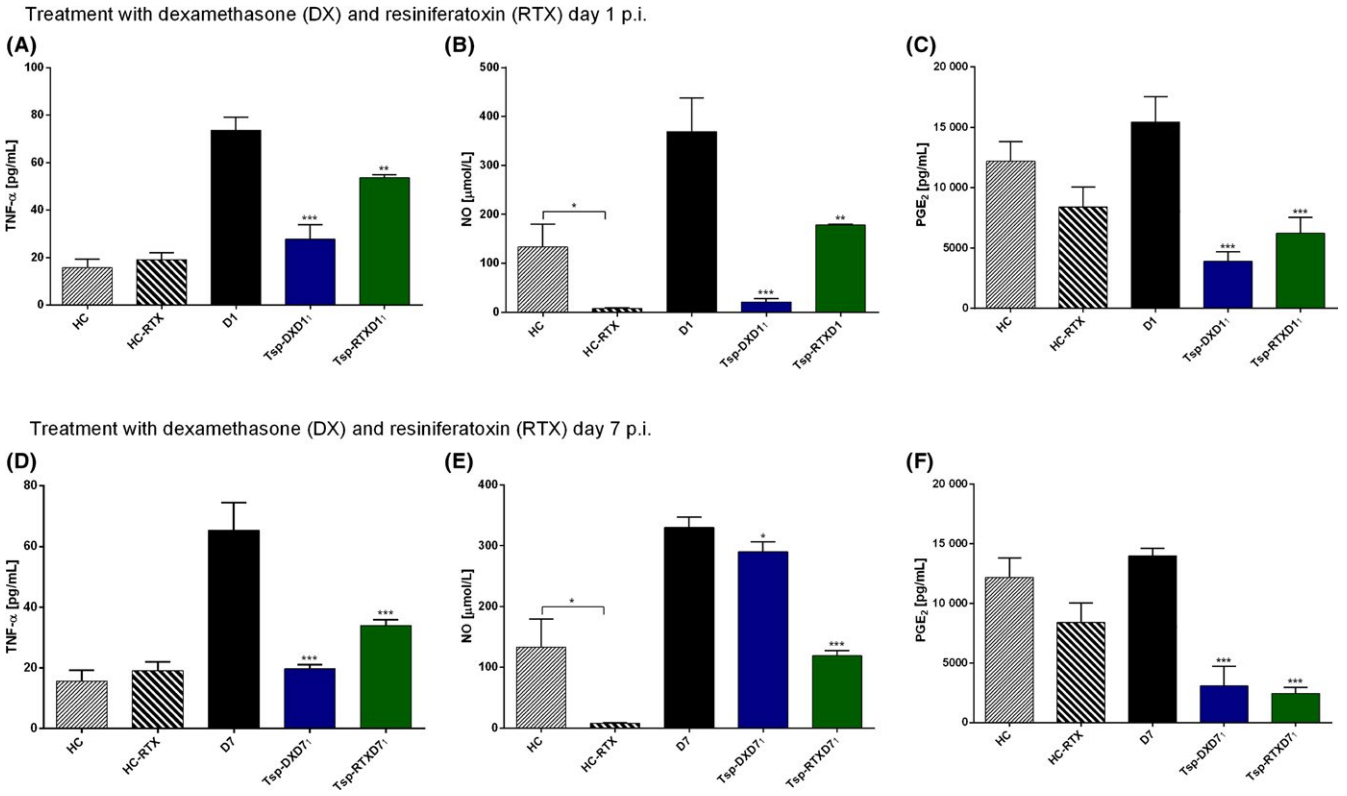

FIGURE 2 Resiniferatoxin decreases serum pro-inflammatory mediators in *Trichinella spiralis* infected rats. Levels of (A, D) TNF-α, (B, E) NO and (C, F) PGE_2 in serum of rats treated with DX (blue bars) and RTX (green bars) on days 1 and 7 of *T. spiralis* infection were measured by ELISA. CITsp (black bars), HC (striped thin bars) and HC-RTX (striped wide bars) groups are shown. Values are presented as group means ± SD, indicating the level of significance (*P<.05, **P<.01 and ***P<.001)

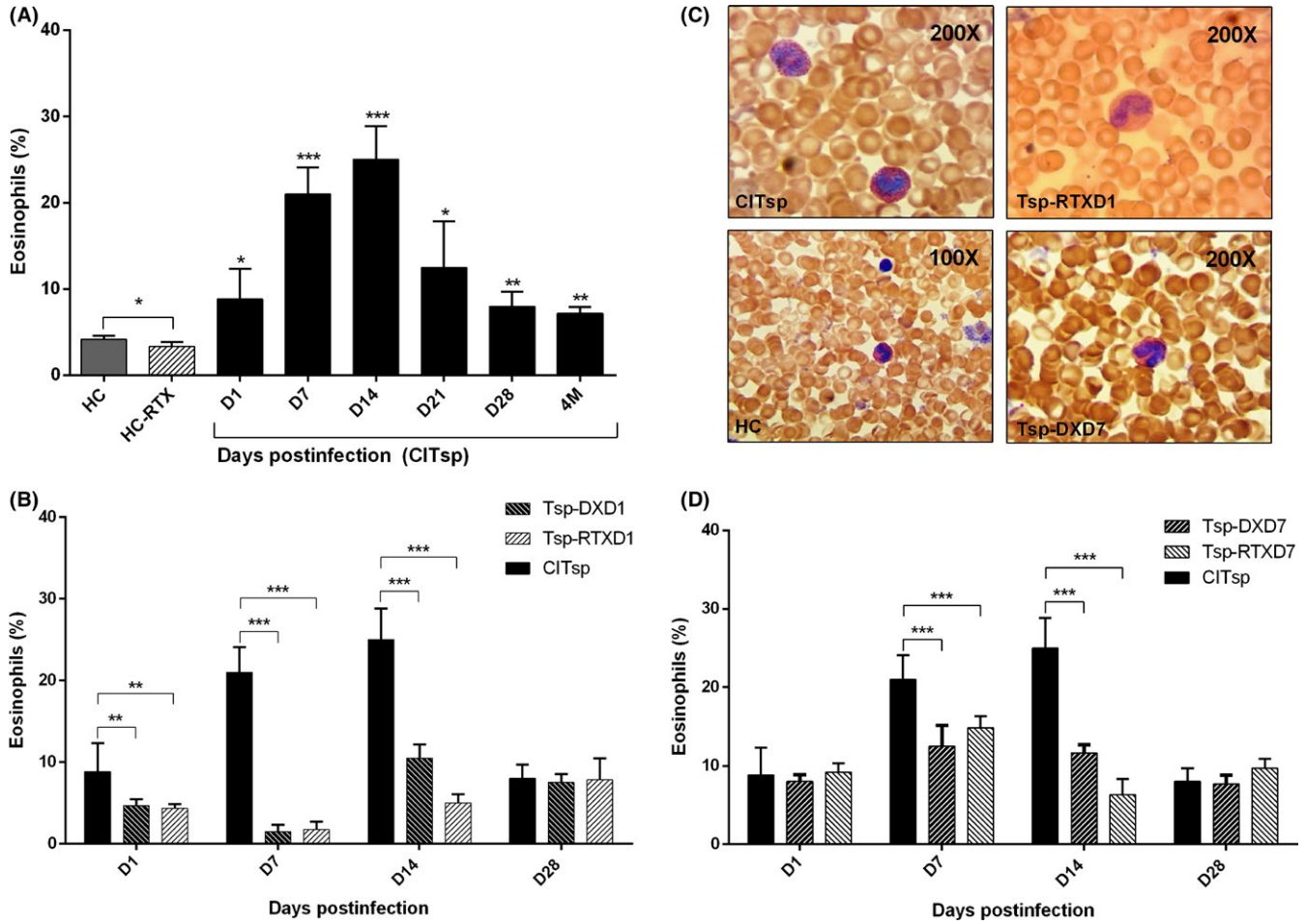

FIGURE 3 Resiniferatoxin decreases the percentage of eosinophils in the blood in *Trichinella spiralis* infected rats. (A) percentage of eosinophils in the blood during *T. spiralis* infection. Groups HC (grey bar), HC-RTX (striped bar) and CITsp (black bars) are shown. (B) percentage of eosinophils on days 1, 7, 14 and 28 of *T. spiralis* infection in both rats treated with RTX (striped thin bars) and DX (striped wide bars) on day 1. (C) percentage of eosinophils on days 1, 7, 14 and 28 of *T. spiralis* infection both in rats treated with RTX (striped thin bars) and DX (striped wide bars) on day 7. (D) photomicrographs of eosinophils in the blood of the HC group (day 1, 100×), CITsp group (day 7, 200×), Tsp-RTXD1 (day 1, 200×) and Tsp-DXD7 (day 7, 200×). The percentage of eosinophils in the blood was determined by Wright's stain method. Values are presented as group means ± SD, indicating the level of significance (*P<.05, **P<.01 and ***P<.001)

3.4 | Intestinal pathology is reduced by treatment with resiniferatoxin during the intestinal phase of *Trichinella spiralis* infection

The small intestine pathology developed by the infected control group $CITsp_1$ on day 7 p.i., is illustrated in Figure 4, where it can be seen that there was a significant decrease (P<.001) and erosion of the villi as well as hyperplasia of the intestinal crypts. Adult females of *T. spiralis* were found in duodenum, in contrast to the HC group, which did not present intestinal pathology (Figure 4).

The groups treated with RTX ($Tsp\text{-}RTXD1_1$) and DX ($TspDXD1_1$) on day 1 p.i., showed a marked reduction in intestinal pathology, as indicated by the significant reduction in the hyperplasia of the intestinal crypts (P<.05 and P<.01 respectively) and the significant increase (P<.05, P<.01 and P<.001) and reconstitution of the intestinal villi. However, in spite of the reduced intestinal pathology, adult females of *T. spiralis* were still observed in both duodenum and jejunum (Figure 5).

Finally, the $CITsp_2$ group did not show intestinal pathology, similar to the HC group (Figure 4). Similarly, groups $Tsp\text{-}RTXD7_1$ and $TspDXD7_1$, treated on day 7 p.i. with RTX and DX respectively, did not present intestinal pathology, like those of the HC and $CITsp_2$ groups. However, in spite of the reduced intestinal pathology, adult females of *T. spiralis* were still observed in both duodenum and ileum (Figures 4 and 6).

3.5 | Resiniferatoxin lowers the implantation of L1 and the parasite burden of *Trichinella spiralis*

The effect of treatment with DX and RTX during the intestinal phase on the implantation of L1 and the parasite burden of *T. spiralis* was evaluated. We observed that after administering DX to the subgroup $Tsp\text{-}DXD1_2$ on day 1 p.i., the implantation of *T. spiralis* at day 28 p.i., increased significantly in diaphragm and leg (70 ± 4 and 17 ± 3 L1 respectively, P<.05) when compared to the $CITsp_3$ group (59 ± 7 and 11 ± 2 L1 respectively). We did not find significant differences

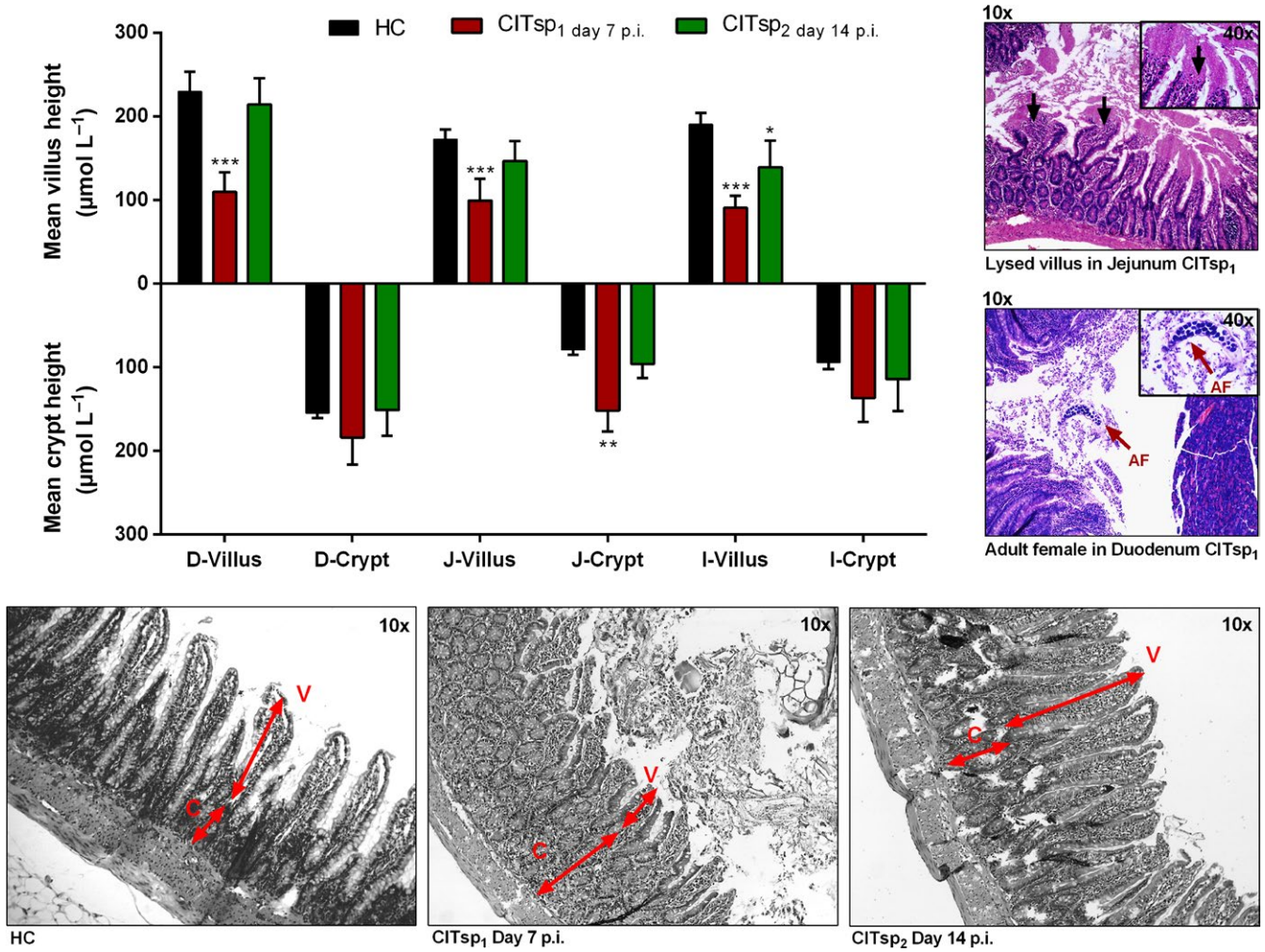

FIGURE 4 Micrographs of small intestinal pathology in *Trichinella spiralis* infected rats. The length (in µmol/L, red arrows) of the intestinal crypts (C) and villi (V) of the duodenum (D), jejunum (J) and ileum (I) portions of the small intestine obtained from the healthy control group (HC, black bars), the *T. spiralis* infected control group at day 7 p.i., ($CITsp_1$, red bars) and the *T. spiralis* infected control group at day 14 p.i., ($CITsp_2$, green bars). Values are presented as group means ± SD, indicating the significant differences (**P*<.05, ***P*<.01 and ****P*<.001) of the villus/crypt lengths of the *T. spiralis* infected control groups ($CITsp_1$ and $CITsp_2$) compared to HC group

between both tongue (37 ± 6 L1) and masseter (32 ± 3 L1) muscles and the $CITsp_3$ group (40 ± 5 and 30 ± 2 L1 respectively). In contrast, when RTX was administered to the subgroup Tsp-$RTXD7_2$ at day 1 p.i., we observed that by day 28, the implantation of *T. spiralis* in diaphragm, tongue, leg and masseter decreased significantly (34 ± 10, 18 ± 4, 12 ± 5, 7 ± 3 L1 respectively, *P*<.05) with respect to the $CITsp_3$ group (Figure 7A). On the other hand, when we administered DX to the subgroup Tsp-$DXD7_2$ at day 7 p.i., we observed that by day 28 p.i., the implantation of *T. spiralis* in diaphragm (84 ± 7 L1, *P*<.001), leg (20 ± 4 L1, *P*<.001) and masseter (37 ± 4 L1, *P*<.01) increased significantly when compared to the $CITsp_3$ group. However, RTX was administered to the group Tsp-$RTXD7_2$ on day 7 p.i., resulting in a decreased implantation of *T. spiralis* in diaphragm, tongue, masseter and leg at day 28 p.i., (8 ± 2, 4 ± 1, 4 ± 1 and 3 ± 1 L1 respectively, *P*<.001) as can be seen in Figure 7B.

Regarding the parasite burden, in the Tsp-$DXD1_2$ group, treated with DX at day 1 p.i., we observed a non-significant increase in the parasite burden (2417 ± 258 L1) to compared to the $CITsp_3$ group (2, 024 ± 292 L1). In contrast, when RTX was administered to the Tsp-$RTXD1_2$ group on day 1 p.i., the parasite burden decreased significantly (1042 ± 368 L1, *P*<.001, Figure 8A). On the other hand, the parasite burden increased after administering DX on day 7 p.i., to the Tsp-$DXD7_2$ group (3750 ± 570 L1, *P*<.001) in comparison to the $CITsp_3$ group. However, when we applied RTX to the Tsp-$RTXD7_2$ subgroup at day 7 p.i., we observed a decrease in the parasite burden (542 ± 188 L1, *P*<.001) with respect to the $CITsp_3$ group (Figure 8B).

Finally, IgM and IgG antibodies against *T. spiralis* antigens were detected in serum from rats during *T. spiralis* infection using the Dot-ELISA technique (Table 1). We detected anti-*T. spiralis* IgM antibodies at the third (day 21 p.i.) and fourth (day 28 p.i.) weeks of *T. spiralis* infection in CITsp group (Table 1a). Anti-*T. spiralis* IgG antibodies were detected at the second (day 14 p.i.), third (day 21 p.i.) and fourth (day 28 p.i.) weeks of *T. spiralis* infection in CITsp group (Table 1b). As it can be observed in Table 1a, groups CITsp, Tsp-DXD1, Tsp-DXD7 and Tsp-RTXD1 were positive for IgM antibodies on day 28 p.i., in contrast to the Tsp-RTXD7 group, which were negative for IgM. Similarly, groups

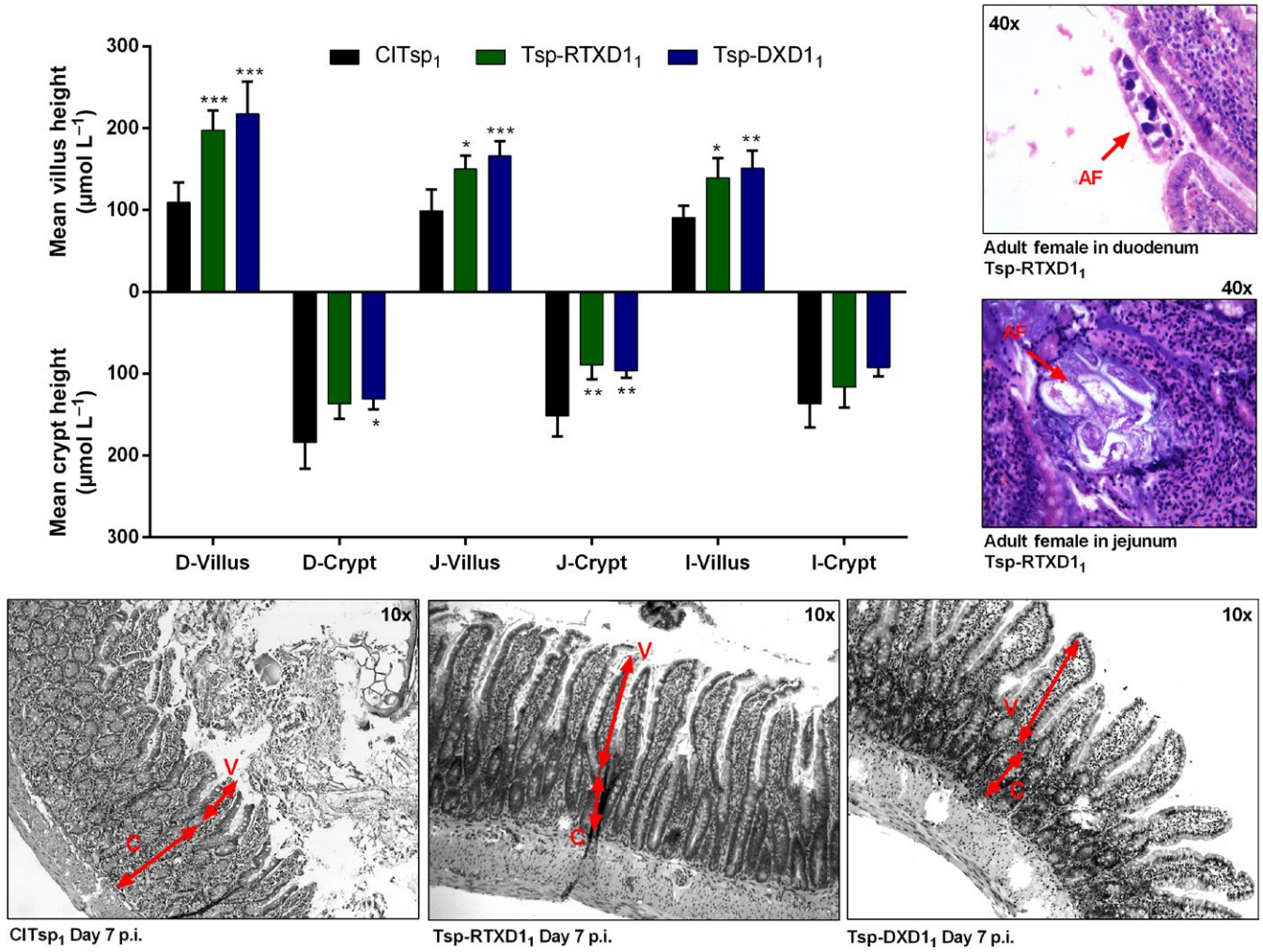

FIGURE 5 Micrographs of small intestinal pathology on day 7 p.i., in control and experimental groups. The length (in µmol/L, red arrows) of the intestinal crypts (C) and villi (V) of the duodenum (D), jejunum (J) and ileum (I) portions of the small intestine obtained from the *Trichinella spiralis* infected control group ($CITsp_1$, black bars), the *T. spiralis* infected control group treated with DX on day 1 p.i., ($Tsp\text{-}DXD1_1$, blue bars) and the *T. spiralis* infected group treated with RTX on day 1 p.i., ($Tsp\text{-}RTXD1_1$, green bars). Values are presented as group means ± SD, indicating the significant differences (*P<.05, **P<.01 and ***P<.001) of the villus/crypt lengths of the groups treated with DX and RTX compared to *T. spiralis* infected control group ($CITsp_1$)

CITsp, Tsp-DXD1 and Tsp-DXD7 were positive for IgG at day 28 p.i., in contrast to groups Tsp-RTXD1 and Tsp-RTXD7, which were negative for IgG (Table 1b).

4 | DISCUSSION

Following previous reports suggesting that pro-inflammatory mediators are involved in the intestinal pathogenesis of *T. spiralis* infection, in this study, we evaluated for the first time whether treatment with RTX is capable of lowering the levels of TNF-α, NO and PGE_2 in rat serum during infection by *T. spiralis*.

TNF-α, a potent pro-inflammatory cytokine produced by several types of immune cells such as dendritic cells, macrophages, Th1 lymphocytes, mastocytes, etc., exert its biological effects in a pleiotropic manner, thus having a key role in the pathogenesis of inflammatory diseases.[38-40] Diverse studies have associated the production of TNF-α with the development of intestinal pathology during Trichinellosis. For instance, it has been shown that TNF receptor 1 (TNRF1)-deficient mice are still capable of expelling *T. spiralis*, although a reduction in intestinal pathology was observed.[18] Another study showed that TNF-α derived from mastocytes is required for mastocytosis as well as for the generation of the Th2 immune response, which are both needed for the expulsion of *T. spiralis*.[41] In addition, the soluble form of TNF-α plays a critical role in the protection against the parasite through the Th2 immune response, since the absence of soluble TNF-α in transgenic mice delayed the expulsion of *T. spiralis* significantly, along with a reduction in the intestinal pathology and mastocitosis.[42]

Regarding NO, it is well known that it acts mainly as an effector molecule against both intracellular and extracellular parasites.[17] Previous reports indicate that NO also participates in the induction of intestinal pathology and in the inflammatory response during *T. spiralis* infection. It has been shown that *T. spiralis* L1 antigens produce an increase in both the expression of iNOS and the resulting production of

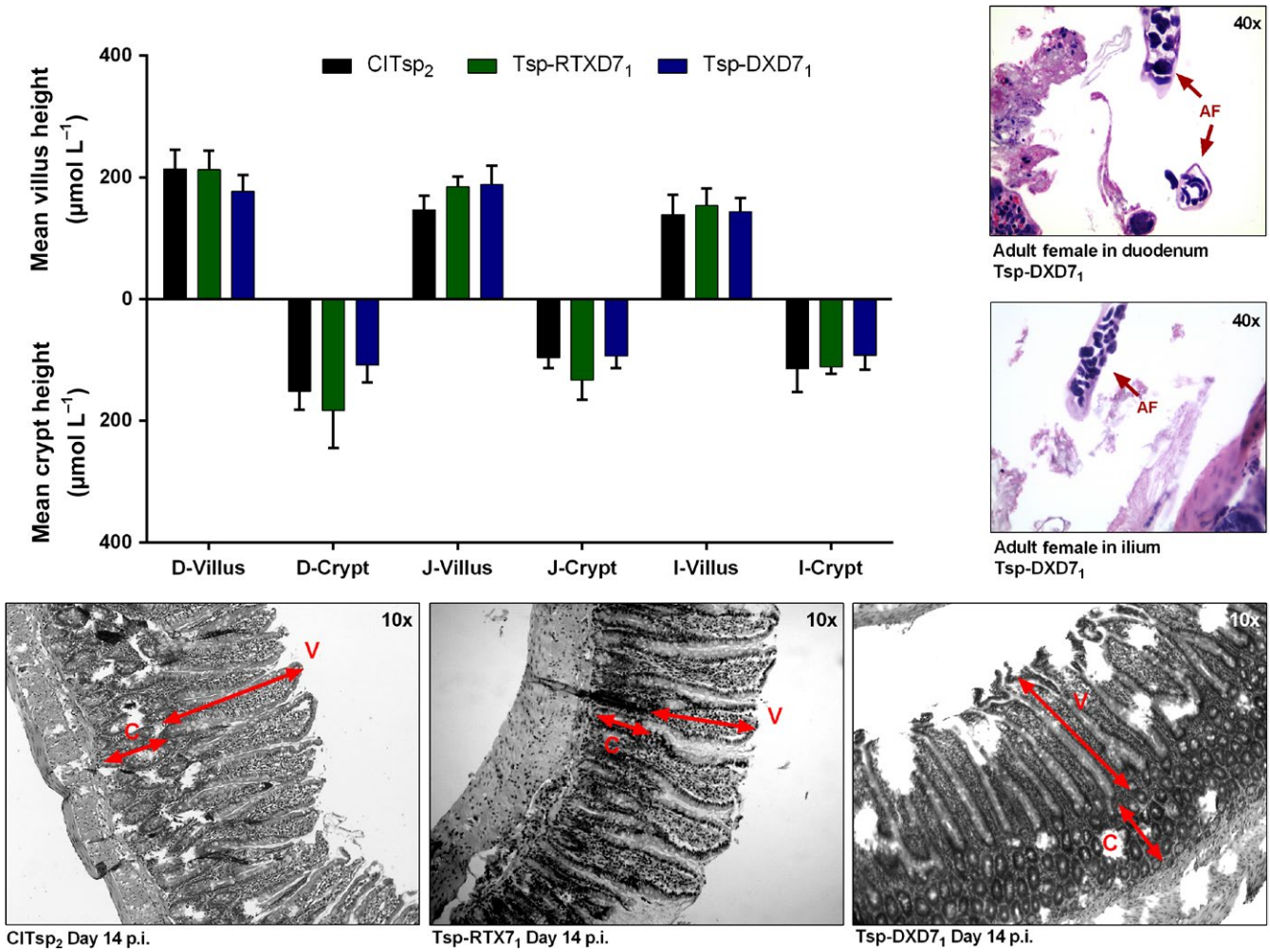

FIGURE 6 Micrographs of small intestinal pathology on day 14 p.i., in control and experimental groups. The length (in µmol/L, red arrows) of the intestinal crypts (C) and villi (V) of the duodenum (D), jejunum (J) and ileum (I) portions of the small intestine obtained from the *Trichinella spiralis* infected control group ($CITsp_2$, black bars), the *T. spiralis* infected control group treated with DX on day 7 p.i., ($Tsp\text{-}DXD7_1$, blue bars) and the *T. spiralis* infected group treated with RTX on day 7 p.i., ($Tsp\text{-}RTX7_1$, green bars). Values are presented as group means ± SD, indicating the significant differences (**P*<.05, ***P*<.01 and ****P*<.001) of the villus/crypt lengths of the groups treated with DX and RTX compared to *T. spiralis* infected control group ($CITsp_2$)

NO.[43] Furthermore, a reduced humoral response (IgG1, IgE), a reduction in the expression of IL-4 and IL-5 (both associated with the Th2 immune response), a reduced mastocytosis and accumulation of fluids in the intestine, were observed in iNOS knockout mice (C57BL/6) infected with 400 L1 of *T. spiralis*. However, it should be mentioned that this study was not able to find significant differences in the expulsion of the parasite, although the iNOS knockout mice did show a reduced intestinal pathology when compared to wild type mice.[19]

PGE_2 is the most abundant prostaglandin produced in the body, and it is responsible for a variety of biological functions. It is synthesized through the metabolism of the arachidonic acid by the two isoforms of the cyclooxygenase (COX), -1 and -2 in a constitutive manner or in response to an inflammatory stimulus respectively. In physiological conditions, PGE_2 is an important mediator of several biological functions, such as the regulation of the immune response in the intestinal mucosa, mainly by maintaining the integrity of the gastrointestinal tract.[44,45] Dysregulated PGE_2 synthesis has been associated with a wide range of pathological conditions. For instance, during an inflammatory response, PGE_2 is particularly relevant since it participates in all the processes leading to the typical signs of inflammation: redness, swelling and pain. The first two (redness and swelling) are a result of an increased blood flow to the inflamed tissue driven by both an augmented arterial dilation and a higher microvascular permeability mediated by PGE_2.[44] During trichinellosis, PGE_2 has been related to the acute phase of infection, since its release together with other mediators such as histamine, serotonin and bradykinin, leads to an increased permeability of the capillaries and a leak of fluids and albumins in the surrounding tissue.[20,46]

Based on these studies about the role of TNF-α, NO and PGE_2 in the intestinal phase of *T. spiralis* infection and their relation with the development of the intestinal pathology, our results support the hypothesis that the production of these pro-inflammatory mediators is associated with the development of the intestinal pathology during *T. spiralis* infection. We observed that the levels of serum TNF-α, NO

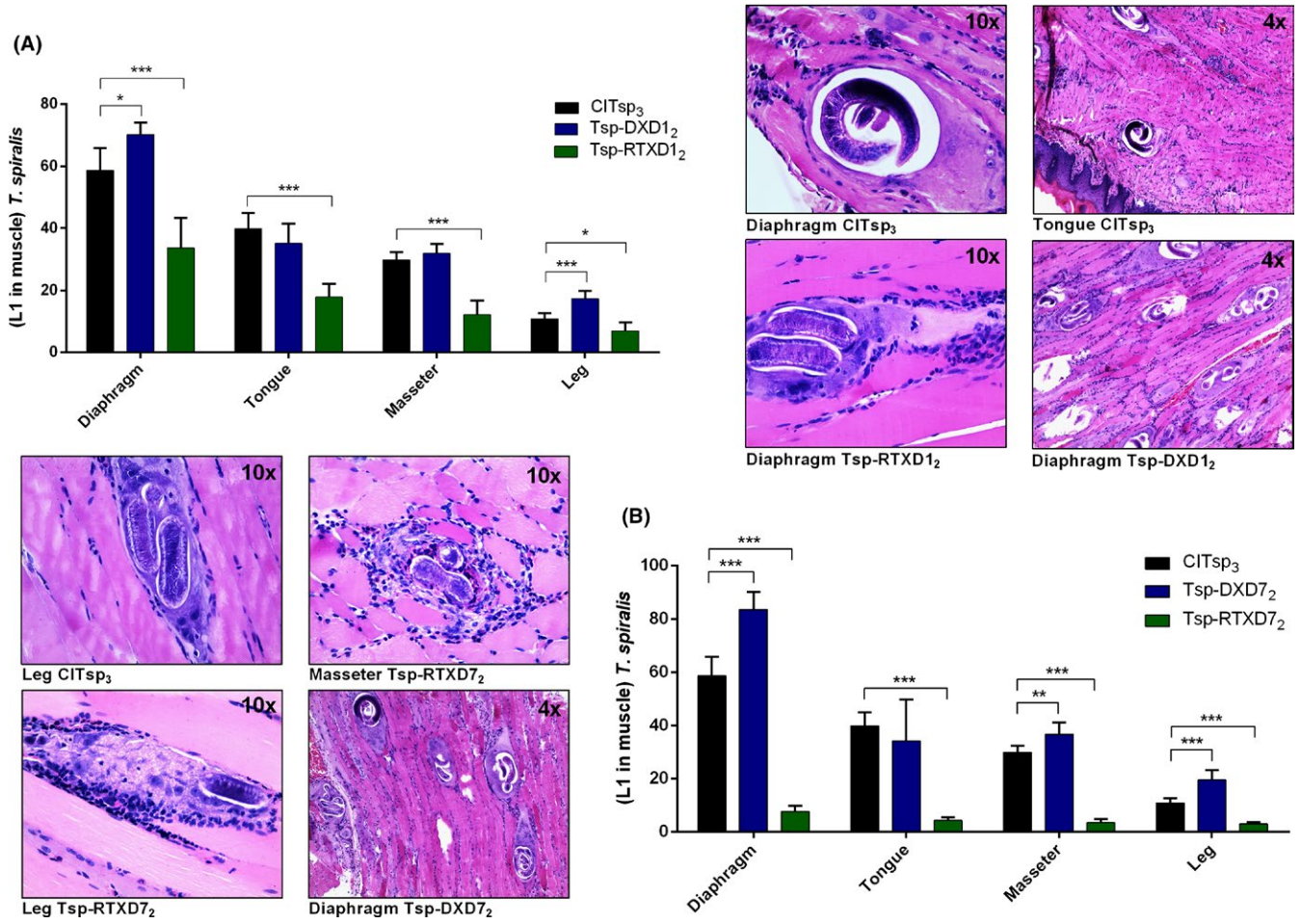

FIGURE 7 Infective larvae (L1) of *Trichinella spiralis* detected in striated muscular tissue of rats treated with DX and RTX on day 1 (A) and 7 (B) after *T. spiralis* infection. The number of implanted L1 in diaphragm, tongue, masseter and leg is shown for the $CITsp_3$ (black bars), Tsp-$DXD1_2$ and Tsp-$DXD7_2$ (blue bars) and Tsp-$RTXD1_2$ and Tsp-$RTXD7_2$ (green bars) groups. Muscle tissues were observed under an optical light microscope using a 4× objective. Values are presented as group means ± SD, indicating the level of significance (**P*<.05, ***P*<.01 and ****P*<.001)

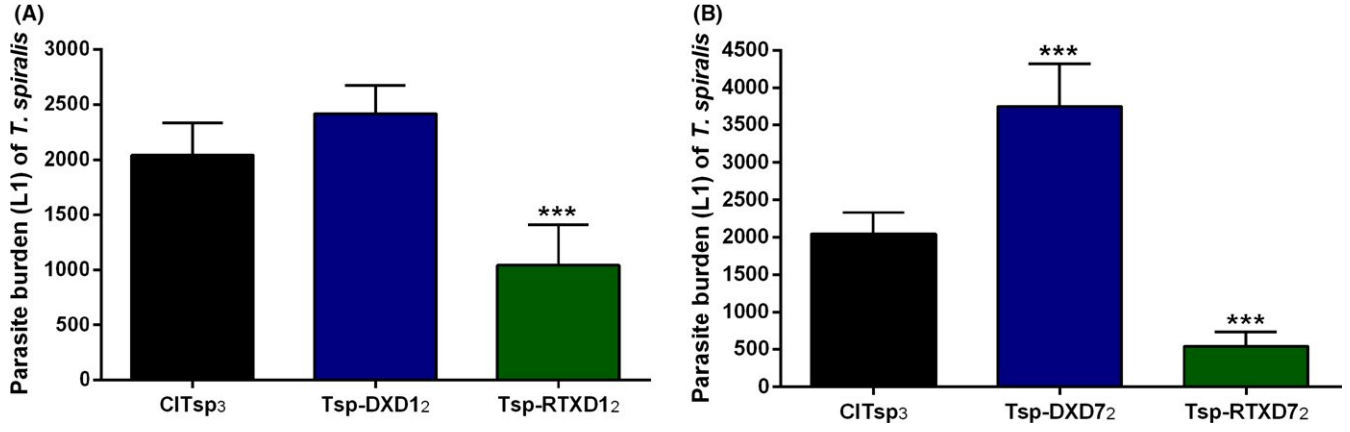

FIGURE 8 Determination of parasite burden in rats infected with *Trichinella spiralis* treated with DX and RTX at days (A) 1 and (B) 7 p.i. The parasite burden per 15 g of infected tissue of the CITsp (black bar), Tsp-$DXD1_2$ and Tsp-$DXD7_2$ (blue bars) TSP-$RTXD1_2$ and Tsp-$RTXD7_2$ (green bars). Values are presented as group means ± SD, indicating the level of significance (**P*<.05, ***P*<.01 and ****P*<.001)

and PGE_2 increased simultaneously to the development of the intestinal pathology during the intestinal phase of infection. Treatment with both DX (control anti-inflammatory) and RTX produced a decrease in the levels of serum TNF-α, NO and PGE_2, along with a reduction in the hyperplasia of the intestinal crypts and the reconstitution of the intestinal villus. This can be explained by the fact that steroidal anti-inflammatory agents (i.e. DX) suppress the expression of pro-inflammatory genes by immobilizing transcription factors such as

TABLE 1 IgM/IgG antibodies detection against *Trichinella spiralis* antigens in serum from rats during *T. spiralis* infection using the Dot-ELISA technique

Infection days	CITsp	Tsp-DXD1	Tsp-DXD7	Tsp-RTXD1	Tsp-RTXD7
(a) IgM detection					
D1	–	–		–	
D7	–		–		–
D14	–				
D21	+				
D28	+	+	+	+	–
(b) IgG detection					
D1	–	–		–	
D7	–		–		–
D14	+				
D21	+				
D28	+	+	+	–	–

NF-kB[47] and the activator protein-1 (AP-1) through a protein-protein interaction,[48,49] therefore preventing the transcription of both pro-inflammatory cytokines (e.g. TNF-α) and enzymes (e.g. iNOS, COX-2 and cytosolic phospholipase A, cPLA2). Finally, it results in the inhibition of the synthesis of NO and PGE_2 respectively.[50]

It is known that RTX performs most of its biological functions through the TRPV1 receptor by blocking and desensitization of both thermal nociception and pain, thus having an analgesic effect.[26,51] However, in vitro studies have demonstrated that RTX has an important anti-inflammatory effect, since it inhibits the expression of NF-kB in ML-1a cells stimulated with TNF-α in a dose-dependent manner.[27] Similarly, it was also demonstrated that RTX inhibits the expression of iNOS and COX-2 in RAW264.7 macrophages stimulated with LPS and IFN-γ, thus resulting in a decrease in PGE_2 and NO.[28] Another study based on a model of ischaemic acute renal failure showed that treatment with RTX prevented renal damage by inhibiting the inflammatory response by simultaneously inducing a decrease in the expression of renal TNF-α and an increase in plasma IL-10.[30] Our results agree with these studies since our data indicate a similar pharmacological effect of RTX during infection with *T. spiralis* (i.e. a significant decrease in the levels of serum TNF-α, NO and PGE_2). Based on these observations, we hypothesize that in our model, the decrease in TNF-α, NO and PGE_2 produced by treatment with DX and RTX, is associated with a reduction in intestinal pathology.

Given that eosinophils are prominent during inflammatory processes associated with helminth infection,[52] we evaluated the number of blood eosinophils during infection with *T. spiralis*. T-cells are stimulated by *T. spiralis* antigens in order to produce cytokines such as IL-4 and IL-5, which induce terminal differentiation and proliferation of eosinophils in the infected host,[53] thus promoting the inflammatory response that facilitates, along with the hypercontractility of the intestinal muscle cells, the expulsion of *T. spiralis*.[8] In our study, we observed a significant increase in the number of blood eosinophils during infection with *T. spiralis*, although it should be noted that the number of blood eosinophils decreased significantly following administration of DX and RTX. This can be explained by considering that glucocorticoids inhibit the survival of inflammatory cells (e.g. eosinophils[50]). It has been shown that DX induces apoptosis[54] and inhibits the survival of eosinophils in a dose-dependent manner,[55] which is consistent with our results on the pharmacological effect observed in DX treatment. Moreover, we showed that treatment with RTX significantly decreased the number of blood eosinophils. It is important to mention that, to our knowledge, this is the first report showing that treatment with RTX decreases the number of eosinophils in blood. Given that previous studies have associated the survival of eosinophils to the production of NO[56] and TNF-α,[57,58] it is reasonable to hypothesize that the observed decrease in the number of blood eosinophils is related to the lowering effects of RTX on the levels of NO and TNF-α, although further studies are needed to confirm this hypothesis.

Previous studies have shown that treatment with glucocorticoids favours the *T. spiralis* infection. One study showed that in rats treated with betamethasone were more susceptible to infection as shown by the increased parasite burden when compared to the infected control group.[22] Similar results were obtained in another study, which showed that treatment with DX increased the proportion of apoptotic and necrotic lymphocytes, as well as the number of larvae in the muscle tissue was slightly higher in mice treated with DX than in the control group.[23] Our results coincide with these investigations, because in our study we observed that treatment with DX during the intestinal phase of infection increased significantly both the implantation and parasite burden of *T. spiralis*. This is perhaps the consequence of DX systemic suppression of immune response.

On the other hand, we showed for the first time that treatment with RTX significantly decreased both the parasite burden and implantation of *T. spiralis*. Perhaps this decrease in parasite burden is under the influence of treatment with RTX, first associated with the decrease in the number of eosinophils, given that several studies have shown that the absence of eosinophils decrease the parasite burden [59] and eosinophils may influence the immune response in a manner that would sustain chronic infection and insure parasite survival in the host,[60-62] and secondly that the RTX is exerting an immunomodulatory effect preventing muscle invasion of *T. spiralis*. However, the

underlying mechanisms of the antagonistic effect of RTX on the life cycle of the parasite remains as an open question for future research.

It is known that specific early antibodies are related to the *T. spiralis* antigens present in the host at the beginning of the infection.[63] Based on these observations, we assessed whether there is a clear relationship between the parasite burden during treatment with RTX and DX and the humoral response of the infected host (i.e. rat) by detecting the anti-*T. spiralis* IgG and IgM antibodies. These antibodies are known to be present in the host at the beginning of the infection[64] since the polyclonal activation of the T lymphocytes, and particularly of the B-lymphocytes, is responsible of the high levels of the IgM and IgG immunoglobulins observed in infected animals.[65] IgM is one of the most important classes of immunoglobulins for it is the first one expressed in the membrane of the B cells in its monomeric form during their development. In addition, it is the first immunoglobulin produced (in its pentameric form) during the primary response to an antigen.[65] IgM antibodies are associated with a primary immune response and are used frequently to diagnose an acute exposition to an immunogen and/or a pathogen.[66] It has been previously reported that during trichinellosis, specific anti-*Trichinella* IgM antibodies can be found from the second week after infection.[67,68] In our study, we detected anti-*T. spiralis* IgM antibodies at the third and fourth weeks after infection (Table 1a). Similarly, we found anti-*T. spiralis* antibodies at week four following treatment with DX and RTX, which resembles the results obtained from the control group. These results seem to indicate that both treatments are not related to the synthesis of anti-*Trichinella* IgM antibodies.

On the other hand, IgG is the most abundant immunoglobulin in mice and humans. This family of molecules is composed of four subclasses: IgG1-4 in humans and IgG1, IgG2a, IgG2b and IgG3 in mice.[66] During trichinellosis, the IgG-specific antibodies are involved in the inflammatory response to infection, showing an increase during the muscular phase.[69] IgG antibodies have been found at the third week,[34] and even 3 years after infection.[63,68] Here, we detected anti-*Trichinella* IgG antibodies both at the second (Table 1b) and fourth week after infection in our infected control group. After treatment with DX, we were able to find anti-*T. spiralis* IgG antibodies at the fourth week. In contrast, following treatment with RTX, we did not find anti-*T. spiralis* IgG antibodies at the fourth week after infection. Based on these results and on the previous reports mentioned above, we hypothesize that the diminished humoral response after treatment with RTX could be associated with a decrease in the parasite burden due to a lower antigenic expression (not detectable in this model). Further studies are needed to confirm this hypothesis.

In conclusion, our current opinion according with our results is that treatment with RTX shows a similar anti-inflammatory effect to that produced by DX, since both lowered the serum levels of pro-inflammatory mediators such as TNF-α, NO and PGE_2, as well as the number of blood eosinophils, which are associated with a diminished intestinal pathology. It is worth noting that treatment with RTX poses the advantage that it prevents the side effects produced by steroidal anti-inflammatory drugs in the muscular phase of infection with *T. spiralis*, which can be explained by the fact that RTX decreases both the parasite burden and the humoral response, thus protecting the host against *T. spiralis*. Future research should be focused on gaining understanding about the mechanisms underlying the effects of RTX on the intestinal immune response during *T. spiralis* infection, which will allow us to assess the use of RTX as a possible treatment for inflammatory diseases.

ACKNOWLEDGEMENTS

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