

several: to understand cell differentiation, to find molecules responsible for causing specific diseases, and to speed up the production of meat, fish, and silk.

Some attempts have been made to create transgenic mosquitoes to make them non-vectors of germs or to reduce the germs' transmission ability [13, 14]. Anopheline mosquitoes are the primary targets for transgenes, because they transmit malaria parasites. Some laboratories, including ours, have succeeded in producing transgenic mosquitoes with lower levels of malaria parasites in the digestive tract after blood meals on malaria-infected animals [15, 16]. The goal of those attempts is to control disease transmission through genetic modification of the mosquitoes [17].

We here present a new attempt to produce a useful protein in the mosquito salivary gland (SG) by adding a new gene to a mosquito chromosome. When mosquitoes attach to the skin surface, they try to find blood vessels from which they can take blood. At that time, they first deposit their saliva in the skin [1]. Mosquito saliva contains many molecules, such as vasodilators, platelet inhibitors, an anesthetic substance, and so on [18]. These substances dilate the blood vessel, allowing the mosquito to insert its proboscis into the vessel. A mosquito can have a blood meal without the blood coagulating and without being noticed. On the other hand, humans develop anti-saliva protein antibodies after experiencing several mosquito bites [19, 20]. Our idea is to put a gene encoding a useful protein into a mosquito chromosome, causing it to make the useful protein in its saliva and to inject the protein, via the saliva, into animals or human beings upon blood feeding. We expect that the host would develop antibodies to the recombinant protein as a reaction. If these transgenic (TG) mosquitoes, whose saliva contains a vaccine protein against a disease, were spread in an area where people are suffering from the disease, people who are daily bitten by them would develop antibodies to the vaccine protein, ultimately vaccinating the community from the disease. In this situation, mosquitoes would play the role of vaccine deliverers [21].

In field conditions of malaria-endemic areas, for instance, nearly 100 mosquitoes bite an individual per night, but more than 50% of the mosquitoes are nulliparous females (non-oviposit females) [22]. Even in malaria-holoendemic areas, the percentage of mosquitoes infected with malaria parasites in the SG is less

than 4% among collected Anopheline mosquitoes [23]. Indeed, it is important to reduce malaria-infective mosquitoes, but people in malaria-endemic areas are bitten by thousands of non-infective mosquitoes each year. These non-infective mosquitoes could work as vaccine distributors.

We discovered one molecule of a platelet inhibitor in the SG of a model mosquito, *Anopheles stephensi*, cloned the gene, and named it the Anopheline anti-platelet protein (AAPP) [24]. This molecule is expressed only in the female SG. We used the upper stream area of the AAPP gene (*aapp*) as a promoter for the vaccine protein. For the vaccine protein, we chose circumsporozoite protein (CSP) of the malaria parasite. This molecule has been studied as a malaria vaccine candidate [25, 26]. In a rodent malaria model, a monoclonal antibody to CSP neutralizes the infectivity of sporozoite, an infective form of malaria parasite from the mosquito [27], and a synthetic peptide induces sufficient antibodies to protect immunized mice from sporozoite challenge [28]. CSP is now thought to be a promising vaccine candidate molecule [29], and recombinant proteins composed of CSP are being evaluated in malaria-endemic areas [30]. Thus, we decided to produce a TG mosquito containing the promoter area of *aapp* followed by the CSP gene (*csp*). We expected that the TG mosquitoes would express CSP in the SG and inject the recombinant CSP (rCSP) into the skin of mice when the TG mosquitoes fed on them.

Materials and Methods

Mosquitoes, mice, and parasites. *Anopheles stephensi* SDA 500 strain was reared in our laboratory under conditions of 26°C room temperature, 50–70% relative humidity, and light control of 14 h bright and 10 hours dark. Female BALB/c mice were purchased from SLC (Shizuoka, Japan). A rodent malaria parasite, *Plasmodium berghei* ANKA strain, was maintained by cyclical passage through BALB/c mice and *An. stephensi* mosquitoes.

Recombinant CSP of *P. berghei* (rPbCSP). The rPbCSP was expressed as His-tagged at the C-terminal by the baculovirus expression system, and purified with a nickel column (Qiagen, Hilden, Germany) as described elsewhere [31]. The rPbCSP is expressed with two molecular weight sizes, 70 and

82kDa [31]. When mice are immunized with rPbCSP three times, they become protected against parasite challenge (unpublished data). The protein concentration of rPbCSP was measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum immunoglobulin was used as a standard for the protein concentration.

Preparation of anti-CSP antibody. For anti-CSP antibody production, 0.5ml of rPbCSP solution (50 µg/ml) and 0.5ml of alum, Al(OH)₃ (16mg/ml), were well mixed and 0.2ml was injected intraperitoneally into each of four BALB/c mice. Injection was done 3 times at intervals of 2 weeks. Anti-CSP antibody was monitored by ELISA, as shown below, and the mice were sacrificed to take their sera. The sera were used as the anti-CSP antibody.

Preparation of anti-saliva antibody. To prepare anti-saliva antibody, 4 female BALB/c mice were each bitten by 100 female mosquitoes every 2 weeks for a 10-week period (5 biting sessions). Development of an anti-saliva antibody in the mice was confirmed by ELISA as shown below, and sera were taken from the mice. The sera were used as the anti-saliva antibody.

Evaluation for antibody titer by ELISA. For the evaluation of anti-CSP antibody, 8 wells of a 96-well assay plate were coated with 100 µl of rPbCSP (1 µg/ml) in 0.05M carbonate buffer (pH9.6), and 8 wells were filled with 100 µl of carbonate buffer without antigen. The plate was incubated at 4°C overnight.

For the evaluation of anti-saliva antibody, 10 pairs of SG were collected from female mosquitoes, destroyed in 1.0ml of carbonate buffer by sonication (1 sec × 5 times), and centrifuged (8,000rpm for 3min). The supernatant was used as the SG antigen. Eight wells were each coated with 100 µl of SG antigen.

Two microliters of blood was collected from the tail of each mouse 7 days after the injection of rPbCSP or mosquito biting. The blood was mixed with 0.8ml of phosphate-buffered saline containing 1% bovine serum albumin (BSA/PBS) and centrifuged at 8,000rpm for 3min. The supernatant was used as an 800-fold-diluted serum.

After the antigen solution was removed and washed twice, the wells of an ELISA plate were blocked with

150 µl of 1% BSA/PBS for 30min. After removing the blocking solution, 100 µl of the mouse serum diluted 800-fold was distributed into each of 2 antigen wells and 2 no-antigen wells and incubated for 2h. As the second antibody, rabbit anti-mouse IgG conjugated with horseradish peroxidase (HRP) (Bio-Rad Laboratories) diluted 3,000-fold was distributed into each of the same wells and incubated for 1h. After the plate was washed, the substrate solution, a mixture of 0.04% 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma-Aldrich, St. Louis, MO, USA), 0.05% H₂O₂ in 0.05M phosphate, and 0.1M citrate buffer (pH4.5) was added. After 20–60min, a green color appeared. The absorption was measured at a wavelength of 405 nm by a microplate reader, Spectra Max M5 (Molecular Devices, Tokyo, Japan). The mean absorbance of antigen wells minus the mean absorbance of no-antigen wells was taken as the OD value for the antigen.

Plasmid construction (Fig. 1). The anopheline anti-platelet protein gene (*aapp*) promoter region and its signal peptide coding region of *An. stephensi* [24] (1,746 bp) were amplified with a primer pair of AAPP-F-*AscI* (5'-GGCGCGCCTTATAAGACGGAGCTCATTGTCGCTCGTC-3') and AAPP-R-*SmaI* (5'-CCCGGGCGGCCGTGCGGATACGATCAGCGCAAGGC-3'), then cloned into the pCR-BluntII TOPO vector (Invitrogen, Carlsbad, CA, USA) (plasmid a). The open reading frame of the *P. berghei* CSP gene without a signal peptide coding region or a C-terminal GPI-anchor domain coding region was amplified with a primer pair of PbCS-F-*SmaI* (5'-CCCGGGCAAATAAAATCATCCAAGCCCAAAGGAAC-3') and PbCS-R-*SpeI* (5'-ACTAGTTATTTATCCATTTTACAAATTCAGTATCAATATC-3'), then cloned into the vector (plasmid b). The 3' non-coding region of the AAPP gene was amplified with a primer pair of 3UTR-F-*SpeI* (5'-ACTAGTGAAACACACCCGTTAACGACAC-3') and 3UTR-R-*XbaI* (5'-TCTAGATATTC AAAGGTCCACAAATGTC-3'), then cloned into the vector (plasmid c). The inserts of plasmids a, b, and c were serially cloned into the pENTR4 vector (Invitrogen) (donor vector).

The pBac [3xP3-EGFP] vector [11] and helper plasmid were kindly provided by Professor A. S. Raikhel (Department of Entomology, University of California at Riverside, USA). The pBac [3xP3-EGFP] vector was digested with *AscI*, blunted with a

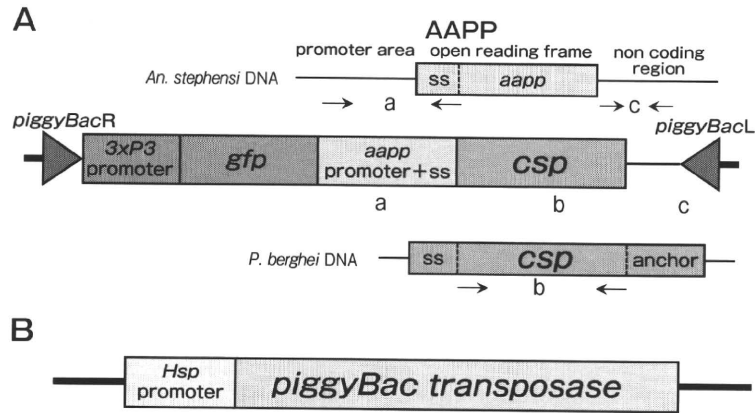


Fig. 1 Construction of plasmids injected into mosquito eggs. **A**, Two DNA fragments (a and c) were amplified from *A. stephensi* DNA. One DNA fragment (b) was amplified from *P. berghei* DNA. Three fragments were inserted in tandem in a plasmid vector downstream from the GFP gene. A promoter, 3xP3, was adopted to induce GFP expression in the eyes of mosquitoes as a selection marker. AAPP: anopheline anti-platelet protein. ss: signal sequence. CSP: circumsporozoite protein; **B**, To improve uptake of the recombinant plasmid in the mosquito chromosome, a piggyback helper plasmid was added when microinjection was performed. Hsp: heat-shock protein.

Klenow fragment, and dephosphorylated. The reading frame cassette A (Invitrogen) was then cloned into the vector (destination vector). The insert in the donor vector was cloned into the destination vector by Gateway LR clonase reaction (Invitrogen). The resulting plasmid (pBac-AAPP-PbCSP; Fig. 1A) was expected to drive CSP gene expression under the AAPP promoter. The pBac-AAPP-PbCSP plasmid was mixed with piggyBac helper (Fig. 1B) and microinjected into the eggs of *A. stephensi* as described below.

Microinjection of the plasmids into mosquito eggs. The mosquito microinjection was performed as described elsewhere [32]. In brief, blood-fed *An. stephensi* mosquitoes were allowed to lay eggs on a wet filter sheet 72–84 h after a blood meal. Eggs were laid and injected with plasmids within 90 min after oviposition. Injection was done by glass needles (Eppendorf, Hamburg, Germany) with a mixture of the pBac-AAPP-PbCSP (500 ng/μl) and piggyBac helper (300 ng/μl) in injection buffer (5 mM KCl, 0.1 mM Na₂HPO₄, pH 6.8). After injection, the eggs were placed in water and observed for hatching. Hatched larvae were analyzed on a fluorescence microscope at a wavelength of 490 nm to detect GFP expression.

Selection of transgenic mosquito. We selected GFP-expressing larvae and made them emerge (G0). One G0 adult female (or male) expressing GFP was put in a cage containing 5 wild-type

males (or females). After mating and blood feeding, each female was allowed to lay eggs individually. Hatched larvae were observed under a fluorescence microscope, and GFP-expressing larvae were isolated as a G1 strain. Among the same batch of G1, mosquitoes were allowed to mate, feed, and lay eggs. Hatched larvae were observed and GFP-expressing larvae were isolated as the G2 of the strain.

Selection of CSP-expressing strain. Ten pairs of SG were collected from G2 female adults in each strain, destroyed in 1.0 ml of carbonate buffer by sonication (1 sec × 5 times), and centrifuged (8,000 rpm for 3 min). The supernatant was used as the SG antigen. Then, 100 μl of SG antigen was distributed into each of four wells of an ELISA plate and allowed to stand at 4°C overnight. After blocking with 1% BSA/PBS, anti-CSP antibody diluted 800-fold was poured into 2 wells, and normal mouse serum diluted 800-fold was poured into 2 other wells. Incubation with the secondary antibody and the subsequent procedures were the same as described above.

Western blotting. Ten pairs of salivary glands were collected from both TG female mosquitoes and wild-type female mosquitoes. Samples were separated on a 10% SDS-polyacrylamide gel under reducing conditions (with 2% 2-mercaptoethanol), transferred onto a nitrocellulose (NC) sheet, and probed with mouse anti-CSP antibody diluted 800-fold. The NC sheet was next incubated with anti-mouse IgG conju-

gated with HRP (Bio-Rad Laboratories) diluted 3,000 fold, then reacted with substrate, SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL, USA). Positive bands were visualized in a Lumino Image Analyzer (LAS-1000) (Fuji Film, Tokyo, Japan).

Immunization of mice via biting by CSP-expressing transgenic mosquitoes. Two female BALB/c mice were bitten by each of 50 TG female mosquitoes every 2 weeks over a 10-week period (5 biting sessions). Two other mice were bitten by wild-type female mosquitoes under the same protocol. Two microliters of mouse blood was collected from the tail vein 7 days after the final mosquito biting. Antibody assay to CSP and SG was carried out by ELISA as described above.

Comparison of CSP contents in the SG before and after blood feeding. A pair of SG of the TG female mosquitoes before blood feeding was transferred in 0.5ml of carbonate buffer, destroyed, and centrifuged as above. Another TG female mosquito was dissected within 10 min after full blood feeding, and a pair of SG were isolated and treated as described above. Each antigen solution was added to 4 wells, 100 μ l per well. Anti-CSP antibody (800-fold) was reacted with the 2 wells and anti-saliva antibody (800-fold) was reacted with the other 2 wells. Incubation with the secondary antibody and the subsequent procedures were the same as described above. Eight SG samples in each group were tested.

Results and Discussion

Isolation of TG mosquito. We injected recombinant plasmid into 461 mosquito eggs, from which 109 larvae hatched and 103 adults emerged (G0 adults: 56 males and 47 females). We crossed these G0 males with wild-type females and allowed them to lay eggs. We also crossed G0 females with wild-type males and allowed them to lay eggs. GFP-expressing larvae were selected and allowed to emerge. From the G0 male group we established 1 strain, and from the G0 female group we established 4 strains. The rate of establishment for 5 strains from the 461 egg injections (1.1%) was reasonable compared with our previous experiments [16, 33] and with the results obtained by other groups [15, 32].

As shown in Fig. 2, larvae and adult TG mosqui-

toes expressed GFP in the eyes as expected, because the *3xP3* promoter works specifically in the eyes [11]. Unexpectedly, GFP was expressed not only in the eyes but also in the SG (Fig. 2C). *3xP3* marker protein is typically used for *Drosophila* (a small fly) transgenesis, and expression of a transgene in the SG of *Drosophila* has not been confirmed. Thus, this is the first observation that the *3xP3* promoter works in the SG of a mosquito. The SG cells may contain some transcriptional factors to attach to the *3xP3* promoter. GFP expression in female SG was observed among all strains of CS1 to CS5. In the SG, the highest expression of GFP was observed in the distal region of the lateral lobes in all SGs (Fig. 2C). In contrast, GFP was not always expressed in the median lobes. This tendency was similar to the results of a previous report [33].

Recombinant CSP expression in the SG. We expected that rCSP would be expressed in the SG of TG mosquitoes. ELISA was performed to confirm rCSP expression. Ten pairs of SG from each strain of TG female mosquitoes were collected and used as ELISA antigens. Only one of the 5 strains (CS2) showed strong reactivity against anti-CSP antibody (Fig. 3). This means that the *3xP3* promoter worked in all 5 strains, but that the *aapp* promoter worked only in strain CS2. The exact reason for this is unknown, but may be related to the insertion site in the mosquito chromosome. The insertion site may allow the *3xP3* promoter, but not the *aapp* promoter, to work. A possible explanation for this is that some of the transcriptional factors that attach to the *aapp* promoter to start AAPP expression react mostly with the intrinsic *aapp* promoter. The inserted *aapp* promoter cannot react with these factors, and thus rCSP may be expressed in only small amounts.

Western blotting analysis and estimation of rCSP expression. Western blotting was performed with 10 pairs of SG dissected from transgenic mosquitoes of the CS2 strain. A band of 38kDa reacted with anti-CSP in the TG mosquito lane (Fig. 4). The molecular size was as we expected for rCSP. This indicates that we succeeded in producing a foreign protein of rCSP in the mosquito SG. A comparison of the density of the 38kDa band with those of the positive control bands of a series of different amounts of rPbCSP revealed that the amount of rCSP in the CS2 lane was equivalent (in reactivity

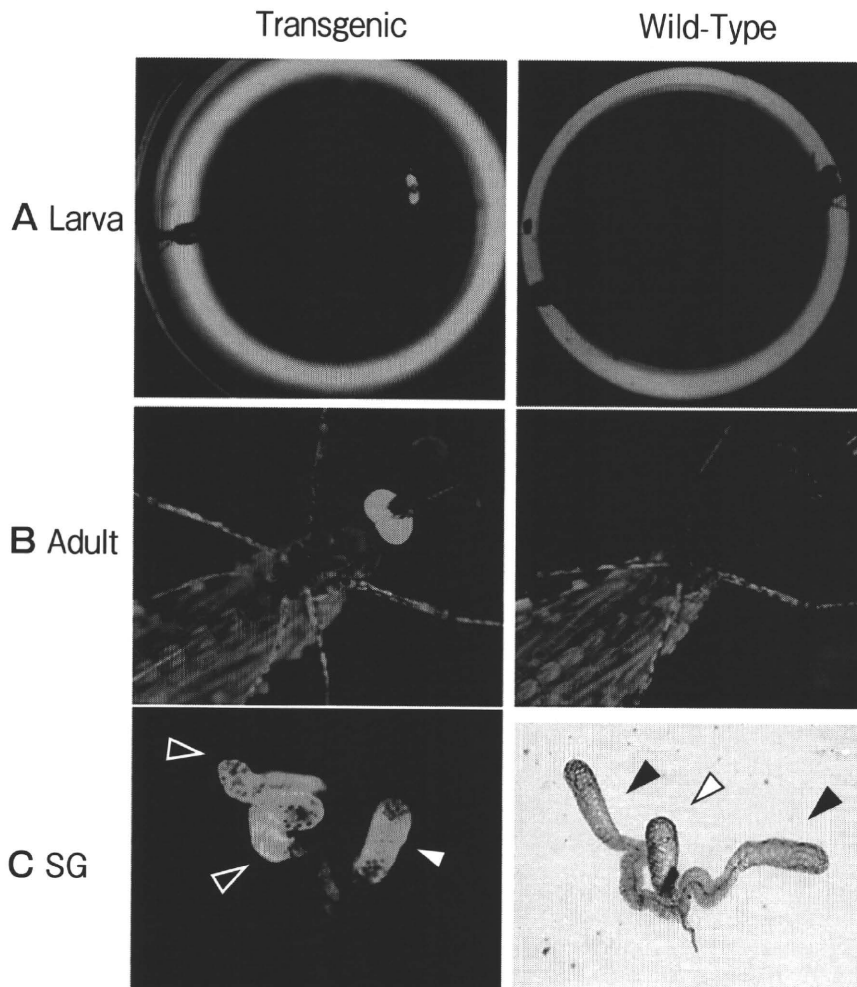


Fig. 2 Transgenic mosquito of *A. stephensi* expressing GFP. **A**, Larvae expressing GFP in the eyes. Since each larva was placed into its own wells of a 96-well plate, auto-fluorescence appeared from the well edge; **B**, Adult female expressing GFP in the eyes; **C**, Unexpectedly, GFP was expressed in the SG of transgenic mosquitoes. As a reference, the SG of a wild-type mosquito was taken at the phase condition. Arrow heads (▶) show the lateral lobes of SG, and the other arrow heads (▷) show the median lobes of SG. No GFP expression was observed in the SG of wild-type mosquitoes.

with the anti-CSP antibody) to 400ng rPbCSP. This, in turn, indicated that approximately 40ng of rCSP was expressed in one pair of SG in the TG mosquito.

Besides the 38kDa band, some other faint bands appeared in the CS2 lane. The molecular sizes of these bands were 26, 48, 64, and 80kDa. Even in the reducing conditions (with 2% 2-mercaptoethanol), some intermolecular re-binding may have occurred. There is another possibility that rCSP binds to some molecules of the SG components such as ubiquitins, proteasome-associated cofactors [34].

Anti-CSP antibody in mice bitten by TG mos-

quitoes. Two Female BALB/c mice were each bitten by 50 TG female mosquitoes of the CS2 strain every 2 weeks over a 10-week period. The mice developed anti-SG antibody but did not develop anti-CSP antibody (Fig. 5). In our prediction, 20% of rCSP (about 8ng of rCSP per mosquito) in the SG should be injected into a mouse during a single blood feeding. In each blood feeding, a mouse received 50 TG mosquito bites and received a total of 400ng of rCSP in the skin. As these blood feedings were conducted 5 times, each mouse was injected with 2,000ng of rCSP. From these injections, the mice

should have developed anti-CSP antibody in the serum. However, no antibody against rPbCSP was detected in the mice. Only anti-SG antibody was detected in the mouse serum. In our experience, 2 injections each of 1,000ng rPbCSP are sufficient to develop anti-rPbCSP in mice (unpublished observation). Thus, we consider the possibility that rCSP was not secreted as we had expected in the saliva of the TG mosquito and thus was not injected into the skin during blood feeding.

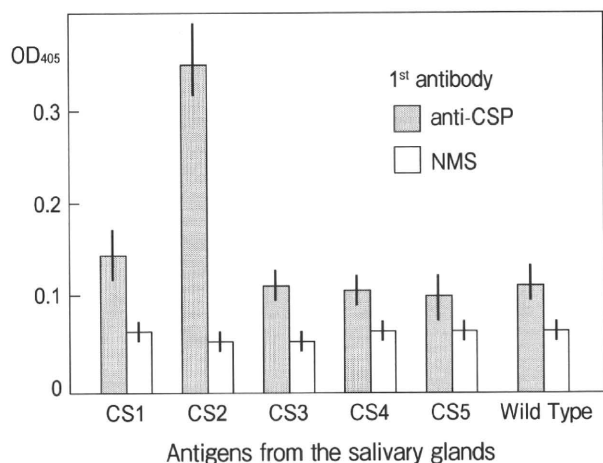


Fig. 3 Evaluation of CSP expression in SG among 5 mosquito strains. SG was taken from the 5 strains and tested as an antigen. Anti-CSP was used as the first antibody. SG from the CS2 strain reacted highly to anti-CSP. NMS (normal mouse serum) was used as a control antibody. The test was conducted 3 times.

Comparison of CSP contents in the SG before and after blood feeding. We performed ELISA with antigens of the SG before and after blood feeding. The amount of rCSP in the SG after blood feeding did not decrease, although the amount of total saliva protein did decrease (Fig. 6). This indicates that the TG mosquitoes consume a lot of saliva during blood feeding and that the saliva does not contain rCSP.

We used the *aapp* promoter for rCSP expression

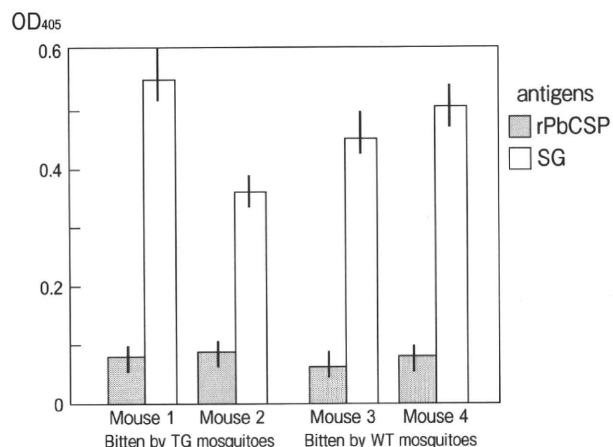


Fig. 5 Anti-CSP antibody and anti-saliva antibody evaluated by ELISA. Mice 1 and 2, which were bitten by TG mosquitoes of the CS2 strain, did not develop anti-CSP antibody. All 4 mice developed anti-saliva antibody. The test was conducted 3 times.

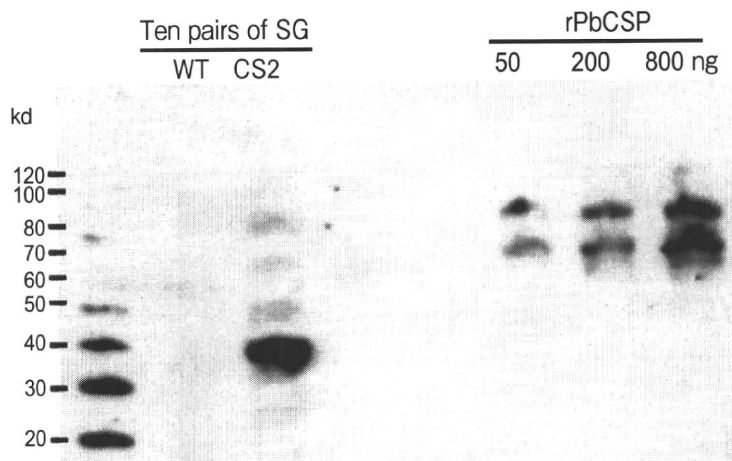


Fig. 4 Western blotting analysis of CS2 strain salivary glands. A 38kDa band appeared after the reaction with anti-CSP antibody. The left lane contains molecular weight markers. Series of rPbCSP (70kDa and 82kDa) with different amounts were run on the same gel in the right 3 lanes.

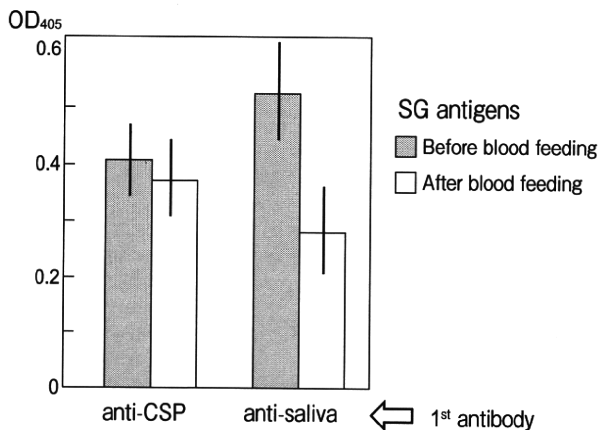


Fig. 6 Comparison of CSP contents in the SG before and after blood feeding. Both SG antigens ($n = 8$) were filled in the ELISA plate. The reactivity of anti-CSP to both SGs was similar, whereas the reactivity of anti-saliva to the SGs after blood feeding was significantly reduced ($p < 0.05$).

because AAPP was confirmed to be secreted in the saliva and injected into the skin during blood feeding [24]. However, we obtained the disappointing result that the TG mosquito did not properly release the foreign protein into the mouse skin. One possible explanation is that rCSP may have been captured by some components of the SG cells, such as ubiquitins [35]. As shown in Fig. 4, rCSP was expressed as some larger molecules in Western blot analysis. This figure suggests the possibility of “captured rCSP” in the SG. According to this hypothesis, rCSP cannot be secreted in the saliva because of its molecular characteristics, even if it has a secretion signal. We are preparing a new construct using the *aapp* promoter and *aapp* secretion signal with other foreign protein genes to test this hypothesis.

In summary, we presented a transgenic mosquito expressing a foreign protein in the SG. The expressed protein was rCSP, a promising malaria vaccine candidate protein. The amount of rCSP in one pair of SG was estimated to be 40 ng. If 20% of rCSP in the SG is injected into a mouse during a single blood feeding band there are 50 mosquito bites on the mouse, 8×50 ng of CSP should be injected and anti-CSP antibody should be developed in the mouse after several episodes of blood feeding. Since we did not succeed in preparing such a TG mosquito, we will develop new constructs for producing alternative TG mosquitoes.

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Involvement of CD8⁺ T cells in protective immunity against murine blood-stage infection with *Plasmodium yoelii* 17XL strain

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When developing malaria vaccines, the most crucial step is to elucidate the mechanisms involved in protective immunity against the parasites. We found that CD8⁺ T cells contribute to protective immunity against infection with blood-stage parasites of *Plasmodium yoelii*. Infection of C57BL/6 mice with *P. yoelii* 17XL was lethal, while all mice infected with a low-virulence strain of the parasite 17XNL acquired complete resistance against re-infection with *P. yoelii* 17XL. However, the host mice transferred with CD8⁺ T cells from mice primed only with *P. yoelii* 17XNL failed to acquire protective immunity. On the other hand, the irradiated host mice were completely resistant to *P. yoelii* 17XL infection, showing no grade of parasitemia when adoptively transferred with CD8⁺ T cells from immune mice that survived infection with both *P. yoelii* XNL and, subsequently, *P. yoelii* 17XL. These protective CD8⁺ T cells from immune WT mice had the potential to generate IFN- γ , perforin (PFN) and granzyme B. When mice deficient in IFN- γ were used as donor mice for CD8⁺ T cells, protective immunity in the host mice was fully abrogated, and the immunity was profoundly attenuated in PFN-deficient mice. Thus, CD8⁺ T cells producing IFN- γ and PFN appear to be involved in protective immunity against infection with blood-stage malaria.

Key words: CD8 T cells · Immune responses · Infectious diseases · Malaria · Memory cells



Supporting Information available online

Introduction

Malaria is one of the main global infectious diseases, and results in 300–500 million clinical cases and one million deaths

annually, mostly among young children in sub-Saharan Africa ([http://rbm.who.int/wmr2005/\[1\]](http://rbm.who.int/wmr2005/[1])). The development of resistance to drugs in parasites and vectors poses one of the greatest threats to malaria control and has been linked to recent increases in malaria morbidity and mortality, so vaccine development is urgently required. To achieve this purpose, it is essential to elucidate the detailed protective mechanisms of the hosts against infection.

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Antibodies and T cells play crucial roles in protective immunity against malaria parasites. Antibodies specific for merozoites prevent invasion of those parasites into RBC [2–5]. These antibodies, attached to the parasites or parasitized RBC, lyse the parasites and RBC in a complement-dependent manner. Another function of these antibodies is to mediate phagocytosis and digestion by monocytes/macrophages [3–5]. CD4⁺ T cells act as Th cells for B-cell differentiation and antibody production; on the other hand, Th cells activate phagocytosing macrophages and kill the parasites. Thus, CD4⁺ T cells should play key roles in protective immunity against both liver and blood-stage malaria. Strangely, however, clinical vaccine trials using antigenic peptide for malaria have resulted in failure in some cases [6], despite the fact that activation of CD4⁺ T cells and antibody production have been efficiently induced in vaccine trials.

CD8⁺ T cells have been proposed to be essential for protective immunity against liver-stage malaria [2–5]. On the other hand, several studies have concluded that CD8⁺ T cells do not contribute to protective immunity against blood-stage parasites, mainly because of the absence of MHC class I molecules on infected erythrocytes, by which antigens are presented to CD8⁺ T cells [7–10]. For example, Vimetz *et al.* have reported that adoptive transfer of CD8⁺ T cells from immune animals does not confer protective immunity to blood-stage infection in the host [7]. However, some studies have stressed the existence of CD8⁺ T cells specific for blood-stage malaria parasites. First, CD8⁺ T-cell clones that proliferate and produce IFN- γ in response to blood-stage malarial antigens in an HLA-restricted manner are generated in patients who live in a malaria-endemic area [11]. Second, experimental infections of non-immune volunteers with an ultra-low dose of infected RBC induce immunity to subsequent challenge in the absence of detectable antibody responses. Those who acquire immunity show proliferative responses in CD8⁺ T cells [12].

There are few studies showing that CD8⁺ T cells protect against blood-stage parasites. However, there are some reports in murine models that CD8⁺ T cells contribute to the pathology of experimental cerebral malaria. Mice that are depleted in, or do not have, CD8⁺ T cells, are protected against experimental cerebral malaria [13]. CD8⁺ T cells might contribute *via* perforin (PFN)-dependent destruction of cerebral microvascular endothelial cells [14]. These malaria Ag-specific CD8⁺ T cells are induced by cross-presentation from dendritic cells [15]. In the present study, we evaluated the contribution of CD8⁺ T cells in immunity against blood-stage parasites.

Results

CD8⁺ T cells from *Plasmodium yoelii* 17XNL-recovered mice fail to transfer protection against PyL

We infected C57BL/6 mice with the blood-stage form of the rodent malaria parasite *P. yoelii* 17X, which has two substrains. One substrain, PyL, is highly virulent in mice and causes lethal

infection; the other, PyNL, causes a self-limiting, non-lethal infection [16]. Mice infected with 25 000 PyNL-parasitized RBC (PyNL-pRBC) spontaneously recovered within 4 wk of infection (data not shown). Mice that recovered from infection with PyNL (PyNL-recovered mice) were then infected with PyL. These mice were highly resistant to the lethal infection and allowed no parasite growth, while control mice showed rapid parasite growth and died within 10 days (Fig. 1A). Thus, infection with PyNL functions as a live vaccination against lethal infection with PyL. To examine the involvement of CD8⁺ T cells in protective immunity to blood-stage infection with PyL, we injected PyNL-recovered mice with anti-CD8 mAb to deplete CD8⁺ T cells prior to infection with PyL. However, depletion of CD8⁺ T cells, as well as CD4⁺ T cells, did not attenuate protection in PyNL-recovered mice (data not shown). We next performed adoptive transfer experiments. CD8⁺ T cells purified from PyNL-recovered mice were transferred to the syngeneic recipient mice. In order to exclude the influence of host lymphocytes, we irradiated the recipients with 5.5 Gy of γ radiation. One week after cell transfer, these mice were infected with PyL. These recipient mice failed to control infection with PyL, similar to those transferred with CD8⁺ T cells from uninfected control mice (Fig. 1B). These results were very similar to those of previous studies [6–9], which indicates that CD8⁺ T cells do not contribute to protection against blood-stage malaria parasites.

CD8⁺ T cells from highly immunized mice transfer protection against PyL

PyNL-recovered mice showed sterile immunity against infection with PyL. However, CD8⁺ T cells from these mice did not transfer protection to the recipient mice. Several factors, such as presence or absence of antibodies in the circulation, might account for this discrepancy. Among these factors, we speculate that CD8⁺ T cells

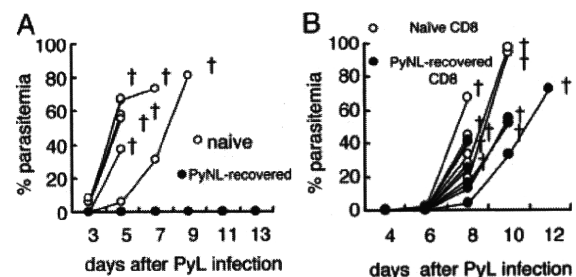


Figure 1. CD8⁺ T cells from PyNL-recovered mice failed to transfer protection against PyL. (A) The kinetics of parasitemia in PyNL-recovered (closed circles) or naive (open circles) mice were monitored after infection with 25 000 PyL-pRBC. (B) CD8⁺ T cells (8×10^6) purified from PyNL-recovered (closed circles) or naive (open circles) mice were transferred to the syngeneic irradiated recipient mice, followed by infection with PyL. Each symbol represents a value from an individual mouse. Daggers indicate death. Similar results were obtained from at least four experiments.

are hardly activated during primary infection, as prime-boost regimens are commonly used to fully activate antigen-specific CD8⁺ T cells [17]. To assess this possibility, PyNL-recovered mice were further infected twice with PyL (immune mice, Fig. 2A). Although PyNL-recovered mice showed no signs of infection, or even parasite growth, when boosted with PyL as shown in Fig. 1A, serum antibodies specific for PyL were significantly increased after the boosts (Fig. 2B), which indicates successful reactivation of parasite-specific immunity. CD4⁺ or CD8⁺ T cells purified from immune mice were transferred to irradiated recipients, which were subsequently infected with PyL and monitored for the course of infection (Fig. 2C and D). All recipients transferred with CD4⁺ or CD8⁺ T cells from control mice died as parasites grew. Mice that received CD4⁺ T cells from immune mice showed low parasitemia and survived with no parasite recrudescence (Fig. 2C). Surprisingly, CD8⁺ T cells from immune mice transferred protection against infection with PyL as well as CD4⁺ T cells (Fig. 2D). Furthermore, transfer of CD8⁺ T cells from immune mice also conferred protection against PyL in RAG2 KO recipient mice (Supporting Information Fig. S1B). To exclude the possibility that small contaminating population of other cells was responsible for the protection, we sorted the three groups of cells from immune mice for adoptive transfer experiment, using MACS cell separation system; the first group was CD8⁺ cells, the second was CD4⁻ (CD4 depleted, negative selection) CD8⁺ cells and last one was CD4⁻ CD8⁻ (CD4, CD8 depleted, negative selection) cells. Although CD4⁻ CD8⁺ T cells

from immune mice transferred protection against infection with PyL, mice depleted of both CD4 and CD8 T cells failed to control the infection and died from high parasitemia (Supporting Information Fig. S1A), confirming that contaminants do not contribute to protection. These results clearly demonstrate that CD8⁺ T cells play a protective role against blood-stage malaria parasites.

Immunophenotype characterization of CD8⁺ T cells in immune mice

Mice that received CD8⁺ T cells from immune mice showed low parasitemia and survived with no parasite recrudescence. This protective immunity against malaria parasites might induce activation of CD8⁺ T cells and generate memory CD8⁺ T cells. We analyzed the phenotype of CD8⁺ T cells. Naive T cells express CD62L (L-selectin), which is known to be a homing receptor, and shed this molecule to migrate from lymphoid organs to inflammatory sites after activation [18]. CD44 appears to be the most reliable marker that is expressed at high levels in all memory T cells of mice, irrespective of their activation status [19]. CD8⁺ T cells can be classified into three groups according to their expression patterns of CD62L and CD44: CD62L^{hi}CD44^{lo}, CD62L^{hi}CD44^{hi} and CD62L^{lo}CD44^{hi} patterns represent naive, central memory, and effector memory phenotypes, respectively [19]. Spleen cells from recipient mice transferred with the

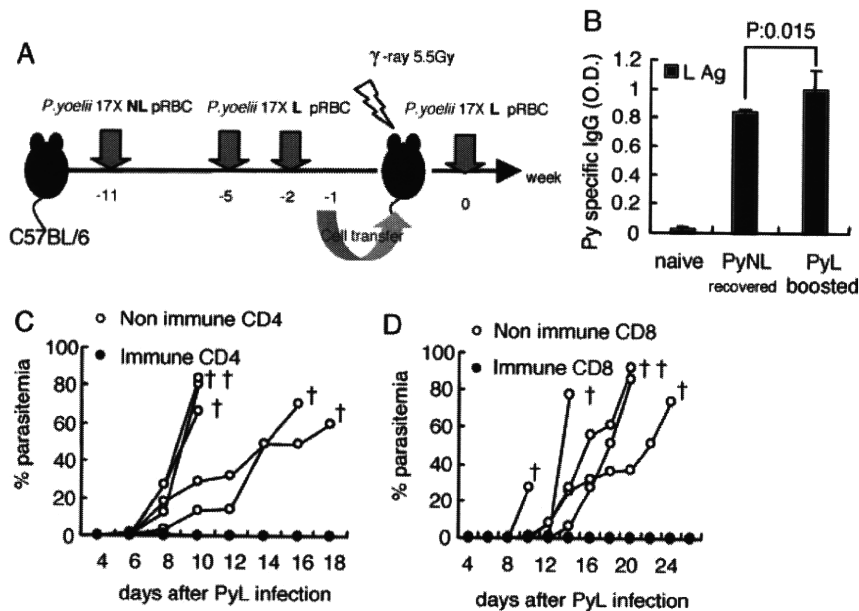


Figure 2. CD8⁺ T cells from highly immunized mice transferred protection against PyL. (A) Protocol of immunization, cell transfer and challenge infection. (B) Antibody titers specific for malaria parasites in sera collected from mice were measured. Data are the means+SD of OD₄₁₅ values of 200-fold-diluted sera from six mice in each group. Parasitemia of the recipients transferred with CD4⁺ (C) or with CD8⁺ (D) T cells purified from control (open circles) or immune (closed circles) mice were evaluated as in Fig. 1B. One representative of at least two repeated experiments is shown. Daggers indicate death.

indicated cells were analyzed 7 days after infection with PyL (Fig. 3A right panels). Cells from the recipients left uninfected were also analyzed (Fig. 3A left panels). Immune mice contained twice as many CD62L^{lo} and effector memory CD8⁺ T cells than naive control mice (Fig. 3A). Also, the numbers of CD8⁺ T cells expressing CD30, which is known to be a marker of memory T cells, increased in immune mice by as much as 16.7% versus 5.8% in naive mice (data not shown). In response to infection with PyL, the numbers of effector memory CD8⁺ T cells predominantly increased in recipients transferred with CD8⁺ T cells from immune mice (Fig. 3A). Furthermore, infection of these mice markedly increased the numbers of CD8⁺ T cells that express granzyme B, PFN or IFN- γ , all of which are important molecules for the protective role of CD8⁺ T cells. In sharp contrast, infection of recipients transferred with CD8⁺ T cells from non-immune mice did not affect the expression of these molecules (Fig. 3B), although CD8⁺ T cells were slightly activated, as evaluated by an increase in the size of the CD62L^{lo} population (Fig. 3A). These results demonstrate that the immunization protocol can effectively generate memory CD8⁺ T cells that respond quickly to become effector cells.

IFN- γ from CD8⁺ T cells is important to protect against infection with PyL

We evaluated the role of IFN- γ in protective immunity mediated by CD8⁺ T cells. IFN- γ is a pro-inflammatory cytokine that is intimately involved in the innate and acquired immune responses. This cytokine has important roles in immunity against blood-stage malaria, because a previous study has demonstrated

that IFN- γ KO mice are highly susceptible to infection with *Plasmodium chabaudi* AS-pRBC [20]. First, the irradiated recipients transferred with CD8⁺ T cells from immune mice were treated with neutralizing antibodies to IFN- γ prior to infection with PyL. The recipients injected with anti-IFN- γ suffered from high parasitemia and died quickly, while those injected with irrelevant antibodies showed only a slight increase in parasitemia (Fig. 4A). To confirm the importance of IFN- γ secreted from CD8⁺ T cells, we employed IFN- γ KO mice as immune donors, because the recipient-derived cells might have secreted the cytokine. IFN- γ KO mice were much more susceptible to infection with PyNL, and 25–50% of these mice survived (data not shown). However, once recovered from the infection, IFN- γ KO mice acquired sterile immunity to infection with PyL. CD8⁺ T cells from immune IFN- γ KO mice were transferred to the irradiated recipients prior to infection with PyL. The recipients transferred with CD8⁺ T cells from immune IFN- γ KO mice failed to control the infection similar to those transferred with CD4⁺ T cells from immune IFN- γ KO mice (Fig. 4B). These results suggest that IFN- γ is important to CD8⁺ T-cell-mediated protection against blood-stage malaria.

Macrophages play essential roles in CD8⁺ T-cell-mediated protection against PyL

Among the pivotal roles of IFN- γ , activation of macrophages is supposed to be one of the major mechanisms for activating anti-malarial immunity. Macrophages play a critical role in innate immunity against malaria due to their ability to phagocytose pRBC in the absence of cytophilic or opsonizing parasite-specific antibodies [21]. In adaptive immunity, upon

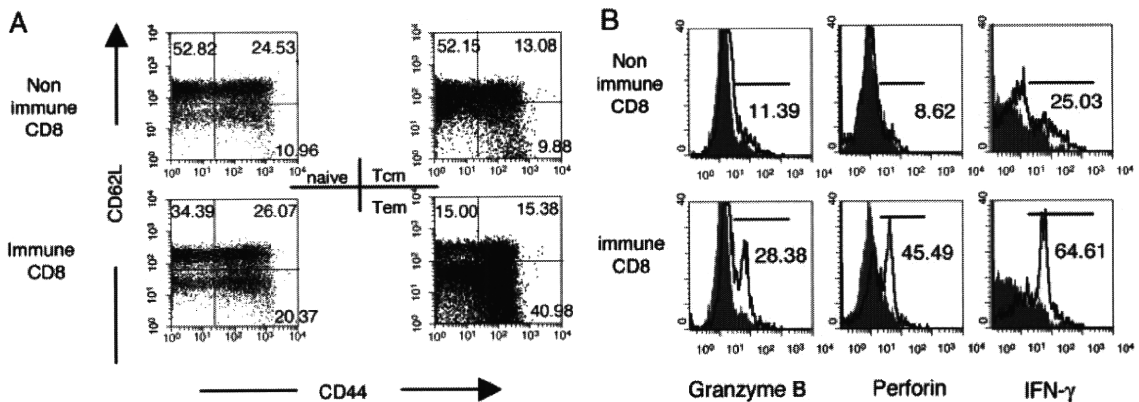


Figure 3. Immunophenotype and expression of effector molecules of protective CD8⁺ T cells. (A) Spleen cells from recipient mice transferred with the indicated cells were analyzed 7 days after infection with PyL (right panels). Cells from the recipients left uninfected were also analyzed (left panels). Gated CD8⁺ T cells from mice were classified into central memory (Tcm), effector memory (Tem) and naive cells by staining with CD62L and CD44. Numbers represent the percentage of cells in the corresponding quadrants. (B) Spleen cells from recipient mice transferred with non-immune (upper panel) or immune (lower panel) CD8⁺ T cells were collected 7 days after PyL infection. Expression of granzyme B, PFN and IFN- γ in CD8⁺ T cells from spleen was analyzed by intracellular staining with the corresponding antibodies. The expression profiles of CD8⁺ T cells from the infected recipients (solid lines) were plotted over those of CD8⁺ T cells from uninfected naive mice (shaded areas). Numbers indicate the percentages of CD8⁺ T cells positive for the indicated marker in infected recipients. The percentages of CD8⁺ T cells from uninfected mice positive for granzyme B, PFN, and IFN- γ were 4.6, 9.5 and 11.5 %, respectively.

stimulation with IFN- γ , macrophages function as effector cells that can mediate antibody-dependent cellular inhibition or the production of anti-parasitic molecules. Thus, we evaluated the role of macrophages in irradiated recipients transferred with CD8⁺ T cells from immune mice. Carrageenan (CGN), a sulfate polygalactose preferentially phagocytosed but undigested in macrophages, was administered to the recipients to impair macrophages [22]. This manipulation completely abolished the protective ability of CD8⁺ T cells (Fig. 4C). These results indicate that macrophages, presumably activated by IFN- γ secreted from CD8⁺ T cells, are responsible for parasite eradication in recipient mouse transferred with immune CD8⁺ T cells. Indeed, the potential of macrophages for phagocytosing pRBC was profoundly impaired in recipients transferred with CD8⁺ T cells from immune IFN- γ KO mice compared with those from recipients transferred with immune WT mice (Fig. 4D).

PFN from CD8⁺ T cells contribute partially to protection against PyL

CD8⁺ T cells from immune mice expressed effector molecules related to cytotoxicity. We investigated the involvement of these molecules in the mechanism of protective immunity induced by CD8⁺ T cells against blood-stage parasites. PFN KO mice were infected with PyNL. PFN KO mice were more susceptible to infection with PyNL than WT mice, and only half of them survived (Fig. 5A), which suggests that PFN has a protective role. Mice surviving infection with PyNL were resistant to boosting with PyL to the same degree as WT mice, and we employed these mice as immune mice. We compared the activation and phenotype of CD8⁺ T cell from immune WT and immune PFN KO mice that were subsequently used in the adoptive transfer experiments (Supporting Information Fig. S2A). The activation maker (CD62L lo cells) was almost the same between WT and

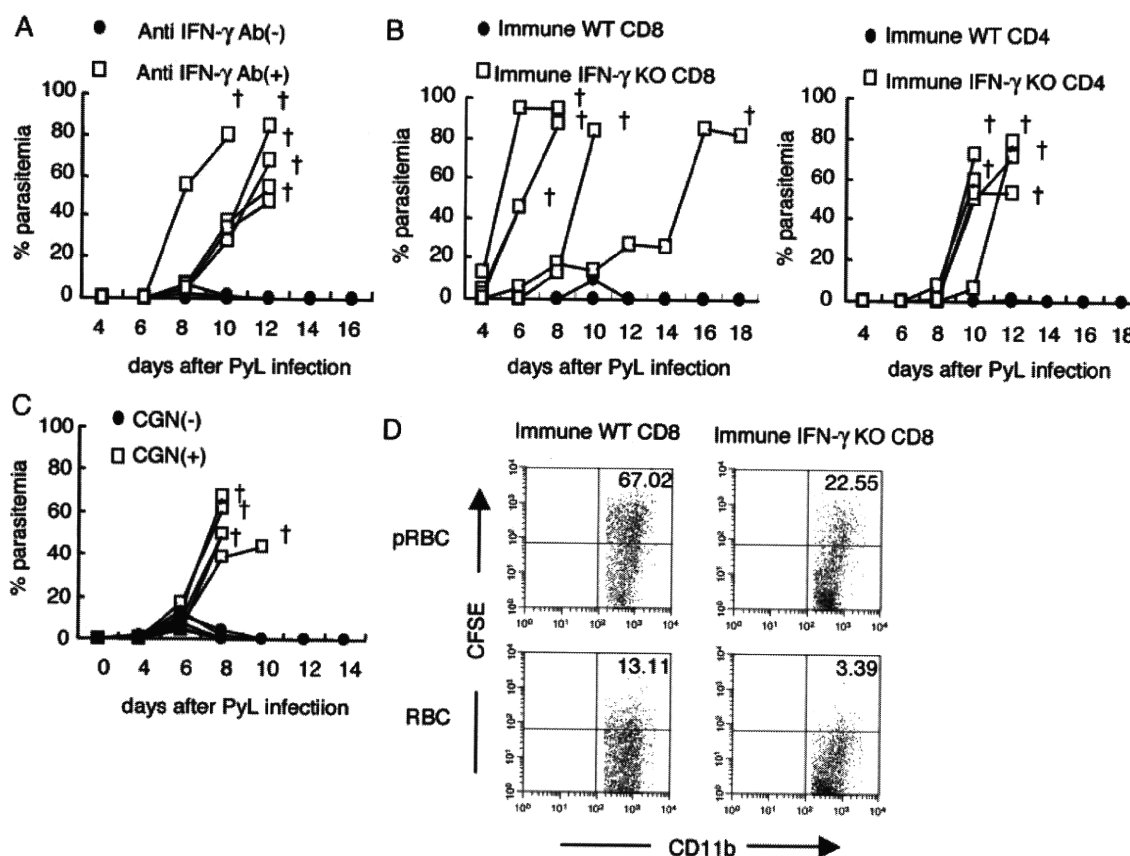


Figure 4. IFN- γ from CD8⁺ T cells was essential for protection against blood-stage malaria. (A) Parasitemia in recipients transferred with immune CD8⁺ T cells treated with anti-IFN- γ (open squares) or irrelevant antibodies (closed circles) was evaluated as in Fig. 1B. (B) CD8⁺ (left panel) or CD4⁺ (right panel) T cells from IFN- γ KO (open squares) or WT (closed circles) mice were transferred to the irradiated recipients, followed by infection with PyL. (C) Macrophages played an essential role in CD8⁺ T-cell-mediated protection against PyL. The irradiated recipients transferred with CD8⁺ T cells from immune mice were treated with CGN to impair macrophage function (open squares), and parasitemia was evaluated. Daggers indicate death. (D) Macrophage uptake assay. Splenic CD11b macrophages were removed from day 7 post-PyL-infected recipients that were transferred with CD8⁺ T cells from immune WT or immune IFN- γ KO mice. Macrophages were co-cultured with CFSE-labeled pRBC or RBC. The percentage of CD11b⁺ cells undergoing phagocytosis is presented. One representative of at least two repeated experiments is shown.

PFN KO (Supporting Information Fig. S2C). Furthermore, the number of transferred effector memory cells that are thought to be a key player in protection against infection was also the same between WT and PFN KO (Supporting Information Fig. S2B). These results indicated that activation and phenotype of transplanted immune PFN KO CD8⁺ T cells are very similar to immune WT CD8⁺ T cells even though PFN KO mouse are susceptible to PyNL infection. When infected with PyL, the irradiated recipients transferred with CD8⁺ T cells from immune PFN KO mice showed higher parasitemia at an early stage of infection than those transferred with CD8⁺ T cells from immune WT mice, and some mice in the former group failed to control the challenge infection (Fig. 5B). However, CD4⁺ T cells from these donors protected recipients from infection with PyL, similar to those from immune WT mice (Fig. 5B). These results indicate that the cytotoxicity-related molecule PFN contribute, at least in part, to the protective immunity to blood-stage malaria infection conferred by CD8⁺ T cells.

Discussion

In the present study, we found that CD8⁺ T cells play roles in the protective immunity against blood-stage infection with highly virulent *P. yoelii* in C57BL/6 mice. Irradiated naive mice transferred with CD8⁺ T cells from these mice were resistant to infection with PyL. IFN- γ and macrophages were essential for the protective immunity dependent on CD8⁺ T cells. Additionally, cytotoxicity-related molecules expressed by CD8⁺ T cells, such as PFN, appeared to also contribute to this protective

immunity. It is noteworthy that the protective immunity mediated by CD8⁺ T cells was not acquired when recipient mice were treated with CGN, a macrophage blocker, after challenge with the parasite. Based on those observations, the role of CD8⁺ T cells in protective immunity would be exerted by a two-step mechanism. As a first step, these T cells would activate macrophages through the release of IFN- γ . These macrophages should potentially phagocytize the infected erythrocytes and digest them. If the parasites were not completely eliminated by the first step mechanism, CD8⁺ T cells might kill the infected macrophages via the PFN-granzyme system in the second step.

We used irradiated or RAG2-deficient mice as recipients for transfer with immune CD8 T cells. It is well established that T cells transferred into such immunodeficient hosts will expand in a non-physiologic manner (homeostatic proliferation) that may result in artificial activation [23]. Moreover, irradiated hosts likely represent a highly inflammatory environment, "hormesis effects" [24]. Similarly, RAG-deficient mice have robust innate immune systems to partially compensate for the lack of adaptive immunity [25]. It is quite possible that these unexpected effects are responsible for the protection observed here. However, only CD8⁺ T cells from immune mice but not from naive mice (even after a single infection) were potent in transferring protection. These results clearly indicate that protection through the transfer of immune CD8⁺ T cells is unlikely due to hormesis or homeostatic proliferation and is antigen-specific. In terms of antigen specificity, we determined whether immune CD8⁺ T cells transfer protection against an irrelevant protozoan parasite, *Toxoplasma gondii*. Mice transferred with immune CD8⁺ T cells were susceptible

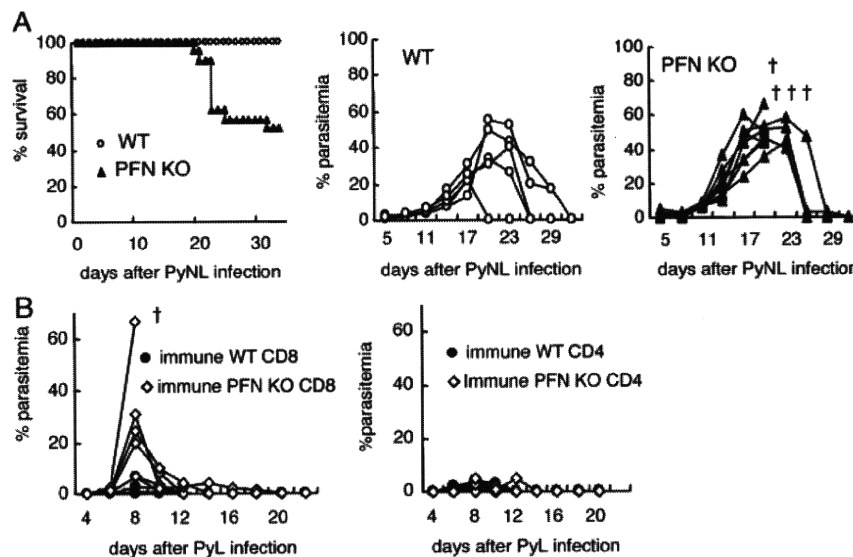


Figure 5. Molecules associated with CTL activity partially contributed to protection against blood-stage malaria. (A) Survival rates and parasitemia in PFN KO (closed triangles) mice were monitored after infection with 25 000 PyNL-pRBC. (B) The recipient mice transferred with CD8⁺ or CD4⁺ T cells from immune PFN KO mice were infected with PyL, and parasitemia was evaluated as in Fig. 1B. One representative of at least two repeated experiments is shown. Daggers indicate death.

to the infection similar to those with naive CD8⁺ T cells (data not shown).

Malaria parasites reside within RBC to evade host immunity. Merozoite-specific antibodies in the circulation readily bind to free merozoites and may inhibit invasion of the parasites into RBC, but these antibodies cannot reach merozoites once they have invaded RBC. Furthermore, blood-stage parasites should escape recognition by CD8⁺ T cells since RBC do not express MHC class I molecules on their surface, leading to the conclusion that CD8⁺ T cells could not contribute to protective immunity against blood-stage parasites [6–9].

RBC have no MHC class I molecules. Nevertheless, CD8⁺ T cells specific for malaria antigens are activated during blood-stage malaria [10, 11]. Taken together with our observation that the numbers of activated CD8⁺ T cells increased after infection with PyL, antigens derived from malaria parasites must be presented on MHC class I molecules on APC including macrophages. The activation mechanism of CD8⁺ T cells may be explained by cross-presentation of APC that have engulfed pRBC as other particle antigens [26].

CD8⁺ T-cell-dependent protective immune responses have been shown to correlate well with IFN- γ production and cytotoxic activity. Our results showed important roles of IFN- γ in protection mediated by CD8⁺ T cells. Macrophages were also important in this protection, strongly suggesting that CD8⁺ T cells contribute to the elimination of blood-stage parasites by activating macrophages with IFN- γ , and that the activated macrophages would phagocytose and digest pRBC.

On the other hand, cytotoxic activity exerted by PFN might play some roles in the protective immunity in the late stage of the protective immunity. Macrophages activated with IFN- γ phagocytose antigen abundantly, and are exhausted after phagocytosis reaches its limit [27]. As a final step, CD8⁺ T cells direct pRBC-phagocytosed monocytes/macrophages to undergo apoptosis via the PFN-granzyme B pathway, and completely eliminate them. A previous study reported that CD8⁺ T cells mediate loss of macrophages in the spleen, which is the most important organ for removal of pRBC by macrophages [28]. Thus, cytotoxic activity exerted by PFN and granzyme B in CD8⁺ T cells might be directed to these macrophages, resulting in the elimination of parasites and recruitment of fresh macrophages.

Although great efforts have been made to develop vaccines against malaria, practical vaccine strategies have not yet been established. One of the causes for this failure may be that host protective immunity and the immune evasion mechanisms of the parasites have not been fully understood. We clearly showed the involvement of CD8⁺ T cells in protective immunity against blood-stage infection. In addition, blood-stage parasites seem to evade CD8⁺ T cell immunity. That is, dendritic cells that interact with pRBC selectively impair the cell cycles of CD8⁺ T cells, but not CD4⁺ T cells [29]. In developing vaccines for blood-stage malaria, establishment of strategies for activating CD8⁺ T cells specific for the parasites or infected erythrocytes may be essential.

Materials and Methods

Mice and parasites

C57BL/6 mice were obtained from Kyudo (Tosu, Japan), RAG2-deficient mice were obtained from Central Laboratory of Experimental Animals (Kawasaki, Japan), PFN-deficient mice were obtained from Jackson Immuno Research Laboratories (West Grove, PA, USA), IFN- γ -deficient mice were provided by Dr. A. Nakane (Hirosaki University), and Ly5.1C57BL/6 mice were obtained from Sankyo Lab Service (Tokyo, Japan) with the permission of Dr. H. Nakachi (Tokyo University). Age- and sex-matched groups of WT and mutant mice were used. All experiments using mice were reviewed by the Committee for Ethics on Animal Experiments in the Faculty of Medicine, and carried out under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government. Blood-stage parasites of PyL or PyNL, generous gifts from Dr. M. Torii (Ehime University) and Dr. K. Yui (Nagasaki University), were obtained after fresh passage through a donor mouse 2–3 days after inoculation with frozen stock. Mice were infected with 25 000 pRBC i.p., which were purified as previously described, and used for infection.

Determination of parasitemia

Blood samples were collected from the experimental mice by bleeding via the tail vein at the time indicated. Thin blood films were prepared and fixed with methanol followed by staining with Giemsa solution (Sigma-Aldrich, St. Louis, MO, USA). Parasitemia was determined by counting the percentages of pRBC under a microscope.

Reagents

FITC-, PE-, and PE-Cy5-anti-CD8, FITC-anti-CD4, FITC-anti-CD62L, PE-anti-CD30, PE-anti-granzyme B, and FITC-anti-PFN (eBioscience, San Diego, CA, USA) were used for flow cytometry. Purified anti-CD16/32 (2.4G2) antibodies were obtained from eBioscience. Anti-PE- and anti-FITC microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) were used for MACS cell purification. mAb to IFN- γ (R4-6A2) purified from the ascites fluid of hybridoma-injected athymic nude mice was used for *in vivo* treatment. CGN type IV (Wako, Osaka, Japan) was used for macrophage blockade.

Purification of cells and adoptive transfer

Spleens were aseptically removed from mice and prepared as single-cell suspensions. RBC were lysed with NH₄Cl, and the cells

were washed twice in fresh medium. CD4⁺ or CD8⁺ T cells were purified with positive selection using a MACS cell separation system (Miltenyi Biotech) according to the manufacturer's protocols; in some experiments negative and positive selection were done. The purity of the separated cells was usually >95%. Eight million purified cells were adoptively transferred i.v. to syngeneic recipients that had been irradiated with 5.5 Gy of γ -radiation (GammaCell) or to RAG2 KO mice.

Determination of antibodies specific for PyL

Serum antibodies specific for PyL were measured by ELISA. The 96-well microtiter plates were coated with 50 μ L PyL antigen solution (5 μ g/mL). Diluted serum samples were added to the wells, and then incubated at room temperature for 2 h. PyL-specific antibodies were detected by horseradish peroxidase-conjugated anti-mouse IgG (Zymed, San Francisco, CA, USA). Enzymatic activity was visualized using a substrate. Absorbance at 415 nm was measured using a spectrophotometer.

Flow cytometry

Spleen cell suspensions were stained with combinations of fluorochrome-labeled antibodies. For intracellular staining, cells were stimulated with 50 ng/mL PMA and 1 μ g/mL calcium ionophore in the presence of 1 μ g/mL brefeldin A, in complete medium at 37°C for 5 h. These cells were then incubated with anti-CD16/32 (Fc-block) and stained with surface markers, followed by fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin. Then, the cells were stained with second antibodies. Stained cells were analyzed by a FACS Calibur cytometer (Becton Dickinson, San Jose, CA, USA), and the data were analyzed using CellQuest Pro software (Becton Dickinson).

In vivo neutralization of IFN- γ and macrophage inhibition

To neutralize IFN- γ , mice received i.p. injection with 500 μ g of anti-IFN- γ antibody 1 day prior to infection, and every 2 wk thereafter. To impair macrophages [22], mice were injected i.p. with 1 mg CGN on 2, 5, 8, 11 and 14 days after infection.

Macrophage uptake assay

The macrophage phagocytosis assay was a modification of a previous method [26]. To stain pRBC or normal RBC, cells (10⁷ cells/mL) were incubated with 10 μ M CFSE (Molecular Probes, CA, USA) in PBS for 15 min at 37°C. CFSE staining was stopped by adding excess complete medium and washing cells three times with medium. CD8⁺ T cells from immune WT or immune IFN- γ KO mice were transferred to the recipients. Splenic CD11b macrophages

were then removed from mice at day 7 post PyL infection for a macrophage uptake assay. Splenic CD11b⁺ cells were purified with positive selection using a MACS cell separation system (Miltenyi Biotech) according to the manufacturer's protocols. Splenic CD11b macrophages (10⁶ cells/well) were seeded with CFSE-labeled pRBC or normal RBC in a 1:20 ratio, at a final volume of 200 μ L for 4 h at 37°C. Following co-culture, non-ingested RBC were removed by lysis with NH₄Cl lysing buffer, and the remaining macrophages were washed twice with PBS, FcR-blocked and then stained with PE-labeled anti-CD11b mAb, in sorting buffer consisting of PBS with 1% FBS and 0.05% sodium azide (Sigma-Aldrich, St. Louis, MO, USA). The capacity of macrophages to uptake CFSE-labeled pRBC or normal RBC was analyzed by a FACS Calibur cytometer (Becton Dickinson), and the data were analyzed using CellQuest Pro software (Becton Dickinson).

Statistical analysis

Statistical evaluation of differences between experimental groups was done by analysis of variance and two-tailed unpaired Student's *t*-tests; *p*<0.05 was considered statistically significant

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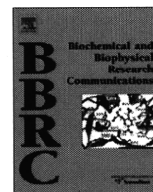
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Abbreviations: CGN: carrageenan · PFN: perforin · pRBC: parasitized RBC · PyNL: *P. yoelii* 17XNL

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Development of experimental cerebral malaria is independent of IL-23 and IL-17

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ABSTRACT

Cerebral malaria (CM) is the most severe complication of *Plasmodium* infection. Although inappropriate immune responses to *Plasmodium falciparum* are reported as the major causes of CM, the precise mechanisms for development remain unclear. IL-23 and IL-17 have critical roles in the onset of autoimmunity and inflammatory diseases triggered by microbial infections. Thus, we investigated the influence of IL-23 and IL-17 on experimental CM (ECM) using *Plasmodium berghei* ANKA infection of C57BL/6 mice. Both IL-23 deficient mice and wild-type (WT) mice developed ECM. IL-17 deficient mice also developed ECM, while IL-17 producing cells other than CD4⁺ T cells (Th17) were increased in WT mice that developed ECM. In conclusion, this study showed that IL-23 and IL-17 are not involved in ECM development.

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1. Introduction

Malaria is a major life-threatening parasitic disease. Each year, 500 million cases and 2 million deaths are reported and 40% of the world's population is at risk of malaria infection [1]. Cerebral malaria (CM) associated with *Plasmodium falciparum* infection is responsible for almost all malaria deaths and is characterized by impaired consciousness/coma and generalized convulsions [2,3]. Approximately 1% of *P. falciparum* infected patients develop CM. The majority of these cases occur in young children in sub-Saharan Africa, of which, 10–20% are fatal and the remaining survivors acquire permanent neurological damage [4,5]. Histologically, multifocal capillary obstruction with parasitized red blood cells (RBC) and leukocytes is observed in brain tissue from patients that die of CM [6] and suggests *P. falciparum* infection may trigger vascular and immune system dysfunction. However, the precise mechanisms causing CM are not fully understood.

After the discovery of CD4⁺ T cells producing IL-17 (Th17), a new subset of effector T helper cells, the immune responses involv-

ing IL-23 and IL-17 have been investigated in detail. IL-23 is a heterodimeric cytokine comprised of a p40 subunit and a unique p19 subunit [7]. Recently, it was revealed that IL-23 is critical for the development of Th17 [8] and for immune cell activation [9,10]. IL-17 is a proinflammatory cytokine secreted by immune cells and mediates granulopoiesis, infiltration of neutrophils and recruitment of T cells into peripheral tissues via the induction of chemokine and cytokine expression [11,12].

Many studies using *IL-23 p19*^{-/-} mice (P19KO) and *IL-17*^{-/-} mice (17KO) suggest that IL-23 and IL-17 play a critical role in the onset of autoimmune diseases, such as experimental autoimmune encephalitis, collagen induced arthritis and inflammatory bowel diseases [7,13–15]. Furthermore, as both cytokines contribute to the development of arthritis caused by *Borrelia burgdorferi* infection [16] and brain damage caused by *Toxoplasma gondii* infection [17], IL-23 and IL-17 may be associated with the immunopathology triggered by microorganism infection.

Infection of susceptible mouse strains with *Plasmodium berghei* ANKA (PbA) allows for the development of experimental CM (ECM) that shares some characteristics with CM. Immune cells, including T cells, antigen-presenting cells (APC), NK cells and neutrophils, play critical roles in the development of ECM [18–21]. During infection, these cells activate and cooperate with each other to allow T cell migration into brain. This results in blood–brain barrier (BBB) disruption and is a key pathological feature of ECM. IL-23 is

Abbreviations: IL-23, Interleukin-23; IL-17, Interleukin-17; RBC, red blood cell; Th, T helper cells; NK cells, natural killer cells; Treg, regulatory T cell.

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strongly associated with activation of APC and NK cells and also with development of Th17 that is capable BBB disruption [8–10]. IL-17 is involved in the activation and recruitment of neutrophils [11] and upregulation of CXCL 9, 10, and 11, which are ligands of CXCR3 essential for the migration of CD8⁺ T cells into brain [12,22]. Given these findings, it is possible that IL-23 and/or IL-17 may contribute to development of ECM.

We therefore examined the role of IL-23 and IL-17 in ECM development using P19KO and 17KO mice. Our results demonstrated that P19KO mice developed ECM similarly to wild-type (WT) mice. 17KO mice also developed ECM while IL-17-producing cells other than CD4⁺ T cells increased in WT mice during ECM. Thus, our results conclude that IL-23 and IL-17 may not be involved in ECM development.

2. Materials and methods

2.1. Mice and parasites

C57BL/6 mice were purchased from Kyudo (Tosu, Japan). Age and sex-matched groups of WT mice, P19KO mice and 17KO mice were used for experiments. All experiments that involved mice were reviewed by the Committee for Ethics on Animal Experiments in the Faculty of Medicine and were conducted under the control of the Guidelines for Animal Experiments in the Faculty of Medicine, Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government. Blood-stage parasites of *P. berghei* ANKA (PbA) were a generous gift from Dr. M. Torii (Ehime University), obtained after fresh passage through a donor mouse, 2–3 days after inoculation with frozen stock. Mice were infected with 50,000 parasitized RBC (pRBC) via intraperitoneal injection.

2.2. Determination of parasitemia

Blood samples were collected from the tail vein of experimental mice at the time indicated. Thin blood films were prepared and fixed with methanol before being stained with Giemsa solution (Sigma–Aldrich, St. Louis, MO, USA). Parasitemia was determined by counting the percentage of infected RBC (pRBC) using light microscopy.

2.3. Preparation of brain-sequestered leukocytes (BSL) and spleen cells

BSL were prepared according to the previously described methods [23,24]. Briefly, sacrificed mice were intracardially perfused with PBS to remove both circulating, nonadherent RBC and leukocytes from the brain. The brain was then removed and crushed in RPMI1640 supplemented with 100 IU/mL penicillin, 100 mg/mL streptomycin, 20 mM HEPES, 2 mM L-glutamine, 100 mM 2-mercaptoethanol and 10% inactivated fetal bovine serum (complete medium). The tissue suspension was centrifuged at 400 g for 5 min and the pellet was resuspended with HEPES buffer, supplemented with 0.05% collagenase (Roche Applied Science, Indianapolis, IN, USA) and 2 U of DNase (Sigma–Aldrich, St. Louis, MO, USA)/ml. The mixture was stirred at room temperature for 60 min, then passed through a sterile gauze and centrifuged at 80 g for 30 s to remove debris. The supernatant was deposited on a 30% Percoll (Sigma–Aldrich, St. Louis, MO, USA) to remove brain cells and centrifuged at 1400 g for 10 min. The pellet was resuspended with medium to form single-cell suspensions. Residual RBC in single-cell suspensions prepared from the brain and spleen were lysed with NH₄Cl. Cells were then washed twice with fresh medium before being used for experiments. CD4⁺ cells were purified with positive selection using a MACS cell separation system (Miltenyi Biotec) according to the manufacturer's protocols. Separated cell purity was generally >95%.

2.4. Real-time RT-PCR

Total RNA was extracted from BSL, spleen cells and CD4⁺ cells before being reverse-transcribed to cDNA. mRNA that encoded genes of interest was quantified from cDNA by evaluating SYBR Green dye incorporation (Takara, Tokyo, Japan) using a real-time PCR system GeneAmp7000 thermal cycler (Applied Biosystems, Foster City, CA, USA). PCR was performed according to the manufacturer's protocols. Expression levels of target genes were determined as the difference of Ct between the β-actin-encoding gene and target gene using the following formula: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{reference gene}}$. PCR primers used were as follows: for IL-23p19, 5'-TGGCTGTG CCTAGGAGTAGCA-3' and 5'-TTCATCTCTTCTCTTAGTAGATTC ATA-3'; for IL-17, 5'-TCATCTGTGTCTCTGATGCTGTG-3' and 5'-TCG CTGCCTTCACTGT-3'; for RORγt, 5'-AGCAGTGAATGTGGCCTAC-3' and 5'-GCACTTCTGCATGTAGACTG-3'; and for β-actin, 5'-TGGAACTCTGTGGCATCCATGAAAC-3' and 5'-TAAAACGCAGCTCAGTAACAG TCCG-3'.

2.5. Flow cytometry

BSL and spleen cells were stained with a combination of fluorochrome-labeled antibodies. For intracellular staining, cells were stimulated with 50 ng/ml PMA and 500 ng/ml calcium ionophore in the presence of Golgi plug (BD Bioscience, San Jose, CA, USA), in complete medium at 37 °C for 4 h. Cells were then incubated with anti-CD16/32 (Fc-block) and stained with FITC-anti-CD4 (eBioscience, San Diego, CA, USA) followed by fixation/permeabilization with BD cytofix/cytoperm (BD Bioscience, San Jose, CA, USA) according to manufacturer's protocols. Cells were stained with PE-anti-IL-17 or PE-anti-IFN-γ (BD Bioscience, San Jose, CA, USA) and analyzed using a FACS Caliber cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed using CellQuest Pro software (Becton Dickinson).

2.6. Histology

For histological analysis of cerebral pathology, brains from mice developing ECM were perfused with PBS and carefully removed and fixed in formaldehyde solution (4 v/v%). The 5-μm tissue sections were prepared and stained with hematoxylin and eosin (HE).

2.7. Statistical analysis

Statistical differences between experimental groups were evaluated using a two-tailed unpaired Student's *t*-test. Survival analysis was determined using Kaplan Meier survival analysis. *P* < 0.05 was considered statistically significant.

3. Results and discussion

3.1. IL-23 is not involved in the onset of ECM

C57BL/6 mice infected with PbA developed ECM that was characterized by neurological manifestations, such as ataxia, paralysis (monoplegia, hemiplegia and tetraplegia) and coma as early as 7 or 8 days after infection, and death occurred between 7 and 10 days after infection [25]. We first investigated the expression of IL-23 during ECM to assess the involvement of IL-23. BSL and spleen cells were collected and p19 mRNA expression was analyzed by quantitative RT-PCR. No significant difference in the expression of p19 mRNA was observed between mice developing ECM and uninfected control mice (Fig. 1A and B). Thus, PbA infection of mice did not induce IL-23 in both tissues sampled. Furthermore, to directly investigate the contribution of IL-23 to the

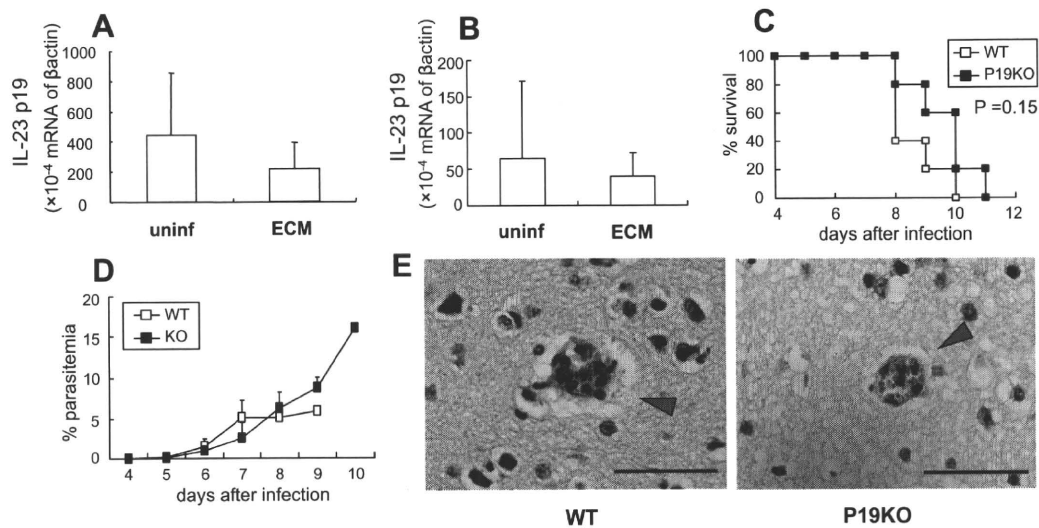


Fig. 1. ECM induced by PbA infection was independent of IL-23. mRNA encoding IL-23p19 in BSL (A) and spleen cells (B) isolated from mice developing ECM was quantified using RT-PCR. Values are relative to amounts of mRNA encoding β -actin. Data represent the mean \pm SD of four mice. Survival rates (C) or parasitemia (D) of WT and P19KO mice infected with 50,000 PbA-pRBC were monitored. Each group is comprised of five mice. One representative of at least two repeated experiments is shown. Brains from WT (left panel) and P19KO mice (right panel) developing ECM were analyzed for histological examination (E). Representative histological sections from areas around the blood vessels of cerebrum with HE staining are shown (original magnification, $\times 1000$). Arrowheads indicate accumulation of mononuclear cells and erythrocytes. Scale bars equal 50 μ m.

development of ECM, P19KO mice were infected with PbA. Infected P19KO as well as WT mice showed severe neurological symptoms and died within 11 days when the parasitemia was lower than 10% (Fig. 1C, D), suggesting that P19KO mice developed ECM. To confirm development of ECM in these mutants, histological analyses of brains isolated from P19KO mice developing ECM were performed. Both P19KO and WT mice showed cerebral blood vessels sequestered with mononuclear cells and erythrocytes, a typical sign of ECM induced by PbA (Fig. 1E). No sequestered vessel was observed before infection (data not shown). These results clearly demonstrated that the development of ECM does not require IL-23.

3.2. IL-17 does not contribute to ECM development

To assess whether IL-17 contributes to ECM, we analyzed IL-17 producing cells using flow cytometry. IL-17 producing cells were increased in the brain and spleen of mice developing CM when compared to uninfected control mice (Fig. 2A and C). The emergence of Th17 during ECM was also investigated as an assessment of IL-17 production. Mice that developed ECM showed a higher frequency and absolute number of CD4⁺ IFN- γ ⁺ Th1 cells (IFN- γ ⁺ CD4⁺ cells (Th1)) in the brain and spleen when compared to uninfected control mice (Fig. 2B and C), which supported previous study [26]. By contrast, there were no differences in the frequency and absolute number of Th17 between mice that developed ECM and uninfected control mice (Fig. 2A and C). Furthermore, we analyzed the expression of mRNAs encoding IL-17 and a “master-regulator” transcription factor of Th17, ROR γ t, in CD4⁺ cells purified from BSL and spleen cells. Mice that developed ECM showed lower mRNA expression levels compared to uninfected mice (Fig. 3A and B). These results demonstrate that IL-17-producing cells other than Th17 developed during ECM. Finally, to directly investigate the contribution of IL-17 to onset of ECM, 17KO mice were infected with PbA. Both infected 17KO mice and infected WT mice developed neurological symptoms and died (Fig. 4A) and produced no differences in parasitemia kinetics (Fig. 4B). Histological examination of brain sections revealed that 17KO as well as WT mice showed leukocyte-packed vessels (Fig. 4C). These results demonstrate that the development of ECM is independent of IL-17.

3.3. General discussion

As observed during our study, infection with PbA did not induce the expression of IL-23 and a lack of IL-23 did not affect the development of ECM. Moreover, IL-17 deficient mice also develop ECM, while IL-17 producing cells increased in WT mice affected with ECM. These results suggest that both cytokines are unnecessary for the development of ECM.

Th17 development was not observed during infection with PbA primarily because IL-23, which is critical for Th17 expansion, was not secreted. We confirmed that expression of mRNA encoding IL-17 or ROR γ t in CD4⁺ T cells decreased in mice that developed ECM and that the frequency and cell number of Th17 were not increased. In addition, environments under malaria infection favor to induce Treg [29,30], consequently might disturb Th17 differentiation as a counter-regulatory action [31]. By contrast, we observed that the frequency of Th1 was increased in WT mice that developed ECM (Supplementary Fig. 1). Expression of mRNA encoding T-bet or IFN- γ in CD4⁺ T cells from mice that developed ECM were higher than those from uninfected mice (data not shown). These findings suggested that Th1 rather than Th17 may be of importance in ECM development and supports previous suggestions that Th1 are dominant during ECM [26].

IL-17 is known to suppress pathogenic Th1 responses during inflammatory bowel disease [27,28], suggesting that augmented induction of Th1 covers or compensates lack of IL-17-mediated pathogenesis in ECM development. However, there was no difference between WT and 17KO mice in Th1 induced by infection with PbA (Supplementary Fig. 1). Given these results, it appears that IL-17 does not suppress Th1 responses during ECM and thus does not contribute to ECM development.

In addition to the ECM produced by PbA infection, hepatic injury observed in mice infected with *P. berghei* NK65 (PbNK) is also reported to be immunopathological [1,32]. Our preliminary results revealed that P19KO mice or 17KO mice infected with PbNK displayed elevated serum levels of enzymes released from the damaged hepatocytes, as was the case for WT mice (data not shown). Thus, these cytokines do not appear to be involved in ECM development or liver injury by infection with PbNK. Immunopathologies