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Original Article

The immunomodulatory effects of rolipram abolish drug-resistant latent phase of *Toxoplasma gondii* infection in a murine model

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ABSTRACT

Background: Latent toxoplasmosis always has the risk of reactivation leading to significant sequelae. The available medications, for chronic toxoplasmosis, are awfully limited by resistance of *Toxoplasma* cysts. Therefore, there is a growing necessity for novel therapeutic approaches. Agents increasing cAMP levels and downregulating proinflammatory cytokine could inhibit *Toxoplasma* conversion to the bradyzoite stage. This study explores a potential immunomodulatory effect of rolipram, a PDE4 inhibitor, on the course of experimental toxoplasmosis and links this role to deterrence of the resistant chronic phase of the disease.
Materials and methods: Mice infected with low pathogenic strain of *Toxoplasma gondii* were treated with rolipram for three weeks. The effect of rolipram was evaluated through tissue injury scoring, brain cyst count, specific IgG titers as well as TNF-α, IFN-γ and IL-12 assays.
Results: Rolipram was partially able to prevent the progression to chronic toxoplasmosis. *Toxoplasma* brain cyst burden showed a 74% reduction while *Toxoplasma*-induced inflammatory foci per liver area and nucleated cells per inflammatory focus were significantly reduced: 57.14% and 61.3% respectively. Significant reduction of TNF-α (84.6%), IFN-γ (76.7%) and IL-12 (71%) levels was demonstrated along with significant inhibition of anti-*Toxoplasma* antibody response.
Conclusion: Rolipram efficiently modulated the *Toxoplasma*-induced immunological changes with a consequent remission of chronic toxoplasmosis. This study is the first to report the utilization of PDE4 inhibitors as possible immune modulators of chronic phase of *Toxoplasma* infection.

1. Introduction

Toxoplasmosis is a cosmopolitan infection caused by *Toxoplasma gondiii*, an obligatory intracellular parasite. It is estimated that 30–50% of the world population are infected with the parasite, representing one of the most prevalent infections among humans [1]. Latent toxoplasmosis always has the risk of reactivation, in immunocompromised patients, leading to acute encephalitis [2] or relapsing ophthalmitis in immunocompetents [3]. Current therapy relies on agents that can suppress active infection with no effect on the latent phase of the disease [4]. The available medications are awfully limited by resistance of *Toxoplasma* cysts and drug toxicity to the children, pregnant

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women and immunocompromised patients [5]. Interference with the pathways mediating tachyzoite–bradyzoite interconversion is crucial for modulating the pathogenesis of *Toxoplasma* infection.

Cyclic nucleotide phosphodiesterases (PDEs) are critical modulators of cellular levels of cAMP catalyzing cyclic nucleotide hydrolysis [6]. Agents causing high cAMP levels have been reported to inhibit *Toxoplasma* conversion to the bradyzoite stage [7]. PDE4 inhibitors could interfere with tachyzoite–bradyzoite interconversion due to suppression of cytokines; TNF-α, IFN-γ and IL-12, having pivotal roles in this transition [8,9]. While proinflammatory cytokines, especially IFN-γ and TNF-α, are critical for an effective checking of *Toxoplasma* growth and dissemination, they are damaging when overproduced [10]. Complete neutralization or inhibition of pro-inflammatory cytokines could lead to an exacerbated acute disease. Neutralization of IL-12 has shown to result in an overwhelming *Toxoplasma* proliferation and a severe acute stage in infected mice [11].

This study represents an alternative approach for better management of latent toxoplasmosis. The purpose of the study was to explore a potential role of rolipram, a PDE4 inhibitor, in aborting the progression to chronic toxoplasmosis. The roles played by pro-inflammatory cytokines to mediate this progression were highlighted.

2. Materials and methods

2.1. Animals

Female Swiss albino mice, 6–8 weeks old and weighting 25–30 g (from a local KAU university vendor) were kept under standard laboratory conditions. The mice had free access to standard diet and water throughout the experiment. Animal experimentations were performed in accordance with the Code of Ethics of EU Directive 2010/63/EU for animal experiments [12]. Mice were divided into three groups (10 mice each). An infection control group (GI) were challenged with *T. gondii* (KSU strain). The second group (GII) received, day 7 post infection and for three weeks, 10 mg/kg/day rolipram (Sigma, St. Louis, MO, USA) by oral gavage. A drug-control group (GIII) received rolipram (as in GII) without a parasitic challenge. A single rolipram concentration representing the highest but safe therapeutic dosage [13], was used in the study.

2.2. Parasitic challenge

Mice of GI and GII were challenged with intraperitoneal (i.p.) injection of 20 *Toxoplasma* tissue cysts in a total volume of 0.1 ml sterile brain emulsion of a Swiss albino mouse infected two months earlier. The low pathogenic, cyst-forming KSU strain of *T. gondii* (a generous gift from Ain-shams Diagnostic and Research Unit, Cairo, Egypt) was used.

2.3. Rolipram preparation

Rolipram was initially dissolved, at a concentration of 1 mg/10 μl DMSO. A final concentration of 1 mg/ml was reached by dilution with an appropriate volume of phosphate-buffered saline (PBS). The final concentration of the vehicle (DMSO) was 1%. Mice of relevant groups were treated with rolipram as mentioned above.

2.4. Blood and tissue sampling

On day 14 post-infection (PI), 3 mice/group were anesthetized and then euthanized by cervical dislocation. Liver excision and blood sampling were done using standard procedures. Blood sampling was repeated at day-28 PI (after completion of rolipram treatment), for the rest of mice (7 mice/group). On day 50 PI, 7 mice/group were euthanized and similar processes of blood sampling and brain harvesting were performed.

Blood samples were centrifuged, sera were separated and then stored at −80 °C for later analytical assays. Recovered brains were immediately homogenized for cyst counting. Liver samples were fixed in buffered formalin (10%) for histological examination.

2.5. Brain cyst counting

Isolated brains, from all mice groups (day-50 PI), were crushed individually in a mortar. The grinded brain tissue was dispersed in 2 ml saline by passing it through needles with decreasing gauge sizes. The number of cysts, per individual brain, was then counted microscopically in 10 ml of the brain emulsion and the entire number of cysts per brain was calculated.

2.6. Histopathological examination

Formol-fixed liver samples, harvested on day-14 PI, were immersed in paraffin, cut into 5 μm-thickness sections and stained with hematoxylin and eosin. The liver sections were assessed microscopically for inflammatory foci and nucleated cells. Inflammatory foci, defined as isolated cluster of 6–60 nucleated cells, were quantified as previously described [14]. They were counted, at a magnification of ×100, in 8 mm^2 surface area of liver tissue. Nucleated cells/inflammatory focus were counted at a ×400 magnification.

2.7. Antibody assays

Dye test (DT) was performed on serum, recovered from all remaining mice (7/group) at day-50 PI. A micro-modified method of Sabin-Feldman assay was performed [15]. In a microtiter plate, a mixture of 2×10^6 tachyzoites in 50% accessory factor was added to assigned volumes of mice sera to get serial four-fold dilutions. Tachyzoites, of RH strain, were freshly recovered by peritoneal lavage of mice infected two days earlier while accessory factor was a *Toxoplasma*-negative serum checked for specific antibodies in a previous dye test. After 1 h incubation at 37 °C, titers were read as the sera dilutions showing 50% parasite killing. End-points were determined, after the addition of methylene blue, using a phase contrast microscopy. Titers ≥1/16 were considered positive.

2.8. Cytokine assays

Commercially available ELISA kits for mouse cytokines (R&D Systems, MN, USA) were used to determine the levels (pg/ml) of TNF-α, IFN-γ and IL-12-P40 in mice sera collected at day-14 and 28 PI. The assay was run in duplicates following the manufacturer's instructions. The lower sensitivity limits of TNF-α, IFN-γ and IL-12 assays were 5 pg/ml, 2 pg/ml and 7.8 pg/ml respectively. Cytokine levels below the assay's threshold of detection were assigned hypothetical values of the lower limit of sensitivity of each assay.

2.9. Statistical analysis

The results are demonstrated as mean ± SD. Several comparisons, related to different variables among all groups, were made using SPSS software, version 18 (SPSS Inc., Chicago, IL). Two-tailed Student (*t*-) test was used and $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Scored liver pathology

Toxoplasma-infected mice treated with rolipram developed significantly fewer inflammatory foci than *Toxoplasma*-infected group. Number of cells per focus significantly regressed and the inflammatory foci became smaller and less frequent. The inflammatory foci/liver area were decreased to less than one-half (57% reduction), and the numbers of nucleated cells/inflammatory focus were decreased by 61%, in rolipram-treated mice compared to infected control mice (Fig. 1).

3.2. Brain cyst burden

The results showed also a mean suppression of 74% of *T. gondii* brain cyst formation under the influence of rolipram treatment. The number of brain tissue cysts, has been significantly ($p < 0.001$) decreased, almost four fold, in rolipram-treated mice compared to non-treated ones (Fig. 2A).

3.3. Anti-Toxoplasma antibody

The reduction in parasitic load and tissue inflammatory reactions was reflected in a highly significant ($p < 0.001$) reduction of anti-*Toxoplasma* antibody titers (Fig. 2B). The results showed a 61% suppression of antibody response to *T. gondii* infection mediated by rolipram treatment.

3.4. Cytokines profile

Compared to that of the infection control, TNF-α only showed a highly significant ($p < 0.001$) reduction while a just significant ($p < 0.05$) suppression of both IFN-γ and IL-12 levels was demonstrated at day-14 PI (Fig. 3A). The cytokine pattern was considerably different at day-28 PI (Fig. 3B), where a highly significant ($p < 0.001$) reduction of all tested cytokines, in rolipram-treated infected mice, was demonstrated. Percentages of reduction as high as 84.6%, 76.7% and 71% were demonstrated for TNF-α, IFN-γ and IL-12 respectively.

4. Discussion

We have explored the immunomodulatory role of rolipram, a selective PDE4 inhibitor, in a murine model of chronic toxoplasmosis. The current results complement and explain a previous report [16] confirming that rolipram, is both necessary and sufficient to mitigate the classic immunological and pathological patterns of *Toxoplasma* infection.

Many reasons were behind our choice to use rolipram. While a selective PDE4 inhibitor, rolipram inhibits all PDE4 subtypes with comparable potencies [17]. Rolipram has a highly selective potency in brain tissue, where PDE4 are mainly found [18], and where *T. gondii* prefers to establish its chronic state of infection [19].

Two main concerns raised while applying a PDE4 inhibitor to modulate *T. gondii* infection. First, is an expected exacerbation of the acute stage which could lead to a lethal outcome. The second concern was a possible reactivation of the chronic stage leading to a fatal toxoplasmic encephalitis. PDE4 inhibitors have a suppressant effect on pro-inflammatory cytokines [8] which have critical roles in both resisting acute infection and stabilizing the chronic stage of toxoplasmosis [9]. Previous studies reported an overwhelming proliferation of the parasite and a severe acute *T. gondii* infection in mice on neutralizing IL-12 and other proinflammatory cytokines [11]. Other reports showed that neutralization of TNF-α in a chronically infected mice led to reactivation of chronic toxoplasmosis and a fatal outcome of the disease [20]. Additionally, mice deficient in certain TNF receptors developed fatal toxoplasmic encephalitis [21]. However both concerns did not come true in our study. We suggest that TNF-α, IFN-γ and IL-12 levels, while reduced, helped rolipram-treated mice to avoid an exacerbated acute phase. Conversely, it is possible that their levels were not low enough to deter the transition to a chronic but interestingly incomplete state.

The results interestingly showed that rolipram efficiently induced a remission of chronic toxoplasmic lesions, with a significant reduction of liver pathology and brain cysts. This finding is explicable because potent cAMP-mediated anti-inflammatory effects of rolipram, have been demonstrated in various cellular and animal models [22]. Rolipram opposed the TNF-α-induced loss in barrier integrity of the corneal endothelium [23].

Rolipram-induced mitigation of liver injury, shown in this study, is also explainable and complementary to what we have reported earlier [16]. It could be, in part, due to significant regression in TNF-α release with subsequent prevention of its tissue damaging effect. However, the observed marked reduction of IFN-γ and IL-12 levels could have a share in this effect. Interferon-γ and IL-12 interplay with TNF-α to mediate *Toxoplasma*-associated pathology. Thus their observed down-regulation might have mediated the anti-inflammatory action of rolipram. Furthermore, IFN-γ is a critical TNF-α sensitivity priming factor. Appropriate priming of hepatocytes with IFN-γ, makes them more susceptible to TNF-α damaging effect

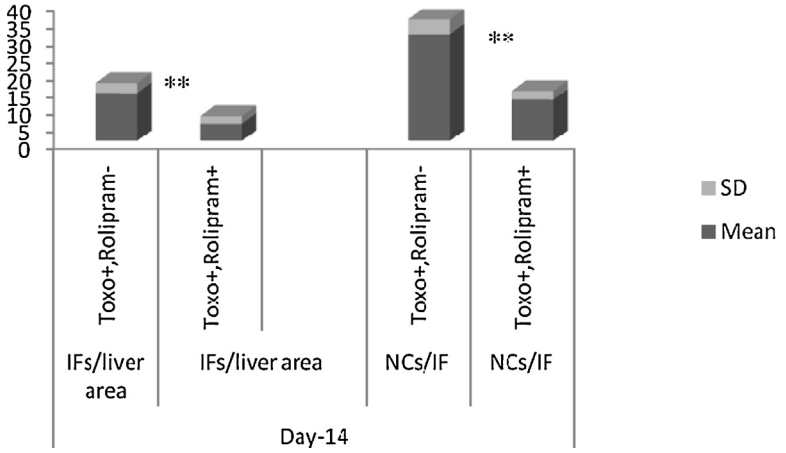

Fig. 1. Scored liver pathology to show the modulatory role of rolipram in *T. gondii*-infected mice (day-14 PI). Number of inflammatory foci (IF) shown is the mean of that counted in a liver tissue area $1 \times 8\ mm^2$. Number of nucleated cells (NCs) shown per IF is the mean of 20 foci. Each number represents the mean of three mice. **Highly significant difference, $p < 0.001$.

[24]. Rolipram suppresses nitric oxide production independent of its inhibitory effect on TNF-α or IL-12 secretion [25] suggesting a role for the anti-oxidant action of rolipram in the mitigation of the observed liver pathology.

Investigation of the individual role of PDE4 subtypes was beyond the scope of our study. However, we speculate that rolipram inhibition of phosphodiestrase B-subtype (PDE4B) might mediate the beneficial effects observed in

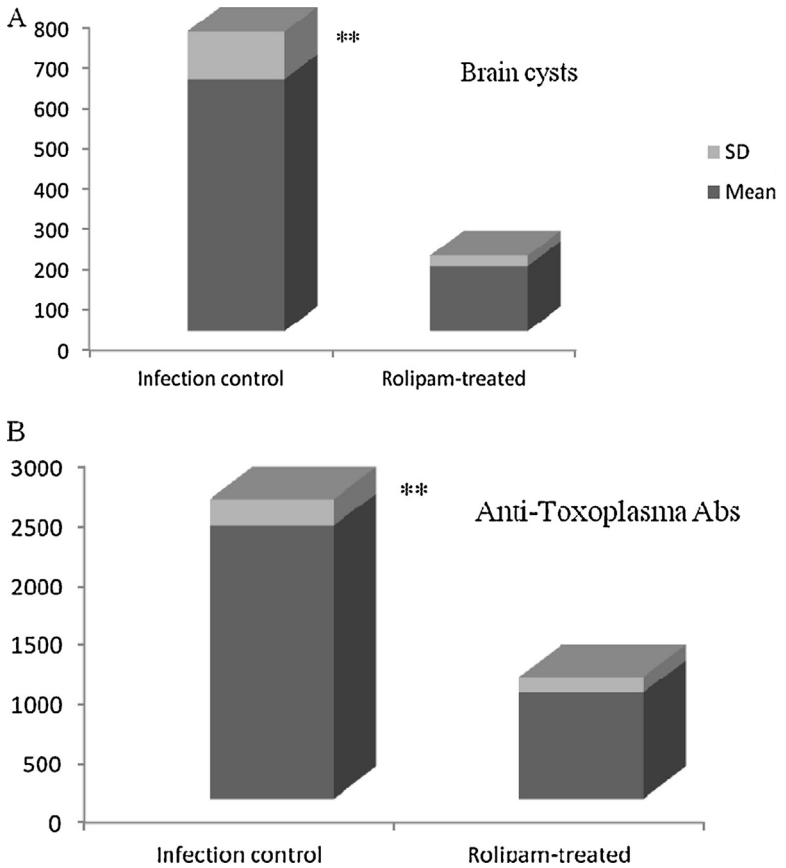

Fig. 2. Effect of rolipram on *T. gondii*-infected mice at day-50 PI. Each number represents the mean of seven mice. (A) Brain tissue cyst burden. Number of brain cysts shown is the calculated mean of an overall cyst burden in mice brains counted in 10 ml brain emulsion per mouse. (B) Mean titers of total anti-*Toxoplasma* antibodies assayed by DT in sera of infected mice. **Highly significant difference, $p < 0.001$.

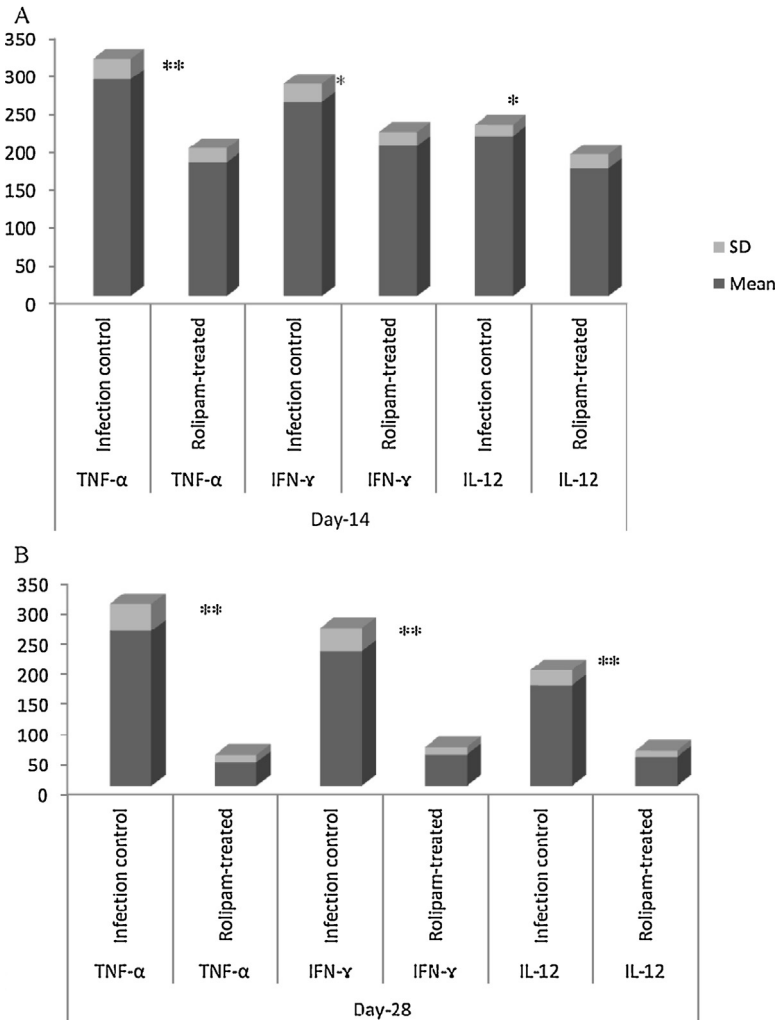

Fig. 3. Effect of rolipram on TNF-α, IFN-γ and IL-12 (pg/ml) release in *T. gondii*-infected mice at day-14 (A) and day-28 (B) PI. Numbers represent means for 3–7 mice. *Significant difference, $p < 0.05$, **highly significant difference, $p < 0.001$.

this study. PDE4B was previously considered to be essential for lipopolysaccharide-activated TNF-α responses [16].

Many factors might be involved in partial resolve of pathology and incomplete inhibition of the chronic phase. However, we cannot exclude that the rolipram has just achieved a partial block of TNF-α, IFN-γ or IL-12 release. We think that rolipram was able to keep cytokines at low levels enough to resist the infection while still able to inflict a form of tissue injury that is considerably mitigated.

Parasite's strain variability might have a share in the pathology-mitigating effects of rolipram, demonstrated in our study. Low pathogenic, cyst-forming strains of *T. gondii* are particularly suitable for cAMP manipulation by PDE4 inhibitors. This finding was not observed in virulent, poorly differentiating strains [7]. Therefore, it is likely to observe different treatment responses with different infecting strains of the parasite.

Despite its potency as an anti-inflammatory drug, previous clinical studies reported undesirable adverse effects of rolipram mainly severe nausea and vomiting [26]. The intolerable emetic action of rolipram might be due to inhibition of the D-subtype (PDE4D) [27]. Therefore, a selective PDE4B inhibitor, sparing PDE4D, would serve as better anti-*Toxoplasma* drug with no emetic action. Moreover, considering the unmet need for new therapies and the interest in a short course of treatment, the necessity for strict selectivity might be diminished if the drug was efficacious in a shorter course time compared to current medications.

Conclusion

A novel approach, to halt transition to the drug-resistant chronic toxoplasmosis rather than treating it, was introduced. Our findings are the first to link the anti-inflammatory action of PDE4 inhibitors to the modulation of the course of toxoplasmosis presenting rolipram as a useful modulator of *Toxoplasma*-induced tissue injury.

Conflict of interest

The authors declare that there is no conflict of interest. The authors also confirm that no part of this work has been submitted or published elsewhere.

Ethical consideration

The work with laboratory animals, described in this study, has been carried out in accordance with the Code of Ethics of EU Directive 2010/63/EU for animal experiments.

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