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Original Article

Toxoplasma gondii Suppresses Th2-Induced by *Trichinella spiralis* Infection and Downregulates Serine Protease Genes Expression: A Critical Role in Vaccine Development

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Abstract

Background: *Toxoplasma gondii* coinfection can modify host immune responses and the severity and spread of other parasites. We investigated how *T. gondii* and *Trichinella spiralis* infections counter-regulate each other's immune responses.

Methods: The parasite burden, the expression of *T. gondii* rhoptry kinase ROP18 and *T. spiralis* putative serine protease (TsSP), the IgG1 and IgG2a responses, besides histopathological and immunohistochemical staining with iNOS and arginase were used to evaluate the dynamics of coinfection.

Results: Through their effects on host immune responsiveness, coinfection with *T. gondii* modified the virulence of *T. spiralis* infection. Coinfected animals with high and low doses of *T. gondii* demonstrated significant reductions in the *T. spiralis* burden of 75.2% and 68.2%, respectively. TsSP expression was downregulated in both groups by 96.2% and 86.7%, whereasROP18 expression was downregulated by only 6% and10.6%, respectively. In coinfected mice, elevated levels of *T. gondii*-specific IgG2a antibodies were detected. Th1 induced by *T. gondii* inhibits the Th2 response to *T. spiralis* in coinfected animals with high iNOS expression andlow-arginine1 expression.

Conclusion: *T. gondii* infection induces a shift toward a Th1-type immune response while suppressing a helminth-specific Th2 immune response, paving the way for developing novel vaccines and more efficient control strategies.

Introduction

Coinfection with many parasite or pathogen species is likely to be the norm rather than the exception in most biological systems, including human societies with limited medical access. The interactions of many common parasites and pathogens are poorly understood, despite the fact that co-infection research is common for diseases. Coinfection must be investigated because it can alter both treatment and susceptibility to infection (1). Furthermore, coexposure to multiple pathogens is a natural occurrence (2), and coinfection affects more than one-sixth of the world's population (3). The interaction between two or more co-infecting parasites can have various repercussions. It may contribute to the aggravation or amelioration of the pathophysiology of either infection by modifying the immune profile and/or parasite interactions (4).

Currently, more than 2 billion people are infected with helminth parasites, which cause serious, neglected tropical diseases in both humans and animals (5). Helminths can infect and remain in their hosts for extended periods of time, causing chronic infections with severe health consequences for the host. They suppress the immune response to maintain their life cycle (6).

Trichinella spiralis is the primary agent responsible for trichinosis, which affects 12 million individuals worldwide (7). The immune evasion of *T. spiralis* and stage-specific antigen diversity present obstacles to the development of effective vaccines. In most nematode-endemic regions, toxoplasmosis, malaria, and tuberculosis typically co-occur (8).

The parasite *Toxoplasma gondii* infects warm-blooded vertebrates via the gastrointestinal tract, causing a dormant stage in muscle and brain tissue (9). In some regions, *T. gondii* seroprevalence can approach 70%, with most immunocompetent individuals being asymptomatic and at a high risk of coinfection with other parasites found in the general population (10). In contrast to helminth infections, *T. gondii* infection is controlled by an entirely different immune response.

IgG1 and IgE antibodies are characteristic of helminth infections, which are characterized by the activation of CD4+ T helper 2 (Th2) cells that produce the cytokines interleukin IL-13, IL-9, IL-5, and IL-4 (11). In contrast, protozoal infections induce a Th1 response that produces pro-inflammatory mediators such as IFN, nitric oxide, and IL-12. These two types of immune responses counter-regulate each other; therefore, enhancing one type of immune response inhibits the other (12).

T. gondii possesses a pathogenicity locus referred to as *T. gondii* rhoptry kinase (ROP18), a highly polymorphic rhoptry protein kinase that is significantly expressed in type I and II strains (13). Serine protease is a proteolytic enzyme that contributes to parasite tissue penetration, larval development, and survival, while TsSP is a sensitive and specific early diagnostic marker for trichinellosis detection (14).

In this study, the parasite burden, ROP18, TsSP expression, and antibody response (IgG1 & IgG2a) in mice infected with a single or mixed dose of both parasites were analyzed to gain a better understanding of the dynamics of coinfection with *T. gondii* and *T.* spiralis besides to how the distinct immune responses induced by each parasite counter-regulate the other. Histopathological evaluations, iNOS, and arginase immunohistochemical staining were performed.

Materials and Methods

Animals

Male Swiss albino mice were raised in the animal house under specified pathogen-free conditions using 8-week-old Swiss albino mice

purchased from the Animal House at Faculty of Medicine, Zagazig University, Egypt.

The tests were approved by the Animal Ethics Committee for Animal Protection and conducted in accordance with the National Animal Protection Guidelines (ZU-IACUC/3/F/165/2022).

T. gondii Infection

The *T. gondii* ME49 strain was obtained from the Department of Parasitology, Faculty of Medicine, Zagazig University, and then maintained through continuous passage in cyst-infected mice. The parasite cysts were obtained from infected mice with chronic infections. Following animal sacrifice, the brain of a mouse was homogenized in saline. The cyst count in the homogenate was determined using light microscopy (15). The infection was administered orally to mice at either a low dose of 10-tissue cysts/animal (16) or a high dose of 100-tissue cysts/mouse (17).

T. spiralis infection

The employed *T. spiralis* isolate was initially isolated from diseased pork in Cairo and maintained in the laboratory of the Medical Parasitology Department at Faculty of Medicine, Zagazig through repeated passages in mice. Muscle larvae were obtained from experimentally infected mice with *T. spiralis* 42 days post infection (dpi) (18). A high dose of 400 larvae per mouse was administered orally (19).

Experiment

Six groups of ten mice each, were categorized as controls: group I; naive mice, group II; mice inoculated with an elevated dose of *T. gondii*, group III; mice infected with a low dose of *T. gondii*, group IV; and mice infected with *T. spiralis*. Furthermore, group V consisted of mice that were coinfected with high doses of *T. gondii* and *T. spiralis*, whereas group VI consisted of mice that were coinfected with low doses of *T. gondii* and *T. spiralis*. All mice groups were infected at the same time.

Parasitological assessment

Survival rate

Throughout the duration of the experiment, the daily death rate of mice was recorded, and the survival rate was determined.

Collection of T. spiralis larvae in muscles

On day 42-post infection, the muscular phase of infection and the burden of muscle larvae were examined using artificial digestion (20, 21). The sediment was tallied using a stereomicroscope at 40× magnification.

Toxoplasma gondii brain cyst burden

At the chronic phase (42 days post infection) phase, brains with tissue cysts were obtained from each group, homogenized, stained with Giemsa stain, and counted under a microscope (22).

T. gondii ROP18 and T. spiralis serine protease

The QIAamp RNeasy Mini kit (Qiagen, Germany, GmbH) was used to extract RNA from tissue Animal Tissues protocol of the manufacture kits was followed.

Oligonucleotide primers

The used primers were supplied by Metabion (Germany). Primer sequences for gene amplification were *T. gondii* ß actin forward: TCCCGTC-TATCGTCGGA-AAG, reverse CCATTCCGAC-CATGATAC (23), *T. gondii* ROP18 forward: CGCTGGTGAGAGGTGCAC, reverse: GAC-CGTCTTTCAAGAGGAGG (24), *T.spiralis* GAPDH forward: GCAGC-TATGGATGTTCAGGTG, reverse: TAC-GGCTGACAGCATGATTT (25), *T. spiralis* serine protease: forward: CTT TTCAAGTGCTTATTTCTC, reverse TATTACCCGCTTTTCTGAA (14).

Analysis of the SYBR green rt-PCR results.

Standard curves and Ct of the targeted genes were determined by Agilent MX3005P software. The obtained threshold of each sample was compared with that of the positive control to es-

timate the variation of gene expression consistent with Yuan et al. (26).

Determination of antigen- specific IgG sub-types

The ELISA RayBio® method identified specific IgG1 and IgG2a antibodies against *T. gondii* and *T. spiralis*. The optical density at 450 nm was measured using a Biotech ELx800 microplate reader (Biotech, USA).

Histopathological assessment

Samples of formalin-preserved muscle and brain samples were fixed and dehydrated. The 4–5 um paraffin sections were stained with Hematoxylin and eosin (27).

Immunohistochemistry (Arginase and iNOS expression)

Standard immunohistochemical procedures were used, and the tissue slices were microwaved to distinguish the antigen's epitopes (28). Immunostaining requires a two-step process. Firstly, the binding of the primary antibody to the related antigen, followed by visualization of reaction by a link antibody to which are attached different enzyme systems. In addition, the biotin-streptavidin (BSA) system to view the markers (28). Diaminobenzidine (DAB) was used as a chromogen because it permits a permanent preparation, while Hematoxylin was used as a contrast dye. The sections were then treated with one to two drops of the ultrasensitive monoclonal primary antibody [against inducible nitric oxide synthase (INOS) and arginase (ARG)].

Statistics

The results were displayed as the mean ±SEM. Statistical analysis was performed using GraphPad Prism. In order to determine the level of significance, the Mann–Whitney U-test or Kruskal–Wallis and Dunn's multiple comparison tests were used.

Results

The *T. spiralis* larval load was significantly reduced in GV (coinfected with *T. spiralis* and a high dose of *T. gondii*) and GVI (coinfected with *T. spiralis* and a low dose of *T. gondii*) compared with the *T. spiralis*-infected GIV control. The *T. gondii* brain cyst burden of the coinfected GV compared to the control GII infected with high doses of *T. gondii* and the coinfected GVI compared to the control GIII infected with low doses of *T. gondii* did not differ significantly between the two groups (Fig.1A, B).GV had the greatest reduction (75.2%) of *T. spiralis* larvae, followed by GVI with 68.2%, whereas GVI and GV had only modest reductions of *T. gondii* brain cyst burdens at10.2% and 8% (Fig.1H).

T. spiralis putative serine protease (TsSP) expression was downregulated by 96.2% in GV and 86.7% in GVI compared with GIV, a statistically significant difference. The expression of *T. gondii* rhoptry kinase (ROP18) between the coinfected GV and the control GII, as well as between the coinfected GVI and the control GIII, did not differ statistically (Fig.1 C,D), while there was a negligible downregulation of ROP18 gene expression in GV and GVI (Fig.1H).

There was a highly statistically significant difference in the IgG2a antibody levels of the targeted coinfected GV, a statistically significant difference in the coinfected GVI, and no statistically significant difference in the IgG2a antibody levels of the GIV in comparison with the normal GI. Additionally, the serum levels of IgG1antibodies in the targeted coinfected GV and GVI did not differ significantly from those of the GI. GIV exhibited a high statistically significant difference (Fig.1 E, F).

Infection with *T. spiralis* larvae is associated with a mild inflammatory response of muscle tissue with preserved longitudinal cross striations and peripheral multinuclear arrangement (light blue arrows) in GV (Fig.2B).

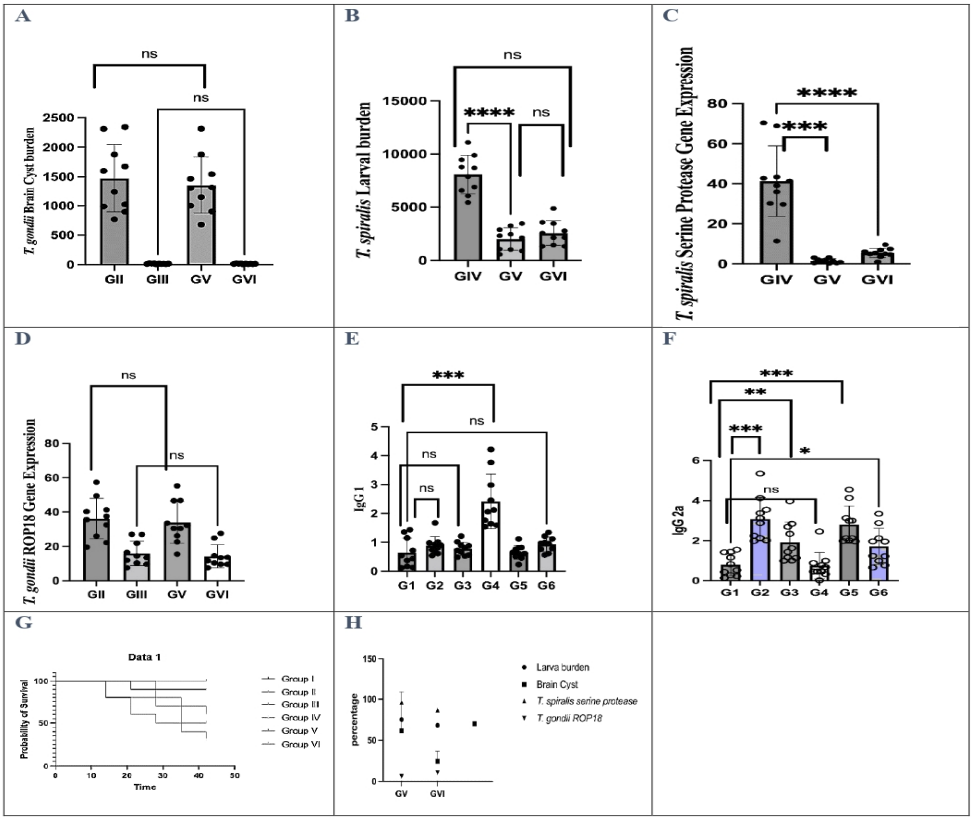

Fig. 1: A dominant Th1 response elicited by *T. gondii* inhibits T. spiralis Th2 response, reducing *T. spiralis* larval burden, downregulating serine protease gene expression, and decreasing IgG2a antibody response in co-infected groups

In contrast to the control group GIV, which exhibited a severe *T. spiralis* infestation and a strong hypersensitive inflammatory reaction, GVI exhibited a moderate inflammatory reaction of mixed cellular type (Fig. 2C). In the vicinity of the parasite (dark blue arrows), muscle fibers exhibit pressure atrophy (light blue arrow), hyaline degeneration, marked congestion, edema, allergic myositis, and necrosis (black and yellow arrows) (Fig.2 A).In brain tissue, the embedded *T. gondii* bradyzoites appeared as dark bluish structures in the aggregated mass, whereas the control groups GII and GIII displayed vague cyst-like structures (black arrows) (Fig.2D). Additionally, the cerebral hemisphere demonstrated severe tissue reactions, including neuronal degeneration, neutrophils demyelination (dark blue arrow), microgliosis (yellow arrow), and focal periventricular oligodendrogliosis (light blue arrows), in addition to meningeal hyperemia with round cell infiltration (lymphocytic meningitis) (black arrows) in GV and GVI (Fig. 1E).

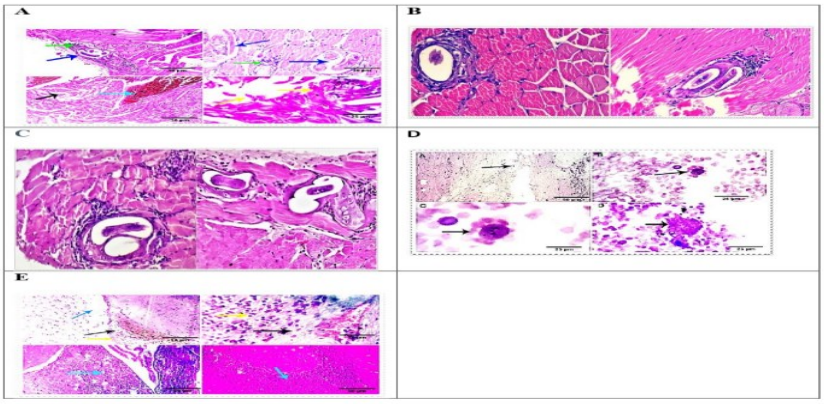

Fig. 2: Photomicrograph of H&E from muscle and brain, with scale bars of 25 and 50 um

The production of iNOS was examined in brain and muscle tissues. Brain sections revealed with strong expression in sub-meningeal round cells (lymphocytes), other leukocytes (neutrophils and microglial cells), and some degenerated glial and neuronal cells. Additionally, the vascular endothelial cells were highly expressed (blue arrows). Muscle sections displayed strong immunoreactivity in degenerated muscle fibers, infiltrating inflammatory cells, and dilated intramuscular capillary endothelium (blue arrows) (Fig. 3C). Similar immunoreactivity was observed in control groups of brain tissue infected with *T. gondii* GII and GIII (Fig. 3A), whereas GVI showed moderate expression in brain tissue with high expressed vascular endothelial cells (blue arrows) (Fig.3E). In contrast, muscle sections from the control group infected with *T. spiralis* GIV exhibited low iNOS expression (Fig. 2B).

Arginase activity was extremely low in microglial cells and some degenerated neuronal cells, with mild reactivities in some degenerated muscle fibers in the targeted coinfected GV mice (yellow arrows) (Fig.3D). Brain tissue control groups infected with *T. gondii* GII and GIII exhibited comparable immunoreactivity (Fig.3A). In GVI, low expression was observed in sub-meningeal infiltrated round cells (lymphocytes) and in some degenerated glial and neuronal cells, as well as mild reactivity in some degenerated muscle fibers and mildly dilated intramuscular capillary endothelia (blue arrows) (Fig.3F). In contrast, the strong expression was observed in the degenerated muscle fibers and vascular endothelial cells of GIV (blue arrows) (Fig.3B).

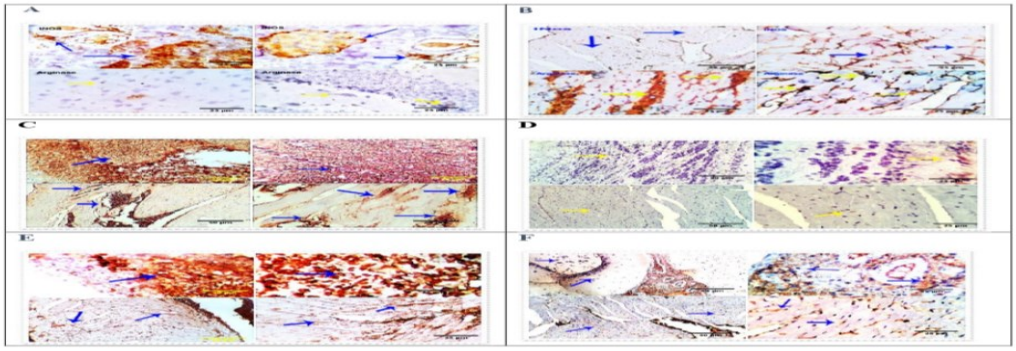

Fig. 3: Immunostained photomicrograph of iNOS and Arginase (scale bars 25um, 50 um)

Discussion

The nature of the immune response to infection is influenced by the nature of the infecting organism. In addition to classical macrophage activity, *T. gondii* induces a Th1-immune response. *T. spiralis* induces Th2 responses. Given the prevalence of coinfections with these parasites in the field (30), it is crucial to understand how the unique characteristics of each parasite's immune responses may affect or counter-regulate the immune responses of the other. In this study, *T. gondii* infection inhibited the local and systemic Th2 responses normally induced by infection with *T. spiralis*. The *T. spiralis* larval load was significantly reduced in the coinfected groups with high and low doses of *T. gondii* compared to the *T. spiralis*-infected GIV by 75.2% and 68.2%, respectively. The prevalence of *T. gondii* brain cysts decreased negligibly in both groups.

This study suggests that *T. gondii* infection can inhibit the host's ability to generate a Th2-polarized immune response to coinfection with *T. spiralis*. The development of a Th2-polarized immune response to certain helminth infections was inhibited in mice coinfected with malaria (31, 32). Our findings support these findings. These results suggest that a robust Th1-targeted immune response to *T. gondii* infection may inhibit the development of Th2-polarized immune responses to coinfection with helminths (33). As long as *T. spiralis* has no effect on the Th1 response of *T. gondii*; the effect of *T. spiralis* on the toxoplasmosis parasite burden in coinfected mice was insignificant. The observed decrease in Th2 responses may be attributable to perturbed priming and polarization events at various stages, such as insufficient stimulation of naive CD4+ helper T cells and altered dendritic cell function. Additionally, infection with *T. gondii* impairs the formation and function of naive T cells (34).

TsSP and ROP18 expression were measured to confirm that a decrease in the parasite load correlates with a decrease in parasite virulence. ROP18 plays a crucial role in determining the virulence of *T. gondii* infections (35). TsSP expression was significantly reduced in muscle tissue of the targeted coinfected GV (96.2%) and GVI (86.7%) compared to the control GIV. Although there was a slight shift in ROP18 expression in the brain tissue of the targeted GV and GVI by 6% and 10.6%, respectively, there was no significant difference. TsSP plays a role in the invasion of the intestinal epithelial cells of the host by *T. spiralis*; therefore, a decrease in TsSP expression indicated a decrease in the burden of muscle larvae (25, 36).

We examined isotype-specific antibody reactions against the investigated parasites to establish that the standard Th2 and Th1 responses were initiated in our targeted coinfection scenario, as IgG antibodies are essential for preventing the parasite infection. Compared to GI, the levels of IgG2a antibodies in the targeted coinfected GV and GVI groups increased. These results suggest that *T. gondii* enhances the humoral response mediated by the major subclass antibody IgG2a. However, throughout the experiment, IgG1 levels were negligible. These findings imply that infection with *T. gondii* induces an IgG2a (Th1)-dominant antibody response. These results may be attributed toTh1-polarizing cytokines that protect against intracellular pathogens (37) and prevent the transition of T cells into Th2 responses, decreasing Th2-type cytokine secretion (38). Our findings are consistent with those of Bokken et al. (39), who found that coinfection with *T. gondii* and *T. spiralis* in pigs may result in the production of specific serum antibody responses.

Coinfection with *T. gondii* and *T. spiralis* altered the normal pathological results of *T. spiralis*, corroborating our findings. Surprisingly, mild *T. spiralis* infection was observed in the targeted GIV and GV coinfected muscle tissues. Compared to the control GIV, the inflammatory response gradually diminished to the point where the cells resumed their normal architectures (Fig.

1B and C). Consistent with our findings, Xu et al. (40) demonstrated that *T. gondii* eased the liver fibrosis induced by *Schistosoma japonicum*. Moreover, Miller et al. (30) established that *T. gondii* inhibited *Fasciola hepatica* infection responses.

The activation of macrophages was determined by examining the immunohistochemical staining expression of iNOS and arginase. The muscle sections of the targeted GV demonstrated high iNOS expression and very low arginine-1expression. This can be explained by the transition from a Th2-dominant to a *T. gondii* Th1-dominant response, as well as the production of macrophage-derived NO from arginine by the iNOS (41). During *T. spiralis* mono-infection, alternatively activated macrophages produce a substantial amount of arginase-1. Reduced protection against *T. gondii* in coinfected animals is associated with an increase in M1 cell activation in mice infected with type II *Toxoplasma*, while classically activated macrophages (CaMs) increase iNOS expression (42). This study demonstrates how macrophage polarization and the relative levels of iNOS and arginase-1 production in response to the parasite infection may influence the host's ability to co-infect with different parasites. Most human toxoplasmosis cases are caused by genotype II, which ME49 corresponds to in the current study. Therefore, we believe that our mouse-model-based findings are useful for understanding the relationship between *T. gondii* and *T. spiralis* infections in humans (43).

Conclusion

Infection with *T. gondii* induces a shift toward a Th1-type immune response while suppressing a helminth-specific Th2 immune response. Our findings suggest that the clear antagonistic activity of *T. gondii* against *T. spiralis* is due to cross-immunity. *T. gondii* induces robust Th1 responses, which are characterized by early, classical macrophage activation and the production of inflammatory mediators, including IFN-c, NO, and IL-12. These reactions are sufficient to prevent alternative macrophage activation and, consequently, the Th2 responses associated with *T. spiralis* infection. Moreover, these findings may also facilitate the creation of new vaccines.

Conflict of interest

The authors disclose no conflicts of interest.

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