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***Toxoplasma gondii* infection induces the formation of host’s nuclear granules containing poly (A)-binding proteins**

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Running title: nuclear granules formed upon *Toxoplasma* infection

Abstract

To study a mechanism by which human host cells respond to an infection of *Toxoplasma gondii*, we monitored the level of poly-(A) binding protein (PABP), an indicator of translation. Here we report an observation of the relocalization of PABPs in human host cells upon *T. gondii* infection. Notably the aggregates of PABPs formed upon infection are mainly found in the nucleus, which is different from the response found in an exposure to a heat shock. The treatment of the infected monolayers with pyrimethamine inhibits the multiplicity of the parasite, and reverses the relocalization of PABP aggregates. This active interaction between the infected mammalian host cells and *T. gondii* appears to be different than that caused by viral infection.

Keywords: Cellular response; PABPs; *Toxoplasma* infection; nuclear granules; host-parasite interaction

41 Introduction

42 *Toxoplasma gondii* is an obligate intracellular parasitic protozoan of the phylum
43 Apicomplexa, which includes clinically and agriculturally important pathogens, *Plasmodium*, the
44 organism responsible for malaria; *Eimeria* and *Cryptosporidium*, enteric pathogens; and
45 *Neospora* and *Theileria*, systemic veterinary pathogens. Having felines as its definitive host, the
46 sexual reproduction of *T. gondii* occurs in the feline intestinal epithelial lining. Un-sporulated
47 oocysts are passed in the fecal matter at which point the process of sporogony takes place outside
48 the definitive host and results in sporulated oocysts containing infective sporozoites (Tenter et al.
49 2000). Following ingestion by an intermediate host, which includes various warm-blooded
50 animals including humans, the sporozoites will lyse from the oocyst and will differentiate into the
51 tachyzoite form of the parasite. In its intermediate host cell, the rapidly growing tachyzoite form
52 reproduces asexually with a doubling time of about six to eight hours until the lysis of the host
53 cell. Approximately seven to ten days after infection of its intermediate host, tachyzoites will
54 begin to differentiate into the quiescent bradyzoite form, which forms tissue cysts mainly in
55 muscle and central nervous system tissue. Upon entering the digestive tract, ingested tissue cysts
56 will burst leading to release of the bradyzoites, which will subsequently infect the intestinal
57 epithelium and differentiate into the tachyzoite form of the parasite (Black and Boothroyd 2000).

58 It has been estimated that about one-third of the population are affected by *T. gondii*
59 infection; the infection of which could lead to the development of clinical toxoplasmosis. The
60 disease's prevalence is affected by several factors, including socioeconomic status of the area.
61 Most of cases in humans are the result of ingestion of oocysts in food or water, which has been
62 contaminated with cat feces, or ingestion of tissue cysts in raw or undercooked meat. Congenital
63 infection results when a woman becomes infected while she is pregnant and the infective
64 tachyzoites are transmitted through the placenta to the fetus. This results in the possibility of a

variety of issues for the infected child, including blindness as well as the possibility of severe neurological conditions including hydrocephalus, and may even result in spontaneous abortion. In most patients, toxoplasmosis is asymptomatic (Montoya and Liesenfeld 2004). The tachyzoite form of the parasite is repressed by the host's immune system, which causes differentiation of the parasites into the slow-dividing and latent bradyzoite form (Blader et al. 2015). However, in immunocompromised individuals, including those with HIV/AIDS or those undergoing chemotherapeutic treatment, the disease can be life-threatening and usually results from reactivation of latent infection. In these cases, patients can suffer from a variety of health issues including chorioretinitis, encephalitis, acute respiratory failure or pneumonitis (Montoya and Liesenfeld 2004). The treatment of *T. gondii* infection includes the combination of pyrimethamine and sulfonamide; however, the treatment is not effective against tissue cysts and has toxic side effects (Black and Boothroyd, 2000). A better understanding of host-parasite interaction is required in the development of preventative treatments and therapies of chronic toxoplasmosis.

Poly-adenosine binding proteins (PABPs) play an important role in eukaryotic cells' response to infection and stress. PABPs are an abundant family of RNA binding proteins found in eukaryotes, which bind to polyadenylated RNA with both strong specificity and affinity for the poly (A) tail (Wigington et al. 2014). This class of proteins recognizes the 3' poly (A) tail, which is added to mRNA precursors during 3'-end processing in the nucleus, and is essential for stabilizing mRNA, for efficient export of mRNA to the cytosol, and for the initiation of translation in the cytoplasm. Although PABPs do not display catalytic activity, they are multifunctional scaffolds involved in all metabolic pathways of the mRNA (Eliseeva et al. 2013). In eukaryotes there are cytoplasmic and nuclear PABPs, each with their respective roles. In metazoans, multiple genes encode PABPs (Goss and Kleiman 2012). In humans, there are seven

PABPs, including one nuclear termed PABPN1, four cytoplasmic PABPs which include PABPC1, PABPC3, PABPC4 and PABPC4L, in addition to an embryonic protein called ePABP (or PABPC1L), and an X chromosome encoded protein termed PABPC5 (Eliseeva et al. 2013). The cytoplasmic PABPs bind the poly (A) tail of mRNA and interacts with the initiation factor eIF4G, circularizing the mRNA for translational initiation. Therefore, PABPBs are considered a marker for monitoring translation (Kühn and Wahle 2004).

Under stressful conditions, PABPs can be relocalized to multiple and/or distinct sites (Gray et al. 2015). For instance, following UV irradiation both PABPC1 and PABPC4 briefly localize to cytosolic stress granules, and subsequently to the nucleus (Burgess et al. 2011). It was postulated that their nuclear relocalization may have a role in destruction of the transcriptome (Gray et al. 2015). In addition, the Rotavirus infection has been shown to cause displacement of PABP to the nucleus (López et al. 2016). It is also possible that the relocalization of PABPs reflects mRNA localization and turnover. Here we report an observation of the relocalization of PABPs in human host cells upon *T. gondii* infection. Notably the aggregates of PABPs formed upon infection are mainly found in the nucleus, which is different from the response found in an exposure to a heat shock. The treatment of the infected monolayers with pyrimethamine inhibits the multiplicity of the parasite, and reverses the relocalization of PABP aggregates. This active interaction between the infected mammalian host cells and *T. gondii* appears to be different than that caused by viral infection, further confirming that PABPs is an ideal protein marker to decipher an innate protective response caused by infection.

Materials and methods

Mammalian cell and parasite cultures

Human fibroblasts (ATCC-SCRC-1041), were cultured using a media consisting of Dulbecco's Modified Eagle Medium (Invitrogen, 12100046) supplemented with 10% Cosmic Calf Serum (HyClone, H3008704N), and 0.5x antibiotic-antimycotic reagent (Invitrogen, 15240-062). *T. gondii*, RHΔHX strain (ATCC-50174), was maintained by infecting confluent human fibroblast monolayers and culturing using a media, consisting of Minimum Essential Medium (Invitrogen, 61100061) supplemented with 1% dialyzed fetal bovine serum (ThermoFisher Scientific, Hyclone, SH3007903) and 0.5x antibiotic-antimycotic. The parasites were subcultured every 2-3 days following lysis from the monolayers. Both human fibroblast and parasite cultures were maintained in a 37°C incubator with 5% CO₂ until used.

Western blot analysis

The lysates were denatured in 1x SDS loading dye, and then boiled before resolving on a 10% SDS-PAGE. Resolved proteins were transferred to a nitrocellulose membrane, and blocked in 5% (w/v) non-fat skim milk in TBST for 1 hour. The blot was then incubated in PABPC3-polyclonal antibody (PABPC3, 1: 1,000, ABM, Y058722) in 2% (w/v) non-fat skim milk in TBST. The blot was subsequently washed with TBST and then incubated in HRP-conjugated goat-anti-rabbit antibody in 2% (w/v) non-fat skim milk in TBST. The secondary antibody used for this study was HRP-conjugated goat anti-rabbit (Rockland Immunochemicals Inc., #611-1322) at a 1: 10,000 dilution. The blot was again washed with TBST and then incubated with Chemiluminiscent HRP Substrate Kit (Millipore, #WBKLS0500) and imaged using the FluorChem Q Imager (Alpha Innotech) with AlphaView-FluorChem Q software.

Treatment conditions

Heat shock treatment was performed by incubating human fibroblast monolayers at 42°C in 5% CO₂ incubator for 2 hours. Treatments with pyrimethamine (Sigma-Aldrich, P7771) were carried out 24 hours post-infection by adding pyrimethamine into the culturing media of infected monolayers (1 µM final concentration).

Immunofluorescence assay

Human fibroblast monolayers were grown on coverslips (22 x 22 mm). The coverslips with infected and non-infected human fibroblast monolayers were washed with 1x PBS, fixed with 3% paraformaldehyde for 5 minutes, and then treated with 0.25% triton in 1x PBS for 10 minutes. Non-specific binding was prevented by incubating in 5% BSA (Fisher Scientific) or 5% equine serum (HyClone, SH30074). An antiserum raised against human polyclonal PABPC3 (1:500, ABM, Y058722), and FITC-conjugated goat anti-rabbit secondary antibody (1:1000, Rockland, 611-102-122) were used. Hoechst 33342 (Invitrogen, H1399) was used for nuclear staining. The coverslips were mounted onto microscope slides with Fluoromount-G (Electron Microscopy Sciences, 17984-25), and viewed by fluorescence microscopy (Leica DMI 6000 fluorescence microscope with Leica Application Suite Advanced Fluorescence Software). Approximately 100 independent fields of view were taken, and the images were analyzed by CellProfiler 2.2.0 (Dao et al. 2016; Jones et al. 2008). Data were compiled into Microsoft Excel spreadsheets, then averages and standard errors were calculated for each infection time point.

Results and discussion

In eukaryotes, PABP is known to be a component of stress granules (Anderson and Kedersha 2008). To ensure that the PABP detection used in the study would allow for the

monitoring of stress-induced granule formation, monolayers of human fibroblasts were subjected to a heat shock treatment under conditions known to cause cellular stress responses, and analyzed by immunofluorescence assay. The aggregations of PABPs were detected in both the cytoplasm and nucleus of the host cells (Figure 1A). Following heat shock treatment, PABP-aggregates were noticeably increased when compared to the control. To determine whether the increase was due to the formation of PABP-containing mRNP granules or an increase in total PABPs, Western blot analysis was performed using cell lysates prepared from the control and heat shocked human fibroblast monolayers, and revealed by a polyclonal antibody raised against human PABPC3, which has been shown to detect PABP homologs in different cell types (Marque et al. 2005). Here, resolved protein bands were detected around 60 and 80 kDa, while the predicted molecular masses of human PABPs without post-translational modification (UniProt Consortium) are 70.7 kDa (PABPC1), 70.0 kDa (PABPC3), 70.8 kDa (PABPC4), 41.9 kDa (PABPC4L), 32.7 kDa (PABPN1), 68.4 kDa (PABPC1L), and 43.3 kDa (PABPC5). The discrepancy of sizes of revealed proteins and predicted molecular mass is not surprising. Human PABPC1 has at least 14 post-translational modifications, including the acetylation of lysine residues, the methylation of glutamate, aspartate, lysine and arginine residues, and the dimethylation of lysine residues, found throughout its functional domains (Brook et al. 2012). We observed no differences between the intensities of both PABP revealed bands detected in the control and heat shock treatments (Figure 1B). It thus indicated that 2-hour heat shock treatment did not increase *de novo* PABP synthesis, but induced formation of PABP-aggregates.

Heat-shock and parasite infection

Naturally at any given time, human cells could simultaneously be assaulted by multiple cellular stresses (e.g. heat or cold shock, nutrient deprivation, and infection). We questioned how

this stress-induced response would affect the host's ability to cope with an infection caused by *T. gondii*. To address this, we compared the progression of *T. gondii* multiplication following an infection of host cells with and without prior heat-shock treatments. As an intracellular obligate parasite, *T. gondii* replicates within a parasitophorous vacuole by the mode of endodyogenous replications. Herein to follow the progression of *T. gondii* infection, we assessed the number of parasites per vacuole formed in infected host monolayers. With or without prior heat shock treatment, the host cells allowed a similar *T. gondii* replication. Twenty-four hours post-infection, 35-40% of vacuoles carried 4 parasites per vacuole (Figure 1C). It thus indicated that the intracellular multiplication of *T. gondii* was not affected by the host cells' stress response under the conditions used.

***Toxoplasma* infection and the host cell's stress response**

While assessing the host cell's responses to a heat shock and *T. gondii* infection, we observed a significant increase in the formation of PABP-granules in the nucleus, referred to here as nuclear PABP-granulation, in infected human fibroblasts at 24 hours post-infection, as compared to uninfected host monolayers (Figure 2). To determine the effect of parasitic infection on the nuclear PABP-granulation as a host cell's stress response, we performed a time-course analysis. We monitored nuclear PABP-granulation at different intervals following an infection over a period of 48 hours, or until *T. gondii* infection caused the host cells to lyse. Figure 3A shows representative images of infected human fibroblasts. Hoechst staining was used to identify the boundaries of the nucleus and to allow for the quantification of the fluorescence signals derived from the nuclear PABP-granulation. Approximately 100 – 300 independent fibroblasts were analyzed and their fluorescence signals revealed by a polyclonal PABPC3 antibody were recorded for each time point as relative fluorescence units (RFU, Figure 3B). When an average fluorescence signal per nucleus

was plotted as a function of time since infection, we found a linear relationship with the increase intensity due to the nuclear granule formation with the rate of 0.31 hour^{-1} (Figure 3B). To determine whether the increase of PABP-fluorescence signals in the nucleus was the result of more PABP accumulation in each granule, or more granules per nuclei, we further performed a morphometric analysis using CellProfiler software. The number and size of nuclear granules were analyzed for each time point. On average, both number and size of nuclear granules increased following *T. gondii* infection (Figure 3C, Supplementary Figures S1 and S2). While the average size of nuclear PABP granules was unchanged at the end of the first hour post-infection, the number of granules was slightly increased. At 7 hours post-infection, both average size and average number of nuclear PABP granules were increased $\sim 20\%$. At 24 hours post-infection, the average number of nuclear PABP granules was at its peak of ~ 15 granules/nucleus, while the average size was $\sim 13 \mu\text{m}^2$. At the end of 48 hours post-infection, the average size of the granules reached its peak of $\sim 15 \mu\text{m}^2$ (Figure 3C, hatched bars) At this time point, some human fibroblasts were infected with freshly released *T. gondii* (secondary infection). It is possible that the number of granules has reached its maximal level, and that the secondary infection caused the granule number to increase further (Figure 3C, dark bars). A gradual increase in the quantity of PABP-granules during the few hours following infection could indicate that the infection elicits a defense mechanism, in which mRNAs are accumulated in the nuclear compartment. The longer the parasites proliferate inside the host cell, more translational materials (mRNAs and various RNA binding proteins) are sequestered into larger mRNP granules. As infection progressed, so did the granulation of PABP in the host nucleus. However, it remains to be investigated whether a same phenomenon takes place in different cell types, including muscle, kidney or liver cells, and/or with a higher parasite load. In human fibroblasts used here, we postulate that these nuclear granules are part of host-parasite interaction, and that is similar to cytoplasmic stress granules. To fight infection, the translational machinery and

mRNA are repressed, until the stress is relieved. These observations paralleled findings with Rotavirus, which also causes displacement of host PABP to the nucleus upon infection (López et al. 2016). However, while Rotavirus infection inhibits the formation of stress granules in the host (Montero et al. 2007), *T. gondii* infection appears to be inducing the formation of nuclear granules in a time-dependent manner.

Relocalization of PABPs following *T. gondii* infection

Since the accumulation of PABPs in the nucleus of human fibroblasts was observed to be directly correlated with the progression of *T. gondii* infection, we questioned whether the removal of the parasites may reverse this relocalization of PABP. Cultures of human fibroblasts were infected with *T. gondii* and allowed to grow for 24 hours prior to the addition of pyrimethamine, an antiparasitic agent. Pyrimethamine is a potent dihydrofolate reductase inhibitor with an IC₅₀ of 0.25 to 1.5 µM (Ben-Harari et al. 2017). Pyrimethamine treatments of *T. gondii*-infected monolayers were analyzed at different intervals (12-48 hours) for the accumulation of nuclear PABP-granules. A decrease in nuclear PABP granulation was observed after 12 hours of pyrimethamine treatment (Figure 4). The results confirmed that the infection and growth of *T. gondii* cause a stress-induced response in the host leading to the formation of nuclear PABP-containing granules.

During the invasion of the host cell by Apicomplexan parasites, specialized secretory organelles, including micronemes, rhoptries, and dense granules, release their protein contents into the host cell. These proteins are speculated to interact with those of the host cell leading the alteration of host cell's biology (Gilbert et al. 2006). For example, members of the dense granule protein family called GRA proteins of *T. gondii* could elicit changes in the host's gene expression. Once successfully invading the host cell, *T. gondii* replicates within a parasitophorous vacuole and continues to secrete their proteins into the parasitophorous vacuole and then into the host cell. Two

members of GRA proteins, namely GRA16 and GRA24, are able to cross the membrane of the parasitophorous vacuole and migrate to the host nucleus (Bougdour et al. 2014). The interaction of these proteins with host cell proteins affects the regulation of host cell's gene expression through disrupting certain signaling pathways, which may be a potential cause for this nuclear PABP-granulation. Upon infection, the parasite also secretes from its rhoptries TgPP2C-hn, a protein phosphatase, into its host. This protein then traverses to the nucleus where its target molecules are not yet known. The secretion of this phosphatase, a common enzymatic effector of cell signaling pathways, is another example of a way that the parasite interacts with and potentially modulates its host (Gilbert et al. 2006). As a nuclear directed protein, this phosphatase may provide another possible explanation for the infection-induced nuclear granulation seen in this study. To further elucidate a mechanism by which *T. gondii* manipulates the host's cellular response, one could utilize a chemical compound known to specifically inhibit invasion or egress of the parasite from the host cells. For example, cytochalasin D, an inhibitor of actin polymerization, can be used in causing *T. gondii* to secrete its rhoptry factor, but inhibiting parasite invasion (Caldas et al. 2013). The experiment would address whether the host cell's PABP granulation is directly correlated with the rhoptry secretion and/or *T. gondii* invasion.

Conclusion

This study describes an observation of a host-parasite interaction, where *T. gondii* infection causes granulation of the RNA-binding protein, PABPs, in the host cell. This stress response differs from a heat shock treatment that generally causes both cytoplasmic and nuclear granulation of PABPs in eukaryotes. *T. gondii* infection specifically led to pronounced nuclear PABP granulation, which increased over the period of infection. In addition, the removal of the parasites by

chemotherapeutic agent reversed the process of nuclear granulation, confirming that the infection and growth of the parasites was causing PABP to aggregate in the nucleus in a time-dependent manner. Prospective work would be to study the molecular pathway leading to this stress-induced response to further understand the dynamic interaction between host and pathogen. The better understanding of how *T. gondii* manipulates the host's response will help elucidate host-parasite interaction, and open an avenue to disrupt and prevent the parasite infection.

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Conflicts of interest

The authors declare no competing financial interest.

Author contributions

KF, MR, and YA performed experiments, and analyzed data. SA conceived and coordinated the study. SR and SA conceptualized and designed the experiments. KF, MR and SA wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

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Figure legends

Figure 1 Formation of stress-induced PABP-mRNP granules. (A) Following a two-hour heat shock treatment at 42°C, PABP-containing aggregates were detected throughout the nucleus and the cytosol of human fibroblasts. Micrometer bars represent 10 µm. (B) Western blot analysis revealed similar level of PABPs in human fibroblasts (HF) with and without a heat shock treatment. Protein lysates were resolved on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with PABPC3-antisera. The sizes of protein markers are indicated, and an arrow identifies the revealed PABP at ~ 80 kDa. (C) Replication of *T. gondii* in human fibroblasts was observed at 24 hours post-infection of the human fibroblasts with and without a heat shock treatment. Number of parasites per vacuole were counted from ~100 independent vacuoles, and their average values were plotted. The experiments were done in replicates, and similar results were detected.

Figure 2 Human fibroblasts in response to *T. gondii* Infection. Representative images of *T. gondii* infected and uninfected human fibroblasts are shown. PABPC3-antisera reveal the locations of PABPs in the nucleus and cytosol of *T. gondii* as well as the nucleus of the human fibroblasts. Arrow indicates infected host cell nucleus and arrow-head indicates uninfected host cell nucleus. Micrometer bars represent 10 µm.

Figure 3 Time-course analysis of PABP-containing granules following *T. gondii* infection. (A) Representative images of *T. gondii* infected human fibroblasts from different time points post-infection. (B) Independent images were taken with a constant exposure time, and resolution to allow for the measurement of fluorescent intensity of PABP-aggregates formed within the nucleus of the fibroblasts. Average fluorescence intensity was plotted as a function of post-infection time (1 – 48 hours). Due to low fluorescence signals detected in uninfected fibroblasts (control), the

exposure time and resolution were altered for visualization; the fluorescence intensity obtained from the control (time = 0 hour) was thus not included in the plot. (C) Morphometric analysis was performed to obtain the number and size of infection-induced PABP granules. The average size (solid bars) and the average number (hatched bars) were obtained from independent 100 – 300 nucleus were plotted as a function of post-infection time (see supplementary Figures S1 and S2 for the data distribution for the number and size of granules).

Figure 4 Pyrimethamine treatment of *T. gondii*-infected fibroblasts. *T. gondii* was allowed to infect human fibroblasts for 24 hours prior to the treatment with pyrimethamine (1 μ M). Representative images are shown. It was observed that the presence of pyrimethamine diminished the parasite growth and decreased the formation of nuclear PABP granules.

Figure 1

Figure 1 Formation of stress-induced PABP-mRNP granules. (A) Following a two-hour heat shock treatment at 42°C, PABP-containing aggregates were detected throughout the nucleus and the cytosol of human fibroblasts. Micrometer bars represent 10 μm . (B) Western blot analysis revealed similar level of PABPs in human fibroblasts (HF) with and without a heat shock treatment. Protein lysates were resolved on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with PABPC3-antisera. The sizes of protein markers are indicated, and an arrow identifies the revealed PABP at ~ 80 kDa. (C) Replication of *T. gondii* in human fibroblasts was observed at 24 hours post-infection of the human fibroblasts with and without a heat shock treatment. Number of parasites per vacuole were counted from ~ 100 independent vacuoles, and their average values were plotted. The experiments were done in replicates, and similar results were detected.

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Figure 2

Figure 2 Human fibroblasts in response to *T. gondii* Infection. Representative images of *T. gondii* infected and uninfected human fibroblasts are shown. PABPC3-antisera reveal the locations of PABPs in the nucleus and cytosol of *T. gondii* as well as the nucleus of the human fibroblasts. Arrow indicates infected host cell nucleus and arrow-head indicates uninfected host cell nucleus Micrometer bars represent 10 μ m.

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Figure 3

Figure 3 Time-course analysis of PABP-containing granules following *T. gondii* infection. (A) Representative images of *T. gondii* infected human fibroblasts from different time points post-infection. (B) Independent images were taken with a constant exposure time, and resolution to allow for the measurement of fluorescent intensity of PABP-aggregates formed within the nucleus of the fibroblasts. Average fluorescence intensity was plotted as a function of post-infection time (1 – 48 hours). Due to low fluorescence signals detected in uninfected fibroblasts (control), the exposure time and resolution were altered for visualization; the fluorescence intensity obtained from the control (time = 0 hour) was thus not included in the plot. (C) Morphometric analysis was performed to obtain the number and size of infection-induced PABP granules. The average size (solid bars) and the average number (hatched bars) were obtained from 100 – 300 independent nucleus were plotted as a function of post-infection time (see supplementary Figures S1 and S2 for the data distribution for the number and size of granules).

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Figure 4

Figure 4 Pyrimethamine treatment of *T. gondii*-infected fibroblasts. *T. gondii* was allowed to infect human fibroblasts for 24 hours prior to the treatment with pyrimethamine (1 μM). Representative images are shown. It was observed that the presence of pyrimethamine diminished the parasite growth and decreased the formation of nuclear PABP granules.

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