European Journal of Immunology

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

Takashi Imai[1,2], Jianying Shen[1], Bin Chou[1], Xuefeng Duan[1], Liping Tu[1], Kohhei Tetsutani[1], Chikako Moriya[1], Hidekazu Ishida[1], Shinjiro Hamano[1], Chikako Shimokawa[1], Hajime Hisaeda[1] and Kunisuke Himeno[1]

[1] Department of Microbiology and Immunology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan
[2] Laboratory for Immunochaperones, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan

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]). 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.

Correspondence: Dr. Takashi Imai
e-mail: t-imai@rcai.riken.jp

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 microvasculor 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

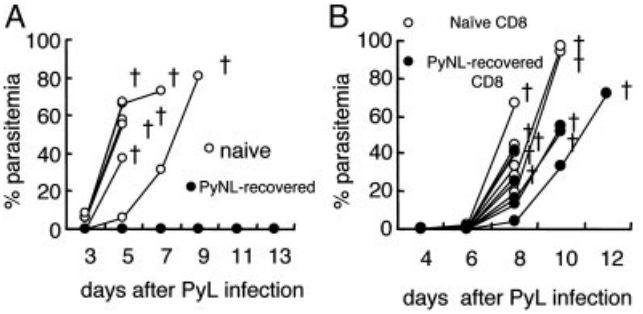

Figure 1. $CD8^+$ T cells from PyNL-recovered mice failed to transfer protection against PyL. (A) The kinetics of parasitemia in PyNL-recovered (closed circles) or naive (open circles) mice were monitored after infection with 25 000 PyL-pRBC. (B) $CD8^+$ T cells (8×10^6) purified from PyNL-recovered (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

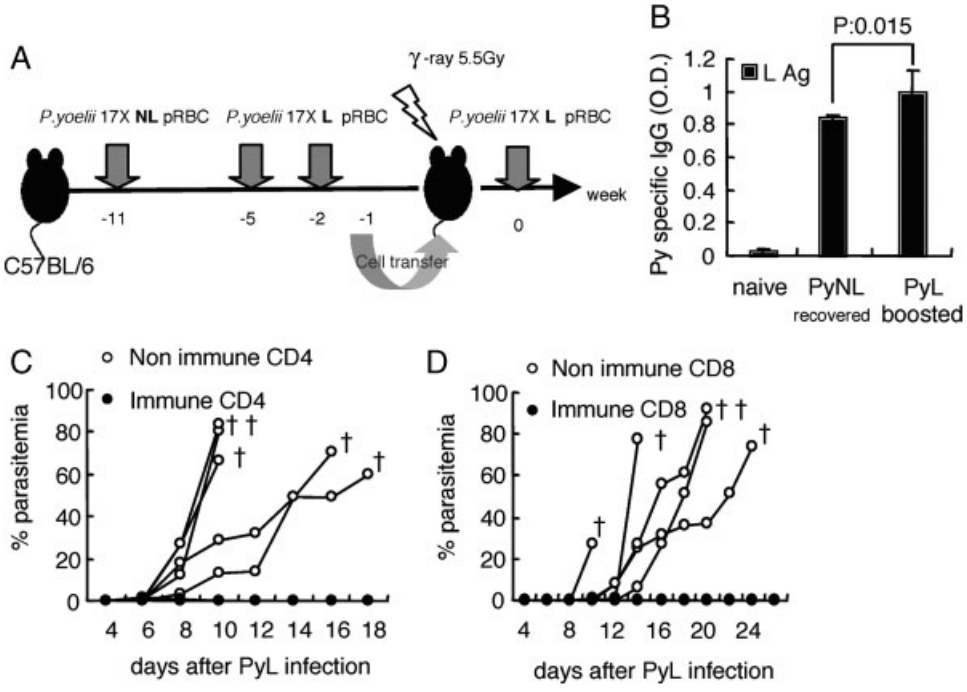

Figure 2. $CD8^+$ T cells from highly immunized mice transferred protection against PyL. (A) Protocol of immunization, cell transfer and challenge infection. (B) Antibody titers specific for malaria parasites in sera collected from mice were measured. Data are the means+SD of OD_{415} values of 200-fold-diluted 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

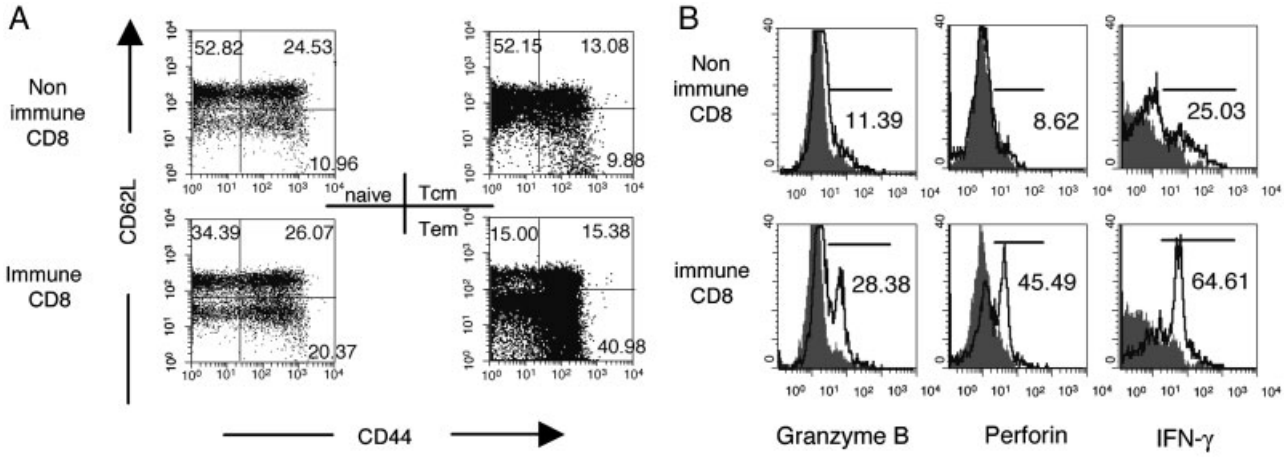

Figure 3. Immunophenotype and expression of effector molecules of protective CD8$^+$ T cells. (A) Spleen cells from recipient mice transferred with the indicated cells were analyzed 7 days after infection with PyL (right panels). Cells from the recipients left uninfected were also analyzed (left panels). 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

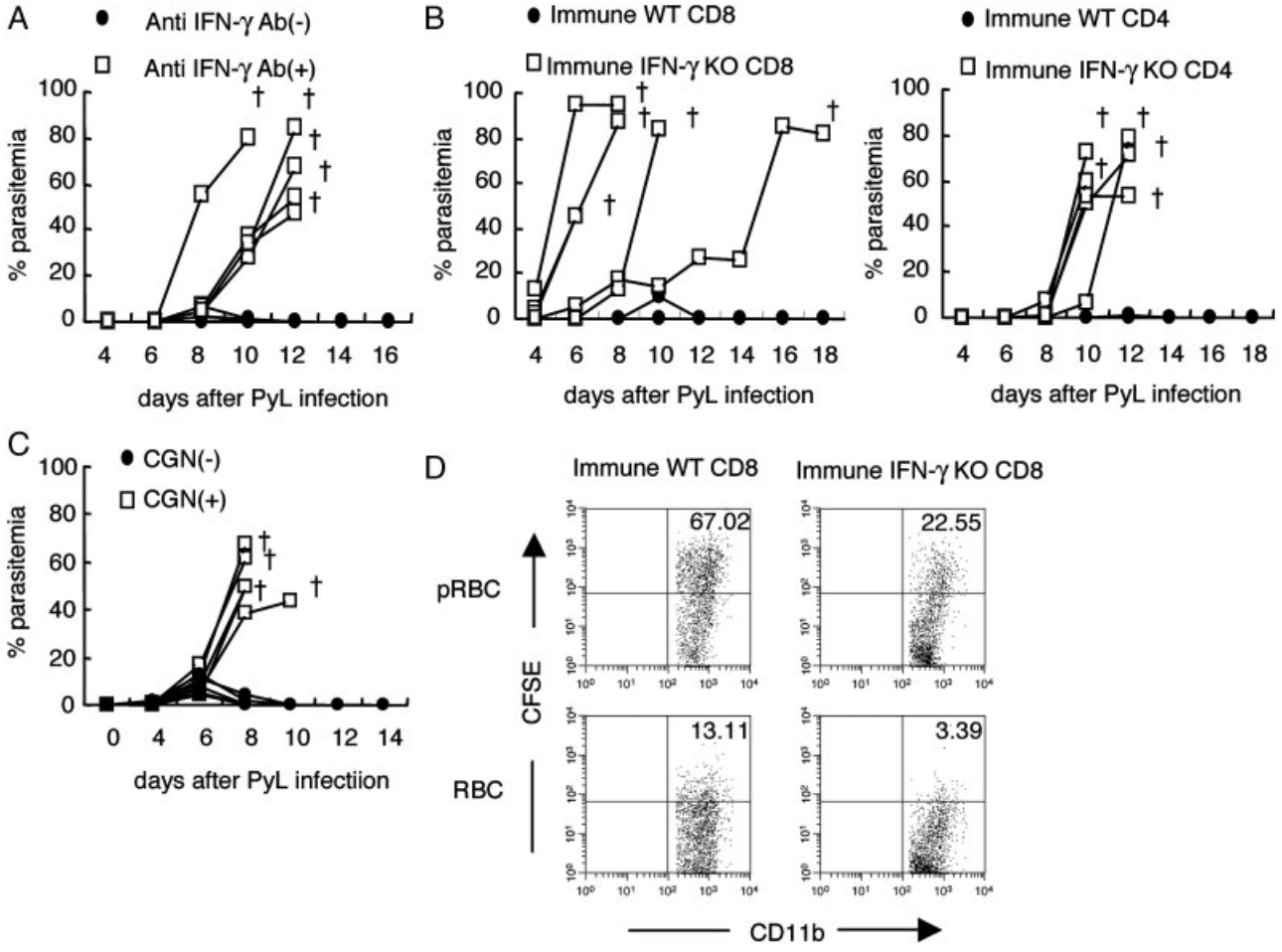

Figure 4. IFN-γ from $CD8^+$ T cells was essential for protection against blood-stage malaria. (A) Parasitemia in recipients transferred with immune $CD8^+$ T cells treated with anti-IFN-γ (open squares) or irrelevant antibodies (closed circles) was evaluated as in Fig. 1B. (B) $CD8^+$ (left panel) or $CD4^+$ (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 potently 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

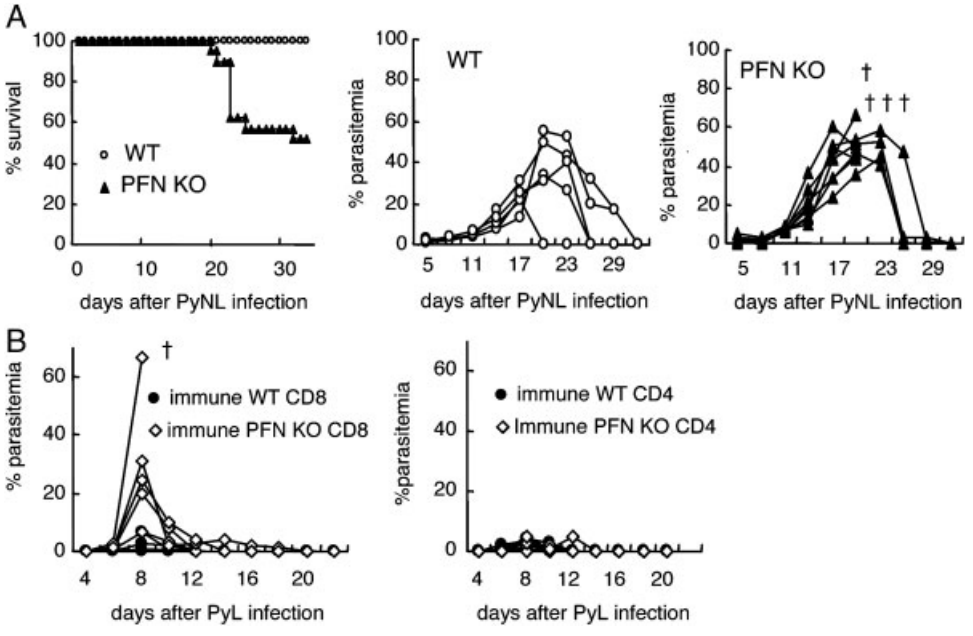

Figure 5. Molecules associated with CTL activity partially contributed to protection against blood-stage malaria. (A) Survival rates and parasitemia in PFN KO (closed triangles) mice were monitored after infection with 25 000 PyNL-pRBC. (B) The recipient mice transferred with CD8$^+$ or CD4$^+$ T cells 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. Nakauchi (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 NH_4Cl, 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 μL PyL antigen solution (5 μg/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 μg/mL calcium ionophore in the presence of 1 μg/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 μg 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 (10^7 cells/mL) were incubated with 10 μM 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 an MACS cell separation system (Miltenyi Biotech) according to the manufacturer's protocols. Splenic CD11b macrophages (10^6 cells/well) were seeded with CFSE-labeled pRBC or normal RBC in a 1:20 ratio, at a final volume of 200 μL for 4 h at 37°C. Following co-culture, non-ingested RBC were removed by lysis with NH_4Cl 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

Acknowledgements: This work was supported by grants from the Ministry of Education, Science Sport and Culture of Japan to K.H. (18015040, 19590429), to H.H. (18590400, 19041056), and from The Uehara Memorial Foundation to H.H.

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

References

1 **WHO World malaria report.** 2005. http://rbm.who.int/wmr.

2 **Hafalla, J. C., Cockburn, I. A. and Zavala, F.,** Protective and pathogenic roles of CD8+T cells during malaria infection. *Parasite Immunol.* 2006. **28**: 15–24.

3 **Wizel, B., Houghten, R. A., Parker, K. C., Coligan, J. E., Church, P., Gordon, D. M., Ballou, W. R. and Hoffman, S. L.,** Irradiated sporozoite vaccine induces HLA-B8-restricted cytotoxic T lymphocyte responses against two overlapping epitopes of the Plasmodium falciparum sporozoite surface protein 2. *J. Exp. Med.* 1995. **182**: 1435–1445.

4 **Marsh, K. and Kinyanjui, S.,** Immune effector mechanisms in malaria. *Parasite Immunol.* 2006. **28**: 51–60.

5 **Good, M. F. and Doolan, D. L.,** Immune effector mechanisms in malaria. *Curr. Opin. Immunol.* 1999. **11**: 412–419.

6 **Herrera, S., Herrera, M. A., Corredor, A., Rosero, F., Clavijo, C. and Guerrero, R.,** Failure of a synthetic vaccine to protect Aotus lemurinus against asexual blood stages of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* 1992. **47**: 682–690.

7 **Vinetz, J. M., Kumar, S., Good, M. F., Fowlkes, B. J., Berzofsky, J. A. and Miller, L. H.,** Adoptive transfer of CD8+T cells from immune animals does

not transfer immunity to blood stage *Plasmodium yoelii* malaria. *J. Immunol.* 1990. **144**: 1069–1074.

8 **Jayawardena, A. N., Murphy, D. B., Janeway, C. A. and Gershon, R. K.,** T cell-mediated immunity in malaria I The Ly phenotype of T cells mediating resistance to *Plasmodium yoelii*. *J. Immunol.* 1982. **129**: 377–381.

9 **Suss, G., Eichmann, K., Kury, E., Linke, A. and Langhorne, J.,** Roles of CD4- and CD8-bearing T lymphocytes in the immune response to the erythrocytic stages of *Plasmodium chabaudi*. *Infect. Immun.* 1988. **56**: 3081–3088.

10 **van der Heyde, H. C., Manning, D. D., Roopenian, D. C. and Weidanz, W. P.,** Resolution of blood-stage malarial infections in CD8+cell-deficient beta 2-m0/0 mice. *J. Immunol.* 1993. **151**: 3187–3191.

11 **Sinigaglia, F., Richard, J. and Pink, L.,** Human T lymphocyte clones specific for malaria (Plasmodium falciparum) antigens. *EMBO J.* 1985. **4**: 3819–3822.

12 **Pombo, D. J., Lawrence, G., Hirunpetcharat, C., Rzepczyk, C., Bryden, M., Cloonan, N., Anderson, K. *et al.*,** Immunity to malaria after administration of ultra-low doses of red cells infected with Plasmodium falciparum. *Lancet* 2002. **360**: 610–617.

13 **Belnoue, E., Kayibanda, M., Vigario, A. M., Deschemin, J. C., van Rooijen, N., Viguier, M., Snounou, G. and Renia, L.,** On the pathogenic role of brain-sequestered alphabeta CD8+T cells in experimental cerebral malaria. *J. Immunol.* 2002. **169**: 6369–6375.

14 **Nitcheu, J., Bonduelle, O., Combadiere, C., Tefit, M., Seilhean, D., Mazier, D. and Combadiere, B.,** Perforin-dependent brain-infiltrating cytotoxic CD8+T lymphocytes mediate experimental cerebral malaria pathogenesis. *J. Immunol.* 2003. **170**: 2221–2228.

15 **Lundie, R. J., de Koning-Ward, T. F., Davey, G. M., Nie, C. Q., Hansen, D. S., Lau, L. S., Mintern, J. D. *et al.*,** Blood-stage Plasmodium infection induces CD8+T lymphocytes to parasite-expressed antigens, largely regulated by CD8alpha+dendritic cells. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 14509–14514.

16 **Langhorne, J.,** The immune response to the blood stages of Plasmodium in animal models. *Immunol. Lett.* 1994. **41**: 99–102.

17 **Schneider, J., Gilbert, S. C., Hannan, C. M., Degano, P., Prieur, E., Sheu, E. G., Plebanski, M. and Hill, A. V.,** Induction of CD8+T cells using heterologous prime-boost immunisation strategies. *Immunol. Rev.* 1999. **170**: 29–38.

18 **Williams, M. A. and Bevan, M. J.,** Effector and memory CTL differentiation. *Annu. Rev. Immunol.* 2007. **25**: 171–192.

19 **Krishnan, L., Gurnani, K., Dicaire, C. J., van Faassen, H., Zafer, A., Kirschning, C. J., Sad, S. and Sprott, G. D.,** Rapid clonal expansion and prolonged maintenance of memory CD8+T cells of the effector (CD44highCD62Llow) and central (CD44highCD62Lhigh) phenotype by an archaeosome adjuvant independent of TLR2. *J. Immunol.* 2007. **178**: 2396–2406.

20 **Su, Z. and Stevenson, M. M.,** Central role of endogenous gamma interferon in protective immunity against blood-stage Plasmodium chabaudi AS infection. *Infect. Immun.* 2000. **68**: 4399–4406.

21 **Shear, H. L., Nussenzweig, R. S. and Bianco, C.,** Immune phagocytosis in murine malaria. *J. Exp. Med.* 1979. **149**: 1288–1298.

22 **Zhang, M., Hisaeda, H., Sakai, T., Ishikawa, H., Hao, Y. P., Nakano, Y., Ito, Y. and Himeno, K.,** Macrophages expressing heat-shock protein 65 play an essential role in protection of mice infected with *Plasmodium yoelii*. *Immunology* 1999. **97**: 611–615.

23 **Brown, I. E., Blank, C., Kline, J., Kacha, A. K. and Gajewski, T. F.,** Homeostatic proliferation as an isolated variable reverses CD8+T cell anergy and promotes tumor rejection. *J. Immunol.* 2006. **177**: 4521–4529.

24 **Ren, H., Shen, J., Tomiyama-Miyaji, C., Watanabe, M., Kainuma, E., Inoue, M., Kuwano, Y. and Abo, T.,** Augmentation of innate immunity by low-dose irradiation. *Cell Immunol.* 2006. **244**: 50–56.

25 **Grundy, M. A. and Sentman, C. L.,** Immunodeficient mice have elevated numbers of NK cells in non-lymphoid tissues. *Exp. Cell Res.* 2006. **312**: 3920–3926.

26 **Ing, R., Segura, M., Thawani, N., Tam, M. and Stevenson, M. M.,** Interaction of mouse dendritic cells and malaria-infected erythrocytes: uptake, maturation, and antigen presentation. *J. Immunol.* 2006. **176**: 441–450.

27 **Cannon, G. J. and Swanson, J. A.,** The macrophage capacity for phagocytosis. *J. Cell Sci.* 1992. **101**: 907–913.

28 **Beattie, L., Engwerda, C. R., Wykes, M. and Good, M. F.,** CD8+ T lymphocyte-mediated loss of marginal metallophilic macrophages following infection with Plasmodium chabaudi chabaudi AS. *J. Immunol.* 2006. **177**: 2518–2526.

29 **Pouniotis, D. S., Proudfoot, O., Bogdanoska, V., Scalzo, K., Kovacevic, S., Coppel, R. L. and Plebanski, M.,** Selectively impaired CD8+but not CD4+ T cell cycle arrest during priming as a consequence of dendritic cell interaction with plasmodium-infected red cells. *J. Immunol.* 2005. **175**: 3525–3533.

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

Full correspondence: Dr. Takashi Imai, Laboratory for Immunochaperones, RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan
Fax:+81-92-642-6118
e-mail: t-imai@rcai.riken.jp

Received: 16/4/2009
Revised: 30/11/2009
Accepted: 7/1/2010
Accepted article online: 25/1/2010

