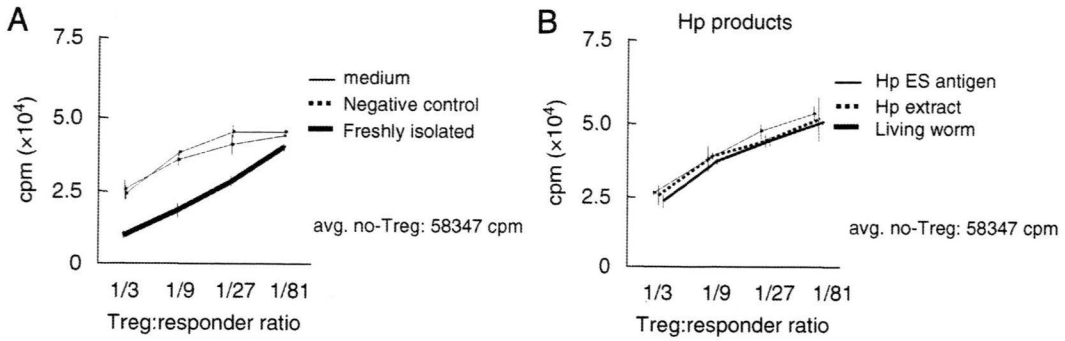

Figure 5. Stimulation of Treg with Hp products or living Hp worms *in vitro*. Splenic Treg isolated from mice at 5 days after Py infection were cultured with Hp products (100 μg/mL) or living Hp worms using a transwell system in the presence of DCs. Cultured Treg were used for suppressive analyses as described in the legend for Fig. 3C. Data are presented as the means ± SE of triplicate samples in a representative of 3–5 repeated experiments. There are no significant differences among the Treg conditioning treatments by Student's t-test.

responses in mice co-infected with Hp may prohibit the development of protective Th1 responses such as the production of IFN-γ.

Infections with helminths have also been reported to activate Treg [25, 28], and Hp suppresses allergic responses to innoxious Ag or protective immunity against pathogens in a Treg-dependent manner [29]. Su *et al.* [18] described the induction of regulatory cytokines, such as TGF-β and IL-10, during co-infection with Hp and *P. chabaudi*. We observed as well that transcription of TGF-β and IL-10 of Treg isolated from co-infected mice was slightly increased compared with those from non-infected animals or infected with either Py or Hp (data not shown). It would be interesting to evaluate the importance of these regulatory cytokines in *in vitro* suppressive function analysis, as done in Fig. 3C for instance.

The ES Ag of Hp were reported to be immunosuppressive toward T cells [30, 31]. However, we observed that infection with Hp alone did not activate Treg *in vivo* and that neither Hp products nor living worms directly activated Treg *in vitro*. Previous report suggested that Hp activates Treg *via* DC [32], but our *in vitro* results showed that Hp did not induce significant activation of Treg even in the co-culture with DC, suggesting less probability of the pathway between Hp *ex vivo* and strongly activated Treg, either dependently or independently of DC. One possibility may be that Hp infection takes longer than 2 wk to activate Treg by itself. Su *et al.* [18] described that mortality of co-infected mice increased if *P. chabaudi* infection was performed later than 2 wk after Hp infection. However, when we compared the effect of interval from preceding Hp to following Py infection on the induction of Treg between 2 and 6 wk (data not shown), Treg from both sets of co-infected mice showed similar and significant suppression compared with those from Py-infected mice, while Treg from Hp-infected mice did not show significant suppression. Taken together, our results suggest that Hp infection does not directly induce Treg activation, but probably conditions the host to stand by for Treg activation on exposure to incoming Ag /pathogens and/or enhances Treg activation by other stimulations such as Py.

Finally, our results demonstrate that co-infection with intestinal nematodes deteriorates the course of malaria, which is supposed to reflect the infectious state in malaria-endemic areas. Although we did not address whether treatment with anti-helminthic drugs improves malaria, chemotherapy against helminths would be included in global malaria control.

Materials and methods

Mice and parasites

Male 8- to 10-wk-old C57BL/6 mice were purchased from Kyudo (Tosu, Japan). All experiments using mice were conducted according to the guidelines for animal experimentation of Kyushu University.

Hp was kindly provided by Dr. J. F. Urban, Jr. (Beltsville Human Nutrition Research Center, US Department of Agriculture, Beltsville, MD, USA) and maintained by *in vivo* passages using male ICR mice. For infection, feces containing eggs were incubated on wet filter paper to allow the eggs to develop into infective larvae. Mice were infected orally with 200 infective larvae by gastric intubation. Infection was confirmed by Hp egg detection in feces before and after Py infection.

pRBC (2.5×10^4 cells/mouse) were commonly injected i.p., and the percent parasitemia (ratio of pRBC to total RBCs) was monitored by microscopic evaluation of thin blood films stained with Giemsa solution. For depletion of $CD25^+$ T cells *in vivo*, mice were injected i.p. with 100 μg of anti-CD25 Ab 1 day before and 1 day after Py infection. Each infection experiment was repeated three times with 5–7 mice *per* group.

Reagents

PE-anti-CD25 (PC61.5), allophycocyanin-anti-CD4 (GK1.5) and FITC-anti-Foxp3 (FJK-16s) staining kits, as well as FITC-anti-CD11c

(N418) Ab were obtained from eBioscience (San Diego, CA, USA). Anti-PE, anti-allophycocyanin and anti-FITC microbeads (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) were used for cell purification. Monoclonal rat anti-mouse CD25 (7D4) IgM purified from the ascites of hybridoma-injected athymic nude mice was used for *in vivo* treatments.

Preparation of parasites and their products

Worm products were prepared as described previously [30, 31] with modifications. Adult Hp worms were collected from the upper gastrointestinal tract of infected mice at 3–4 wk after larvae infection. The worms were washed three times with PBS containing ampicillin (150 μg/mL; Sigma, St. Louis, MO, USA) and streptomycin (50 μg/mL; Sigma), and approximately 500–1000 worms were incubated in 0.5 mL of ampicillin/streptomycin/PBS for 24–48 h at 37°C. The culture supernatant was collected by centrifugation at 10 000 × *g* for 10 min at 4°C and used as the Hp ES Ag. Worms were mechanically homogenized after or before incubation, and frozen and thawed 3–5 times. The supernatant was collected by centrifugation at 10 000 × *g* for 10 min at 4°C and used as the Hp extract. As a negative control, non-Hp-infected mouse intestinal contents were prepared in the same manner as for Hp ES Ag. All products were filtered through 0.45-μm pore filters, quantified by the BCA™ Protein Assay (Pierce, Rockford, IL, USA) and stored at –80°C until use. The products were pretreated with polymyxin B (50 μg/mL; Sigma) for 30 min before dilution with cell culture medium to a final concentration of 100 μg/mL, including 12.5 μg/mL polymyxin B. When required, living worms were freshly prepared by washing three times with PBS containing ampicillin and streptomycin and pretreated with polymyxin B for 30 min at room temperature. Hp products were prepared for each cellular experiment.

pRBC were isolated using a Percoll enrichment technique [33]. Briefly, blood from Py-infected mice was collected into heparinized PBS and passed through a cellulose column to remove leukocytes and platelets. After addition of the RBC solution to 63% v/v Percoll/PBS and centrifugation, pRBC were collected from the interphase. pRBC were freshly prepared for each cellular experiment. When required, isolated pRBC were frozen and thawed three times, and the supernatant was collected by centrifugation at 10 000 × *g* for 10 min at 4°C for use as a coating Ag in ELISA.

Cell purification

Cell purification was performed using a magnetic cell sorting system (Miltenyi Biotech GmBH) according to the manufacturer's instructions. Spleens of mice were reduced to single cell suspensions by hemolysis with 0.86% NH_4Cl. To purify $CD4^+CD25^-$ cells, the suspensions were incubated with FITC-anti-CD4 and PE-anti-CD25 Ab, followed by the addition of anti-PE microbeads and removal of $CD25^+$ cells. Anti-FITC microbeads were added to the flowthrough and $CD4^+CD25^-$ cells were obtained. DCs were purified using an FITC-anti-CD11c Ab. Treg were purified using a PE-anti-CD25 Ab, and 90% of them were confirmed to express CD4, CD25 and Foxp3 by flow cytometry. The purity of each cell subset usually exceeded 90%.

Cell culture

Purified T cells were cultured with pRBC in the presence of $CD11c^+$ cells in 200 μL of RPMI1640 supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 mM HEPES, 50 mM $NaHCO_3$, 2 mM L-glutamine, 100 μM 2-mercaptoethanol and 10% inactivated fetal bovine serum on round-bottomed 96-well plates. ConA was added as an assay control at a final concentration of 2.5 μg/mL. $CD11c^+$ cells were irradiated with 30 Gy before coculture with other cells. Typically, 1×10^5 T cells, $1–2 \times 10^5$ pRBC and 1×10^4 $CD11c^+$ cells were cocultured *per* well. Cultures were performed for 68–76 h at 37°C in air supplemented with 5% CO_2, including 10–16 h of coculture with ^{3}H-thymidine (1 μCi/well). Cells were harvested onto glass-fiber filter mats, dried and measured for their ^{3}H-thymidine uptake using a liquid scintillation counter. When required, the supernatant was collected before the addition of ^{3}H-thymidine and kept at –80°C until use. Each triplicate experiment was repeated 3–6 times.

Treg suppression assay

To analyze Treg functions, purified $CD4^+CD25^-$ cells (1×10^5 cells/well) from uninfected or infected mouse spleens stimulated with soluble ConA (2.5 μg/mL) and $CD11c^+$ cells (1×10^4 cells/well) from uninfected mouse spleens were cultured with a variety of freshly isolated or preconditioned Treg at various populations for 72 h and incubated with ^{3}H-thymidine (1 μCi/well) for the last 8–12 h. Radioactivity was measured using a liquid scintillation counter.

When required, purified Treg isolated from uninfected or Py-infected mouse spleens were preconditioned with $CD11c^+$ cells from uninfected mouse spleens under various conditions for 72 h. Conditioning with living Hp worms was performed using a transwell system (0.2-μm Anopore membrane; Nalge Nunc International, Rochester, NY, USA), in which the cells were in the lower chamber and 10 worms were in the upper chamber. Conditioned Treg were washed once, counted for living cells by Trypan blue staining and used for suppression assays as described above. Each triplicate experiment was repeated 3–5 times.

ELISA

The IFN-γ concentrations in the above-described cell culture supernatants were measured using a Mouse IFN-γ ELISA

Development Kit, DuoSet (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Each duplicate experiment was repeated three times. The anti-malaria Ab titers in the sera of mice were measured by ELISA by the OD at 440 nm using HRP-anti-mouse IgG(H+L), HRP-anti-mouse IgG1 and HRP-anti-mouse IgG2a Ab as previously described [34]. Sera were collected and kept at –80°C until analysis. Malaria Ag was prepared as described above and used as a coating Ag. Each triplicate experiment was repeated three times.

Flow cytometry

Splenocytes were prepared as single cell suspensions by hemolysis with 0.86% NH_4Cl. After staining of cell surface molecules or intracellular staining of Foxp3, the cells were evaluated using a FACSCalibur (BD, Franklin Lakes, NJ, USA) according to the manufacturer's instructions and the data were analyzed with the CellQuest Pro software (BD). Each infection experiment using 2–3 mice was repeated three times.

Statistical analysis

Student's *t*-test was used for statistical analyses. Values of $p<0.05$ were considered significant.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Barry, A. E., Leliwa-Sytek, A., Tavul, L., Imrie, H., Migot-Nabias, F., Brown, S. M., McVean, G. A. V. *et al.***, Population genomics of the immune evasion (*var*) genes of *Plasmodium falciparum*. *PLoS Pathogens* 2007. **3**: e34.

2 **Healer, J., Murphy, V., Hodder, A. N., Masciantonio, R., Gemmill, A. W., Anders, R. F., Cowman, A. F. *et al.***, Allelic polymorphisms in apical membrane antigen-1 are responsible for evasion of antibody-mediated inhibition in *Plasmodium falciparum*. *Mol. Microbiol.* 2004. **52**: 159–168.

3 **Walther, M., Tongren, J. E., Andrews, L., Korbel, D., King, E., Fletcher, H., Andersen, R. F. *et al.***, Upregulation of TGF-beta, FOXP3, and $CD4^+CD25^+$ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity* 2005. **23**: 287–296.

4 **Hisaeda, H., Maekawa, Y., Iwakawa, D., Okada, H., Himeno, K., Kishihara, K., Tsukumo, S. and Yasutomo, K.**, Escape of malaria parasites from host immunity requires $CD4^+CD25^+$ regulatory T cells. *Nat. Med.* 2004. **10**: 29–30.

5 **Xu, H., Wipasa, J., Yan, H., Zeng, M., Makobongo, M. O., Finkelman, F. D., Kelso, A. and Good, M. F.**, The mechanism and significance of deletion of parasite-specific $CD4^+$ T cells in malaria infection. *J. Exp. Med.* 2002. **195**: 881–892.

6 **Urban, B. C., Ferguson, D. J. P., Pain, A., Willcox, N., Plebansk, M., Austyn, J. M. and Roberts, D. J.**, *Plasmodium falciparum*-infected erythrocytes modulate the maturation of dendritic cells. *Nature* 1999. **400**: 73–77.

7 **March, K. and Snow, R. W.**, Malaria transmission and morbidity. *Parasitologia* 1999. **41**: 241–246.

8 **Shankar, A. H.**, Nutritional modulation of malaria morbidity and mortality. *J. Infect. Dis.* 2000. **182**: S37–S53.

9 **Barnes, K. I., Durrheim, D. N., Little, F., Jackson, A., Mehta, U., Allen, E., Dlamini, S. S. *et al.***, Effect of artemether-lumefantrine policy and improved vector control on malaria burden in KwaZulu-Natal, South Africa. *PLoS Med.* 2005. **2**: e330.

10 **Abu-Raddad, L. J., Patnaik, P. and Kublin, J. G.**, Dual infection with HIV and malaria fuels the spread of both diseases in sub-Saharan Africa. *Science* 2006. **312**: 1603–1606.

11 **World Health Organization**, 2004. Malaria cases (per 100,000) by country, latest available data. Accessed on 28th January 2009.

12 **World Health Organization**, 2006. Soil-transmitted helminth (STH) infections are widely distributed in tropical and subtropical areas – 2006. Accessed on 28th January 2009.

13 **Jenkins, S. J., Hewitson, J. P., Jenkins, G. R. and Mountford, A. P.**, Modulation of the host's immune response by schistosome larvae. *Parasite Immunol.* 2005. **27**: 385–393.

14 **Hoerauf, A., Satoguina, J., Saeftel, M. and Specht, S.**, Immunomodulation by filarial nematodes. *Parasite Immunol.* 2005. **27**: 417–429.

15 **Maizels, R. M. and Yazdanbakhsh, M.**, Immune regulation by helminth parasites: cellular and molecular mechanisms. *Nat. Rev. Immunol.* 2003. **3**: 733–744.

16 **Nacher, M., Singhasivanon, P., Yimsamran, S., Manibunyong, W., Thanyavanich, N., Wuthisen, P. and Looareesuwan, S.**, Intestinal helminth infections are associated with increased incidence of *Plasmodium falciparum* malaria in Thailand. *J. Parasitol.* 2002. **88**: 55–58.

17 **Nacher, M., Gay, F., Singhasivanon, P., Krudsood, S., Treeprasertsuk, S., Mazier, D., Vouldoukis I. and Looareesuwan, S.**, *Ascaris lumbricoides* infection is associated with protection from cerebral malaria. *Parasite Immunol.* 2000. **22**: 107–113.

18 **Su, Z., Segura, M., Morgan, K., Loredo-Osti, J. C. and Stevenson, M. M.**, Impairment of protective immunity to blood-stage malaria by concurrent nematode infection. *Infect. Immun.* 2005. **73**: 3531–3539.

19 **Noland, G. S., Urban, J. F., Jr. , Fried, B. and Kumar, N.**, Counter-regulatory anti-parasite cytokine responses during concurrent *Plasmodium yoelii* and intestinal helminth infections in mice. *Exp. Parasitol.* 2008. **119**: 272–278.

20 **Tetsutani, K., Ishiwata, K., Torii, M., Hamano, S., Hisaeda, H. and Himeno, K.**, Concurrent infection with *Heligmosomoides polygyrus* modulates murine host response against *Plasmodium berghei* ANKA infection. *Am. J. Trop. Med. Hyg.* 2008. **79**: 819–822.

21 **Gause, W. C., Urban, J. F., Jr., and Stadecker, M. J.**, The immune response to parasitic helminths: insights from murine models. *Trends Immunol.* 2003. **24**: 269–277.

22 **Monroy, F. G. and Enriquez, F. J.**, *Heligmosomoides polygyrus*: a model for chronic gastrointestinal helminthiasis. *Parasitol. Today* 1992. **8**: 49–54.

23 **Adachi, K., Tsutsui, H., Kashiwamura, S., Seki, E., Nakano, H., Takeuchi, O., Takeda, K. et al.,** *Plasmodium berghei* infection in mice induces liver injury by an IL-12-and toll-like receptor/myeloid differentiation factor 88-dependent mechanism. *J. Immunol.* 2001. **167**: 5928–5934.

24 **Shear, H. L., Srinivasan, R., Nolan, T. and Ng, C.,** Role of IFN-γ and nonlethal malaria in susceptible and resistant murine hosts. *J. Immunol.* 1989. **143**: 2038–2044.

25 **Finney, C. A. M., Taylor, M. D., Wilson, M. S. and Maizels, R. M.,** Expansion and activation of $CD4^+CD25^+$ regulatory T cells in *Heligmosomoides polygyrus* infection. *Eur. J. Immunol.* 2007. **37**: 1874–1886.

26 **Ohata, J., Miura, T., Johnson, T. A., Hori, S., Ziegler, S. F. and Kohsaka, H.,** Enhanced efficacy of regulatory T cell transfer against increasing resistance, by elevated Foxp3 expression induced in arthritic murine hosts. *Arthritis Rheum.* 2007. **56**: 2947–2956.

27 **Couper, K. N., Blount, D. G., de Souza, J. B., Suffia, I., Belkaid, Y. and Riley, E. M.,** Incomplete depletion and rapid regeneration of $Foxp3^+$ regulatory T cells following anti-CD25 treatment in malaria-infected mice. *J. Immunol.* 2007. **178**: 4136–4146.

28 **Watanabe, K., Mwinzi, P. N., Black, C. L., Muok, E. M., Karanja, D. M., Secor, W. E. and Colley, D. G.,** T regulatory cell levels decrease in people infected with *Schistosoma mansoni* on effective treatment. *Am. J. Trop. Med. Hyg.* 2007. **77**: 676–682.

29 **Wilson, M. S., Taylor, M. D., Balic, A., Finney, C. A. M, Lamb, J. R. and Maizels, R. M.,** Suppression of allergic airway inflammation by helminth-induced regulatory T cells. *J. Exp. Med.* 2005. **202**: 1199–1212.

30 **Telford, G., Wheeler, D. J., Appleby, P., Bowen, J. G. and Pritchard, D. I.,** *Heligmosomoides polygyrus* immunomodulatory factor (IMF), targets T-lymphocytes. *Parasite Immunol.* 1998. **20**: 601–611.

31 **Rzepecka, J., Lucius, R., Doligalska, M., Beck, S., Rausch, S. and Hartmann, S.,** Screening for immunomodulatory proteins of the intestinal parasitic nematode *Heligmosomoides polygyrus*. *Parasite Immunol.* 2006. **28**: 463–472.

32 **Segura, M., Su, Z., Piccirillo, C. and Stevenson, M. M.,** Impairment of dendritic cell function by excretory-secretory products: a potential mechanism for nematode-induced immunosuppression. *Eur. J. Immunol.* 2007. **37**: 1887-1904.

33 **Tosta, C. E., Sedegah, M., Henderson, D. C. and Wedderburn, N.,** *Plasmodium yoelii* and *Plasmodium berghei*: isolation of infected erythrocytes from blood by colloidal silica gradient centrifugation. *Exp. Parasitol.* 1980. **50**: 7–15.

34 **Matsuoka, Y.,** Malaria ELISA. In **Tanabe, K., Waki, S., Kojima, S.** and **Kita, K.** (Eds.), *Malariology Laboratory Manuals*. Kinokuniya, Tokyo 2000, pp. 123–126.

Abbreviations: **ES:** excretory/secretory · **Foxp3:** forkhead box p3 · **Hp:** *Heligmosomoides polygyrus* · **pRBC:** *Plasmodium yoelii*-parasitized erythrocyte · **Py:** *Plasmodium yoelii* 17XNL

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Efficient protective immunity against *Trypanosoma cruzi* infection after nasal vaccination with recombinant Sendai virus vector expressing amastigote surface protein-2

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ABSTRACT

Chagas' disease, caused by infection with the protozoan parasite *Trypanosoma cruzi* (*T. cruzi*), is intractable showing a high mortality rate, and the development of effective vaccines is much desired. To examine the efficacy of a new mode of recombinant viral vaccine, we constructed two non-transmissible Sendai viruses (rSeV/dF) encoding the full-length parasite antigen amastigote surface protein-2 (ASP2) or ASP2 fused with a mono-ubiquitin on its N-terminus (UASP2). C57BL/6 mice immunized intranasally with rSeV/dF expressing either ASP2 or UASP2 showed significantly suppressed parasitemia and could be protected from lethal *T. cruzi* challenge. Depletion of $CD8^+$ T cells around the time of infection with *T. cruzi* completely abolished this protection, confirming that acquired immunity against the infection of *T. cruzi* is dependent on $CD8^+$ T cells. We also demonstrated that the protective immunity correlated with higher secretion of interferon-γ (IFN-γ) by spleen cells on *in vitro*-specific or non-specific stimulation. Increased CTL activity was also confirmed by degranulation or CTL assays. Interestingly, the control virus, rSeV/dF-GFP, induced even a higher IFN-γ production from spleen cells following non-specific but not specific stimulation *in vitro*, suggesting that SeV may also be a good adjuvant when used as a vaccine vehicle. Taking together, the current findings indicate that recombinant Sendai virus expressing the ASP2 or UASP2 antigens of *T. cruzi* are interesting candidates for the development of a new mode of recombinant viral vaccine against Chagas' disease.

1. Introduction

Infection with the protozoan parasite *Trypanosoma cruzi* causes Chagas' disease, a neglected disease that affects more than 16 million inhabitants in Latin America. More than 300,000 new patients become infected with *T. cruzi* every year, and approximately 21,000 people die annually, despite the use of drugs that have diminished morbidity and mortality rates in the past. Drugs used for the treatment are not effective in chronically infected individuals and parasites naturally resistant to chemotherapy have been described in various regions of Latin America [1,2]. Approximately 30% of infected individuals either have or will develop cardiac, peripheral nervous system or digestive system complications within 10–30 years after infection. Some individuals will suffer sudden cardiac death. These disease sequelae will continue to place a large economic burden on endemic countries, and hence Chagas' disease control, treatment and premature disability and death constitute a tremendous economic burden for Latin America. Although controversy between the hypothesis of autoimmune and parasite persistence for the reason of symptoms observed in the chronic phase of the disease still exists, parasite persistence is now considered to be the main factor. Based on this, containment of parasite growth and survival may be the best route to avoid chronic phase immunopathology. Thus, vaccination should be seriously considered as an alternative approach in therapy and prophylaxis of Chagas' disease.

Several studies have attempted to develop a vaccine using live attenuated [3] or killed *T. cruzi* parasites [4]. Even related trypanosomatids which share common antigens with *T. cruzi*, such as the tomato parasite *Phytomonas serpens* [5], have been tested as inducers of cross-protection. Unfortunately, most of these immunizations were only able to delay manifestations of the disease

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or, at best, decrease the associated pathology. In addition to live vaccines, subunit vaccines have been tried to induce protective immunity. Antigens, such as TSA1, trans-sialidase, ASP2, and cruzipain, have been tested as genetic vaccines as well as recombinant protein-based vaccines [6–20]. Genetic vaccines are supposed to effectively activate $CD8^+$ T cells because antigens are produced in the cytosol compartment. In particular, recombinant viral vaccines display the universal adjuvant effect attributed to their pathogen-associated molecular patterns, resulting in remarkable induction of effective immunity [21]. Indeed, clinical trails of new recombinant viral vaccine candidates for malaria, tuberculosis, or acquired immunodeficiency syndrome have shown unprecedented levels of cellular immunity against the recombinant products expressed by viral vectors, compared with previous vaccine formulations based on purified recombinant proteins or plasmid DNA vaccines [22–25].

Sendai virus (SeV) is a negative-strand RNA virus and a member of the *Paramyxoviridae* family, whose genomic replication and transcription are carried out in the cytoplasm using its own RNA polymerase. We have developed a clinically available, non-transmissible, and cytoplasmic recombinant viral vector based on SeV-lacking the fusion (F) gene (rSeV/dF). This vector could limit additional adjuvant effects to the first round of infection, and is not expected to induce prolonged generalized systemic immune-stimulatory effects that could have deleterious consequences [26]. Importantly, this mode of vector has been under clinical evaluation in Kyushu University Hospital. Ten patients with critical limb ischemia have already been received rSeV/dF expressing an angiogenic factor, basic fibroblast growth factor (bFGF/FGF-2) without significant and direct adverse events (Yonemitsu et al. unpublished data).

Protective immunity against *T. cruzi* is likely to be highly dependent on the induction of $CD8^+$ T cell responses and interferon (IFN-γ), similarly to that against the pre-erythrocytic stages of *Plasmodium* species and *Toxoplasma gondii*. Indeed, our previous studies demonstrated that vaccination of mice with naked DNA encoding antigen fused to mono-ubiquitin at the N-terminus induced protective $CD8^+$ T cells specific for the antigen expressed by the DNA vaccine [27–29], suggesting that the use of the ubiquitin fusion sequence was advantageous for the induction of $CD8^+$ responses.

In this study, we tested the ability of rSeV/dF encoding the amastigote surface protein (ASP)-2 fused with or without a mono-ubiquitin on its N-terminus to induce protective $CD8^+$ T cells following a lethal challenge with *T. cruzi* parasites. Results indicate that these vectors promote the development of efficient protective immunity against *T. cruzi* infection, suggesting a promising strategy for the development of a vaccine for Chagas' disease.

2. Materials and methods

2.1. Animals and parasites

Female C57BL/6 (B6) mice purchased from Kyudo (Tosu, Japan) were used for experiments at 8–10 weeks of age. All experiments using mice were reviewed by the Committee for the Ethics on Animal Experiment in the Faculty of Medicine, and carried out under the control of the Guidelines for Animal Experiment in the Faculty of Medicine, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government. The Tulahuen strain of *T. cruzi* was maintained by weekly passaging in B6 mice. For challenge infections, mice were inoculated with 1000 blood-derived trypomastigotes at the base of the tail 2 weeks after immunization. Parasitemia levels were evaluated by counting the number of bloodstream trypomastigotes in 5 μl blood from the tail veins under light microscopy.

2.2. Preparation of SeV and immunization

ASP2 cDNA was amplified by reverse-transcriptional PCR (RT-PCR) from total RNA isolated from liver section clinically separated from *T. cruzi* infected mice. ASP2 DNA was constructed into pcDNA3.1(−) (Invitrogen) or pcDNA-Ub vector by inserting into XhoI and AflII sites before preparation of SeV. Preparation and recovery of F-defective and non-transmissible recombinant SeV used in this study were preformed as previously described [26,30]. Briefly, LLC-MK2 cells stably expressing the F-gene were transfected with a plasmid mixture containing pSeV18+b(+)/F-target gene (enhanced GFP (EGFP), ASP2 or UASP2), pGEM-NP, pGEM-P, and pGEM-L. The transfected cells were collected, resuspended, and lysed by three cycles of freezing and thawing. Subsequent genomic RNA-nuclear protein complex transfection was preformed by cationic lipid into F-expressing LLC-MK2/F7 cells. Virus yield is expressed in PFU and cell infectious units (CIU). B6 mice were administrated intranasally with 5×10^6 CIU SeV-GFP, SeV-ASP2 or SeV-UASP2 in 20 μl PBS, respectively.

2.3. In vivo depletion of T-cell subsets

Anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 2.43) were injected intraperitoneally at 0.25 mg/mouse on days −1 and 1 of infection of *T. cruzi*. Depletion of each T-cell subset was confirmed by flow cytometry on day 2 of infection. Typically, over 98% of the appropriate cell subset was depleted.

2.4. Flow cytometry

To detect intracellular IFN-γ, 1×10^6 spleen cells were stimulated with 50 ng/ml PMA and 1 μg/ml calcium ionophore in the presence of 1 μg/ml brefeldin A at 37 °C for 5 h. These cells were then stained with APC-conjugated anti-mouse CD3ε (clone 145-2C11), FITC-conjugated anti-mouse CD8α (clone 53-6.7), followed by fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin. The cells were then stained with PE-conjugated anti-mouse IFN-γ (clone XMG1.2). For CD107a mobilization assays, spleen cells stimulated with 20 μg/ml of the ASP2 CD8 epitope peptide (VNHSFTLV) were stained with PE-anti-mouse CD107a (clone eBio1D4B) in the presence of the epitope followed by surface staining. After washing twice, stained cells were analyzed using a FACSCalibur (BD Biosciences) and the list data were analyzed using CellQuest Pro software (BD Biosciences).

2.5. ELISA

IFN-γ in the supernatants from splenocytes cultured with the ASP2 CD8 epitope peptide for 3 days were measured using a DuoSet sandwich ELISA (R&D Systems) according to the manufacturer's instructions.

2.6. Cytotoxicity assay

Mice were sacrificed 10 days after *T. cruzi* infection, and their spleen cells (4×10^7) were co-cultured with the ASP2 CD8 epitope peptide (4 μg/ml) in six-well culture plates in complete RPMI 1640 medium. After 3 days of culture, cells were collected for the analysis of CTL activity as described previously [31].

2.7. Statistical analysis

Differences between groups were analyzed for statistical significance using Excel software with unpaired Student's *t*-tests. For

survival curves, Kaplan–Meier plots were performed. Probability below 0.05 was considered to be statistically significant.

3. Results

3.1. Vaccination with rSeV/dF expressing ASP2 induced protective immunity against T. cruzi

To develop effective vaccines against *T. cruzi*, the causative agent of Chagas' disease, we constructed a rSeV/dF expressing amastigote surface protein (ASP)-2 and evaluated its capacity to induce protective immunity. C57BL/6 mice were immunized with rSeV/dF expressing ASP2 (rSeV/dF-ASP2) or ubiquitin-fused ASP2 (rSeV/dF-UASP2) via the intranasal route. Two weeks after immunization, mice were challenged with 1000 blood-stream trypomastigotes. Mice immunized with control rSeV/dF expressing GFP (rSeV/dF-GFP) as well as unimmunized mice displayed an increasing parasitemia from day 7 after infection, with peak parasitemia around 24 days immediately prior to death (Fig. 1A). In contrast, mice that received rSeV/dF-ASP2 or rSeV/dF-UASP2 displayed significantly lower parasitemia on days 7, 14, 17 or on days 7, 14, 17, 20, and 24, respectively, compared with those immunized with rSeV/dF-GFP. In a clear correlation with the results of parasitemia, unimmunized and rSeV/dF-GFP-immunized mice died quickly, and only about 20% of mice survived. In contrast, more than 90% of mice immunized with both rSeV/dFs expressing ASP2 survived until the end of the experiment (Fig. 1B).

3.2. Protection induced by vaccination with rSeV/dF expressing ASP2 was dependent on CD8⁺ T cells

In previous studies it was demonstrated that protective immunity does not correlate with the levels of antibodies specific for *T. cruzi* [10,17,18]. Instead, IFN-γ-producing CD4⁺ and CD8⁺ T cells have been demonstrated to be linked to protection against *T. cruzi*

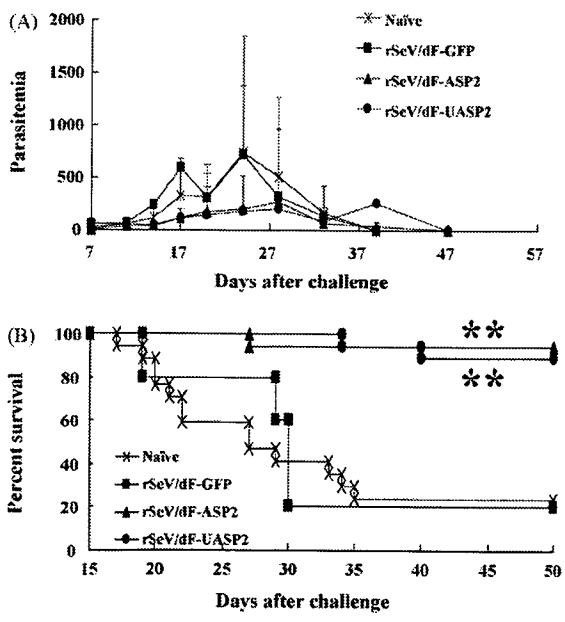

Fig. 1. Parasitemia and mortality in C57BL/6 mice after challenge infection with *T. cruzi*. B6 mice immunized with the indicated rSeV/dF were challenged with 1000 blood-form *T. cruzi* trypomastigotes. Infection was monitored on the basis of parasitemia (A) and survival (B). (A) Each value represents the mean ± SD from more than five mice in each group. (B) Results are expressed as the percentages of surviving mice in each group. ** indicates statistical significance at $p < 0.01$ compared with unimmunized or rSeV/dF-GFP immunized groups with Kaplan–Meier plot analyses.

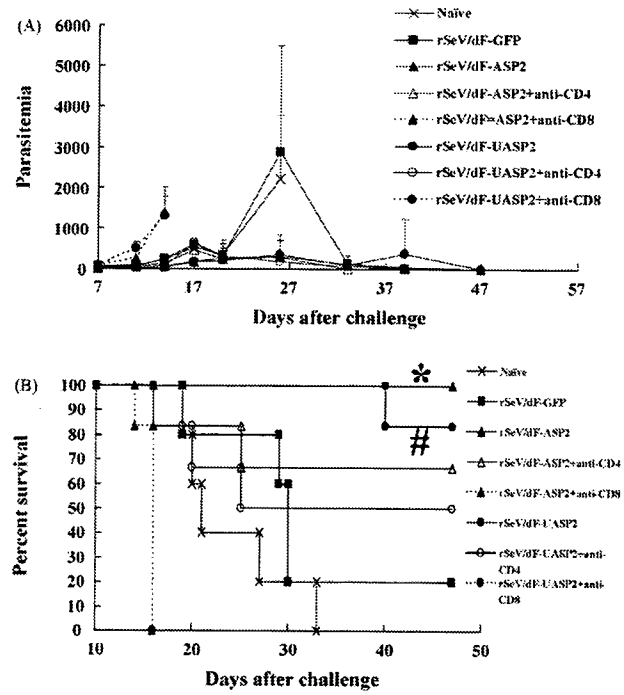

Fig. 2. Crucial role of CD8⁺ T cells in anti-parasite immunity induced by rSeV/dF immunization. Mice vaccinated with rSeV/dF-ASP2 or rSeV/dF-UASP2 were depleted of CD4⁺ or CD8⁺ T cells and challenged with *T. cruzi*. The course of infection was monitored as in Fig. 1. * or # indicates statistical significance at $p < 0.01$ or $p < 0.05$, respectively, compared with unimmunized or rSeV/dF-GFP immunized groups with Kaplan–Meier plot analyses.

[17,18,32,33]. Therefore, to investigate the impact of CD4⁺ and CD8⁺ T cells in protective immunity induced by rSeV/dF-based vaccination, we depleted CD4⁺ or CD8⁺ T cells from mice immunized with rSeV/dF-ASP2 or with rSeV/dF-UASP2 immediately before the challenge infection. Again, vaccination with ASP2-expressing viruses protected mice from otherwise lethal infection with *T. cruzi*.

Depletion of CD4⁺ T cells showed a mild effect in the protective immunity, since mice depleted of CD4⁺ T cells showed neither significant increase in parasitemia nor decrease in survival rate (Fig. 2A and B). In contrast, depletion of CD8⁺ T cells completely abolished the protective immunity induced by rSeV/dF expressing ASP2. After depletion of CD8⁺ T cells, mice immunized with rSeV/dF-ASP2 or rSeV/dF-UASP2 showed great exacerbation in parasitemia and died very quickly even compared with unimmunized mice.

These results indicate that CD8⁺ T cells were essential for the protective immunity against infection with *T. cruzi* following rSeV/dF-based vaccination.

3.3. CD8⁺ T cells were activated following vaccination with rSeV/dF-ASP2 and rSeV/dF-UASP2

We further analyzed several parameters related to the activation of CD8⁺ T cells against *T. cruzi*.

To determine the production of IFN-γ by CD8⁺ T cells, spleen cells cultured with PMA in the presence of Ca^{2+} ionophore were analyzed for their production of IFN-γ using flow cytometry. Whereas no significant difference was found in the IFN-γ expression level of CD4⁺ T cells between rSeV/dF-immunized and -unimmunized mice (Fig. 3A), production of this cytokine by CD8⁺ T cells strongly increased in mice immunized with rSeV/dF expressing ASP2, irrespective of fusion with the ubiquitin sequences. The number of IFN-γ⁺ CD8⁺ T cells was also significantly increased

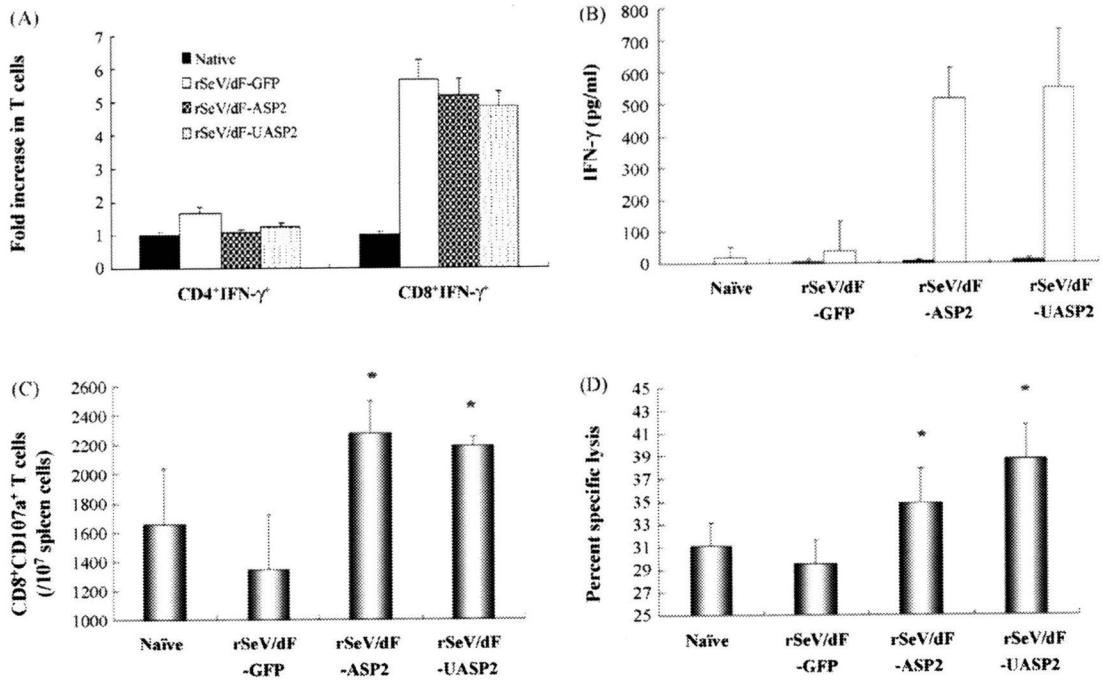

Fig. 3. Functional analyses of $CD8^+$ T cells in mice immunized with rSeV/dF. (A–C) Spleen cells isolated from mice 2 weeks after immunization were analyzed. (A) Spleen cells from the indicated mice were stimulated with PMA and ionomycin for 5 h *in vitro*, and then stained with fluorochrome-labeled antibodies. Values represent the mean ± SD from triplicate cultures. (B) The levels of IFN-γ in supernatants from splenocytes cultured with (open bars) or without (filled bars) the APS2 CD8 epitope peptide were determined by ELISA. Values represent the mean ± SD from more than 4 samples. (C) Spleen cells were cultured with the ASP2 CD8 epitope peptide for 3 days. For the last 4 h, cells were incubated with PE-conjugated anti-mouse CD107a to allow antibodies to bind CD107a during exocytosis. Cells were then stained with cell surface markers and analyzed. Values represent the mean ± SD from triplicate cultures. (D) Spleen cells isolated from rSeV/dF-vaccinated mice 10 days after challenge infection with *T. cruzi* were stimulated with the ASP2 CD8 epitope peptide for 3 days. These effector cells were then co-cultured with ^{3}H-labeled EL-4 cells pulsed with the ASP2 epitope peptide at an effector-to-target ratio of 100:1. * indicates statistical significance at $p < 0.05$ compared with unimmunized or rSeV/dF-GFP-immunized groups using a Student's *t*-test.

in rSeV/dF-immunized mice compared with unimmunized mice (Fig. 3A). These antigen-non-specific responses could be attributed to inoculation with rSeV/dF, confirming an excellent adjuvant effect of this virus vector on activating $CD8^+$ T cells.

Next, in order to study antigen-specific responses, we cultured splenocytes with a peptide corresponding to the CD8 epitope of ASP2 and measured the production of IFN-γ in culture supernatants by ELISA. As expected, production of IFN-γ in mice immunized with rSeV/dF-ASP2 or rSeV/dF-UASP2 was remarkably enhanced in the presence of the epitope peptide but not in unimmunized and rSeV/dF-GFP-immunized control mice (Fig. 3B).

We next evaluated cytotoxic activity of $CD8^+$ T cells. It is well known that $CD8^+$ T cells are able to lyse target cells via the granule exocytosis pathway [34] and degranulation closely correlates with cytotoxicity [35]. We therefore evaluated the exocytosis of intracellular vesicles by measuring the exposure of CD107a (also known as lysosome associated membrane protein-1, LAMP-1), a protein normally localized on the luminal surface of granules [36]. As shown in Fig. 3C, significantly increased numbers of $CD8^+$ $CD107a^+$ T cells were detected in the splenocytes from mice immunized with rSeV/dF-ASP2 or rSeV/dF-UASP2.

Finally, to confirm that these observations might reflect cytotoxic ability, we examined CTL activity against H-2^b-bearing EL4 cells pulsed with the CD8 epitope peptide of ASP2 (Fig. 3D). Compared with unimmunized or rSeV/dF-GFP immunized mice, those vaccinated with rSeV/dF-ASP2 or rSeV/dF-UASP2 showed prominent CTL activity.

These results indicate that immunization with rSeV/dF-ASP2 and rSeV/dF-UASP2 could efficiently activate functional ASP2-specific $CD8^+$ T cells responsible for protection against lethal *T. cruzi* infection.

4. Discussion

In this report, we used recombinant rSeV/dF vectors expressing the amastigote antigen to develop an effective strategy for the induction of protective immunity mediated by $CD8^+$ T cells against *T. cruzi*. Our results clearly demonstrate that vaccination with rSeV/dF expressing ASP2 induced $CD8^+$ T cells specific for ASP2 and protected mice from infection with *T. cruzi*.

The development of effective vaccines against *T. cruzi* is desired because the current therapeutic agents are not enough to achieve complete cures. Two distinct approaches to developing vaccines against intracellular pathogens are encountered; one is to inhibit the invasion of pathogens into their host cells, and the other is to kill intracellular pathogens and/or host cells themselves after invasion. Antibodies to molecules expressed on the surface of pathogens outside host cells would be the principal effector. From this point of view, trypomastigote surface antigen-1 and other trans-sialidase antigens have been tried for vaccine development against *T. cruzi* [6–15]. However, once pathogens invade host cells antibodies are ineffective as they cannot reach their targets. Therefore, alternative immune responses are also needed to kill intracellular pathogens. $CD4^+$ T cells play major roles in restricting pathogens via the secretion of IFN-γ which enhances the production of nitric oxide, a toxic substance for intracellular parasites, and the expression of MHC class I molecules in infected cells, allowing easier recognition by $CD8^+$ T cells. $CD8^+$ T cells also contribute to the elimination of intracellular pathogens, exhibiting cytotoxic activity against infected cells as well as by producing IFN-γ upon recognition of antigenic epitopes presented in combination with MHC class I molecules. Thus, the major target cells of our vaccine strategy are infected cells. This required us to choose ASP2, exclu-

sively expressed on amastigotes, an intracellular proliferating form of the parasite, rather than molecules expressed by a bloodstream form, trypomastigotes, as the vaccine antigen.

In previous studies, protective immunity against *T. cruzi* was obtained only after multiple vaccinations with naked plasmid DNA [11,15,17,18] or with recombinant viral vectors [32]. We previously demonstrated that at least four repeated vaccinations with a plasmid encoding ASP2 was needed to induce partial protective activity to infection with *T. cruzi* (unpublished data). In the present study, in contrast, a single intranasal vaccination with ASP2-expressing rSeV/dF was sufficient to induce efficient protective effects as well as antigen-specific $CD8^+$ T cells responses. This feature of rSeV/dF vaccination is advantageous in clinical settings, because it may be possible to avoid multiple vaccinations [37–40].

The favorable efficacy of rSeV/dF vectors in the induction of protective $CD8^+$ T cells demonstrated in this study might be due to several factors. For instance, rSeV/dF efficiently infects a wide variety of host cells, including airway epithelium and DCs [41,42], and these cells express large amounts of the vaccine antigen. It is also speculated that a possible adjuvant activity of rSeV/dF may be suitable for activation of $CD8^+$ T cells. Indeed, we found here that mice vaccinated with rSeV/dF contained significant numbers of antigen-non-specifically activated $CD8^+$ T cells, and such adjuvant activities may result from activation of innate immunity. It has been shown that rSeV/dF activates the innate immune system via pattern recognition receptors, including the RNA helicase RIG-I and Toll-like receptors (TLRs), expressed by non-immune and immune cells. Consequently, several cytokines are released, and pro-inflammatory environments would be established.

Priming of antigen-specific $CD8^+$ T cells requires antigen presentation (primary signal) and co-stimulation during antigen presentation (secondary signal) by DCs. Although rSeV/dF could directly infect DCs [41], the major target cells are epithelial cells of the airway. In order to induce an effective priming of $CD8^+$ T cells, cell-associated cross-presentation would be necessary after uptake of rSeV/dF-infected epithelial cells. Some RNA viruses have been reported to promote cross-presentation of myeloid DCs in a TLR3-dependent fashion [43]. Although rSeV/dF is not likely to be recognized directly by TLR3, similar mechanism may operate during vaccination with rSeV/dF. This highly efficient activation of $CD8^+$ T cells may explain why the fusion of ubiquitin to ASP2 did not enhance the vaccine efficacy.

Finally, developing vaccines still faces many challenges. For example, one of the evasion strategies of *T. cruzi* is considered to present simultaneously multiple variable surface antigens. Proteins of the trans-sialidase-like (TS) superfamily, including ASP2, are likely to be the most abundant proteins on the surface of the infective forms of *T. cruzi*, and genes encoding these proteins might represent about 5% of the parasite genome [44]. $CD8^+$ T cells of a single mouse strain infected with different parasite strains recognized distinct peptides as the immunodominant epitope [45–48]. Thus, one of the important tasks for researchers will be how to fight against the diversity of the immunodominant response between strains.

In conclusion, our present work shows that only a single intranasal inoculation of rSeV/dF encoding a *T. cruzi* amastigote antigen, ASP2, is sufficient to induce high and sustained levels of immunity against *T. cruzi*. Our mucosal-delivery, non-transmissible recombinant Sendai virus vector will provide a more efficient, convenient, and safe strategy to develop vaccines against Chagas' disease.

Conflict of interest

Dr. Yonemitsu is a member of the Scientific Advisory Board of DNAVEC Corporation.

Acknowledgments

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References

[1] Urbina JA. Specific treatment of Chagas disease: current status and new developments. Curr Opin Infect Dis 2001;14(6):733–41.
[2] Camandaroba EL, Reis EA, Goncalves MS, Reis MG, Andrade SG. *Trypanosoma cruzi*: susceptibility to chemotherapy with benznidazole of clones isolated from the highly resistant Colombian strain. Rev Soc Bras Med Trop 2003;36(March–April (2)):201–9.
[3] Menezes H. Protective effect of an avirulent (cultivated) strain of *Trypanosoma cruzi* against experimental infection in mice. Rev Inst Med Trop Sao Paulo 1968;10(January–February (1)):1–4.
[4] Basombrio MA. *Trypanosoma cruzi*: partial prevention of the natural infection of guinea pigs with a killed parasite vaccine. Exp Parasitol 1990;71(1):1–8.
[5] Pinge-Filho P, Peron JP, de Moura TR, Menolli RA, Graca VK, Estevao D, et al. Protective immunity against *Trypanosoma cruzi* provided by oral immunization with *Phytomonas serpens*: role of nitric oxide. Immunol Lett 2005;96(January (2)):283–90.
[6] Wrightsman RA, Dawson BD, Fouts DL, Manning JE. Identification of immunodominant epitopes in *Trypanosoma cruzi* trypomastigote surface antigen-1 protein that mask protective epitopes. J Immunol 1994 Oct 1;153(7):3148–54.
[7] Wizel B, Nunes M, Tarleton RL. Identification of *Trypanosoma cruzi* trans-sialidase family members as targets of protective $CD8^+$ TC1 responses. J Immunol 1997;159(December (12)):6120–30.
[8] Costa F, Franchin G, Pereira-Chioccola VL, Ribeirao M, Schenkman S, Rodrigues MM. Immunization with a plasmid DNA containing the gene of trans-sialidase reduces *Trypanosoma cruzi* infection in mice. Vaccine 1998;16(8):768–74.
[9] Wizel B, Garg N, Tarleton RL. Vaccination with trypomastigote surface antigen 1-encoding plasmid DNA confers protection against lethal *Trypanosoma cruzi* infection. Infect Immun 1998;66(11):5073–81.
[10] Fujimura AE, Kinoshita SS, Pereira-Chioccola VL, Rodrigues MM. DNA sequences encoding $CD4^+$ and $CD8^+$ T-cell epitopes are important for efficient protective immunity induced by DNA vaccination with a *Trypanosoma cruzi* gene. Infect Immun 2001;69(9):5477–86.
[11] Garg N, Tarleton RL. Genetic immunization elicits antigen-specific protective immune responses and decreases disease severity in *Trypanosoma cruzi* infection. Infect Immun 2002;70(10):5547–55.
[12] Katae M, Miyahira Y, Takeda K, Matsuda H, Yagita H, Okumura K, et al. Coadministration of an interleukin-12 gene and a *Trypanosoma cruzi* gene improves vaccine efficacy. Infect Immun 2002;70(9):4833–40.
[13] Fralish BH, Tarleton RL. Genetic immunization with LYT1 or a pool of trans-sialidase genes protects mice from lethal *Trypanosoma cruzi* infection. Vaccine 2003;21(June (21–22)):3070–80.
[14] Dumonteil E, Escobedo-Ortegon J, Reyes-Rodriguez N, Arjona-Torres A, Ramirez-Sierra MJ. Immunotherapy of *Trypanosoma cruzi* infection with DNA vaccines in mice. Infect Immun 2004;72(1):46–53.
[15] Chou B, Hisaeda H, Shen J, Duan X, Imai T, Tu L, et al. Critical contribution of immunoproteasomes in the induction of protective immunity against *Trypanosoma cruzi* in mice vaccinated with a plasmid encoding a CTL epitope fused to green fluorescence protein. Microbes Infect 2008;10(3):241–50.
[16] Schnapp AR, Eickhoff CS, Sizemore D, Curtiss 3rd R, Hoft DR. Cruzipain induces both mucosal and systemic protection against *Trypanosoma cruzi* in mice. Infect Immun 2002;70(9):5065–74.
[17] Boscardin SB, Kinoshita SS, Fujimura AE, Rodrigues MM. Immunization with cDNA expressed by amastigotes of *Trypanosoma cruzi* elicits protective immune response against experimental infection. Infect Immun 2003;71(5):2744–57.
[18] Vasconcelos JR, Hiyane MI, Marinho CR, Claser C, Machado AM, Gazzinelli RT, et al. Protective immunity against *Trypanosoma cruzi* infection in a highly susceptible mouse strain after vaccination with genes encoding the amastigote surface protein-2 and trans-sialidase. Hum Gene Ther 2004;15(9):878–86.
[19] Fontanella GH, De Vusser K, Laroy W, Daurelio L, Nocito AL, Revelli S, et al. Immunization with an engineered mutant trans-sialidase highly protects mice from experimental *Trypanosoma cruzi* infection: a vaccine candidate. Vaccine 2008;26(May (19)):2322–34.
[20] Cazorla SI, Frank FM, Becker PD, Corral RS, Guzman CA, Malchiodi EL. Prime-boost immunization with cruzipain co-administered with MALP-2 triggers a protective immune response able to decrease parasite burden and tissue injury in an experimental *Trypanosoma cruzi* infection model. Vaccine 2008;26(April (16)):1999–2009.
[21] Rocha CD, Caetano BC, Machado AV, Bruna-Romero O. Recombinant viruses as tools to induce protective cellular immunity against infectious diseases. Int Microbiol 2004;7(2):83–94.
[22] Webster DP, Dunachie S, McConkey S, Poulton I, Moore AC, Walther M, et al. Safety of recombinant fowlpox strain FP9 and modified vaccinia virus Ankara vaccines against liver-stage *P. falciparum* malaria in non-immune volunteers. Vaccine 2006;24(April (15)):3026–34.
[23] Bejon P, Mwacharo J, Kai OK, Todryk S, Keating S, Lang T, et al. Immunogenicity of the candidate malaria vaccines FP9 and modified vaccinia virus Ankara

encoding the pre-erythrocytic antigen ME-TRAP in 1–6 year old children in a malaria endemic area. Vaccine 2006;24(May (22)):4709–15.
[24] McShane H, Pathan AA, Sander CR, Keating SM, Gilbert SC, Huygen K, et al. Recombinant modified vaccinia virus Ankara expressing antigen 85A boosts BCG-primed and naturally acquired antimycobacterial immunity in humans. Nat Med 2004;10(11):1240–4.
[25] Greenough TC, Cunningham CK, Muresan P, McManus M, Persaud D, Fenton T, et al. Safety and immunogenicity of recombinant poxvirus HIV-1 vaccines in young adults on highly active antiretroviral therapy. Vaccine 2008;26(December (52)):6883–93.
[26] Li HO, Zhu YF, Asakawa M, Kuma H, Hirata T, Ueda Y, et al. A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J Virol 2000;74(14):6564–9.
[27] Zhang M, Obata C, Hisaeda H, Ishii K, Murata S, Chiba T, et al. A novel DNA vaccine based on ubiquitin-proteasome pathway targeting 'self'-antigens expressed in melanoma/melanocyte. Gene Ther 2005;12(13):1049–57.
[28] Duan X, Hisaeda H, Shen J, Tu L, Imai T, Chou B, et al. The ubiquitin-proteasome system plays essential roles in presenting an 8-mer CTL epitope expressed in APC to corresponding CD8+ T cells. Int Immunol 2006;18(5):679–87.
[29] Ishii K, Hisaeda H, Duan X, Imai T, Sakai T, Fehling HJ, et al. The involvement of immunoproteasomes in induction of MHC class I-restricted immunity targeting Toxoplasma SAG1. Microbes Infect 2006;8(4):1045–53.
[30] Hirata T, Iida A, Shiraki-Iida T, Kitazato K, Kato A, Nagai Y, et al. An improved method for recovery of F-defective Sendai virus expressing foreign genes from cloned cDNA. J Virol Methods 2002;104(2):125–33.
[31] Zhang M, Ishii K, Hisaeda H, Murata S, Chiba T, Tanaka K, et al. Ubiquitin-fusion degradation pathway plays an indispensable role in naked DNA vaccination with a chimeric gene encoding a syngeneic cytotoxic T lymphocyte epitope of melanocyte and green fluorescent protein. Immunology 2004;112(4):567–74.
[32] Machado AV, Cardoso JE, Claser C, Rodrigues MM, Gazzinelli RT, Bruna-Romero O. Long-term protective immunity induced against *Trypanosoma cruzi* infection after vaccination with recombinant adenoviruses encoding amastigote surface protein-2 and trans-sialidase. Hum Gene Ther 2006;17(9):898–908.
[33] Araujo AF, de Alencar BC, Vasconcelos JR, Hiyane MI, Marinho CR, Penido ML, et al. CD8+-T-cell-dependent control of *Trypanosoma cruzi* infection in a highly susceptible mouse strain after immunization with recombinant proteins based on amastigote surface protein 2. Infect Immun 2005;73(9):6017–25.
[34] Atkinson EA, Bleackley RC. Mechanisms of lysis by cytotoxic T cells. Crit Rev Immunol 1995;15(3–4):359–84.
[35] Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002;2(10):735–47.
[36] Rubio V, Stuge TB, Singh N, Betts MR, Weber JS, Roederer M, et al. Ex vivo identification, isolation and analysis of tumor-cytolytic T cells. Nat Med 2003;9(11):1377–82.
[37] Santra S, Sun Y, Parvani JG, Philippon V, Wyand MS, Manson K, et al. Heterologous prime/boost immunization of rhesus monkeys by using diverse poxvirus vectors. J Virol 2007;81(16):8563–70.
[38] Webster DP, Dunachie S, Vuola JM, Berthoud T, Keating S, Laidlaw SM, et al. Enhanced T cell-mediated protection against malaria in human challenges by using the recombinant poxviruses FP9 and modified vaccinia virus Ankara. Proc Natl Acad Sci USA 2005;102(March (13)):4836–41.
[39] Steensels M, Bublot M, Van Borm S, De Vriese J, Lambrecht B, Richard-Mazet A, et al. Prime-boost vaccination with a fowlpox vector and an inactivated avian influenza vaccine is highly immunogenic in Pekin ducks challenged with Asian H5N1 HPAI. Vaccine 2009;27(January (5)):646–54.
[40] Roberts DM, Nanda A, Havenga MJ, Abbink P, Lynch DM, Ewald BA, et al. Hexon-chimaeric adenovirus serotype 5 vectors circumvent pre-existing anti-vector immunity. Nature 2006;441(May (7090)):239–43.
[41] Yoneyama Y, Ueda Y, Akutsu Y, Matsunaga A, Shimada H, Kato T, et al. Development of immunostimulatory virotherapy using non-transmissible Sendai virus-activated dendritic cells. Biochem Biophys Res Commun 2007;355(March (1)):129–35.
[42] Yonemitsu Y, Kitson C, Ferrari S, Farley R, Griesenbach U, Judd D, et al. Efficient gene transfer to airway epithelium using recombinant Sendai virus. Nat Biotechnol 2000;18(9):970–3.
[43] Schulz O, Diebold SS, Chen M, Naslund TI, Nolte MA, Alexopoulou L, et al. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature 2005;433(February (7028)):887–92.
[44] El-Sayed NM, Myler PJ, Bartholomeu DC, Nilsson D, Aggarwal G, Tran AN, et al. The genome sequence of *Trypanosoma cruzi*, etiologic agent of Chagas disease. Science 2005;309(July (5733)):409–15.
[45] Martin DL, Weatherly DB, Laucella SA, Cabinian MA, Crim MT, Sullivan S, et al. CD8+ T-cell responses to *Trypanosoma cruzi* are highly focused on strain-variant trans-sialidase epitopes. PLoS Pathog 2006;2(8):e77.
[46] Tzelepis F, de Alencar BC, Penido ML, Gazzinelli RT, Persechini PM, Rodrigues MM. Distinct kinetics of effector CD8+ cytotoxic T cells after infection with *Trypanosoma cruzi* in naive or vaccinated mice. Infect Immun 2006;74(4):2477–81.
[47] Tzelepis F, Persechini PM, Rodrigues MM. Modulation of CD4+ T cell-dependent specific cytotoxic CD8+ T cells differentiation and proliferation by the timing of increase in the pathogen load. PLoS One 2007;2(4):e393.
[48] Tzelepis F, de Alencar BC, Penido ML, Claser C, Machado AV, Bruna-Romero O, et al. Infection with *Trypanosoma cruzi* restricts the repertoire of parasite-specific CD8+ T cells leading to immunodominance. J Immunol 2008;180(February (3)):1737–48.

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【论著】

恶性疟原虫红内期不同发育阶段 PfRON4 基因转录水平分析

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【摘要】 **目的** 分析恶性疟原虫棒状体颈部蛋白 4 基因（PfRON4）在红内期不同发育阶段的转录水平。 **方法** 用山梨醇结合等渗细胞分离液（Percoll）对实验室体外培养的恶性疟原虫进行均一化处理，收集间隔为 6 h 不同发育阶段的疟原虫，提取 RNA。根据 PfRON4 基因及相关基因（PfAMA1 和 PfRhopH2）的序列设计特异性引物，构建标准质粒并制作标准曲线，对 PfRON4 及相关基因的 mRNA 进行定量检测分析。 **结果** 纯化并同步后的疟原虫生长发育较为同步均一，用于定量分析的标准曲线相关性较好，PfRON4、PfAMA1 和 PfRhopH2 的相关系数（r 值）分别为 -1.00、-0.98 和 -0.98。产物熔解曲线分析结果均显示为单一波峰。定量分析结果显示，在恶性疟原虫红内期发育过程中，PfRON4 基因的转录水平在裂殖子入侵红细胞后 36~40 h（即成熟裂殖体阶段）达到高峰。 **结论** 恶性疟原虫 PfRON4 基因在成熟裂殖体阶段高表达。

【关键词】 恶性疟原虫；PfRON4；实时定量 PCR；转录

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Transcription Profile of PfRON4 Gene in Plasmodium falciparum Erythrocytic Stage

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【Abstract】 **Objective** To reveal the transcription profile of PfRON4 gene in Plasmodium falciparum erythrocytic stage. **Methods** P. falciparum schizonts were purified by differential centrifugation on a Percoll-sorbitol gradient, after which the released merozoites were allowed to invade uninfected erythrocytes for 4 hours before the clearance of all remaining schizonts using 5% D-sorbitol. The cultured synchronous parasites were harvested for RNA assay immediately, 24 hours later, and then at every 6th hour. PfRON4 and related genes (PfAMA1 and PfRhopH2) were amplified by real-time PCR for establishing standard curves to evaluate the copy number of genes. **Results** P. falciparum parasites were well sychronized. Those quantative analyses were reliable because the R value of standard curves were more than 0.98 and the melting curve showed a single peak. When parasites were in the schizont stage, PfRON4 gene transcription reached a peak in 36-40 hours after invasion. **Conclusion** The transcription of PfRON4 peaks at mature schizont stage, suggesting that the PfRON4 gene may involve in erythrocyte-invasion of P. falciparum.

【Key words】 Plasmodium falciparum; PfRON4; Real-time PCR; Transcription

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疟疾是当今世界严重的传染性疾病之一[1]，也是危害我国人民身体健康和生命安全的重要传染病[2]。红内期疟原虫在入侵新的红细胞前，裂殖子直接暴露于机体免疫系统，是疟原虫生命周期中最薄弱的环节之一，因此成为研究红内期疫苗的主要靶点。如能揭示疟原虫入侵红细胞的机制并鉴别相关的蛋白分子，就可制定相应的干预措施，从而控制疟原虫在人体内的发育增殖。

最近在刚地弓形虫的研究中，用蛋白质组技术分离得到一组新的棒状体颈部蛋白（TgRON），并发现其部分成员可与顶端膜蛋白抗原 1 (TgAMA1) 形成复合体，在入侵宿主细胞过程中起关键作用[3-5]。恶性疟原虫基因组数据库中也存在棒状体颈部蛋白基因的同源基因（PfRONs）。而且，Alexander 等[6]在恶性疟原虫的棒状体颈部发现 TgRON4 的同源基因——恶性疟原虫棒状体颈部蛋白 4 基因 (PfRON4)，且发现 PfRON4 蛋白可与恶性疟原虫顶端膜蛋白抗原 1 (PfAMA1) 形成复合体。因此，PfRON4 蛋白很可能和 PfAMA1 蛋白一起，在恶性疟原虫入侵宿主细胞的过程中起重要作用。目前尚不清楚 PfRON4 基因在红内期的转录和表达情况。因此，本研究通过对恶性疟原虫 PfRON4 基因及其相关基因的定量检测，来了解红内期不同发育阶段疟原虫中 PfRON4 基因转录水平的变化，从而为进一步研究该基因的功能提供信息。

材料与方法

1 主要试剂及仪器

2×QuantiTect 反应混合物、定量 PCR 试剂盒（QuantiTect SYBR green Ⅰ）、DNA 凝胶回收试剂盒和质粒抽提试剂盒（QIAprep Spin Miniprep）均购自荷兰 Qiagen 公司，大肠埃希菌（E. coli）DH5α 和 pGEM-T 载体购自美国 Sigma 公司，TA 克隆试剂盒购自美国 Progma 公司，RNA 抽提试剂 TRIzol LS 和反转录试剂 Superscript Ⅲ 购自美国 Invitrogen 公司，实时荧光定量 PCR 仪（Light Cycle 480）为德国 Roche 公司产品，普通 PCR 仪（Mastercycler）为德国 Eppendorf 公司产品，紫外分光光度仪（GeneQuant 1300/100）为美国通用公司产品。

2 疟原虫培养及同步处理

恶性疟原虫克隆株 HB3 按照常规条件在实验室进行体外培养[7]。采用等渗细胞分离液（Percoll）非连续密度梯度离心法纯化疟原虫[8]，将收集的富含裂殖体的疟原虫继续培养 4 h，用 5%山梨醇进行同步化处理后继续培养[9]，并于同步后第 24 h 开始，每 6 h 收集 1 次，各取 1 ml 于-80 ℃冻存备用，同时取少量涂制薄血膜，吉氏染色后显微镜下观察疟原虫生长发育情况。

3 总 RNA 的抽提和反转录

取 250 μl 冷冻的恶性疟原虫（红细胞压积为 2%，疟原虫密度约 3%）加入 750 μl TRIzol LS，混匀。按 TRIzol LS 试剂使用说明提取总 RNA，进行 DNA 酶处理，用紫外分光光度仪检测其吸光度（A_{260}/A_{280} 值），检验 RNA 纯度并计算其浓度。反转录采用随机引物法，按Superscript Ⅲ 试剂使用说明操作，获得的 cDNA 于-20 ℃保存备用。

4 标准质粒的构建

根据目的基因 PfRON4 及相关基因（PfAMA1 和 PfRhopH2）的序列，分别设计特异性引物（表 1）。PCR 扩增产物经 1%琼脂糖凝胶电泳检测，DNA 凝胶回收试剂盒割胶纯化 PCR 产物。TA 克隆法将 pGEM-T 载体与纯化后的 PCR 产物连接并转化感受态的 E. coli DH5α。筛选重组质粒，经测序进一步确认。挑选确认的重组质粒，进行大量制备，用质粒抽提试剂盒抽提，紫外分光光度仪准确定量，并进行梯度稀释，作为标准品，-20 ℃保存备用。

表 1 荧光定量 PCR 引物

Table 1 Oligonucleotide sequence for real-time PCR

目的基因 Target gene	引物名称 Primer name	引物序列 Oligonucleotide sequence
PfRON4	fRON4.qF	5′-ctttttacatttattgatatagttaaacc-3′
	fRON4.qR	5′-tatatggctagcaggtgttgcttg-3′
PfAMA1	fAMA1.qF	5′-ggaagaggacagaattattgggaac-3′
	fAMA1.qR	5′-cctgaatcttcttgttggtatgtatg-3′
PfRhopH2	fRhopH2.qF	5′-gtaacaacacttactaaggcagact-3′
	fRhopH2.qR	5′-gtacaaagctacaatattgttagatct-3′

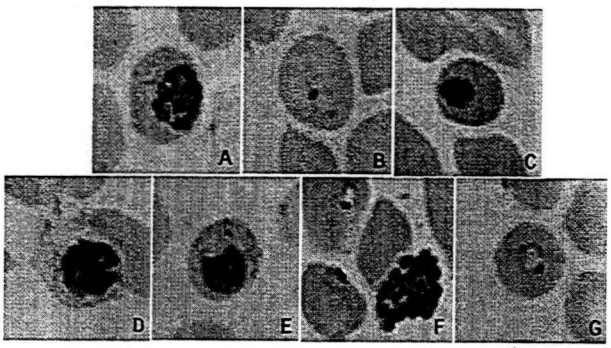
5 定量 PCR 测定及熔解曲线分析

定量 PCR 的反应体系为 2×QuantiTect 反应混合物 5 μl，cDNA 模板 4.5 μl，10 pmol/μl 上、下游引物各0.5 μl。反应条件为 95 ℃ 15 min；92 ℃ 15 s，55 ℃ 15 s，68 ℃ 7 s，共 60 个循环。循环结束后进行熔解曲线分析。在同一次反应中，设置标准质粒反应组（4 个浓度分别 4×10^5、8×10^4、1.6×10^3 和 32 copies/μl），所有反应设 3 个重复。用实时荧光定量 PCR 仪 Light Cycle 软件，根据质粒标准品建立标准曲线，计算待测样品中各目的基因的拷贝数。

结 果

1 处理后不同时期的疟原虫

光镜下观察不同时间收集的恶性疟原虫样品，发现同一时间样品的疟原虫形态高度一致。其中，纯化后大部分原虫为晚期滋养体或裂殖体，经山梨醇同步化处理后均为环状体，培养 24 h 后均为早期滋养体，30 h 后为晚期滋养体，有的疟原虫细胞核已开始分裂；36 h 后均为成熟裂殖体；42 h 后部分裂殖体已破裂，裂殖子入侵新的红细胞；48 h 后所有裂殖子均已入侵新的红细胞并发育为环状体（图 1）。



A：经 Percoll 纯化后，B：同步化处理后，C：处理后 24 h，D：处理后 30 h，E：处理后 36 h，F：处理后 42 h，G：处理后 48 h。
A: After purification, B: Immediately after synchronization, C: 24 h after synchronization, D: 30 h after synchronization, E: 36 h after synchronization, F: 42h after synchronization, G: 48h after synchronization.

图 1 山梨醇同步化处理后不同时期疟原虫形态
Fig. 1 Plasmodium parasites at different stages of sorbitol treatment

2 标准曲线

配套软件根据标准质粒的测试结果自动绘制以初始浓度（拷贝数）的对数 (log concentration) 为 X 轴，初始循环数（cycle number）为 Y 轴标准曲线，PfRON4、PfAMA1 和 PfRhopH2 基因标准曲线的相关系数（r）分别为-1.00、-0.98 和-0.98，表明所得的标准曲线线性关系良好。

3 熔解曲线分析

PfRON4、PfAMA1 和 PfRhopH2 基因的熔解曲线分析结果均显示为单一峰，熔解温度 (melting temperature, Tm) 分别约为 71.8、75.5 和 74.5 ℃，熔解温度均一，峰形较锐利，表明反应中未产生特异性产物。同时，制备标准质粒时 PCR 产物的琼脂糖凝胶电泳结果亦显示只有单一条带，由此双重确认了 PCR 产物的特异性。

4 定量分析结果

结果显示，PfRON4、PfAMA1 和 PfRhopH2 基因的转录水平均在入侵红细胞后 36~40 h 为最高（图 2）。

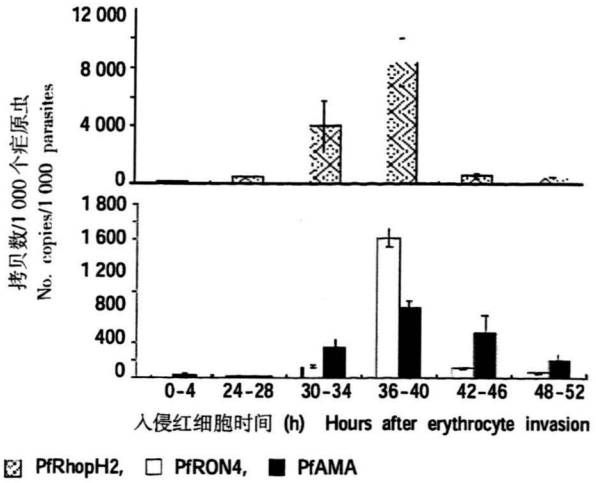


图 2 不同发育阶段红内期疟原虫 PfRON4、PfAMA 和 PfRhopH2 的转录水平
Fig.2 Transcription level of PfRON4, PfAMA and PfRhopH2 in Plasmodium falciparum erythrocytic stage

讨 论

恶性疟原虫红内期体外连续培养技术的建立[7]，为恶性疟分子生物学和免疫学研究提供了丰富的样品，大大促进了相关研究的进展。但要对某一发育阶段的原虫进行研究，获取同步发育的疟原虫仍较困难。本研究采用 Percoll 密度梯度离心纯化结合山梨醇同步化的方法对原虫进行“均一化”处理，先后收集到富含晚期滋养体和裂殖体的疟原虫，继续培养 4 h 后再用 5%山梨醇同步化处理，杀死未入侵红细胞的原虫。继续培养的原虫均为两次处理间入侵红细胞，即仅有 4 h 的“窗口期”，从而得到相对发育较均一的疟原虫，然后进行不同时期转录水平的比较研究。观察不同时间点所收集的原虫，发现各个时间点的原虫形态非常统一，从而证实该方法有效，可获得均一的样品，用于进一步研究。

裂殖子顶端膜抗原 1（AMA1）广泛分布于顶复门寄生虫中，并行使着类似的功能——在入侵宿主细胞中起关键作用。恶性疟原虫裂殖子顶端膜抗原 1（PfAMA1）的单克隆抗体和多克隆抗体都能抑制体外培养疟原虫入侵红细胞[10,11]，在自然感染者血清中提取的抗 AMA1 抗体亦有此抑制作用，鼠疟模型也证实抗 AMA1 抗体具有免疫保护力[12]，因此，AMA1 成为目前最具潜力的红内期疟疾疫苗候选抗原之一。但由于 PfAMA1 在入侵红细胞过程中的具体生物学功能尚不清楚，在一定程度上阻碍了其在疫苗研究中的进展。而最近对刚地弓形虫的研究表明，AMA1 蛋白和

棒状体颈部蛋白 TgRON4 和 TgRON2 能免疫共沉淀，在刚地弓形虫入侵宿主细胞的过程中，它们分布在弓形虫和宿主细胞的联结部位，并随着入侵过程后移，直至虫体完全进入宿主细胞而消失。认为 TgRON4 与 TgAMA1 和 TgRON2 共同参与该"移动连接（moving junction）"的构成[4,5]。而 TgRON4 的同源基因 PfRON4 在恶性疟原虫棒状体颈部的发现，以及 PfRON4 和 PfAMA1 同样形成复合体的现象[6]，提示在疟原虫体内可能也存在类似的情形。最近，本课题组在恶性疟原虫成熟裂殖体提取物中，检测到 PfRON4、PfRON2 和 PfAMA1 形成免疫复合体[13]。因此，PfRON4 很可能与 PfAMA1 和 PfRON2 一起，在恶性疟原虫入侵宿主细胞的过程中起重要作用。本研究通过对 PfRON4 基因的转录水平分析，发现它和其他棒状体蛋白 PfRhopH2 和 PfRON2[13]转录谱类似，在裂殖体时期水平较高，而 PfAMA1 也是在成熟裂殖体时期达到高峰，与推测相吻合：它们在裂殖子中表达并在入侵红细胞过程中起关键作用。但最近对约氏疟原虫的研究发现，其同源基因 PyRON4 的特异性抗体并不能产生免疫保护作用[14]。因此，RON4 能否作为疟疾疫苗候选分子尚需进一步研究。

综上所述，本研究首次对恶性疟原虫 PfRON4 及其相关基因的 mRNA 进行定量检测，发现 PfRON4 的转录水平在成熟裂殖子阶段达到高峰，因此可能在疟原虫裂殖子中表达，从侧面证实了 PfRON4 在疟原虫入侵红细胞过程有重要的作用，但其具体的生物学功能，以及其与 PfRON2 和 PfAMA1 的相互作用机制，有待于进一步研究。

参 考 文 献

[1] Snow RW, Guerra CA, Noor AM, et al. The global distribution of clinical episodes of Plasmodium falciparum malaria [J]. Nature, 2005, 434(7030): 214-217.

[2] Zhou SS, Wang Y, Tang LH. Malaria situation in the People's Republic of China in 2006 [J]. Chin J Parasitol Parasit Dis, 2007, 25(6): 439-441. (in Chinese)
(周水森，王漪，汤林华. 2006 年全国疟疾形势 [J]. 中国寄生虫学与寄生虫病杂志，2007, 25(6): 439-441.)

[3] Bradley PJ, Ward C, Cheng SJ, et al. Proteomic analysis of rhoptry organelles reveals many novel constituents for host-parasite interactions in Toxoplasma gondii [J]. J Biol Chem, 2005, 280 (40): 34245-34258.

[4] Lebrun M, Michelin A, El Hajj H, et al. The rhoptry neck protein RON4 re-localizes at the moving junction during Toxoplasma gondii invasion [J]. Cell Microbiol, 2005, 7(12): 1823-1833.

[5] Alexander DL, Mital J, Ward GE, et al. Identification of the moving junction complex of Toxoplasma gondii: a collaboration between distinct secretory organelles [J]. PloS Pathog, 2005, 1(2): 17.

[6] Alexander DL, Arastu-Kapur S, Dubremetz JF, et al. Plasmodium falciparum AMA1 binds a rhoptry neck protein homologous to TgRON4, a component of the moving junction in Toxoplasma gondii [J]. Eukaryot Cell, 2006, 5(7): 1169-1173.

[7] Trager W, Jensen JB. Human malaria parasites in continuous culture [J]. Science, 1976, 193 (4254): 673-675.

[8] Tharavanij S, Prasertsiriroj V. Simultaneous preparation of merozoites and concentrated schizonts of Plasmodium falciparum [J]. Southeast Asian J Trop Med Pub Hlth, 1981, 12(4): 518-524.

[9] Lambros C, Vanderberg JP. Synchronization of Plasmodium falciparum erythrocytic stages in culture [J]. J Parasitol, 1979, 65(3): 418-420.

[10] Crewther PE, Matthew ML, Flegg RH, et al. Protective immune responses to apical membrane antigen 1 of Plasmodium chabaudi involve recognition of strain-specific epitopes [J]. Infect Immun, 1996, 64(8): 3310-3317.

[11] Deans JA, Knight AM, Jean WC, et al. Vaccination trials in rhesus monkeys with a minor, invariant, Plasmodium knowlesi 66 kD merozoite antigen [J]. Parasit Immunol, 1988, 10(5): 535-552.

[12] Narum DL, Ogun SA, Thomas AW, et al. Immunization with parasite-derived apical membrane antigen 1 or passive immunization with a specific monoclonal antibody protects BALB/c mice against lethal Plasmodium yoelii yoelii YM blood-stage infection [J]. Infect Immun, 2000, 68(5): 2899-2906.

[13] Cao J, Kaneko O, Thongkukiatkul A, et al. Rhoptry neck protein RON2 forms a complex with microneme protein AMA1 in Plasmodium falciparum merozoites [J]. Parasitol Int, 2009, 58 (1): 29-35.

[14] Narum DL, Nguyen V, Zhang Y, et al. Identification and characterization of the Plasmodium yoelii PyP140/RON4 protein, an orthologue of Toxoplasma gondii RON4: the cysteine-rich domain does not protect against lethal parasite challenge infection [J]. Infect Immun, 2008, 76(11): 4876-4882.

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【消息】

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我国当前寄生虫病流行的新特点，是肠道线虫病危害大幅度下降，而食源性寄生虫病呈迅速增长的态势。为此，原卫生部寄生虫病专家咨询委员会副主任委员林金祥等专家，集数十年积累的食源性寄生虫病原实物标本照片及防治工作的实际经验，编辑出版了《食源性寄生虫病图释》。全书共 133 页，收入彩图 860 幅，有食源性寄生虫病的病原形态特征、传播媒介、终宿主等照片，并附以简短的文字说明。本书的亮点是通过阅览，能全面掌握各种食源性寄生虫病的生活史、识别各期病原形态特征、临床症状表现、诊治要点和食品病原的检查方法。全书分总论、各论，总论介绍食源性寄生虫病定义、种类，食物与寄生虫、寄生虫与人及动物的关系，寄生虫对人的危害，食源性寄生虫感染人方式，食源性寄生虫种类。各论按不同的食物源，依次介绍鱼源性、肉源性、贝类、甲壳类、蛙、蛇、昆虫类和植物源性寄生虫病，是一本图文并茂、通俗易懂，集科学性、可读性与一体，雅俗共赏的专业参考书与科普宣教书。该书是高校教学、科研和疾病预防控制、卫生监督等单位的重要参考书，也适合基层专业人员防治和科普宣教之用，可供卫生监督人员、临床医务人员、疾病预防控制工作者工作中参考。本书已由人民卫生出版社出版，定价 63 元。

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Review

Erythrocyte binding ligands in malaria parasites: Intracellular trafficking and parasite virulence

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ABSTRACT

The intracellular trafficking of an Erythrocyte Binding Like (EBL) ligand has recently been shown to dramatically affect the multiplication rate and virulence of the rodent malaria parasite *Plasmodium yoelii yoelii*. In this review, we describe the current understanding of the role of EBL and other erythrocyte binding ligands in erythrocyte invasion, and discuss the mechanisms by which they may control multiplication rates and virulence in malaria parasites.

Contents

1. Multiplication rate and virulence in malaria parasites

The multiplication rate of malaria parasites in the blood of the vertebrate host is often positively correlated with the severity of the disease they cause (Chotivanich et al., 2000). Such multiplication rates vary between species of parasites, and indeed, between strains of the same species (Yoeli et al., 1975; Dondorp et al., 2005; Reilly et al., 2007). Although many factors may influence the virulence of a parasite within a host, there is increasing evidence that such differences can be caused by parasite genetic polymorphism (Walliker et al., 1976; Rowe et al., 1997). Understanding the genetic basis of parasite virulence may pave the way for the design of anti-disease interventions such as novel drugs and vaccines.

In 1975, Yoeli et al. published a report describing the sudden acquisition of "virulence" in a line of the rodent malaria parasite *Plasmodium yoelii yoelii*. Prior to its sudden and dramatic change in character, the parasite line (17X) had exhibited a typical *P. y. yoelii* phenotype in laboratory mice, in which growth is restricted, almost exclusively, to very immature erythrocytes (reticulocytes), and which is characterized by a mild and chronic infection that is rarely fatal and often self-resolving. The parasite line in its original phenotypic state had been cryopreserved in the author's laboratory following its arrival from a colleague at another institution. Upon reconstitution of the line, the parasite exhibited a dramatic alteration in phenotype; its growth remained restricted to reticulocytes for the first 4 days of the infection, at which point it invaded mature erythrocytes, attained parasitemias in excess of 80%, and killed the host by the eighth day of infection. The new "virulent" line was subsequently typed by enzyme markers, which confirmed that it was almost certainly *P. y. yoelii* and not another contami-

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nating species resulting from a laboratory error, and further tests showed that there were no confounding co-infections of bacteria or viruses that may have altered the virulence of the line. Given the sudden way the parasite had acquired its ability to invade and multiply within mature erythrocytes, and combined with the fact that the phenotype was stable through multiple passages in mice, and importantly, also through mosquitoes, the authors speculated that a genetic mutation may have been responsible for the observed alteration in virulence.

Genetic crossing experiments subsequently conducted by Walliker et al. (1976) clearly showed this to be the case. Crosses were made between a clone of the virulent parasite (now named "YM", an acronym of "Yoeli's mouse"), and a genetically unrelated avirulent line (A/C). Of 56 clones of the resulting products of the cross, 34 were recombinants between the two parental types (determined by enzyme type and drug resistance markers) all of which, except one, could be unequivocally assigned either a virulent or avirulent phenotype comparable to the parental strains. The remaining clone exhibited an intermediate virulence phenotype which caused infections in mice in which mature erythrocytes were invaded, and peak parasitemias of 75% were observed, but which did not result in the death of the host. The simple Mendelian fashion in which the virulence phenotype was inherited in the progeny of the cross suggested that the trait was, indeed, genetically controlled, and possibly by a single gene. The authors went on to discuss the feasibility of identifying the product of this gene and determining how it ultimately controls virulence.

Recent work using modern genotyping techniques has shown that the virulent YM parasite is indeed very probably isogenetic with an avirulent parasite type derived from the original 17X isolate, supporting the suggestion that the sudden virulence increase was caused by a genetic mutation (Pattaradilokrat et al., 2008). Several clones were derived from the original 17X isolate, all except one of which appear to be of a single genotype. The virulent strain seems to have appeared independently at least three times from the original isolate, and is also known as 17XL, while the isogenic avirulent clones are known as 17X or 17XNL (Pattaradilokrat et al., 2008). This year, 34 years following Yoeli et al.'s initial observation, two reports appear to have finally identified the major genetic determinant of the multiplication rate differences between the virulent and avirulent *P. y. yoelii* lines. Otsuki et al. (2009) identified a single amino acid substitution in the *P. yoelii* Erythrocyte Binding Like (PyEBL) protein that was responsible for controlling the virulence differences between 17XL and 17XNL. At the same time, Pattaradilokrat et al. (2009) used Linkage Group Selection (LGS) (Culleton et al., 2005) to identify a region on *P. y. yoelii* chromosome 13 which was perfectly linked to the multiplication phenotype in the uncloned progeny of a cross between YM and 33X (a genetically unrelated avirulent *P. y. yoelii* clone). Following communication of Otsuki et al.'s results, they subsequently found that the gene encoding the *P. y. yoelii* PyEBL protein (*pyebl*) marked the trough of the selection valley observed following selection of the progeny for fast multiplying parasites. Combined, these two reports provide strong evidence that the major determinant of the virulence differences between these two parasites is controlled by *pyebl*. Otsuki et al. showed that a single amino acid substitution from a Cys (17XNL) to an Arg (17XL and YM) in PyEBL region 6 (R6) altered its localization within the merozoite from the micronemes to the dense granules. Based on these findings, along with the highly conserved nature of the Cys residues in EBL R6 between different malaria parasite species, the authors suggest that this region is a protein trafficking signal that controls transport of the protein to the micronemes. Thus, the substitution of the second Cys for an Arg abolishes the native conformation of region 6 by disrupting one of the four disulphide bonds formed between the eight Cys residues usually present in this region (Fig. 2) (Withers-Martinez et al., 2008). This subsequently leads to the incorrect trafficking of the protein to the dense granules rather than the micronemes, and dramatically increases parasite multiplication rate and virulence by allowing invasion and development within mature erythrocytes as well as reticulocytes. These results, along with a description of the EBL protein family, are discussed in more detail in the following sections.

2. Erythrocyte Binding Like (EBL) family

It has long been observed that individuals of west and central African origin are completely refractory to infection with *P. vivax*. It was subsequently shown that this protection is caused by the "Duffy-negative" phenotype characteristic of individuals from this region. The Duffy antigen/receptor for chemokines (DARC) is a transmembrane glycoprotein that is present on epithelial cells (Horuk et al., 1997), endothelial cells (Hadley et al., 1994), and erythrocytes. It is encoded by a single copy gene, of which there are four major alleles, *FY*A*, *FY*B*, *FY*X*, *FY*B^{null}* (Zimmerman et al., 1999; Pruenster and Rot, 2006). Combinations of these alleles result in four common phenotypes, *Fy*($a+b-$), *Fy*($a-b+$), *Fy*($a-$ b+^{weak}) and *Fy*($a-$ b−). *FY*A* and *FY*B*, by far the most common alleles among non-African populations, both encode functional proteins and differ from each other by a single amino acid change in the NH_2 extracellular domain. The *FY*B^{null}* allele carries a single nucleotide mutation which impairs promoter activity by disrupting a binding site for the h-GATA-1 erythroid transcription factor (Tournamille et al., 1995). This results in the loss of DARC expression on erythrocytes, but does not affect expression in epithelial or endothelial cells. Individuals who are homozygous for this allele, thus express no DARC protein on the erythrocyte surface. In the late 1980s, a 135–140-kDa *P. vivax* molecule was identified as the ligand that binds to DARC (Haynes et al., 1988; Wertheimer and Barnwell, 1989). Using *P. knowlesi* monkey malaria parasites, the gene encoding *P. knowlesi* Duffy binding protein (PkDBP) was identified in 1990 (Adams et al., 1990) followed closely by the identification of the gene encoding *P. vivax* DBP (PvDBP) (Fang et al., 1991). Interestingly, in addition to the PkDBP (PkDBP-α) protein that specifically recognizes DARC, two paralogous molecules that recognize alternative erythrocyte receptors were also identified in *P. knowlesi*. In *P. falciparum*, a 175-kDa molecule (EBA-175) that recognizes sialic acid on glycophorin A was reported (Camus and Hadley, 1985) and the gene encoding the EBA-175 protein was subsequently sequenced (Sim et al., 1990). Later, the *P. falciparum* genome project revealed other actively expressed paralogs, BAEBL (EBA-140) and JESEBL (EBA-181), that bind to glycophorin C and an unknown receptor, respectively (Gaur et al., 2004). With their distinct binding specificity, such proteins provide alternative erythrocyte invasion pathways in *P. falciparum* and, probably, also in *P. knowlesi*. These erythrocyte binding proteins of *P. vivax*, *P. knowlesi*, and *P. falciparum* share similar protein architecture and their genes were termed the *erythrocyte binding like* (*ebl*) family (Adams et al., 2001; Gaur et al., 2004). Homologous genes have also been identified in other *Plasmodium* species including the rodent malaria parasites (Prasad et al., 2003).

The genes of the *ebl* family encode type I integral transmembrane proteins that possess two Cys-rich regions which are conserved among orthologs (Fig. 1). The N-terminal Cys-rich region (termed region 2 (R2)), which consists of one or two DBL (Duffy-Binding-Like) domains (Adams et al., 1992), is responsible for erythrocyte surface receptor recognition. Recombinant R2 of PvDBP (one DBL domain) and EBA-175 (two DBL domains) expressed on COS cells was shown to bind erythrocytes, indicating that this region is the binding domain of the EBL proteins (Chitnis and Miller, 1994; Sim et al., 1994). The binding specificity of EBL protein members have previously been reviewed in detail (Gaur et al., 2004).

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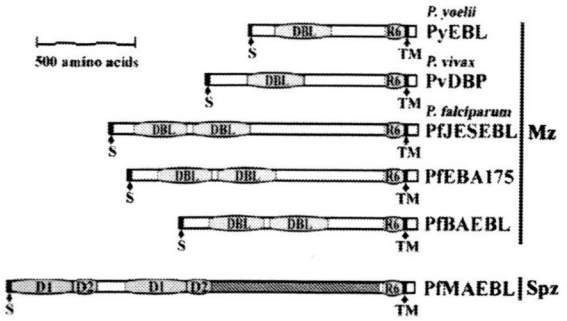

Fig. 1. Schematic representation of the domain architecture of *Plasmodium* EBL family proteins. DBL-EBP expressed at merozoite stage (Mz) and MAEBL expressed at sporozoite stage (Spz) are shown. D1 and D2 are domains similar to AMA-1. DBL, Duffy-Binding-Like domain; R6, region 6; TM, transmembrane region; S, predicted signal peptide sequence. Shaded box indicates non-globular region containing repeats.

3. Modification of EBL region 6 alters EBL localization, erythrocyte-type invasion preference, and, consequently, parasite virulence

The C-terminal Cys-rich region 6 (R6) (or the C-cys domain) is located adjacent to the transmembrane region with the number (eight) and location of Cys residues being highly conserved among known EBL proteins. The crystal structure of EBA-175 R6 reveals structural similarity to the KIX-binding domain of the coactivator CREB-binding protein, which can simultaneously bind to two independent proteins using two different hydrophobic interfaces. Due to this similarity, EBA-175 R6 has been proposed to possess the ability to bind two proteins (Withers-Martinez et al., 2008). An early hint of the function of R6 as a microneme trafficking signal came from an observation that the EBA-175 protein with R6, the transmembrane region, and the cytoplasmic tail removed was not trafficked to the microneme, which is the usual destination of EBL proteins (Gilberger et al., 2003). Furthermore, Treeck et al. (2006) showed that GFP fused with EBA-175 R6 is trafficked to the microneme, confirming that R6 is not only necessary, but also sufficient, for microneme trafficking.

EBL proteins are not confined to merozoites, as sporozoites, which invade mosquito salivary glands and mammalian hepatocytes, also possess an EBL-related protein termed MAEBL (Kappe et al., 1998; Kariu et al., 2002) MAEBL has similar protein structure to other EBLs, but possesses duplicated AMA-1-like domains (D1 and D2) instead of a DBL domain in R2. AMA-1-like domains also exhibit cell-binding abilities (Kappe et al., 1998; Ghai et al., 2002), thus the biological function of MAEBL is likely to be similar to the classic DBL domain-possessing EBL proteins. MAEBL is known to localize in the micronemes of sporozoites and this, combined with the conservation of the number and position of the Cys residues in R6 with other EBLs, it seems likely that MAEBL R6 is responsible for microneme trafficking in sporozoites. A hypothetical escorter protein was proposed to bind to EBL R6 and transport it from the Golgi apparatus to the micronemes in merozoites (Treeck et al., 2006). Such a mechanism (and possibly the escorter protein itself) may very well be conserved between merozoites and sporozoites.

During the characterization of *P. y. yoelii* EBL in order to evaluate a potential rodent malaria model for an EBL-based vaccine, Otsuki and colleagues observed that the second Cys residue in PyEBL R6 was substituted to Arg in the virulent line 17XL, but not in the avirulent line 17X (Fig. 2) (Otsuki et al., 2009). An immunofluorescent assay using a PyEBL-specific antibody revealed that the cellular location of the protein differed between the two lines. Immunoelectron microscopy revealed that PyEBL was trafficked to the dense granules, rather than the micronemes, of the 17XL line. This led them to hypothesize that the observed substitution in R6 may be responsible for the difference in PyEBL localization and potentially for the pathogenic differences between these two lines of *P. y. yoelii*, as modulation of an EBL gene in *P. falciparum* was previously shown to alter its erythrocyte invasion preference (Reed et al., 2000).

In order to evaluate this hypothesis, the PyEBL allelic type was genetically exchanged between the two lines. The replacement of Arg to Cys in the 17XL line (17XL-RtoC) altered the PyEBL localization from a non-apical diffused pattern to an apical pattern. In the 17X line, replacement of Cys to Arg (17X-CtoR) altered the localization from an apical pattern to a non-apical diffused pattern. These results confirmed that the substitution from Cys to Arg in R6 was responsible for the altered localization of PyEBL from the micronemes to the dense granules in the 17XL line. Furthermore, the 17XL-RtoC parasite predominantly invaded young erythrocytes in the same way as the avirulent 17X line, whereas the 17X-CtoR parasite was able to invade a variety of ages of erythrocytes including mature erythrocytes, comparable to the virulent 17XL line, thus demonstrating that the localization of PyEBL is responsible for the erythrocyte-type preference of *P. y. yoelii*.

Allelic replacement also affected parasite virulence. Mice infected with the 17XL-RtoC parasite developed significantly lower parasitemias compared with the parental 17XL line, and the infection was non-lethal, whereas mice infected with the 17XL lines inevitably died by day 7 (Fig. 3). The pattern observed for the 17XL-RtoC parasite was identical to that observed for the avirulent 17X line. Thus, the trafficking of PyEBL to the micronemes caused the virulence of the 17XL line to be reduced to the same level as the avirulent 17X line, suggesting that PyEBL is the critical virulence determinant of the 17XL line. The parasitemia of mice infected with the 17X-CtoR parasite increased significantly compared to that of mice infected with the parental 17X line during the acute phase of infection on days 4–5 post-inoculation. However, the parasitemia did not reach the level observed for the virulent 17XL line, and fell to a level comparable with the 17X line by day 9. This suggests that the 17X-CtoR parasite is able to invade a greater repertoire of erythrocyte types than the 17X line, but may be unable to invade as many types as the 17XL line. This reduced capacity to invade as great an erythrocyte-type repertoire as the 17XL line resulted in a non-lethal infection, in which all infected mice survived. Thus, displacement of PyEBL from the microneme was not sufficient to make this line fully lethal, suggesting the existence of other determinant(s). In support of this, Pattaradilokrat et al. (2009) also found evidence for a second determining factor in their whole genome LGS association study; two markers apparently unlinked to *pyebl* were also shown to be under multiplication rate selection, although they were unable to assign genomic locations to them for technical reasons.

4. Reticulocyte Binding Like (RBL) family; another key player in erythrocyte invasion

Microneme proteins are discharged early in the invasion of erythrocytes by merozoites, and are thought to play a part in the formation of the tight junction between the parasite and the host cell (Iyer et al., 2007a). Conversely, the dense granules only discharge their contents following successful invasion, and are thought to be involved in the modification of erythrocyte structure to aid parasite development (Culvenor et al., 1991). Just how the removal of an EBL protein from the micronemes and its relocation to the dense granules could allow a parasite to invade a larger repertoire of host erythrocytes is unclear.

In *P. falciparum*, EBL expression appears to be co-operationally regulated with another *Plasmodium* ligand encoded by the reticulocyte binding like (*rbl*) multigene family. For example, destruction

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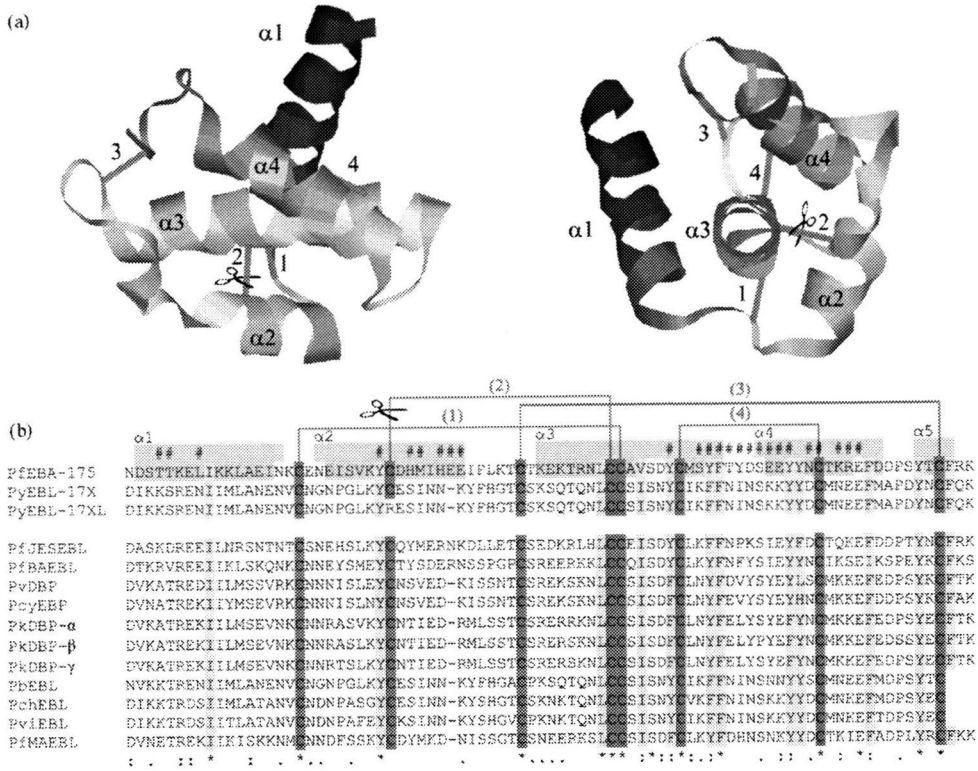

Fig. 2. Predicted structure of PyEBL region 6 (R6). (a) Homology modeling of PyEBL R6 was done with Modeller 9v7 using EBA175 R6 (PDB ID: 2RJI) as a template and visualized using RasMol v2.7. Four predicted disulfide bridges (red bar; 1–4) were added using Adobe Photoshop (Sali and Blundell, 1993; Sayle and Milner-White, 1995). Central α3 helices are surrounded by other helices (α1, α2, and α4). Note that α1 may not be related to other elements. (b) Amino acid sequence comparison of *Plasmodium* EBL region 6. PfEBA-175 (M93397), PfJESEBL (AB080796), PfBAEBL (AF332918), *Plasmodium falciparum* EBLs; PvDBP (DQ156512), *Plasmodium vivax* Duffy Binding Protein; PcyEBP, *Plasmodium cynomolgi* EBP (Y11396); PkDBP-α, -β, and -γ (M90466, M90694, and M90695, respectively), *Plasmodium knowlesi* Duffy Binding Proteins; PyEBL-17X and -17XL, *Plasmodium yoelii* EBL (AB430782 and AB430781, respectively); PbEBL, *P. berghei* EBL (ANKA line, AB430787); PchEBL, *P. chabaudi* EBL (AB430788); PviEBL, *P. vinckei* EBL (AB430789). In the PfEBA-175 sequence, helices are highlighted in grey; residues involved in protein fold stabilization are highlighted in cyan and yellow for nonpolar and polar residues, respectively; all Cys residues are in red; and residues involved in the dimeric interaction are indicated by hash marks. Asterisks indicate the positions where amino acids are identical, and similar amino acids are indicated with colons or periods under the alignment. Disulfide bridges (red lines) are represented above the alignment. Scissors indicate the disulfide bridge that is abolished by substitution from Cys to Arg in *P. y. yoelii* 17XL line. (For interpretation of the references to color in this figure legend, please see the online version of this article).

of the EBA-175 gene locus was shown to increase the transcription level of one such RBL protein, PfRh4, resulting in the alteration of the parasite's invasion phenotype (Stubbs et al., 2005). Members of the *rbl* family are also found in *P. y. yoelii*, comprising at least 14 genes termed *py235* encoding Py235 proteins (Iyer et al., 2007b). Thus Py235 is a potential candidate for the other factor that may be responsible for the difference in virulence between 17XL and the 17X-CtoR parasite. Consistent with this hypothesis is the fact that passive transfer of monoclonal antibodies specific for Py235 or immunization with purified Py235 protein restricted the invasion preference of the normally virulent *P. y. yoelii* YM line to reticulocytes (Freeman et al., 1980; Holder and Freeman, 1981). Further support is provided by work that showed that when Py235 expression is suppressed, the course of infection of the virulent *P. y. yoelii* YM line is altered to an intermediate virulence similar to that observed with the 17X-CtoR parasite. Based on these observations, Otsuki et al. proposed that the removal of EBL from the micronemes may result in free space within this organelle that may be filled with other ligand(s), possibly Py235, which enables the parasite to invade a larger repertoire of erythrocyte types. As different Py235 proteins may have different receptor-specificities, parasite invasion preference and the consequent course of infection may vary, depending on the Py235 member that fills the free space in the micronemes made available by the absence of EBL. Such a switching mechanism for an erythrocyte invasion pathway has been previously proposed for *P. falciparum*, in which removal of one RBL protein, PfRh2b, was proposed to result in the switching of the invasion pathway by redeployment of pre-existing parasite ligands (Duraisingh et al., 2003; Baum et al., 2005). Detailed descriptions of the RBL family including the newly characterized member PfRH5 are described elsewhere (Gaur et al., 2004; Hayton et al., 2008; Rodriguez et al., 2008; Baum et al., 2009).

5. Further questions concerning the control of invasion phenotypes

Although a valuable addition to our understanding of the genetic determinants of multiplication rates and virulence in malaria parasites, the discovery of the influence of PyEBL on the phenotypic difference between avirulent and virulent *P. y. yoelii* parasites leaves many questions unanswered. For example, both *P. y. yoelii* 33X(Pr3) (a pyrimethamine resistant clone of *P. y. yoelii* 33X) and *P. yoelii nigeriensis* exhibit multiplication phenotypes that may be considered "intermediate" between the extremes of 17X and 17XL, yet do not possess the EBL R6 mutation characteristic of 17XL and YM (Fig. 2b) (Otsuki et al., 2009). Furthermore, slowly multiplying and avirulent *P. y. yoelii* parasites such as 33X, 17X and CU are often observed within mature erythrocytes 4 days post-inoculation of mice, but do not appear to be able to mature within them [Walliker et al., 1976 and R. Culleton (*personal observation*)]. There is also evidence for the dramatic influence of host immune status on the ability of the reticulocyte-restricted clones of *P. y. yoelii* to invade

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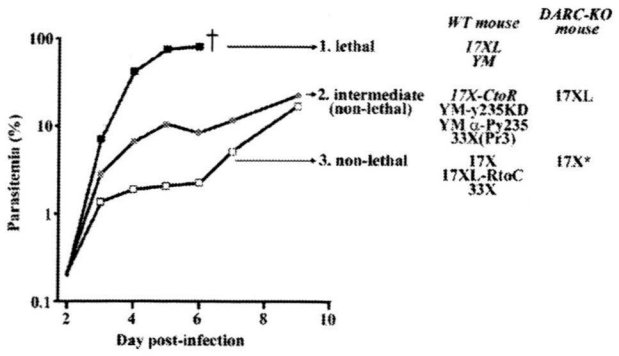

Fig. 3. Course of infection of various *P. yoelii*-derived lines. Three infection courses (1, lethal; 2, intermediate (non-lethal); 3, non-lethal) are based on experiments conducted by Otsuki et al. (2009), in which wild type (WT) BALB/c mice were intravenously inoculated with 1×10^6 parasitized erythrocytes with *P. y. yoelii* 17XL, 17X, 17XL-RtoC, or 17X-CtoR line parasites. Other data were categorized into these 3 groups, by reviewing the conditions and infection courses described in the following publications: DARC-KO mice (C57BL/6 background) were intravenously inoculated with 1×10^5 parasitized erythrocytes with *P. y. yoelii* 17XL and 17X (Akimitsu et al., 2004). Although overall infection of 17X line in DARC-KO mice (asterisk) is similar to that in WT mice (Akimitsu et al., 2004), the invasion of mature Duffy-negative erythrocytes by the 17X line of was dramatically reduced (Swardson-Olver et al., 2002). BALB/c mice were intravenously inoculated with 1×10^7 parasitized erythrocytes with *P. y. yoelii* YM and transgenic YM (YM-y235KD) in which Py235 transcripts were reduced by 10-fold (Iyer et al., 2007b). BALB/c mice were inoculated with *P. y. yoelii* YM and anti-Py235 antibodies were passively transferred when parasitemias reached 0.01–0.1% (YM α-Py235) (Freeman et al., 1980). C57BL/6 mice were intraperitoneally inoculated with 1×10^6 parasitized erythrocytes with *P. y. yoelii* 33X and 33X(Pr3), derived from the avirulent 33X line after ultraviolet light treatment of parasites (Walliker, 1981). All *P. y. yoelii* lines shown possess identical sequences at PyEBL region 6, except 17XL, 17X-CtoR, and YM lines (italicized) which possess an Arg residue instead of the second conserved Cys residue.

mature erythrocytes, with two reports describing the fast multiplication and lethal nature of 17XNL grown in immune-compromised mice (Fahey and Spitalny, 1984; Hisaeda et al., 2004). Interestingly, recent work has shown that variations in the multiplication rate and virulence of two *P. chabaudi adami* clones (DS and DK) are linked to differences in erythrocyte invasion preference (Gadsby et al., 2009). In this system, the erythrocyte invasion preference of the slowly multiplying, avirulent parasite did not appear to be for reticulocytes, but rather for an, as yet, unidentified sub-type of mature erythrocytes.

It has long been known that increases in rodent malaria parasite multiplication rate and virulence may occur as the result of extended serial passage through mice (Mackinnon and Read, 1999), and that this increased virulence may be "reset" by passing such selected lines through mosquitoes (although this does not always occur) (Mackinnon and Read, 2004). An increase in virulence was also observed for *Plasmodium knowlesi* infection of humans following serial passages conducted during the syphilis treatment of programme of the early 20 century (Garnham, 1966). This suggests that at least some aspects of the phenotypic variation in multiplication rates and virulence of malaria parasites are epigenetically controlled. It is also possible that changes at the genome level (such as deletions of genes involved in the sexual and sporogonic cycle) during serial passage may influence parasite virulence.

6. DARC: a potential receptor for PyEBL

Indirect evidence suggests that DARC expressed on the surface of mouse erythrocytes may be the receptor of PyEBL. When DARC-negative mouse erythrocytes were evaluated for their susceptibility to the *P. y. yoelii* 17X line, invasion of mature erythrocytes was dramatically reduced, but there was no alteration in invasion of reticulocytes when compared with DARC-intact erythrocytes. This suggests that although the *P. y. yoelii* 17X line does utilize DARC to invade erythrocytes, there are other receptor(s) on the reticulocyte surface which may be reduce in abundance and disappear altogether during erythrocyte maturation. Invasion into DARC-negative mature erythrocytes is Chymotrypsin-sensitive, but Trypsin-resistant, whereas DARC-negative reticulocyte invasion was abolished by treatment with Chymotrypsin or Trypsin, suggesting that the receptors on mature erythrocytes and reticulocytes are different (Swardson-Olver et al., 2002). An *in vitro* erythrocyte binding assay using radio-labeled parasite-derived protein revealed a 135 kDa erythrocyte binding protein, probably PyEBL, in the *P. y. yoelii* 17X line. This binding was sensitive to Chymotrypsin, but resistant to Trypsin treatments. The existence of Chymotrypsin-sensitive receptors on the erythrocyte surface would explain the inability of the 17X line to invade Chymotrypsin-treated mature erythrocytes (Ogun et al., 2000). An *in vitro* erythrocyte binding assay using a recombinant PyEBL DBL domain expressed on COS cells also showed that the erythrocyte surface receptor was Chymotrypsin-sensitive and Trypsin-resistant (Prasad et al., 2003).

Despite the fact that DARC is expressed on the surface of mature erythrocytes, the *P. y. yoelii* 17X line preferentially invades reticulocytes, even though PyEBL is correctly trafficked to the micronemes in this line. This invasion course is similar to *P. vivax*, which utilizes PvDBP to recognize DARC on erythrocytes, but also preferentially invades young erythrocytes. Galinski et al. (1992) identified two members of the *rbl* family in *P. vivax*, encoding reticulocyte binding protein-1 and -2 (PvRBP-1 and PvRBP-2), which specifically recognize young erythrocytes. They proposed a model in which PvRBP proteins reversibly attach to erythrocyte surface receptors in order to find favorable types of erythrocytes to invade before forming the irreversible tight junction using EBL proteins (Galinski et al., 1992). Thus co-operational function of EBL and RBL proteins may not only be spatially, but also temporally hierarchical.

7. Is PyEBL still functional when localized to dense granules?

It appears likely that dense granule-localized PyEBL remains functional due to the following observations: In wild type mice, the parasitemia of the *P. y. yoelii* 17XL line, in which PyEBL localizes in the dense granules, rapidly increases and causes host death by day 7 post-inoculation. However, when DARC knock-out mice (C56BL/6 background) were infected with the same line, parasitemia increased during the acute phase of infection on days 4–5 after inoculation, but did not exceed 10%, and mice subsequently resolved the infection (Akimitsu et al., 2004). Although Akimitsu et al. speculated that this observation was a result of the change in the immunological status of DARC-KO mice, given that there are no significant effects observed on the infection courses of the other rodent malaria parasite species, *P. berghei*, *P. vinckei*, and *P. chabaudi*, we consider that this observation suggests that DARC still serves as a receptor for PyEBL released from the dense granule.

A subgroup of the dense granules, known as exonemes, were recently reported to discharge their contents immediately prior to schizont rupture (Yeoh et al., 2007), whereas classical dense granules are discharged into a newly formed parasitophorous vacuole space after or during invasion (Torii et al., 1989). Although Otsuki and colleagues failed to detect PyEBL on the surface of the released individual merozoite of 17XL parasites, these parasites were not naturally released (merozoites from artificially ruptured schizonts were analyzed), thus, the timing of the secretion of PyEBL from the dense granules in 17XL is still not fully determined.

Interestingly, even though attempts to disrupt the PyEBL gene locus in both 17X and 17XL failed, suggesting that the protein is

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essential, both lines are able to infect DARC-KO mice. This suggests that PyEBL is essential even when it cannot bind to DARC. As PyEBL R6 appears not to be functional (at least for trafficking to the micronemes) in the 17XL line, we speculate that other regions may possess essential functionality, for example the cytoplasmic tail may form an essential complex with other molecules. Alternatively, low affinity binding or even brief contact of PyEBL to molecules other than DARC may be important. Finally it is potentially possible that DARC is not the receptor for PyEBL.

8. Implications for *P. vivax* virulence

Would a substitution from Cys to Arg in PvDBP R6 alter the invasion preference of *P. vivax* from young erythrocytes to a wider range of human erythrocytes? It is reasonable to postulate that PvDBP with such a mutation in R6 would be trafficked to the dense granule instead of the micronemes. Then, if *P. vivax* has the ability to express RBL member(s) that could recognize a greater repertoire of erythrocyte types, such a parasite would possess the potential for increased multiplication rate and hence greater virulence. This scenario cannot be ignored, as *P. vivax* possesses 10 RBL genes on its genome (Carlton et al., 2008). We suggest that a PvDBP-based anti-*P. vivax* vaccine should be carefully evaluated, taking such a possibility into account. An understanding of the expression pattern and binding specificity of other *P. vivax* RBL proteins is, therefore, important.

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References

Adams, J.H., Blair, P.L., Kaneko, O., Peterson, D.S., 2001. An expanding ebl family of *Plasmodium falciparum*. Trends Parasitol. 17, 297–299.

Adams, J.H., Hudson, D.E., Torii, M., Ward, G.E., Wellems, T.E., Aikawa, M., Miller, L.H., 1990. The Duffy receptor family of *Plasmodium knowlesi* is located within the micronemes of invasive malaria merozoites. Cell 63, 141–153.

Adams, J.H., Sim, B.K., Dolan, S.A., Fang, X., Kaslow, D.C., Miller, L.H., 1992. A family of erythrocyte binding proteins of malaria parasites. Proc. Natl. Acad. Sci. U.S.A. 89, 7085–7089.

Akimitsu, N., Kim, H.S., Hamamoto, H., Kamura, K., Fukuma, N., Arimitsu, N., Ono, K., Wataya, Y., Torii, M., Sekimizu, K., 2004. Duffy antigen is important for the lethal effect of the lethal strain of *Plasmodium yoelii* 17XL. Parasitol. Res. 93, 499–503.

Baum, J., Maier, A., Gm, Good, R.T., Simpson, K.M., Cowman, A.F., 2005. Invasion by *P. falciparum* merozoites suggests a hierarchy of molecular interactions. PLoS Pathog. 1, e37.

Baum, J., Chen, L., Healer, J., Lopaticki, S., Boyle, M., Triglia, T., Ehlgen, F., Ralph, S.A., Beeson, J.G., Cowman, A.F., 2009. Reticulocyte-binding protein homologue 5—an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. Int. J. Parasitol. 39, 371–380.

Camus, D., Hadley, T.J., 1985. A *Plasmodium falciparum* antigen that binds to host erythrocytes and merozoites. Science 230, 553–556.

Carlton, J.M., Adams, J.H., Silva, J.C., Bidwell, S.L., Lorenzi, H., Caler, E., Crabtree, J., Angiuoli, S.V., Merino, E.F., Amedeo, P., Cheng, Q., Coulson, R.M., Crabb, B.S., Del Portillo, H.A., Essien, K., Feldblyum, T.V., Fernandez-Becerra, C., Gilson, P.R., Gueye, A.H., Guo, X., Kang'a, S., Kooij, T.W., Korsinczky, M., Meyer, E.V., Nene, V., Paulsen, I., White, O., Ralph, S.A., Ren, Q., Sargeant, T.J., Salzberg, S.L., Stoeckert, C.J., Sullivan, S.A., Yamamoto, M.M., Hoffman, S.L., Wortman, J.R., Gardner, M.J., Galinski, M.R., Barnwell, J.W., Fraser-Liggett, C.M., 2008. Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. Nature 455, 757–763.

Chitnis, C.E., Miller, L.H., 1994. Identification of the erythrocyte binding domains of *Plasmodium vivax* and *Plasmodium knowlesi* proteins involved in erythrocyte invasion. J. Exp. Med. 180, 497–506.

Chotivanich, K., Udomsangpetch, R., Simpson, J.A., Newton, P., Pukrittayakamee, S., Looareesuwan, S., White, N.J., 2000. Parasite multiplication potential and the severity of falciparum malaria. J. Infect. Dis. 181, 1206–1209.

Culleton, R., Martinelli, A., Hunt, P., Carter, R., 2005. Linkage group selection: rapid gene discovery in malaria parasites. Genome Res. 15, 92–97.

Culvenor, J.G., Day, K.P., Anders, R.F., 1991. *Plasmodium falciparum* ring-infected erythrocyte surface antigen is released from merozoite dense granules after erythrocyte invasion. Infect. Immun. 59, 1183–1187.

Dondorp, A.M., Desakorn, V., Pongtavornpinyo, W., Sahassananda, D., Silamut, K., Chotivanich, K., Newton, P.N., Pitisuttithum, P., Smithyman, A.M., White, N.J., Day, N.P., 2005. Estimation of the total parasite biomass in acute falciparum malaria from plasma PfHRP2. PLoS Med. 2, e204.

Duraisingh, M.T., Maier, A.G., Triglia, T., Cowman, A.F., 2003. Erythrocyte-binding antigen 175 mediates invasion in *Plasmodium falciparum* utilizing sialic acid-dependent and -independent pathways. Proc. Natl. Acad. Sci. U.S.A. 100, 4796–4801.

Fahey, J.R., Spitalny, G.L., 1984. Virulent and nonvirulent forms of *Plasmodium yoelii* are not restricted to growth within a single erythrocyte type. Infect. Immun. 44, 151–156.

Fang, X.D., Kaslow, D.C., Adams, J.H., Miller, L.H., 1991. Cloning of the *Plasmodium vivax* Duffy receptor. Mol. Biochem. Parasitol. 44, 125–132.

Freeman, R.R., Trejdosiewicz, A.J., Cross, G.A., 1980. Protective monoclonal antibodies recognising stage-specific merozoite antigens of a rodent malaria parasite. Nature 284, 366–368.

Gadsby, N., Lawrence, R., Carter, R., 2009. A study on pathogenicity and mosquito transmission success in the rodent malaria parasite *Plasmodium chabaudi adami*. Int. J. Parasitol. 39, 347–354.

Galinski, M.R., Medina, C.C., Ingravallo, P., Barnwell, J.W., 1992. A reticulocyte-binding protein complex of *Plasmodium vivax* merozoites. Cell 69, 1213–1226.

Garnham, P.C.C., 1966. Malaria Parasites and Other Haemosporidia. Blackwell, Oxford, England/Davis, Philadelphia, p. 333.

Gaur, D., Mayer, D.C., Miller, L.H., 2004. Parasite ligand–host receptor interactions during invasion of erythrocytes by *Plasmodium* merozoites. Int. J. Parasitol. 34, 1413–1429.

Ghai, M., Dutta, S., Hall, T., Freilich, D., Ockenhouse, C.F., 2002. Identification, expression, and functional characterization of MAEBL, a sporozoite and asexual blood stage chimeric erythrocyte-binding protein of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 123, 35–45.

Gilberger, T.W., Thompson, J.K., Reed, M.B., Good, R.T., Cowman, A.F., 2003. The cytoplasmic domain of the *Plasmodium falciparum* ligand EBA-175 is essential for invasion but not protein trafficking. J. Cell Biol. 162, 317–327.

Hadley, T.J., Lu, Z.H., Wasniowska, K., Martin, A.W., Peiper, S.C., Hesselgesser, J., Horuk, R., 1994. Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform, which is the Duffy blood group antigen. J. Clin. Invest. 94, 985–991.

Haynes, J.D., Dalton, J.P., Klotz, F.W., McGinniss, M.H., Hadley, T.J., Hudson, D.E., Miller, L.H., 1988. Receptor-like specificity of a *Plasmodium knowlesi* malarial protein that binds to Duffy antigen ligands on erythrocytes. J. Exp. Med. 167, 1873–1881.

Hayton, K., Gaur, D., Liu, A., Takahashi, J., Henschen, B., Singh, S., Lambert, L., Furuya, T., Bouttenot, R., Doll, M., Nawaz, F., Mu, J., Jiang, L., Miller, L.H., Wellems, T.E., 2008. Erythrocyte binding protein PfRH5 polymorphisms determine species-specific pathways of *Plasmodium falciparum* invasion. Cell Host Microbe 4, 40–51.

Hisaeda, H., Maekawa, Y., Iwakawa, D., Okada, H., Himeno, K., Kishihara, K., Tsukumo, S., Yasutomo, K., 2004. Escape of malaria parasites from host immunity requires CD4+ CD25+ regulatory T cells. Nat. Med. 10, 29–30.

Holder, A.A., Freeman, R.R., 1981. Immunization against blood-stage rodent malaria using purified parasite antigens. Nature 294, 361–364.

Horuk, R., Martin, A.W., Wang, Z., Schweitzer, L., Gerassimides, A., Guo, H., Lu, Z., Hesselgesser, J., Perez, H.D., Kim, J., Parker, J., Hadley, T.J., Peiper, S.C., 1997. Expression of chemokine receptors by subsets of neurons in the central nervous system. J. Immunol. 158, 2882–2890.

Iyer, J., Gruner, A.C., Renia, L., Snounou, G., Preiser, P.R., 2007a. Invasion of host cells by malaria parasites: a tale of two protein families. Mol. Microbiol. 65, 231–249.

Iyer, J.K., Amaladoss, A., Genesan, S., Preiser, P.R., 2007b. Variable expression of the 235 kDa rhoptry protein of *Plasmodium yoelii* mediate host cell adaptation and immune evasion. Mol. Microbiol. 65, 333–346.

Kappe, S.H., Noe, A.R., Fraser, T.S., Blair, P.L., Adams, J.H., 1998. A family of chimeric erythrocyte binding proteins of malaria parasites. Proc. Natl. Acad. Sci. U.S.A. 95, 1230–1235.

Kariu, T., Yuda, M., Yano, K., Chinzei, Y., 2002. MAEBL is essential for malarial sporozoite infection of the mosquito salivary gland. J. Exp. Med. 195, 1317–1323.

Mackinnon, M.J., Read, A.F., 1999. Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*. Proc. Biol. Sci. 266, 741–748.

Mackinnon, M.J., Read, A.F., 2004. Immunity promotes virulence evolution in a malaria model. PLoS Biol. 2, E230.

Ogun, S.A., Scott-Finnigan, T.J., Narum, D.L., Holder, A.A., 2000. *Plasmodium yoelii*: effects of red blood cell modification and antibodies on the binding characteristics of the 235-kDa rhoptry protein. Exp. Parasitol. 95, 187–195.

Otsuki, H., Kaneko, O., Thongkukiatkul, A., Tachibana, M., Iriko, H., Takeo, S., Tsuboi, T., Torii, M., 2009. Single amino acid substitution in *Plasmodium yoelii* erythrocyte ligand determines its localization and controls parasite virulence. Proc. Natl. Acad. Sci. U.S.A. 106, 7167–7172.

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