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Original Article

Resistance and Susceptibility to Malarial Infection: A Host Defense Strategy against Malaria

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Abstract

Background: In an effort to understand what limits the virulence of malaria parasites in relation to the host genetic and immunogenic background, we investigated the possibility that the parasite and host genotype crossover interactions constrain virulence.

Methods: Two groups of mice from different genotypes were used (C57BL/6 (B6) and DBA/2 mice). The mice were infected with a virulent parasite line *Plasmodium yoelii 17XL* (*P. yoelii 17XL*). Parasitemia, hematocrit value and lymphocytes yielded by livers and spleens were evaluated. Fluorescence Activated Cell Sorting (FACS) analysis illustrated phenotypic characterization of lymphocytes.

Results: Infection with *P. yoelii 17XL* did not result in the death of DBA/2 mice. In contrast, B6 mice developed significantly high parasitemia and succumbed to death. Using (FACS) analysis, DBA/2 mice were found to experience a marked expansion of interleukin (IL)-2Rβ^{+} CD3^{int} cells and γδ T cells in the liver, especially in the recovery phase. The expansion of unconventional T cells (i.e. B220^{+} T cells) was also marked in DBA/2 mice.

Conclusion: The outcome of murine malaria infections depends on the dynamic interplay between the immune-mediator and the genotype of the host.

Introduction

The genetic and immune-regulatory mechanisms that underlie malaria infections of man or experimental animals are extremely complex. Genetic studies have been undertaken to analyze the complexity and to map the individual genes that are responsible for inter-strain differences in susceptibility (1, 2). Host and parasite genetic factors are known to influence the outcome of malaria infections in experimental animals (3-5).

At the level of immune responses, however, little is known about functionally relevant subphenotypes that distinguish resistant and sus-

ceptible strains. Both cellular and humoral immunity operate to control malaria infections. The mechanisms whereby cells and molecules of the immune system function in immunity to malaria and the pathogenesis of this disease are ill defined. DBA/2 (H-2^d) mice are known to be more resistant than B6 (H-2^b) mice to the non-lethal *17XNL* strain of *P. yoelii* (6, 7). This is an amazing phenomenon because the functions of conventional T cells [thymus-derived T cells or T-cell receptor TCR^{high} cells], especially $CD8^+$T cells, are known to be somewhat lower in DBA/2 mice than in other strains of mice (8). Extrathymic T cells mediate resistance to malarial infection in the liver, but not by conventional T cells in the peripheral organs (9-12). The TCR and interleukin-2 receptor β (IL-2Rβ) phenotype of such extrathymic T cells is TCR^{int} IL-$2R\beta^+$ (13, 14). In contrast, the phenotype of conventional T cells derived from the thymus is TCR^{high} IL-$2R\beta^-$. Athymic nude mice carry only TCR^{int} IL-$2R\beta^+$ cells and lack TCR^{high} IL-$2R\beta^-$ cells (11), the former cells showing potential for protection of athymic nude mice from malarial infection (11). During malaria infection, a great expansion of TCR intermediate cells was detected in the liver of these mice.

In the light of these findings, we aimed to demonstrate how the immune system and the host genotype modulate themselves during malaria infection by comparing the outcome of two groups of mice from different genotype infected with virulent parasite line (*P. yoelii* lethal strain).

Materials and Methods

Mice and parasites

Thirty mice at the age of 8-10 wk were used; the mice were maintained at the animal facility of Niigata University (Niigata, Japan) under specific pathogen-free conditions. Fifteen B6 mice were used (group1) and the other 15 were DBA/2 mice (group 2). B6 mice are common inbred strains of laboratory mice. They are the most widely used models of human disease. B6 mice have a dark brown, nearly black coat. They are more sensitive to noise and odours. MHC Haplotype is H-2^b . DBA/2 mice are the oldest of all inbred strains of mice. DBA/2 mice have a dilute brown; non-agouti coat. They are ideally used for safety and efficacy to test immunology and audiogenic seizues. MHC Haplotype is H-2^d. *P. yoelii 17XL* (lethal strain) were obtained from Niigata University (Niigata, Japan). Parasites were maintained by routine in vivo passages in mice. Mice were infected by intra-peritoneal injection with 10^4 parasitized erythrocytes per mouse (15).

Determination of parasitemia and hematocrit value

Parasitemia was observed by Giemsa-stained thin smears of tail blood every 2 to 3 days throughout the experimental period. The parasitemia was estimated by counting the number of parasitized RBC present in 25 monolayer microscopic fields divided by the total number of erythrocytes present in these fields, and multiplied by 100. The result indicated the percentage of erythrocytes that are infected by malaria parasites. Packed cell volume (PCV) was determined by centrifuging heparinized blood obtained from mice tail in a capillary tube at 10,000 RPM for five minutes. This separates blood into two layers. Volume of packed red blood cells divided by the total volume of blood sample gives PCV. Because a tube was used, PCV was calculated by measuring the lengths of the layers (15).

Preparation of Cells

Mice were killed on the days indicated in the figures after infection. Hepatic mononuclear cells were isolated using a previously described method (16). Briefly, the liver was removed, pressed through 200-gauge stainless steel mesh, and suspended in Eagle's minimal essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 5 mM HEPES and 2% heat-inactivated newborn calf

serum. After washing once with medium, the cells were fractionated by centrifugation in 15 ml of 35% Percoll solution (Pharmacia Fine Chemicals, Piscataway, NJ) for 15 min at 424.8 g. The pellet was resuspended in erythrocyte lysing solution (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA-Na and 170 mM Tris, pH 7.3). The splenocytes were obtained by forcing the spleen through 200-gauge stainless steel mesh. Splenocytes were used after erythrocyte lysing.

Immunofluorescence tests

Fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, peridinin-chlorophyl-protein (PerCP)-, cychrome- or biotin- conjugated monoclonal antibody (mAb) reagents were used. Biotin-conjugated reagents were developed with TRI-COLOR-conjugated streptavidin (Caltag Laboratory, San Francisco, CA) (17). The mAbs used here included anti-CD3 (145-2C11), anti-IL-2Rβ (TM-b1), anti-CD45R/B220 (RA3-6B2), anti-CD4 (PM4-5), anti-CD8 (53-6.7), anti-αβTCR (H57-597), and anti-γδTCR (GL3) mAbs (Pharmingen, San Diego, CA). Cells were analysed by FACScan (Becton-Dickinson Co., Mountain View, CA). To prevent non-specific binding of mAbs, CD16/32 (2.4G2) (Pharmingen) was added before staining with labelled mAb. Dead cells were excluded by forward scatter, side scatter, and propidium iodide gating.

Statistical analysis

Data were analyzed by one factor analysis of variance (ANOVA) or student *t*- test.

Results

Course of P. yoelii 17XL infection in B6 and DBA/2 mice

In both strains, the parasite was detected in circulation on day 3 after infection, parasitemia was very high and the mice succumbed to death between day 7 and day 10 in B6 mice (Fig. 1A). In DBA/2 parasitemia reached a peak on day 15 followed by a sharp reduction in the circulating parasites and all the mice completely recovered between day 20 and day 23 ($P< 0.05$). Hematocrit percentage showed that B6 mice became severely anemic and died probably from anemia (Fig. 1B). However, in DBA/2 mice, there was a clear response to anemia at the peak that rapidly returned to the control level during recovery ($P< 0.05$).

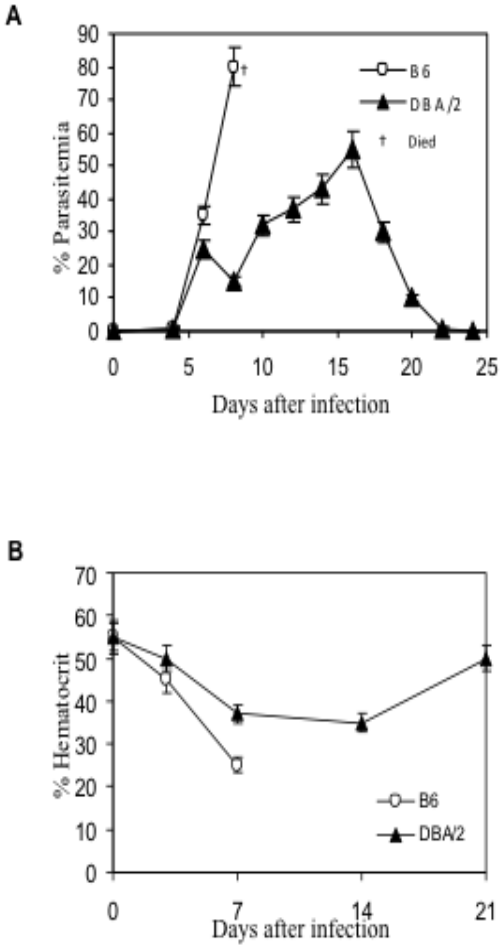

Fig. 1: (A) Time kinetics of parasitemia in B6 and DBA/2 mice during infection with lethal strain *P. yoelii* showing acute phase started on day 3 and severe phase between day 7 and day 10 in B6 mice, and acute phase on day 7 and recovery phase between day 20 and day 23 in DBA/2 mice. (B) Hematocrit percentages during malarial infection showing severe anemia at the severe and peak phase in both B6 and DBA/2 mice consequently ($P< 0.05$)

The number of lymphocytes yielded by liver and spleen during P. yoelii 17XL infection

The number of lymphocytes in the liver and spleen greatly increased throughout stages of infection in both mice strains with marked increase at the recovery phase of DBA/2 mice (Fig. 2) ($P< 0.05$). As shown in Fig. 2, the acute phase occurred on day 3 in all mice, but the severe phase occurred between day 7 and day 10 in B6 mice and the recovery phase occurred between day 20 and 23 in DBA/2 mice.

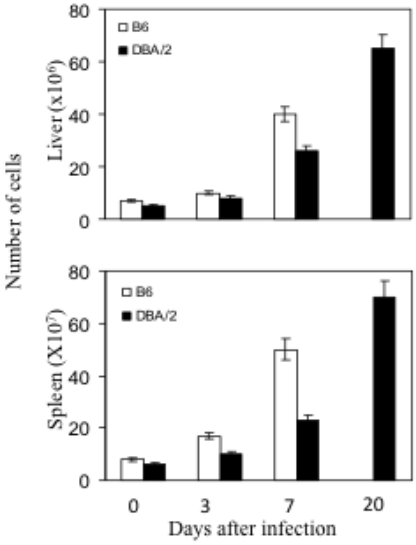

Fig. 2: Number of lymphocytes yielded by the liver and spleen are shown in both B6 and DBA/2 mice during malarial infection ($P< 0.05$)

Expansion of TCR Intermediate (TCR^{int}) cells during malarial infection

We examined what type of lymphocytes expanded in the liver and spleen after malarial infection (Fig. 3). Two colors staining for CD3 and IL-2Rβ was conducted to identify TCR^{int} cells (CD3^{int}IL-2Rβ^{+}) and conventional TCR^{high} cells (CD3^{high}IL-2Rβ^{-}). In the liver of all tested mice, CD3^{int} cells began to expand from acute phase to severe and recovery phase in B6 and DBA/2 mice respectively. However, this response was more prominent in the recovery phase of DBA/2 mice (indicated by arrows). In spleen, the expansion of CD3^{int} cells increased slightly compared to that in the liver (Fig. 3).

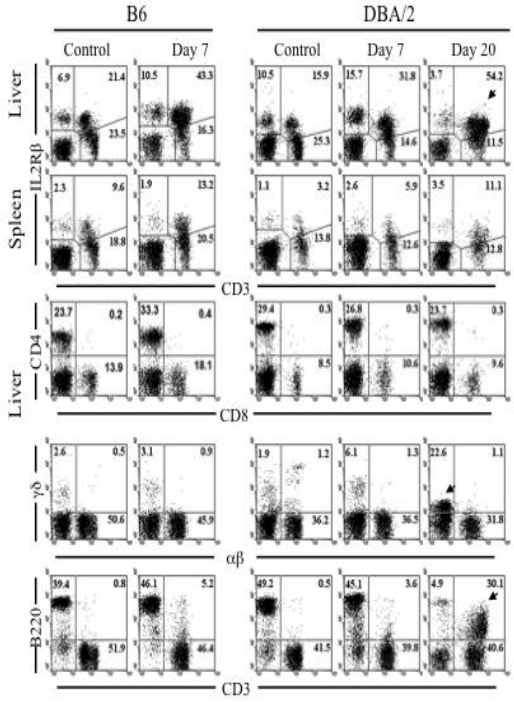

Fig. 3: Identification of interleukin (IL)-2Rβ^{+} CD3^{int} cells, CD4 and CD8 T cells, αβ and γδ T cells and B220^{+} CD3^{+} T cells. Two-color staining for CD3 and IL-2Rβ, for CD4 and CD8, for γδ TCR and αβ TCR and for CD3 and B220 was performed in the liver and spleen. Arrows indicate the expansion of IL-2Rβ^{+} CD3^{int} cells, γδ T cells and B220^{+} CD3^{+}T cells. Numbers in the figure represent the percentages of fluorescence-positive cells in the corresponding areas

The phenotypic pattern of CD4^{+} and CD8^{+} T cells were analyzed by flow cytometry. As shown in Fig. 3, the important observation of this experiment was the detection of double-negative (DN) CD4^{-}CD8^{-}T cells. The total proportion of T cells was greater than the sum proportion of CD4^{+} and CD8^{+} cells in the livers of all tested mice after malarial infection. This result revealed that the majority of extrathymic T cells that appeared after malarial infection were DN CD4^{-} CD8^{-}.

Determination of αβ or γδ type of the expanding T cells during malarial infection

To determine whether αβ or γδ type expanded during malarial infection, two-color staining for αβ TCR and γδ TCR were performed (Fig. 3). The expansion of γδ T cells was only demonstrated in the livers of DBA/2 mice, especially during the recovery phase of infection (indicated by arrows in Fig. 3).

Detection of B220$^+$ T cells in the liver of infected mice

B220$^+$ T cells, namely, T cells with a B-cell marker. To detect this unusual population, two-color staining for CD3 and B220 was performed (Fig. 3). It was confirmed that B220$^+$ CD3$^+$ cells were few in the livers of all tested mice before malarial infection. Somewhat surprisingly, a large proportion of B220$^+$ CD3$^+$ cells appeared in the liver after malarial infection, especially in DBA/2 mice (indicated by arrows).

Further phenotypic characterization of γδ T cells

Three-color staining for γδTCR, αβTCR and the mixture of CD4 and CD8 (or CD4 or CD8) was conducted for gated analysis of CD4, CD8 and DN CD4$^-$CD8$^-$ in the livers of B6 and DBA/2 mice (Fig. 4). The result revealed that the majority of γδ T cells were found to be DN CD4$^-$ CD8$^-$. Interestingly, the reverse was observed in αβ T cells, which were mainly CD4$^+$.

The expression level of γδ T within IL-2Rβ$^+$ CD3^{int} cells

The absolute numbers of IL-2Rβ$^+$ CD3^{int} cells and γδ T cells were calculated (Fig. 5A). Although the largest subset of expanding lymphocytes was IL-2Rβ$^+$ CD3^{int} cells during malarial infection in all mice ($P < 0.05$), there were also marked increases in the numbers of γδ T cells and in DBA/2 mice ($P < 0.05$). Among IL-2Rβ$^+$ CD3^{int} cells, the proportion of γδ T cells increased during the recovery phase in DBA/2 mice (indicated by an arrow in Fig. 5B).

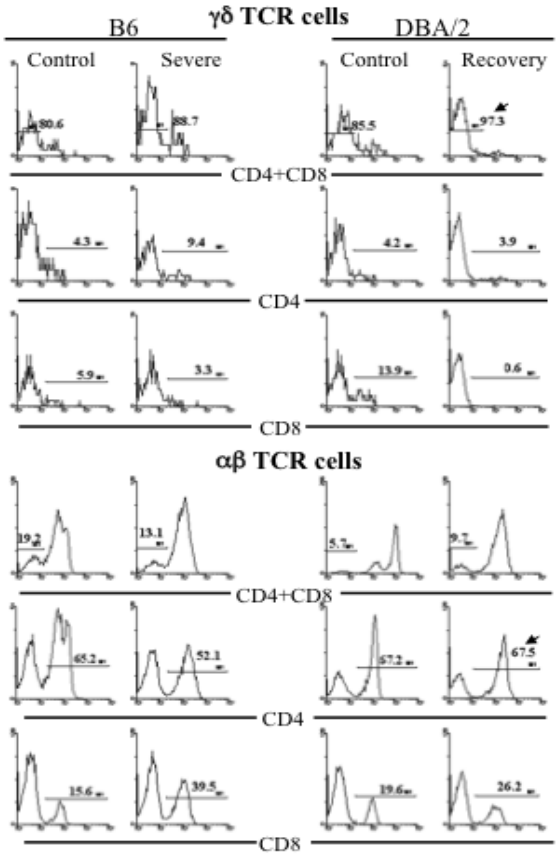

Fig. 4: Phenotypic characterization of γδ T cells in the livers of mice. Three-color staining for cd T-cell receptor (TCR), αβ TCR and a mixture of CD4 and CD8 (or CD4 or CD8) was performed. Numbers in the figure represent the percentages of fluorescence-positive cells in the corresponding areas

Discussion

Survival of *Plasmodium*-infected host is thought to rely almost exclusively on its ability to generate an effector immune response capable of limiting pathogen burden (18, 19). Their implications in susceptibility and resistance to infection are poorly understood and are addressed here by comparing cellular responses in resistant DBA/2 (H-2^d) and susceptible B6 (H-2^b) infected with *P. yoelii 17XL*.

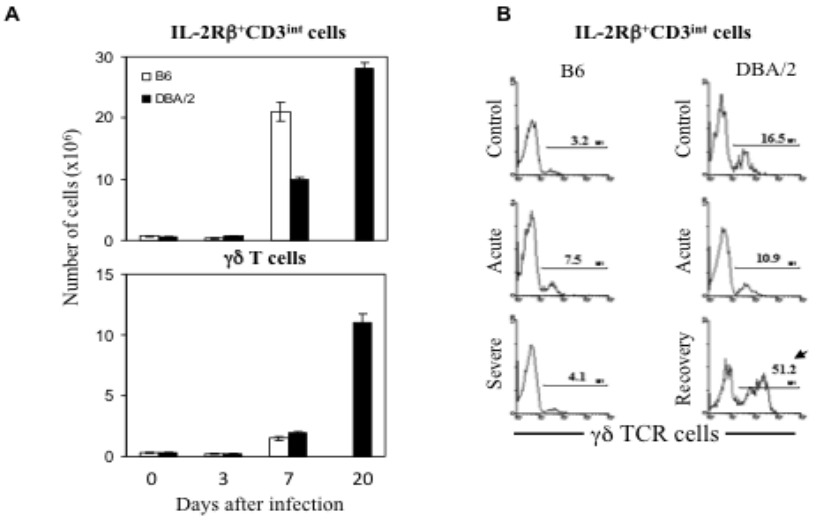

Fig. 5: Phenotypic characterization of interleukin (IL)-2Rβ$^+$ CD3^{int} cells and γδ T cells in the liver after malarial infection. (a) Change in the number of lymphocyte subsets in the liver. (b) Change in the number of γδ T cells among IL-2Rβ$^+$ CD3^{int} cells ($P< 0.05$)

In the present study, we used DBA/2 mice to examine how these mice recovered from virulent strain of malarial infection. In contrast to B6 mice, the genetic background of DBA/2 mice is known to have a functional deficiency of cytotoxic CD8$^+$ αβ T cells (8). Different outcome was observed by comparing DBA/2 and BALB/c mice infected with *Plasmodium chabaudi AS* infection (20, 21)

Concerning susceptibility to infection and course of parasitemia, the two strains presented a significantly different outcome of the infection (i.e., survival versus death). While none or very low deaths occur in resistant mice, 100% of susceptible mice died within 7–10 days after infection and similar results on parameters of anemia. Recent studies (22- 25) confirmed our results about the resistance of DBA2 mice compared with B6 and BABL/c mice infected with highly virulent strain plasmodium (*P. berghei ANKA*). DBA/2 mice infected with *P. berghei ANKA* showed a pattern of survival distinct from the previous two mice strains.

Hypothesizing that the immune responses are likely to differ, we initiated a systematic comparison of the two strains by describing the dynamics of cellular responses to infection. Some of the cellular response to infection was similar in both strains. There is an increase in the lymphoid liver, splenic cellularity, which accounts for the well-known hepatosplenomegaly in both strains (26), and this was maximal when parasites were no longer detected in circulation. The mechanisms and pathological relevance of lymphocyte depletion in primary organs probably associated with the general "stress conditions" of all severe infections (27). It might contribute to dysregulation of immune responses, and to the characteristic anemia in malaria infections (28-30). As many lymphocytes are activated in the periphery, it is likely that increased splenic cellularity might be due to local mitotic activity, rather than to arrival of newly formed lymphocytes from the primary organs (31).

As previously mentioned, protection against malaria is an event of innate immunity mediated by TCR^{int} cells (9-12). This speculation was supported by thymic atrophy, a major consequence of infection (i.e. conventional T cells of thymic origin were suppressed in par-

allel with thymic involution.) (11, 32). In this study, although both B6 mice and DBA/2 mice showed an expansion of IL-2Rβ$^+$ TCR^{int} cells in the liver during malarial infection, the subsets of TCR^{int} cells were completely different in the two strains of mice. αβTCR^{int} T cells expanded in B6 mice, whereas γδTCR^{int} T cells (including some αβTCR^{int} T cells) expanded in the DBA/2 strain of mice in good agreement with earlier studies (26, 33). The current study was confirmed by the previous experiments using athymic nude mice, which carry only TCR CD3^{int} cells of extrathymic origin, malaria infected nude mice showed the expansion of TCR CD3^{int} cells in the liver and the mice recovered from infection (11). This phenomenon was also seen in DBA/2 mice in the present study.

This study confirmed our previous observations concerning resistance of DBA/2 mice to non-lethal strain *p.* yoelii (26). The expansion of CD3^{int} B220$^+$ γδ T cells with the double negative CD4$^-$ CD8$^-$ phenotype in the liver of DBA/2 mice render these mice resistant to malarial infection. B6 mice did not show an expansion of such unconventional T cells, these cells play a compensatory function in a group of mice (DBA/2) already suffer from deficiency of cytotoxic CD8$^+$T cells. One of previous studies, in which the role of T cell subsets was studied in mice depleted of CD4$^+$ or CD8$^+$ T cells, showed that CD4$^+$ but not CD8$^+$ T cells could transfer protection, suggesting that CD8$^+$ T cells are not responsible for blood-stage immunity (34).

Yet another very significant difference in the cellular responses of susceptible and resistant mice was the detection of unconventional γδ T cells. A large expansion of γδ T cells in DBA/2 mice after malarial infection renders these DBA/2 mice resistant to malarial infection (26). Gamma/delta cells function as components of the innate immune system (35, 36). When stimulated by pathogens, these cells acted as a first line of defense and acted by cytokine activation of cells of the adaptive immune response in a manner analogous to activation by NK cells. Humans infected with *Plasmodium falciparum* exhibit a marked increase in the number of peripheral blood γδ T cells (37, 38). Cloned γδ T cells were cytotoxic for *P. falciparum* in vitro (39) and γδ T cells were essential for the expression of cell-mediated immunity in vivo against the murine malarial parasite *P. chabaudi* (40, 41). The experimental results for the elimination of γδ T cells using anti-γδTCR mAb support this speculation (26). γδ T cells cause certain pathologic changes associated with malaria (42).

Other parameters of the cellular responses significantly differed between the two strains. Thus, in *P. yoelii 17XL* infection, while DBA/2 mice respond with elevated numbers of B220$^+$ T cells following the peak of parasitemia, B6 showed a very slight increase in B220$^+$ T cells. The expression of the B220$^+$T cells (T cells carry B-cell marker) was unique. A similar but not identical finding was seen in αβ T cells in the lymph nodes and liver of autoimmune- prone MRL-lpr/lpr mice (43, 44) and in αβ T cells in the appendix of normal mice (45).

Conclusion

Taken together, the genetic deficiency of CD8$^+$ cytotoxic T-cell function in DBA/2 mice seems to induce a compensatory function of primitive lymphocytes. The induction of primordial T and B cells (i.e. innate immunity) is important for the acquisition of resistance against virulent parasite line. It may be argued that B6 mice die although there is marked expansion of intermediate cells. Therefore, death is not related to innate immunity and, in this case, susceptible B6 mice may be died from parasite loads and severe anemia. Further experiments on the importance of primitive T and B cells (innate immunity) in malaria protection in genetically deficient mice should also be conducted.

Acknowledgements

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