



Increased Oxidative Stress and Apoptosis in Splenic Tissue of Lupus-Prone (NZB/NZW) F1 Mice Infected with Live but not Gamma Irradiated *Plasmodium chabaudi*

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ABSTRACT

Spleen is a major immune organ that plays an important role during malaria infection. Systemic lupus erythematosus (SLE) is an autoimmune disease that affects many body organs including spleen. However, the effect of *Plasmodium chabaudi* infection on the spleen of the murine lupus model BWF1 with the comparison between live and gamma irradiated parasite was not studied before. A total of 30 female BWF1 mice were randomly divided into three groups as follows: group (I) control group (lupus uninfected); group (II) live *P. chabaudi* infected group (lupus + live *P. chabaudi* infection); and group (III) irradiated *P. chabaudi*-infected group (lupus + irradiated *P. chabaudi*-infection). All groups were killed at day 14 post infection. Histological and biochemical investigations were performed. Live *P. chabaudi* infection was accompanied with an increase in spleen weight in comparison to either the control or the gamma irradiated *P. chabaudi* infected group. Differential count of WBCs has revealed an increase in lymphocytes, monocytes and granulocytes count in plasma samples of the live *P. chabaudi* infected group in comparison to either the control or the gamma irradiated *P. chabaudi* infected group. Additionally, live *P. chabaudi* infection has resulted in histopathological changes and increased apoptotic cell death in splenic tissue of infected mice. Moreover, infection with live *P. chabaudi* was accompanied with an increase in NO, H₂O₂ and MDA levels in splenic tissue of lupus mice in comparison to either control or gamma irradiated *P. chabaudi*-infected group. Our data reveal that infection of lupus mice with live *P. chabaudi* has several effects on the splenic tissue on the histological and biochemical levels. Parasite irradiation with gamma rays can abrogate these effects.

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Authors' Contributions

MA designed the study and performed the practical work. FAG and SA analyzed the data. GB performed the statistical analysis. SA and AE wrote the article. SA supervised the work.

Key words

Apoptosis, Autoimmune disease, Oxidative stress, *Plasmodium chabaudi*, Systemic lupus erythematosus, Nitric oxide

INTRODUCTION

Systemic Lupus Erythematosus (SLE) is a multisystem autoimmune disease whose aetiology and pathogenesis are incompletely understood (Monova *et al.*, 2011). Dysregulated apoptosis and inadequate removal of apoptotic cells and nuclear remnants cause prolonged exposure of the immune system to nuclear and cell membrane components (Stuart and Hughes, 2002) and, consequently, the progression of autoimmune diseases. Reactive oxygen species (ROS) are among the most important mediators of apoptosis. ROS are short-lived diffusible entities, generated by inflammatory cells, which accumulate in both allergic and non-allergic inflammations (Baldwin *et al.*, 1970). The oxidative damage mediated by ROS results in defects in the control of apoptosis and delayed clearance of apoptotic cells. This may serve to prolong the interaction between ROS and apoptotic cell macromolecules, generating neopeptides that subsequently lead to the formation of a broad spectrum of autoantibodies formation and ultimately to the tissue damage seen in SLE (Ahsan *et al.*, 2003). Nitric oxide (NO) and hydrogen peroxide (H₂O₂) are two of the most

important ROS that have an important role in many health problems (Kullisaar *et al.*, 2013) by influencing discrete signal transduction pathways in various systems and cell types.

Malaria is a parasitic disease that accounts for 1.5–2.7 million deaths annually (Murthy *et al.*, 2014). On the other hand, epidemiological data has revealed that SLE is rarely observed in rural tropical areas of Africa and Asia, where malarial infection is prevalent (Adebajo, 1997). It has been hypothesized, therefore, that genes promoting susceptibility to SLE are beneficial in controlling severe malaria but promote inflammation and the progression of autoimmune conditions in the absence of parasitic infection (Greenwood and Corrah, 2001). Supporting this contention, Greenwood *et al.* (1970) described a higher survival rate in young BWF1 lupus-prone mice infected with *Plasmodium berghei*, while another study revealed that old BWF1 mice, when infected with *P. chabaudi* at the onset of clinical signs of lupus and subsequently treated with chloroquine, showed a temporary remission of the symptoms. Moreover, the injection of immunoglobulins isolated from *P. chabaudi*-infected BALB/c mice produced similar protective effects as the infection its elfin BWF1 mice (Hentati *et al.*, 1994).

In our previous work, we have confirmed that both the kidney and the liver have an important role during infection of female BWF1 lupus mice with *P. chabaudi* (Al-Quraishy *et*

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al., 2013). The spleen is among the major organs affected by malaria. Apoptotic changes are detectable in splenic tissue due to malaria infection, while *Plasmodium chabaudi* infection in mice is associated with striking changes in splenic cell distribution (Achtman *et al.*, 2003) and induces a response that develops in two phases and is characterized by different properties. Migrating monocytes recruited to the spleen also play an important role in the control of blood stage malaria (Sponaas *et al.*, 2009). On the other hand, the involvement of the spleen during the course of SLE was recently confirmed in both human and lupus prone mice (Fleischer *et al.*, 2014). Nevertheless, few studies have investigated the effect of malarial infection on the spleen in lupus mice. In the current study, therefore, we investigated the possible effects of *P. chabaudi* infection (either live or gamma irradiated) on the spleen in BWF1 mice, which represent a murine model of SLE.

MATERIALS AND METHODS

Animals

A total of 30 female BWF1 29-week-old mice were purchased from Jackson Laboratory (Bar Harbor, USA) and maintained and monitored in a specific pathogen-free environment. All animal procedures were performed in accordance with the standards set out in the Guidelines for the Care and Use of Experimental Animals by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The study protocol was approved by the Animal Ethics Committee at King Saud University. All animals were allowed to acclimatize in plastic cages (five animals per cage) inside a well-ventilated room for one week prior to the experiment. The animals were maintained under standard laboratory conditions (temperature of 23°C, relative humidity of 60–70%, and a 12 h light/dark cycle) and were fed a diet of standard commercial pellets and given water *ad libitum*.

Malarial infection

The blood stage forms of *Plasmodium chabaudi* parasites were stored in liquid nitrogen after *in vivo* passage in 3-month-old BALB/c mice according to a previously described protocol (Hentati *et al.*, 1991). Female BWF1 mice (30 weeks old) were infected by i.p. injection of 10⁶ parasitized erythrocytes obtained from an infected mouse of the same strain, as previously described (Hentati *et al.*, 1994). Parasitaemia was monitored by Giemsa-stained thin blood smears. Experimental animals were assigned to three groups (ten mice/group) as follows: group (I) lupus group (Lupus non-infected); group (II) live malaria-infected group (Lupus+ live malaria infection); and group (III) irradiated malaria-infected group (Lupus +irradiated malaria-infection). Group III was infected i.p. with 10⁶ gamma-irradiated red blood cells infected with *P. chabaudi*. Prior to injection, the blood cells were exposed to a dose of 200 Gy gamma-radiation from a Gamma Cell 200 Irradiator

(Atomic Energy of Canada, Ltd., Ottawa, Canada) utilizing a ⁶⁰Co source located at the Research Centre of the College of Science, King Saud University, Saudi Arabia. This radiation dose was applied based on experiments conducted by Ferreira-da-Cruz *et al.* (1997) that provided evidence that a 200-Gy gamma-irradiation dose is able to abolish the original replication of the erythrocytic forms of the Palo Alto *P. falciparum* strain, most likely by inactivating their infectivity. According to their data, 100- or 150-Gy irradiation doses were unable to inactivate the parasite, despite a reduction in parasitaemia.

Sample collection

All animals were sacrificed at day 14 post-infection and blood was collected from the heart in heparinized tubes for the determination of WBCs and to obtain plasma for biochemical studies. Plasma was stored at –80°C until use. The spleen was removed and cut into small pieces in sterile saline. The pieces were fixed in 10% neutralbuffered formalin or 4% paraformaldehyde for TUNEL assays, or suspended in Tris buffer for biochemical studies.

Histological analysis

Pathological evaluation in Hematoxylin and eosin stained tissue sections was done by a pathologist blinded to the experimental regimen. For each histological section per animal not less than ten microscopic fields were randomly selected and examined for any histopathological changes, such as malaria pigment deposition and sequestered RBCs.

TUNEL assay

Spleen tissue was fixed in 4% paraformaldehyde for 4 h at 4°C, washed in phosphate-buffered saline (PBS), and embedded in paraffin blocks. Sections of 7 µm were cut and air-dried for 20 min before staining. Terminal dUTP nick-end labelling (TUNEL) was performed using a commercial TUNEL Apoptosis Detection Kit® FITC-labelled POD (Gen Script, USA) according to the manufacturer's instructions. Apoptotic cells were identified by morphological criteria (cell shrinkage and chromatin condensation and margination). The apoptotic nuclei were stained dark brown and examined and counted under a light microscope.

Cell blood count (CBC)

Whole blood samples were analysed with an automatic Vet abc™ Animal Blood Counter (Horiba ABX, Montpellier, France) using the haematology kits specified for that instrument (Horiba ABX, France) and following the manufacturer's instructions.

Oxidative stress assessment in spleen tissue

Parts of the spleen were weighed and homogenized immediately to give a 50% (w/v) homogenate in an ice-cold medium containing 50mM Tris–HCl and 300 mM sucrose. The homogenate was centrifuged at 500 g for 10 min at 4°C. The supernatant (10%) was used for determination of

NO, H_2O_2 , and MDA using commercial kits (Biodiagnostic, Dokki, Giza, Egypt)

Nitric oxide was assayed according to the technique of Berkels *et al.* (2004). In brief, nitrous acid was formed in an acid medium and, in the presence of nitrite, the formed acid diazotizes sulphanilamide, which was coupled with N- (1-naphthyl) ethylenediamine, and the resulting azo dye could then be measured at 540 nm.

Hydrogen peroxide (H_2O_2) was assayed according to Aebi (1984). In the presence of horse radish peroxidase (HRP), H_2O_2 in tissue homogenate reacts with 3, 5-dichloro-2-hydroxybenzenesulfonic (DHBS) acid and 4-aminophenazone (AAP) to form a chromophore that can be quantified at 240 nm.

Lipid peroxidation was determined by the method of Ohkawa *et al.* (1979). The homogenate was suspended in 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid boiled in a water bath for 30 min. Thiobarbituric acid reactive substances are measured at 535 nm and expressed as malondialdehyde (MDA) equivalents formed.

Statistical analysis

The data was tested for normality using the Anderson-Darling test as well as for homogeneity variances prior to further statistical analysis. The data was found to be normally distributed and is expressed here as the mean $\pm$ standard error of the mean (SEM). Significant differences among the groups were analysed by one- or two-way ANOVA followed by Bonferroni's test for multiple comparisons using PRISM statistical software (Graph Pad Software). The data were also reanalysed by one- or two-way ANOVA followed by Tukey's post-test using SPSS software, version 17. Differences were considered statistically significant at $P < 0.05$

RESULTS

Live *P. chabaudi* infection is associated with an increase in spleen weight and WBCs count

Live *P. chabaudi* infected group has showed infection-associated splenomegaly as indicated by the increase in spleen weight in this group in comparison to that of the control group (Table I). In the gamma irradiated *P. chabaudi*

infected group, however, spleen weight was not significantly changed in comparison to the control group. A differential count of WBCs revealed an increase in lymphocytes, monocytes and granulocytes in plasma samples from the live *P. chabaudi* infected group (Table II) in comparison to the control group. In the gamma irradiated *P. chabaudi* infected group, the plasma level of the three types of WBCs was similar to that of the control group.

Table I.- Effect of *P. chabaudi* infection, either live or gamma irradiated, on the weight and relative weight of spleen of BWF1 lupus mice at day 14 p.i.

Experimental group	Body weight (g)	Spleen weight (g)	Spleen relative weight (g/100 g body weight)
Lupus	41.3 $\pm$ 1.9	0.38 $\pm$ 0.06	0.92 $\pm$ 0.17
Lupus+live <i>P. chabaudi</i>	39.7 $\pm$ 1.4	0.46 $\pm$ 0.02*	1.15 $\pm$ 0.28
Lupus + gamma irradiated <i>P. chabaudi</i>	41.1 $\pm$ 1.5	0.39 $\pm$ 0.03	0.94 $\pm$ 0.19

Mean $\pm$ SEM; n, 6; * $P < 0.05$ for live *P. chabaudi* infected BWF1 mice vs. control.

Table II.- Effect of *P. chabaudi* infection, either live or gamma irradiated, on the differential count of WBCs in plasma samples of BWF1 lupus mice.

	Lupus	Lupus+live <i>P. chabaudi</i>	Lupus + gamma irradiated <i>P. chabaudi</i>
Lymphocytes	4.1 $\times 10^9$ /L	8.4 $\times 10^9$ /L *	4.3 $\times 10^9$ /L
Monocytes	0.4 $\times 10^9$ /L	0.6 $\times 10^9$ /L *	0.3 $\times 10^9$ /L
Granulocytes	1.6 $\times 10^9$ /L	4.8 $\times 10^9$ /L *	1.7 $\times 10^9$ /L

Mean $\pm$ SEM; n, 6; * $P < 0.05$ for live *P. chabaudi* infected BWF1 mice vs. control.

Effect of *P. chabaudi* infection on the histopathology of the spleen in female BWF1 lupus mice

As an important immune organ during malaria infection,

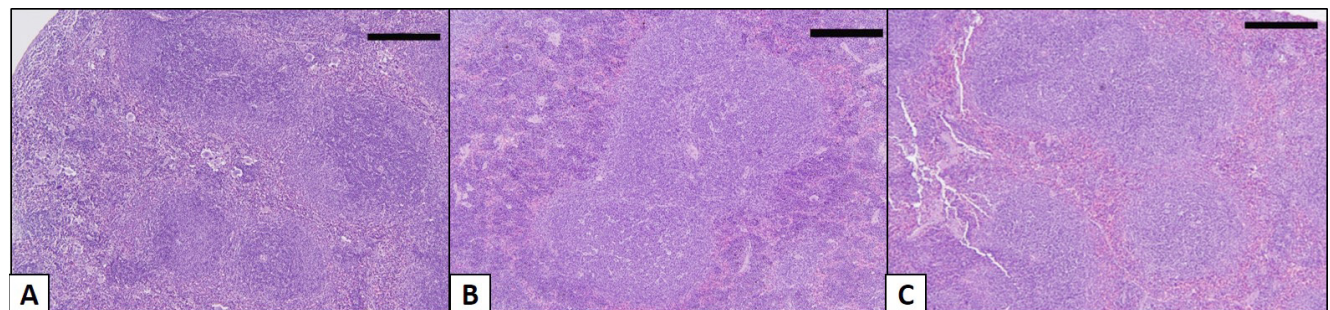


Fig. 1. The effect of malarial infection on the distribution of red and white pulps in splenic tissue of female BWF1 mice. Hematoxylin and eosin (H/E) staining of spleen of lupus (A), live *P. chabaudi* (B) and gamma-irradiated *P. chabaudi* (C) infected group of BWF1 mice (n=10). The organs were removed at day 14 p.i. Sections of *P. chabaudi* infected groups have showed hypertrophied white pulps at the expense of red pulp. Scale bar = 100 μ m.

investigating histopathological changes in splenic tissue due to malaria infection is of special relevance. Figure 1 demonstrates the major histopathological changes in H/E spleen tissue sections of lupus mice after infection with either live or gamma irradiated *P. chabaudi*. The spleen tissue samples of the lupus mice showed a clear distinction between red pulp (RP) and white pulp (WP). Conversely, sections from the *P. chabaudi* infected groups showed hypertrophied white pulp at the expense of red pulp. In all of the three groups the intense proliferation of the cells presented as megakaryocytes in the resting follicles was observed.

Increased apoptotic cell death in splenocytes of BWF1 mice infected with live P. chabaudi

To investigate the apoptotic changes on the histological level, paraffin embedded splenic tissue sections were stained using TUNEL apoptosis detection kits. Histological sections from the live *P. chabaudi* infected group (Fig. 2B) displayed a higher proportion of apoptotic cells in comparison to either the control group (Fig. 2A) or the gamma irradiated *P. chabaudi* infected group (Fig. 2C). To further validate this result, the number of TUNEL-positive nuclei was determined (Table III). The live *P. chabaudi* infected group had the highest number of apoptotic cells in comparison to either the control group or the gamma irradiated *P. chabaudi* infected group.

Live P. chabaudi infection is associated with an increased oxidative stress in splenic tissue of BWF1 mice

The effect of malarial infection on oxidative stress markers in the SLE experimental model was measured by determining the levels of NO, H₂O₂, and MDA in spleen samples from the three experimental groups of female BWF1 mice. We observed a significant increase in the levels of H₂O₂, MDA and NO (Fig. 3) in splenic tissue after live *P. chabaudi* infection compared with the control lupus mice (*P<0.05). Conversely, the gamma-irradiated *P. chabaudi* infected group, had splenic tissue levels of H₂O₂, MDA and NO similar to that of the control group.

Table III.- Incidence and number of apoptotic cells in splenic tissue of control, live and gamma-irradiated *P. chabaudi*-infected BWF1 mice.

Experimental group	No. of apoptotic cells (cm ²)
Lupus	16±3
Lupus + Live <i>P. chabaudi</i>	25±6*
Lupus + Gamma irradiated <i>P. chabaudi</i>	17±5

Mean ± SEM; n, 6; *P<0.05 for live *P. chabaudi* infected BWF1 mice vs. control.

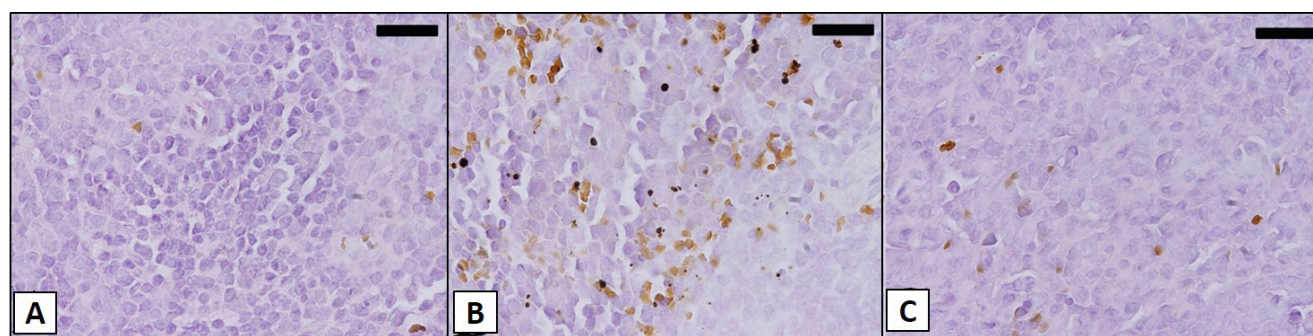


Fig. 2. The effect of malarial infection on apoptotic DNA fragmentation in splenic tissue of female BWF1 mice splenic tissues of the lupus (A), live *P. chabaudi* (B), and gamma-irradiated *P. chabaudi* (C) infected group of BWF1 mice at week 32. Paraffin embedded tissue sections were prepared and investigated using TUNEL apoptosis detection kits. The TUNEL-positive nuclei are markedly different from those observed in the lupus group. Scale bar = 25µm.

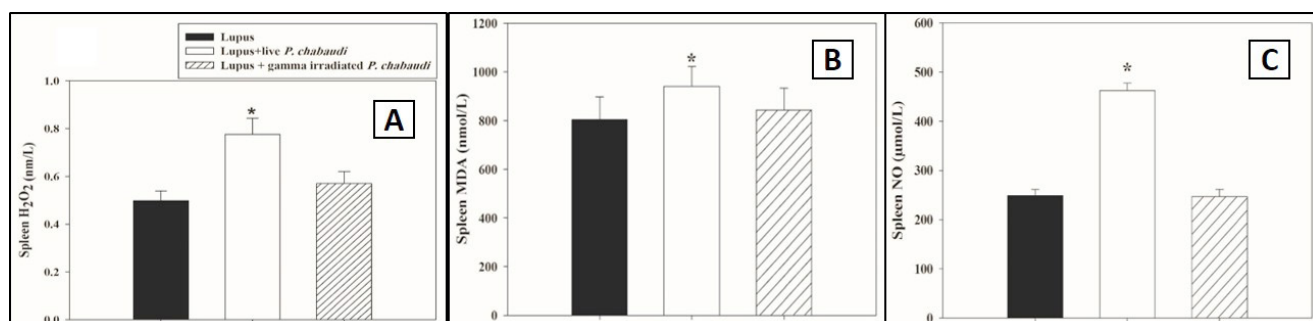


Fig. 3. Effect of live or gamma-irradiated *P. chabaudi* infection on splenic NO, H₂O₂, and MDA levels. The levels of H₂O₂ (a), MDA (b), and NO (c) in splenic tissue of BWF1 lupus mice after experimental infection with either live or gamma irradiated malaria in comparison with the lupus non infected group. The data are the mean ± SEM for 6 mice per group *P<0.05 for live malaria-infected BWF1 mice versus lupus mice.

DISCUSSION

The spleen is a major immune organ (Ebaid *et al.*, 2015) that plays a crucial role during parasitic infections, including malaria (Del Portillo *et al.*, 2012). During malaria infection, oxidative stress has been extensively studied in different organs, including the spleen, in a variety of traditional mouse strains. Oxidative stress has also been documented in both human and murine models of SLE (Lai *et al.*, 2015). In our previous study (Al-Quraishy *et al.*, 2013), we observed that both renal and hepatic tissues of female BWF1 lupus mice were affected by experimental infection with *P. chabaudi*. In the current study, we have extended our data to include splenic tissue. The live *P. chabaudi* infection had several consequences, among which was an increase in spleen weight. Previous studies have reported splenomegaly as a clear symptom of malaria (Legorreta-Herrera *et al.*, 2010). Concomitantly, a significant increase in WBC count after live *P. chabaudi* infection was detected, which is considered to be a well-known immune defence mechanism associated with a number of infections, including malaria. Conversely, in the gamma irradiated *P. chabaudi* infected group, neither the change in spleen weight nor WBC count was significant. The histological alterations in splenic tissue sections after infection with live *P. chabaudi* presented as hypertrophied white pulp, has been reported previously (Achtman *et al.*, 2003). In the current study, live *P. chabaudi* infection resulted in an increase in apoptotic cell death in splenic tissue sections and this is in agreement with previous studies (Keswani and Bhattacharyya, 2013). Nevertheless, the gamma irradiated *P. chabaudi* infected group presented, to some extent, a similar picture to that of the control group. This confirms our assumption that the live parasite is needed for the infection-associated effects.

Oxidative stress is a well-known immune mechanism during malaria infection (Bilgin *et al.*, 2012). NO is a versatile component of the innate immune system, and iNOS-derived NO is involved in both the pathogenesis and control of several types of parasitic infections (Bogdan, 2001). The increased production of endogenous NO during blood-stage malaria infection has been found to correlate with protection against *Plasmodium falciparum*. Additionally, NO can be both cytotoxic and cytostatic to *P. falciparum* *in vitro* (Legorreta-Herrera *et al.*, 2010). Furthermore, NO is able to modulate the immune response via the regulation of apoptosis and the up-regulation of cytokine mRNA expression (Remick and Villarete, 1996). H₂O₂ is another important molecule during malaria infection. According to Brinkmann *et al.* (1984), macrophage activation occurs through mechanisms partially dependent on NO and H₂O₂ and, in the current study, live *P. chabaudi* infection resulted in increased levels of NO and H₂O₂ in splenic tissue. Lipid peroxidation is an important mechanism by which oxidant stress kills the intracellular parasites (Allison and Eugui, 1983). A significant increase in lipid peroxidation markers has been reported in patients with *P. vivax* infection (Fabbri *et al.*, 2013). In the current study, live *P. chabaudi* infection has resulted in an increased level

of lipid peroxidation, as indicated by the increased MDA level in splenic tissue of the live *P. chabaudi* infected group. Taken together, our data confirms that infection of female BWF1 lupus mice with live *P. chabaudi* has deleterious effects on the splenic tissue, presented mainly as increased spleen weight, oxidative stress and apoptotic death. Gamma parasite irradiation can diminish these effects associated with the live parasite infection.

CONCLUSION

Live *P. chabaudi* infection can affect the spleen weight, histology, apoptotic cell death and oxidative stress in splenic tissue. When infecting with the gamma irradiated parasite, these responses are totally different so live parasite infection could have positive effects on the autoimmune disease.

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Statement of conflicts of interest

The authors declare no conflicts of interest.

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