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Experimental cerebral malaria is suppressed by disruption of nucleoside transporter 1 but not purine nucleoside phosphorylase

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ABSTRACT

Protozoan parasites rely on purine nucleosides supplied by the host because they are unable to synthesise purine rings *de novo*. Nucleoside transporter 1 (NT1) and purine nucleoside phosphorylase (PNP) play an essential role in purine salvage in *Plasmodium*. It is unclear whether severe pathology, such as cerebral malaria (CM), develops in hosts infected with *Plasmodium* parasites that lack activity of NT1 or PNP. *Plasmodium berghei* (*Pb*) ANKA-infected mice show features similar to human CM, such as cerebral paralysis and cerebral haemorrhage. Therefore, *Pb* ANKA infection in mice is a good experimental model of CM. In this study, we generated *pbnt1*-disrupted *Pb* ANKA (Δ*pbnt1* parasites) and *pbpnp*-disrupted *Pb* ANKA (Δ*pbpnp* parasites), and investigated the effect of *pbnt1* or *pbpnp* disruption on the outcome of infection with *Pb* ANKA. We showed that the rapid increase of wild-type *Pb* ANKA (WT parasites) in mice early in infection was significantly inhibited by disruption of *pbnt1*. Moreover, Δ*pbnt1* parasite-infected mice showed neither cerebral paralysis nor cerebral haemorrhage, and all mice spontaneously recovered from infection. By contrast, mice infected with Δ*pbpnp* parasites showed features similar to those of mice infected with WT parasites. In this study, we demonstrated that the high virulence of *Pb* ANKA in the asexual phase is suppressed by disruption of *pbnt1* but not *pbpnp*.

1. Introduction

Malaria, caused by protozoan parasites of the genus Plasmodium, is the major parasitic disease in tropical and subtropical regions, including parts of the Americas, Asia and Africa. An estimated 0.6–1 million malarial deaths per year have been reported [1]. Four species of Plasmodium can infect humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium ovale*. Several kinds of drug are used to treat a *Plasmodium*-infected human. However, the increased incidence of drug-resistant parasites [1] has raised the importance of developing effective drugs or vaccines against *Plasmodium* infection.

It has long been recognised that protozoan parasites, including *Plasmodium* spp., are unable to synthesise purine rings *de novo*; instead, they rely on purine nucleosides from the host [2,3]. Nucleosides and nucleobases are transported across the parasite plasma membrane by nucleoside transporters (NTs) of *Plasmodium* spp. [3]. The *P. falciparum* (*Pf*) genome sequencing project revealed four nucleoside transporters, PfNT1, PfNT2, PfNT3 and PfNT4 [4,5]. PfNT1 has been cloned and expressed in *Xenopus* oocytes [6,7] and was shown to transport hypoxanthine, in addition to adenosine and inosine [6–10].

During the asexual phase of malaria parasites, adenosine is converted to inosine by adenosine deaminase (ADA) in the purine salvage pathway. Hypoxanthine is produced from inosine by purine nucleoside phosphorylase (PNP). Hypoxanthine–guanine–xanthine phosphoribosyl transferase (HGXPRT) converts hypoxanthine to inosine monophosphate (IMP), guanine to guanosine monophosphate (GMP), and xanthine to xanthosine monophosphate (XMP). IMP, GMP and XMP are then converted to guanylate and adenylate nucleotides by the action of several other enzymes [3].

It was demonstrated that *Plasmodium* NT1 and PNP are essential for the viability of *Plasmodium* parasites in the host [11,12]. Therefore, *Plasmodium* NT1 and PNP may be targets for anti-malarial drugs. On the other hand, it is unclear whether severe pathology, such as cerebral malaria (CM), is suppressed during infection with parasites lacking activity of *Plasmodium* NT1 or PNP.

Plasmodium berghei (*Pb*) ANKA is a lethal murine malaria parasite strain, and mice infected with *Pb* ANKA show features similar to human CM [13–16]. Therefore, *Pb* ANKA infection in mice is a

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good experimental model of CM. In this study, we generated *pbnt1*-disrupted *Pb* ANKA (Δ*pbnt1* parasites) and *pbpnp*-disrupted *Pb* ANKA (Δ*pbpnp* parasites), and investigated the effect of disruption of *pbnt1* or *pbpnp* on the outcome of infection with *Pb* ANKA. We show that the high virulence of *Pb* ANKA in the asexual phase was suppressed by disruption of *pbnt1* but not *pbpnp*.

2. Materials and methods

2.1. Mice

Female C57BL/6J (B6) mice 5- to 6-weeks old were purchased from CLEA Japan INC (Tokyo, Japan). The experiments were approved by the Experimental Animal Ethics Committee of Kyorin University School of Medicine, Tokyo, and all experimental animals were kept at the animal facility in a specific-pathogen-free unit with sterile bedding, food and water.

2.2. Parasites and infections

Malaria parasites were stored as frozen stocks in liquid nitrogen. Wild-type *Pb* ANKA (WT parasites) is a high-virulence strain and the parasites, which had been cloned by limiting dilution, were obtained from Dr. W.P. Weidanz (University of Wisconsin–Madison, Madison, WI, USA). Using standard methods of reverse genetics in *Pb* [17,18], mCherry, a red fluorescent protein, was integrated into the *c-ssu-rrna* locus on chromosome 5 of WT parasites (*Pb* ANKA-mCherry). Expression of mCherry was controlled by an HSP70 promoter. Parasitised RBCs (pRBCs) of murine malaria parasites were generated in donor mice inoculated i.p. with each frozen stock of parasites. The donor mice were monitored for parasitaemia daily and were bled for experimental infection in ascending periods of parasitaemia. Experimental mice were infected i.v. with 1×10^4 pRBCs of a given parasite.

2.3. Generation of pbnt1- or pbpnp-disrupted Pb ANKA

pbnt1-disrupted *Pb* ANKA (Δ*pbnt1* parasites) and *pbpnp*-disrupted *Pb* ANKA (*Δpbpnp* parasites) were generated by double-crossover homologous recombination (Supplementary Material). In Δ*pbnt1* parasites and Δ*pbpnp* parasites, a selection cassette containing *gfp* and an hDHFR-ts fusion gene was integrated into the target gene (*pbnt1* or *pbpnp*) (Supplementary Material).

2.4. Parasitaemia

Blood was observed by microscopic examination of methanol-fixed tail blood smears stained for 45 min with 3% Giemsa diluted in phosphate buffer (pH 7.2). The number of pRBCs among 250 RBCs was determined when parasitaemia exceeded 10%, whereas 1×10^4 RBCs were examined when mice showed lower parasitaemia. The percentage of parasitaemia was calculated as follows: [(number of pRBCs)/(total number of RBCs counted)] × 100. To examine the parasitaemia of murine malaria parasites expressing GFP in peripheral blood during infection, blood was diluted 1:5000 with FACS buffer and analysed by flow cytometry. Data were analysed with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) using the FlowJo software (version 7.1.3 for Windows).

2.5. Examination of the blood–brain barrier and histopathological examination of brain

Mice were injected i.v. with 0.2 ml of 1% Evans blue (Wako, Osaka, Japan) on days 7 and 14 post-infection. Mice were euthanised and brains perfused with PBS 1 h later. Brains were removed and photographed. They were then weighed and placed in formamide (2 ml) (Wako, Osaka, Japan) (37 °C, 48 h) to extract the Evans blue dye. Absorbance was measured at $\lambda = 620$ nm with a Multiscan FC microplate reader (Thermo Fisher Scientific Inc., Waltham, USA). The Evans blue concentration was calculated from a standard curve and is expressed as μg of Evans blue per g of brain. Brains were obtained from uninfected and infected mice on days 7 and 14 post-infection. Mice were euthanised before their brains were removed. Brains were fixed in 10% buffered formalin and embedded in paraffin. Six-micrometre-thick sections were stained with haematoxylin and eosin (H&E).

2.6. Enzyme-linked immunosorbent assay

Malarial antigens were prepared at the erythrocytic stages as described previously [19,20]. Malaria-specific antibodies were measured in the plasma of mice by using soluble antigens from *Pb* XAT as the capture antigens. Peroxidase-coupled anti-mouse IgG (Zymed, San Francisco, CA) was used to detect specifically bound mouse IgGs. The reaction was visualised using peroxidase-conjugated streptavidin (Zymed) and the substrate, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Wako).

2.7. Statistical analysis

Student's *t*-test was performed using Statcel (OMS Ltd., Saitama, Japan). Survival curves were compared using the log-rank test. All statistical analyses were performed using Statcel (OMS Ltd.). Statistically significant differences were defined as a value of $p < 0.05$.

3. Results

3.1. Effect of disruption of pbnt1 or pbpnp on the outcome of infection with Pb ANKA

To investigate the effect of *pbnt1* or *pbpnp* disruption on the outcome of infection with *Pb* ANKA, mice were infected with wild-type *Pb* ANKA (WT parasites), Δ*pbnt1* parasites or Δ*pbpnp* parasites. Mice infected with WT parasites showed high levels of parasitaemia and neurological signs, such as a cerebral paralysis, and all mice died within 10 days post-infection (Fig. 1A and B). In mice infected with Δ*pbnt1* parasites, parasitaemia on days 6 and 8 post-infection was significantly milder than that in WT parasite-infected mice (Fig. 1A). Δ*pbnt1* parasite-infected mice showed high levels of parasitaemia from day 20 post-infection, but the mice ultimately cleared the parasites by day 90 post-infection (Fig. 1A). During infection, Δ*pbnt1* parasite-infected mice showed neither cerebral paralysis nor depression, and all mice survived (Fig. 1B).

Mice infected with Δ*pbpnp* parasites showed low levels of parasitaemia compared with WT parasite-infected mice on day 4 post-infection (Fig. 1C). However, a rapid increase in parasitaemia was observed in mice infected with Δ*pbpnp* parasites and their levels of parasitaemia on day 7 post-infection were comparable with those in WT parasite-infected mice (Fig. 1C). Ultimately, Δ*pbpnp* parasite-infected mice showed cerebral paralysis and died within 10 days post-infection (Fig. 1D). These results suggest that experimental cerebral malaria (ECM) may be suppressed by disruption of *pbnt1* but not *pbpnp*.

3.2. Disruption of pbnt1 but not pbpnp suppresses the development of ECM

It is known that breakdown of the blood–brain barrier is an indicator of ECM. When *Pb* ANKA-infected mouse with ECM is

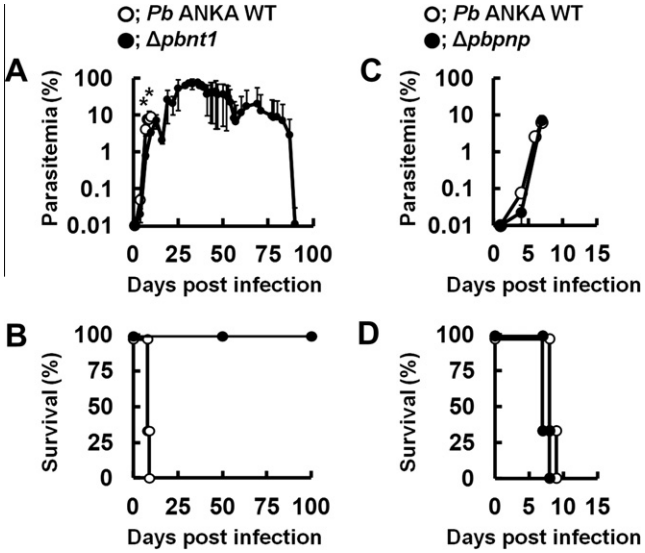

Fig. 1. Effect of disruption of *pbnt1* or *pbpnp* on the outcome of infection with *Pb* ANKA. B6 mice were infected with 1×10^4 pRBCs of WT parasites, Δ*pbnt1* parasites or Δ*pbpnp* parasites. (A) Course of parasitaemia in mice infected with WT parasites and Δ*pbnt1* parasites. Asterisks indicate a statistically significant difference ($p < 0.05$ *vs.* WT parasite-infected mice). (B) Survival rates of mice infected with WT parasites and Δ*pbnt1* parasites. (C) Survival rates of mice infected with WT parasites and Δ*pbpnp* parasites. Results are expressed as the mean ± SD of three mice. Experiments were performed three times with similar results.

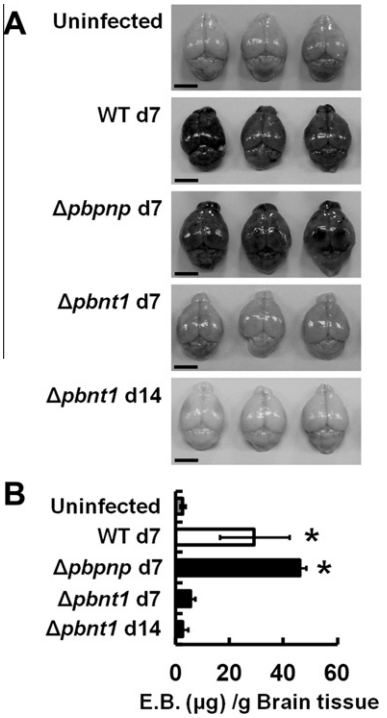

Fig. 2. Breakdown of the blood–brain barrier in infected mice. B6 mice were infected with 1×10^4 pRBCs of WT parasites, Δ*pbnt1* parasites or Δ*pbpnp* parasites. (A and B) Brains were obtained from WT parasite- and Δ*pbpnp* parasite-infected mice on day 7 post-infection, and from Δ*pbnt1* parasite-infected mice on days 7 and 14 post-infection. (A) Brains from mice injected with Evans blue. Scale bar: 5 mm. (B) Quantitative analysis of Evans blue extravasation in the brain. Asterisks indicate a statistically significant difference ($p < 0.05$ *vs.* uninfected mice or Δ*pbnt1* parasite-infected mice). Results are expressed as the means ± SD of 3–5 mice.

injected i.v. with Evans blue, the brain is stained as a result of extravasation of the dye [13,14]. We investigated breakdown of the blood–brain barrier in mice infected with WT parasites, Δ*pbpnp* parasites or Δ*pbnt1* parasites. Brains in mice infected with WT parasites or Δ*pbpnp* parasites were stained on day 7 post-infection (Fig. 2A). The extravasation of Evans blue in brains from Δ*pbnt1* parasite-infected mice on days 7 and 14 post-infection was markedly less than that in mice infected with WT parasites or Δ*pbpnp* parasites, and the levels were comparable to those in uninfected mice (Fig. 2).

Brains were slightly stained in Δ*pbnt1* parasite-infected mice on day 7 post-infection (Fig. 2A). We next performed histopathological analysis of brains from mice infected with WT parasites, Δ*pbpnp* parasites or Δ*pbnt1* parasites to examine in detail whether each group of mice developed ECM. In WT parasite-infected mice, haemorrhage with pRBCs was observed on day 7 post-infection (Fig. 3B and G). Haemorrhage comparable with that in WT parasite-infected mice was also observed in mice infected with Δ*pbpnp* parasites (Fig. 3C and H). By contrast, haemorrhage was not observed in Δ*pbnt1* parasite-infected mice on days 7 and 14 post-infection (Fig. 3D, E, I and J). These results confirm that ECM was suppressed by disruption of *pbnt1*.

3.3. *Pb* ANKA-disrupted pbnt1 induces protective immunity against malaria parasites

Δ*pbnt1* parasite-infected mice spontaneously recovered from infection (Fig. 1A). To examine whether Δ*pbnt1* parasite-infected mice acquire protective immunity against malaria parasites, we first determined the levels of specific IgGs against malaria parasites in Δ*pbnt1* parasite-infected mice (Fig. 4A). As a positive control, plasma from mice infected with *Pb* XAT, a derivative of lethal *Pb* NK65 attenuated by X-ray irradiation [21], was used. Mice that recovered from *Pb* XAT on day 30 post-infection acquire protective immunity against malaria parasites [21,22]. On day 100 post-infection, levels of specific IgGs against malaria parasites in Δ*pbnt1* parasite-infected mice were higher than those in uninfected mice (Fig. 4A). Their levels of specific IgGs against malaria parasites were comparable to those in *Pb* XAT-infected mice on day 30 post-infection (Fig. 4A).

Next, we investigated whether mice that recovered from Δ*pbnt1* parasite infection were resistant to infection with WT parasites (Fig. 4B and C). Naïve mice and mice that recovered from Δ*pbnt1* parasite infection (Δ*pbnt1*-immunised mice) were challenge-infected with mCherry-expressing WT parasites (*Pb* ANKA-mCherry). Mice infected with *Pb* ANKA-mCherry showed a pattern of parasitaemia (Fig. 4B, white circles) similar to that of mice infected with *Pb* ANKA-WT parasites (Fig. 1A, white circles), and all mice died within 10 days post-infection (Fig. 4C). In contrast, Δ*pbnt1*-immunised mice showed low levels of parasitaemia after infection with *Pb* ANKA-mCherry, and all mice spontaneously recovered from the infection (Fig. 4B and C). During challenge infection, Δ*pbnt1* parasites, which express GFP, were not observed in Δ*pbnt1*-immunised mice (data not shown). These results suggest that Δ*pbnt1* parasites induce protective immunity against malaria parasites in mice.

4. Discussion

We aimed to investigate the effect of disruption of *pbnt1* or *pbpnp* on the outcome of infection with lethal *Pb* ANKA. In *P. falciparum*, disruption of *pfnt1* resulted in blockage of growth at the ring stage [8]. A recent study using murine malaria parasites demonstrated that mice infected with *pynt1*-disrupted nonlethal *Plasmodium yoelii* 17X showed significantly lower levels of parasitaemia than mice infected with wild-type nonlethal *P. yoelii* 17X [11]. We showed here that the growth of *Pb* ANKA early in infection was significantly inhibited by disruption of *pbnt1*

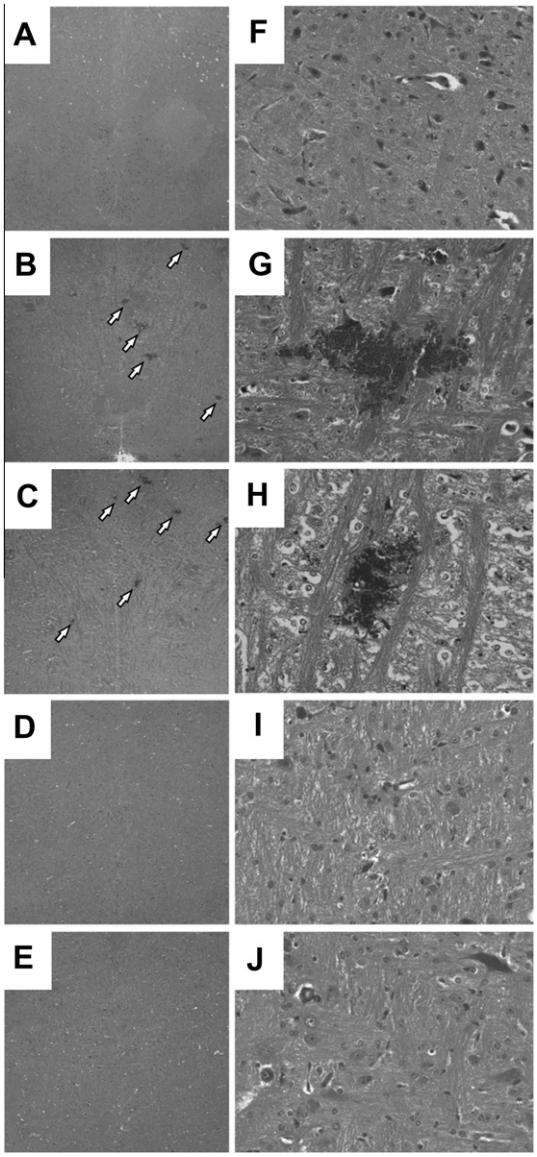

Fig. 3. Haemorrhage in the brain during infection. Mice were infected with malaria parasites as described in the legend to Fig. 2. Brains were obtained from WT parasite- and Δ*pbpnp* parasite-infected mice on day 7 post-infection, and from Δ*pbnt1* parasite-infected mice on days 7 and 14 post-infection, and histological analyses were performed. (A and F) Typical results for uninfected mice. (B and G) WT parasite-infected mice. (C and H) Δ*pbpnp* parasite-infected mice. (D and I) Δ*pbnt1* parasite-infected mice on day 7 post-infection. (E and J) Δ*pbnt1* parasite-infected mice on day 14 post-infection. (A–E) Haemorrhage in the cerebellum was analysed. H&E, 20× magnification. Open arrows indicate haemorrhage. (F–J) H&E, 400× magnification. Experiments were performed twice with similar results and representative data are shown.

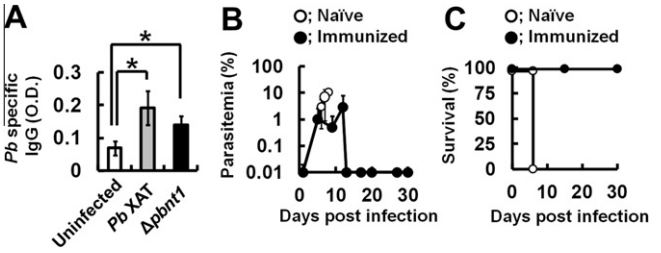

Fig. 4. Induction of protective immunity against malaria parasites in mice infected with Δ*pbnt1* parasites. (A) Plasma levels of *Pb*-specific IgGs in uninfected control mice and *Pb* XAT–infected mice on day 30 post-infection, and in Δ*pbnt1* parasite-infected mice on day 100 post-infection. Plasma was diluted 1:32 for detection of IgGs. Asterisks indicate a statistically significant difference ($p < 0.05$ *vs.* uninfected mice). (B and C) Naïve mice (naïve) and mice that recovered from Δ*pbnt1* parasite infection (immunised) were infected with 1×10^4 pRBCs of mCherry-expressing *Pb* ANKA-WT parasites (*Pb* ANKA-mCherry). (B) Course of parasitaemia after *Pb* ANKA-mCherry infection. (C) Survival rates after *Pb* ANKA-mCherry infection. Results are expressed as the means ± SD of three mice. Experiments were performed twice with similar results.

(Fig. 1). Moreover, Δ*pbnt1* parasite-infected mice showed neither cerebral paralysis nor haemorrhage (Figs. 2 and 3). Our results demonstrate that ECM caused by *Pb* ANKA is suppressed by disruption of *pbnt1*.

Mice infected with Δ*pbpnp* parasites showed features similar to those of mice infected with WT parasites (Figs. 1–3). Our results suggest that PbPNP does not play an essential role in parasite viability in the asexual phase. In purine salvage, PfPNP converts inosine to hypoxanthine. Immucillin-H, a PNP transition state analogue, has been shown to inhibit the growth of *P. falciparum in vitro* [23–25]. However, the inhibition of parasite growth by blockage of PfPNP was reversed by the addition of hypoxanthine, but not inosine, to the culture medium [24]. On the other hand, PfNT1 has been associated with the transport of hypoxanthine, in addition to adenosine and inosine [7,8,10]. In the present study, hypoxanthine would have been supplemented by the action of PbNT1 in asexual-phase Δ*pbpnp* parasites.

In contrast to *Pb* ANKA, the lethal *P. yoelii* YM strain was attenuated by disruption of *pypnp* in the asexual phase [12]. It is possible that the types of purine nucleosides essential for parasite viability might differ between *Pb* ANKA and *Py* YM. Furthermore, *Pb* ANKA-infected mice, but not *P. yoelii* YM-infected mice, develop ECM [26]. The difference in pathogenesis between *Pb* ANKA-infected mice and *Py* YM-infected mice might be associated with the purine salvage pathway.

Δ*pbnt1* parasite-infected mice showed high levels of parasitaemia from day 20 post-infection, but they eventually eliminated parasites by day 90 post-infection (Fig. 1). *Plasmodium* parasites escape from host immune surveillance and disturb immune responses [27,28]. When B6 mice were infected with only a single *Pb* ANKA parasite, infected mice did not develop ECM but showed increased parasitaemia and severe anaemia, and all mice eventually died (data not shown). In contrast, mice resolved Δ*pbnt1* parasite infection and acquired protective immunity against malaria parasites (Fig. 4), suggesting that the Δ*pbnt1* parasites are recognised by host immune surveillance and subsequently induced protective immunity against malaria parasites in the mice.

The growth of *Pb* ANKA in mice was not completely inhibited by *pbnt1* deficiency (Fig. 1). Four NTs of *P. falciparum* (PfNT1, PfNT2, PfNT3 and PfNT4) were identified by genome sequencing [4,5]. These findings raise the possibility that NTs other than NT1 are involved in the growth of *Pb* ANKA during the asexual phase. We have identified orthologues of PfNT1, PfNT2 and PfNT4, but not PfNT3, in the genome of the *Pb* ANKA using PlasmoDB. In asexual-phase parasites, expression levels of *PbNT2* mRNA were comparable to those of *PbNT1* mRNA, but expression levels of *PbNT4* mRNA were significantly lower than those of *PbNT1* mRNA and *PbNT2* mRNA (data not shown). The broad expression pattern of PfNT4 suggests that PfNT4 is associated with purine salvage during replication in hepatocytes of humans or in mosquitos [29]. Therefore, PbNT4 might not be essential for parasite viability during the asexual phase. On the other hand, it has been demonstrated that PfNT2 is localised to the endoplasmic reticulum in parasites [30]. The role of PfNT2 during the asexual phase remains unclear.

In this study, we demonstrated that the purine salvage pathway is important for the growth and virulence of malaria parasites during the asexual phase. Notably, ECM caused by *Pb* ANKA was suppressed by disruption of *pbnt1* but not *pbpnp*. Malarial

pathology is caused by *Plasmodium* parasites in the asexual phase. Therefore, to reduce the risk of severe malaria, it is necessary to understand purine salvage in *Plasmodium* spp.

Author contributions

M.N. designed research; M.N. and Y.Y. performed research; I.K., S.I. and M.Y. contributed reagents/materials/analysis tools; M.N., S.-I.I., S.M., and F.K. analyzed data; and M.N. and F.K. wrote the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.02.004.

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