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Lactobacillus casei ssp. *rhamnosus* enhances non specific protection against *Plasmodium chabaudi* AS in mice

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Abstract

Objective. To evaluate the capacity of *Lactobacillus casei* ssp. *rhamnosus* to enhance resistance against *Plasmodium chabaudi chabaudi* AS. **Material and Methods.** NIH mice were IP injected with viable *Lactobacillus casei* seven days (LC1 group) or 7 and 14 days (LC2 group) before the challenge (day 0) with *Plasmodium chabaudi* parasitized red blood cells (pRBC). Control mice were inoculated with pRBC only. When parasitaemia was resolved, naive mice were injected with spleen cells from each group. The parasitaemia was measured. Nitric oxide (NO[•]) in serum was determined. **Results.** Mice from the LC1 group presented a reduction in parasitaemia, with a prepatent period of five days, parasitaemia lasted 11 days, and the peak was (36.3 % pRBC) on the 12th day post-infection. Mice from the LC2 group showed a prepatent period of five days, parasitaemia lasted eight days, and the peak (30 % pRBC) was of on the 11th day. In the control, the prepatent period was three days, the parasitaemia lasted 15 days, and the peak (51% pRBC) was on day nine. Mice inoculated with spleen cells from the LC2 group showed a prepatent period of 21 days, parasitaemia lasted seven days, and the peak (13.5% pRBC) was on the 26th day. **Conclusion.** *L. casei* enhanced nonspecific resistance to *P. chabaudi*, as indicated by longer prepatent periods, reduced parasitaemia, and reduction in the viability of the parasites recovered from the spleen of infected mice, along with high concentrations of NO[•] in serum.

Key words: lactic acid bacteria; probiotics; non specific protection; *Plasmodium chabaudi* infection; *Lactobacillus casei*; immunity, natural; *Plasmodium chabaudi*

Resumen

Objetivo. Evaluar la capacidad de *Lactobacillus casei* de aumentar la resistencia a la infección con *Plasmodium chabaudi* en ratones. **Material y métodos.** Ratones NIH fueron inyectados intraperitonealmente con *L. casei* viable 7 días (grupo LC1) o 7 y 14 días (grupo LC2) antes del reto (día 0) con glóbulos rojos parasitados (GRP) con *P. chabaudi*. Los testigos fueron inoculados con GRP solamente. Cuando la parasitemia se resolvió, se inocularon ratones limpios con células de bazo de cada grupo. Se midió la concentración de óxido nítrico (NO[•]) en suero. **Resultados.** El grupo LC1 presentó un periodo prepatente de 5 días, una parasitemia de 11 días con el máximo (36.3% de GRP) el día 12. Los ratones del grupo LC2 mostraron un periodo prepatente de 5 días, una parasitemia de 8 días con el pico (30% de GRI) el día 11. En los testigos el periodo prepatente fue de 3 días, la parasitemia de 15 y su máximo (51% de GRI) el día 9. Los ratones que recibieron células de bazo del grupo LC2, mostraron un periodo prepatente de 21 días, una parasitemia de 7 con su máximo (13.5% de GRI) el día 26. **Conclusión.** *L. casei* aumenta la resistencia no específica hacia *P. chabaudi* a juzgar por los periodos prepatentes más largos, las bajas parasitemias, la reducción en la viabilidad y la elevación de la concentración de NO[•] en el suero, que presentaron los ratones estimulados con lactobacilos.

Palabras clave: bacterias lácticas; probióticos; protección no específica; infección con *Plasmodium chabaudi*; *Lactobacillus casei*; inmunidad natural; *Plasmodium chabaudi*

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Lactic acid bacteria (LAB) represent a group of different gram positive microorganisms which are widely used in the dairy industry because they provide a pleasant flavor in milk products and increase their nutritional properties. They have also been recognized as probiotic microorganisms which are defined as mono or mixed cultures of live microorganisms which, when applied to animal and humans, beneficially affect the host by improving the properties of the indigenous microflora. There is much information about the role of probiotic bacteria in the improvement of the intestinal microflora and the modulation of the immune system.^{1,2,3} Beneficial effects have been obtained with mice that received LAB orally,⁴ via intraperitoneal (IP),⁵ and even via intrapleural.⁶ It has been widely shown that LAB enhance protection and reduce pathological effects against: Shiga toxin-producing *Escherichia coli*,⁷ *Salmonella typhim urium*,^{8,9} *Candida albicans*,¹⁰ rotavirus,¹¹ and even against helminthic parasites.¹²

Lactobacillus casei enhances protection against intracellular bacteria by inducing IL-12 and IFN γ production.¹³ These cytokines play a central role in the innate and T helper 1 lymphocytes (Th1) immune responses, which is relevant in cancer prevention.¹⁴ The main effectors cells that participate in the innate immune response are neutrophils, mononuclear cells, and natural killer lymphocytes (NK).

The main sources of IL-12 are activated mononuclear and dendritic cells. IL-12 is a good stimulator of NK and Th1 lymphocytes for producing IFN γ . IFN γ is the main activator of macrophages for producing the required cytokines for activating NK cells and inducing the differentiation of virgins T CD4⁺ to Th1 lymphocytes. IFN γ is also relevant in the control of *Plasmodium chabaudi* infection since IFN γ knockout mice developed significantly higher parasitaemia during acute infection than wild type mice.¹⁵

The aim of the present work was, therefore, to evaluate the protective effect against *Plasmodium chabaudi* infection in NIH mice after IP injection of viable *Lactobacillus casei* ssp. *rhamnosus*; to determine the effect against malaria parasites lodged in the spleen and evaluate its correlation with nitric oxide (NO[•]) production. This work was carried out at the Escuela Nacional de Ciencias Biológicas, IPN, Mexico (ENCB, IPN), from 2004 to 2005.

Materials and Methods

Mice. NIH mice, weighing 22 to 25 g (8 to 10 weeks old), were obtained from a random-breed colony (purchased in BIRMEX S.A. México D.F.). All normal and infected animals were housed and cared for according

to international guidelines for the caring and use of laboratory animals. Unnecessary pain was avoided in infected mice and they were painlessly killed at the end of the experiment. Each experimental group consisted of six mice. The protocol was revised by the Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional Bioethics Committee.

Plasmodium chabaudi chabaudi AS, kindly donated by Dr. Luis Fabila Castillo from ENCB, IPN, was maintained in mice by IP injection, with 10⁵ parasitized red blood (pRBC) every 10 days. After three rounds the strain was preserved in liquid N₂.

Parasitemia was evaluated daily in individual mice on blood smears stained using the Giemsa technique. pRBC cells were counted in 50 microscopic fields (approximately 10⁴ red blood cells) when parasitaemia was incipient, with a total of 200 red blood cells when parasitaemia was abundant.

Lactobacillus casei ssp. *rhamnosus* ATCC 7469. This strain exhibits probiotic properties because it survives in the gastrointestinal tract, elicits an up-regulation of IFN γ levels, and enhances resistance against parasite infections in mice.⁵ The strain was obtained from the strains collection of the Microbiology Department, ENCB-IPN. It was maintained in a Man Rogosa Sharpe broth (MRS: Difco, Detroit Mi, EUA) at 35°C. After 17 hours in the broth, the culture was washed with PBS pH 7.2 at 8 944 x g for 10 min, and adjusted to 1.8X10⁹ viable organisms/0.1 mL in PBS pH 7.2.

Stimulation with Lactic acid bacteria (LAB) and parasite challenge. Two groups of mice were intraperitoneally (IP) injected with 1.8X10⁹ viable *Lactobacillus casei*. The first group was injected seven days before parasite challenge and the second was inoculated twice with the same doses of lactobacilli,¹⁴ and 7 days before IP injection with *Plasmodium chabaudi* 5X10⁴ pRBC (day 0). Some mice were inoculated with PBS only.

Control mice were inoculated with 5X10⁴ *P. chabaudi* pRBC only. When parasitaemia was resolved, three mice from each group were killed, the spleen was dissected, weighed, and a total cellular suspension (75 mg/mL) was prepared in PBS pH 7.2 for inoculating IP naive mice with 0.1 mL.

Serum NO[•] determination. Blood was obtained from the caudal vein of three mice per group at 0, 7, 10, 12, 14, and 21 days post-infection. It was allowed to clot for 2 hours at 4°C, and then centrifuged at 8,944 x g for 10 min. Sera were collected and stored at -20°C until used for determination of NO[•].

For each serum sample, 50 μ L were transferred to a flat bottom plate, and 50 μ L of Greiss reagent were added (25 μ L of 1% sulfanilamide, 25 μ L of 0.1% naphthyl-ethylen-diamine (Sigma Chemical Co., St Louis,

MO, EUA) in 2.5% H_3PO_4) and incubated at room temperature for 15 min. Absorbance was measured at 550 nm (A_{550}) using an ELISA reader (Labsystems Multiskan Plus). NO_2^- concentrations were determined using a standard $NaNO_2$ curve.

Infection with spleen cells from mice that recovered from infection with Plasmodium chabaudi chabaudi AS. Group I mice were inoculated with spleen cells from mice that recovered from an infection with *Plasmodium chabaudi*. Group II was inoculated with spleen cells from mice that recovered from an infection with *Plasmodium chabaudi* before being treated once with *L. casei*; Group III was inoculated with spleen cells from mice that recovered from an infection with *Plasmodium chabaudi* before being treated twice with *L. casei*. Control mice (Group IV) were inoculated with 5×10^4 *P. chabaudi* pRBC only. The findings were confirmed in three repeated trials.

Statistical analysis. Parasitaemia was analyzed using the U-Mann-Whitney test and serum nitric levels were analyzed using a two-way ANOVA; $p < 0.05$ values were considered statistically different.

Results

Stimulation with Lactic acid bacteria (LAB) and parasite challenge

Mice infected with 5×10^4 *P. chabaudi* pRBC (control group) developed parasitaemia with a prepatent period of 4 days, patent period of 15 days, and maximum (about 51%) on day nine postinfection. Mice stimulated once with lactobacilli showed important changes in the early phase of the *P. chabaudi* infection, with a prepatent period of 5 days, a patent period of 11 days, and a maximum parasitaemia (36.3%) on day 12 postinfection; on the 7th day post infection, the parasitaemia level was about 0.4%, whereas in control mice it was about 27.8%. After the peak of parasitaemia, stimulated mice showed a quick drop in the curve ($<10\%$ in 72 hours), while the control mice reached this level in 120 hours (figure 1).

Mice stimulated twice with *Lactobacillus casei* and challenged with *P. chabaudi* showed a prepatent period of 5 days, a patent period of 8 days, and 30% parasitaemia on the 11th day, while the control group showed a prepatent period of 3 days, a patent period of 13 days, and a maximum parasitaemia of 48.5% on day nine post-infection. These mice maintained minimum parasitaemia levels ($<10\%$) during the first eight days post-infection while the control mice showed parasitaemia levels around 40%. After the parasitaemia peak, stimulated mice showed a fast drop with less than

10% pRBC in 48 hours, whereas the control group maintained the same level after 5 days (figure 2).

Infection with spleen cells from mice that recovered from Plasmodium chabaudi chabaudi AS infection

Mice infected with parasites obtained from spleen cells from mice that recovered from a *Plasmodium chabaudi* infection (group I) showed a parasitaemia similar to control mice inoculated with pRBC only (group IV),

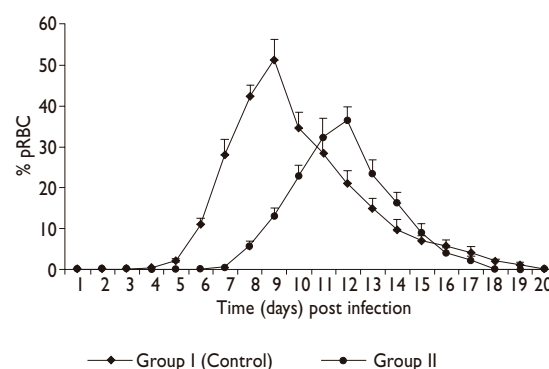


FIGURE 1. PARASITAEMIA IN NIH MICE (N= 6) INOCULATED WITH 5×10^4 *PLASMODIUM CHABAUDI* pRBC (CONTROL) AND TREATED PREVIOUSLY (GROUP II) WITH 1.8×10^9 VIABLE *LACTOBACILLUS CASEI* (DAY -7)

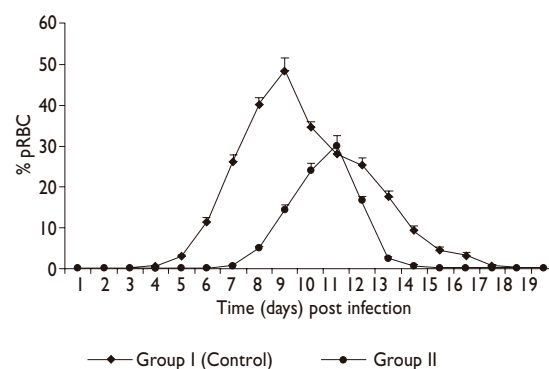


FIGURE 2. PARASITAEMIA IN NIH MICE (N= 6) INOCULATED WITH 5×10^4 *PLASMODIUM CHABAUDI* pRBC (CONTROL) AND TREATED TWICE PREVIOUSLY (GROUP II) WITH 1.8×10^9 VIABLE *LACTOBACILLUS CASEI* (DAYS -14 AND -7)

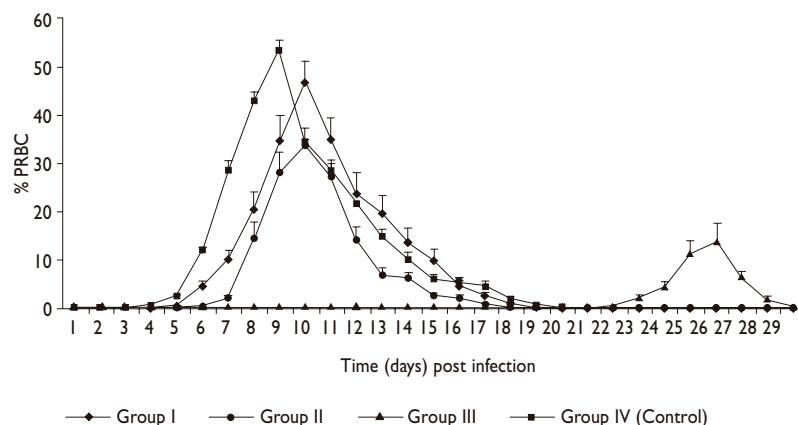


FIGURE 3. PARASITAEMIA IN NIH MICE (N= 6) INOCULATED WITH SPLEEN CELLS FROM MICE: RECOVERED FROM INFECTION WITH *PLASMODIUM CHABAUDI* (GROUP I); RECOVERED FROM INFECTION WITH *PLASMODIUM CHABAUDI* AND TREATED ONCE WITH *L. CASEI* (GROUP II); RECOVERED FROM INFECTION WITH *PLASMODIUM CHABAUDI* AND TREATED TWICE WITH *L. CASEI* (GROUP III); INOCULATED WITH 5×10^4 *PLASMODIUM CHABAUDI* PRBC (GROUP IV, CONTROL). ALL GROUPS WERE INOCULATED WITH A CELLULAR SUSPENSION POOL FROM THREE MICE

with a prepatent period of four days, a patent period of 13 days, and maximum parasitaemia of 46.6% on the 10th day. Group II mice inoculated with spleen cells from mice stimulated once with *Lactobacillus casei* and challenged with *Plasmodium chabaudi* showed a prepatent period of five days, a patent period of 11 days, and a maximum parasitaemia of 33.5% on the 10th day after infection. There was a statistically significant difference ($p < 0.01$) between the control group and the group of mice inoculated with spleen cells from mice stimulated twice with *Lactobacillus casei* before challenging with *Plasmodium chabaudi* (group III), the prepatent period was 21 days, the patent period was seven days, and the maximum parasitaemia was 13.5% on the 26th day (figure 3).

Serum NO determination

In the control (group I), a basal level of NO[•] of 16 μ M was observed, which was increasing with the course of infection. The maximum level reached about 76 μ M on day 10 post-infection, coinciding with the parasitaemia peak, then dropped to the basal level (day 21). Mice stimulated once with lactobacilli and challenged with *P. chabaudi* presented about a 500% increase (92.66 μ M) on day 0 (7 days after stimulation); maximum NO[•] concentration was reached on day 10 (142.33 μ M) (table I). Mice stimulated twice with lactobacilli and chal-

lenged with *P. chabaudi* showed the highest NO[•] levels, on day 0 they had an increase of more than 900% (153.3 μ M) with respect to the basal, and the maximum NO[•] level was on day 10 and declined to 64.3 μ M on day 21

Table I
SERUM NO₂⁻ LEVELS IN MICE INJECTED WITH LACTOBACILLUS CASEI 7 DAYS BEFORE CHALLENGING WITH PLASMODIUM CHABAUDI. ESCUELA NACIONAL DE CIENCIAS BIOLÓGICAS, IPN, MEXICO, 2004-2005

Day	Group I* μ M NO ₂ ⁻ \pm SEM	Group II† μ M NO ₂ ⁻ \pm SEM
0	16.16 \pm 0.60	92.66 \pm 1.45
7	54.33 \pm 1.45	122.33 \pm 1.54
10	75.66 \pm 0.88	142.33 \pm 1.45
12	55.66 \pm 1.20	121 \pm 1.52
14	50 \pm 1.52	105.66 \pm 1.20
21	15 \pm 0.57	30.33 \pm 0.88

Results are an average of three determinations and there is statistical difference between both groups ($p < 0.05$)

* Mice inoculated with 5×10^4 *Plasmodium chabaudi* pRBC

† Mice inoculated with 1.8×10^9 viable *L. casei* 7 days before challenging with 5×10^4 *Plasmodium chabaudi* pRBC (day 0)

Mice treated once with vehicle (PBS pH 7.2) had roughly 15 μ M of NO₂⁻

(table II). Mice inoculated once and inoculated twice with vehicle (PBS pH 7.2) had roughly 15 μM of NO_2^- .

Discussion

Innate immune response is a host's first line of defense against parasites. It determines the course of an infection, curing, and dissemination. Innate immune system components are mechanical barriers, cells, molecules, and cytokines. The IL-12 and $\text{IFN}\gamma$ are cytokines which participate in regulating the innate immune response, and are key to the induction of the adaptive immune response. Macrophages produce IL-12 in response to several microorganisms. IL-12 stimulate the synthesis of $\text{IFN}\gamma$ by NK and T lymphocytes which, in turn, activates more macrophages for destroying the microorganisms. One macrophage destroyer mechanism is the synthesis of reactive metabolites of oxygen and NO^\bullet . $\text{IFN}\gamma$ activates the transcription of genes that codify oxydase and nitric oxide synthase enzymes.

Since the discovery that mammalian cells produce NO^\bullet , considerable attention has been focused on its role in the cell of killing microbes. Recently, macrophages, neutrophils, and mast cells have all been shown to be major producers of this molecule. NO^\bullet is a gaseous water and lipid soluble molecule that regulates several physiological responses in the cardiovascular and

nervous systems. Immune and tissue cells produce NO^\bullet via the inducible nitric oxide synthase (iNOS) pathway in response to infectious and injurious agents.^{16,17}

Some probiotics, such as the *Lactobacillus* and *Bifidobacteria* species, have been found to induce innate immune mechanisms, including enhancement of epithelial barrier function in the intestine, activation of NF- κB and cytokine production in monocytes and natural killer cells, and induction of phagocytic activity in neutrophils.^{1,2} Korhonen¹⁸ investigated the effects of *Lactobacillus rhamnosus* GG probiotic bacteria and found iNOS expression and NO^\bullet production in macrophages in response to *Lactobacillus rhamnosus* GG and $\text{IFN}\gamma$, suggesting that this probiotic has immunomodulatory effects.

It has been shown that NO^\bullet is produced during the infection of mice with *Plasmodium chabaudi* AS. *In vivo* production of NO^\bullet during a *P. chabaudi* AS infection in NIH mice, measured as nitrate in the serum of infected mice, showed a sharp peak of production around peak parasitaemia; treatment of mice with L-NMM (L-Ng-monomethyl arginine), an inhibitor of NO^\bullet production, produced a detectable exacerbation of the peak parasitaemia.^{19,20}

In the present work, it is evident that *L. casei* enhances innate resistance to *Plasmodium chabaudi*, as was showed by lower parasitaemia in mice previously stimulated once or twice with lactobacilli than the control. Stimulated mice had larger prepatent periods and lower parasitaemia than the control (figures 1 and 2).

Parasites recovered from the spleens of mice previously stimulated with *Lactobacillus casei* and challenged with *Plasmodium chabaudi* kept their infective capacity, but the parasitemia was delayed and less intense than in control mice, suggesting that parasites were affected by the non-specific mechanism elicited by viable lactobacilli, and that the spleen plays an important role in the elimination of malaria parasites (figure 3). The precise regulatory mechanisms of iNOS expression *in vivo* and the physiologic role of NO^\bullet in different organs during lethal and non-lethal *P. chabaudi* AS infection are not yet known (Jacobs *et al.*).²¹ Moreover, the tissue site of NO^\bullet production, that is spleen *vs.* liver, appears to be critical and correlates with resistance *vs.* susceptibility to *P. chabaudi* AS, respectively.

Host phagocytic cell activity may play an important role in the control of *P. chabaudi* AS erythrocytic stages early in the infection. Legorreta-Herrera *et al.*,²² could transfer protection against *Plasmodium chabaudi* chabaudi in T cells depleted mice with irradiated spleen cells from hyper immune mice. Furthermore, Mota *et al.*,²³ observed phagocytosis by macrophages of parasitized erythrocytes *in vitro* in acute *Plasmodium chabaudi* infec-

Table II
SERUM NO_2^- LEVELS IN MICE INJECTED WITH
LACTOBACILLUS CASEI 14 AND 7 DAYS BEFORE
CHALLENGING WITH *PLASMODIUM CHABAUDI*. ESCUELA
NACIONAL DE CIENCIAS BIOLÓGICAS, IPN, MEXICO,
2004-2005

Day	Group I* $\mu\text{M NO}_2^- \pm \text{SEM}$	Group II† $\mu\text{M NO}_2^- \pm \text{SEM}$
0	15.66 \pm 0.33	153.33 \pm 1.66
7	51.66 \pm 1.20	223.33 \pm 1.66
10	84.33 \pm 1.76	301.66 \pm 4.40
12	58.33 \pm 0.88	274.33 \pm 2.33
14	58.33 \pm 0.88	142.33 \pm 1.45
21	14.66 \pm 1.45	64.33 \pm 2.33

Results are an average of three determinations, there is statistical difference between both groups ($p < 0.05$)

* Mice inoculated with 5×10^4 pRBC from mice infected with *Plasmodium chabaudi*

† Mice inoculated with 1.8×10^9 viable *L. casei* 14 and 7 days before challenging with 5×10^4 pRBC with *Plasmodium chabaudi* (day 0)

Mice treated twice with vehicle (PBS pH 7.2), showed around 15 μM of NO_2^-

tion. Jacobs *et al*²¹ investigated the production and function of NO' during the early phase of blood stage infection with *Plasmodium chabaudi* AS in two inbred strains of mice that differ in resistance levels to this parasite. They found that the resistant C57BL/6 mice, which clear the infection by week four, have higher iNOS mRNA in the spleen than susceptible A/J mice. In contrast, A/J mice have significantly increased levels of iNOS mRNA in the liver later in the infection, just before death occurs. Furthermore, splenic macrophages recovered from resistant mice produced significantly higher levels of nitrite (NO₂-) in response to LPS than macrophages from susceptible mice. Increased levels of NO' were only detected in the serum of resistant mice at the time of peak parasitaemia. Treatment with the iNOS inhibitor, aminoguanidine, reduced NO' levels in the serum of resistant mice and eliminated resistance of this host to *Plasmodium chabaudi* AS without affecting parasitaemia. These results demonstrate that the ability to produce high amounts of NO' early during the infection with blood stages of *Plasmodium chabaudi* AS correlates with resistance, but that NO' may not be involved in parasite killing. However, Rockett *et al.*¹⁶ showed that a saturated solution of NO' did not inhibit *Plasmodium falciparum* growth, but two oxidation products of NO' (nitrite and nitrate ions) were toxic to the parasite.

In conclusion, the results presented in this work suggest that *Lactobacillus casei* ssp. *rhamnosus* ATCC 7469, when given viable by IP injection, enhances a systemic, non-specific protection with an increase of NO' production (tables I and II). That mechanism probably includes phagocytic activity of macrophages that help to control the early stage of *P. chabaudi* infection, to the erythrocytic phases and the parasites lodged in the spleen.

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