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RESEARCH ARTICLE

Genetic disruption of nucleoside transporter 4 reveals its critical roles in malaria parasite sporozoite functions

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ABSTRACT

All protozoan parasites are lacking the pathway to synthesize purines *de novo* and therefore they depend on their host cells to provide purines. A number of highly conserved nucleoside transporter (NT) proteins are encoded in malaria parasite genomes, of which NT1 is characterized in *Plasmodium falciparum* and *P. yoelii* as a plasma membrane protein that is responsible for salvage of purines from the host, and NT2 is an endoplasmic membrane NT protein. Whereas NT3 is only present in primate malaria parasites, little is known about NT4, which is conserved in all malaria parasite species. Herein, we targeted NT4 gene for deletion in *P. berghei*. NT4 knockout parasites developed normally as blood stages, ookinetes and formed oocysts with sporozoites compared with wild-type (WT) *P. berghei* ANKA parasites. However, *nt4(-)* sporozoites showed significantly decreased egress from oocysts to hemolymph, significant reduction of colonization of the salivary glands, and complete abolishment of infection of the mammalian host by salivary gland and hemolymph sporozoites. Therefore, we identify NT4 as a NT that is important, not for replication and growth, but for sporozoite infectivity functions.

KEYWORDS

Malaria; *P. berghei* ANKA; nucleoside transporter 4; hemolymph sporozoites; oocyst sporozoites; salivary gland sporozoites

1. Introduction

Malaria is a devastating global health problem. It is caused by *Plasmodium* species and primarily affects young children and pregnant women. In 2020, the World Health Organization reported more than 240 million cases around the world [1]. Chemotherapy is the main antimalarial arsenal used to treat malaria. But drug resistance has developed against all available antimalarial drugs in most parts of the world, which hinders the success of antimalarial chemotherapy [2]. Therefore, new intervention strategies are urgently needed to fight against this life-threatening disease.

Plasmodium is a purine-auxotrophic parasite that lacks *de novo* purine synthesis and depends on the host to get the required purines. Parasites contain a special class of protein family called as equilibirative nucleoside transporter (ENT) which are central in the transport of nucleoside from host to parasite. These proteins contain eleven transmembrane domains which are widely present in animals and plants [3]. Genetic and molecular studies targeting ENT were mostly carried out in *Leishmania* and *Trypanosoma*, with some studies also conducted in *Plasmodium* and *Toxoplasma spp*. [4]. *Leishmania donovani* contains two NTs, *Ld*NT1 and *Ld*NT2 with distinct functions. While *Ld*NT1 transports adenosine and pyrimidine *Ld*NT2 is involved in transport of inosine and guanosine nucleosides [5]. *L. major* has NT3, which has an affinity for purine nucleosides but not for pyrimidine nucleosides or nucleobases [6]. *Trypanosoma brucei* reports several ENT proteins termed *Tb*NT2-*Tb*NT10, which are known for the transport of purine nucleosides and nucleobases [7,8]. Four members (NT1, NT2, NT3, and NT4) of the NT gene family have been identified in the genome of *Plasmodium* species, which are known to play important roles in purines acquisition from the host. Gene deletion experiments showed that NT 1 gene is primarily responsible for the import of purines, which is a critical prerequisite for the synthesis of nucleic acids in *Plasmodium* species. Other members of the NT family are also involved in purine transport in the malaria parasite, but their mechanisms of purines salvage are not well understood.

Herein, we performed gene targeting studies of NT4 protein in the rodent malaria parasite *Plasmodium berghei* ANKA. We successfully generated the *Pbnt4(-)* (NT4-deficient *Plasmodium berghei* parasite strain) parasites by double crossover homologous

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recombination. Furthermore, we investigated the role of NT4 at different life cycle stages to explore the importance of NT4 in the parasite life cycle and its significance to be developed as a potential drug target.

2. Materials and methods

2.1. Experimental animals, parasites and mosquitoes

Six- to eight-week-old female CD1 mice were purchased from the Experimental Animal Research Center at Bezmialem Vakif University . All animal experiments described here were approved by the Animal Ethics Committee of Bezmialem Vakif University (protocol number 2020/141). Mice were infected with blood frozen-stocks of *P. berghei* wild-type (*Pb*WT) and *Pbnt4(-)* intraperitoneally. For mosquito infection, *Anopheles stephensi* mosquitoes were blood-fed on *P. berghei* ANKA or *Pbnt4(-)* parasite infected mice as described in our previous study [9].

2.2. Generation of Pbnt4(-) parasites

The NT4 gene of *P. Berghei* ANKA was deleted by double crossover homologous recombination with a modified donor plasmid flanking the mTurquoise2 fluorescent protein and the human *DHFR* cassette. *P. berghei* ANKA parasites were transfected with SacII/KpnI linearized donor DNA plasmid and selected with pyrimethamine. The transfection was done as described earlier [10–12]. The genomic integration of recombinant construct was confirmed by diagnostic PCR using integration specific primers. The sequences of the primers for the preparation of donor DNA plasmid and the integration specific diagnostic PCR are listed in Table S1 (Supplementary Information).

2.3. Determination of blood stage development and male gamete exflagellation

Frozen stocks of *Pbnt4(-)* and *Pb*WT were injected intraperitoneally (IP) into naive female CD1 donor mice. The mice were bled, and 20,000 infected erythrocytes were prepared in incomplete RPMI media in doses of 150 µL, and each mouse in groups of 3 mice for each genotype received one dose intravenously. The parasitemia was monitored for 10 days post infection by Giemsa staining of thin blood smears and counting 50 fields (1000x magnification). To calculate exflagellation events, a group of 5 mice per genotype were used., Each mouse was intravenously (IV) injected with 10,000,000 infected erythrocytes, of *Pbnt4(-)* or *Pb*WT. On day 3 post infection, 2 µL of blood was collected from the tail vein of the mouse with a heparinized needle and immediately diluted (1:50) in incomplete ookinete medium (RPMI 1640 supplemented with 25 mM HEPES, 50 µg/mL hypoxanthine, 2 g/L $NaHCO_3$, and 100 µM xanthurenic acid). The diluted blood samples were placed in a hemocytometer and incubated at room temperature for 10 min. The exflagellation centers in *Pbnt4(-)* and *Pb*WT were counted at 400x magnification. At the same time, blood from the same tail puncture was smeared and stained with Giemsa to determine the ratio of male to female gametocytes.

2.4. In vitro ookinete quantifications

Exflagellation-positive *Pbnt4(-)* and *Pb*WT mice (n = 5) were bled by cardiac puncture, and 500 µL of blood was immediately mixed with 4.5 mL of complete ookinete culture medium in a 6-well tissue culture plate. The ookinete culture was incubated at 20–21°C for 24 hours and the ookinetes were extracted. The samples were centrifuged at 300 g for 5 min at 4 °C. The supernatant was discarded, and the pellet was resuspended with 50 mL of RBCs lysis buffer and incubated on ice for 8 minutes. Ookinetes were harvested by centrifugation at 300 g for 10 minutes at 4 °C and washed with PBS twice using the same conditions. The number of ookinetes was determined by microscopy 400x magnification using a hemocytometer [9].

2.5. Oocysts, oocyst sporozoites, hemolymph sporozoites and salivary gland sporozoites quantification

Mosquito cages were blood-fed with highly exflagellated *Pbnt4(-)* and *Pb*WT infected mice. On days 10, 15, 18, and 21 after feeding, at least 20 mosquitoes of each genotype were dissected to get midguts for determining the number of oocysts and oocyst sporozoites per midgut as described previously [9]. On days 15, 18, and 21 post-infection, oocyst sporozoites, and salivary gland sporozoites were extracted in sterile incomplete RPMI medium containing 3% BSA (Bovine Serum Albumin) for *Pbnt4(-)* and *Pb*WT infected mosquitoes. The dissected midguts and salivary glands were crushed with a pestle and centrifuged at 3000 rpm for 2 min. This step was repeated twice to ensure the proper release of all sporozoites. The number of sporozoites was counted using a hemocytometer. On days 18 and 21 post infection, hemolymph was collected from mosquitoes in isterile incomplete RPMI medium containing 3% BSA to determine the number of hemolymph sporozoites.

2.6. Sporozoite infectivity to the rodent host

Pbnt4(-) sporozoites was determined by IV injection of the extracted sporozoites into CD1 mice. On days 18 and 21 post infection, sporozoites of *Pbnt4(-)* and *Pb*WT were extracted from salivary glands and prepared for

IV mouse injection with doses of 10,000 sporozoites from each genotype, in 150 µL of incomplete RPMI medium with 3% BSA per dose. Hemolymph sporozoites for both *Pbnt4(-)* and *Pb*WT were extracted on day 21 and IV injected into mice similarly, but doses of 100,000 sporozoites were prepared in 150 µL of incomplete sterile RPMI medium with 3%BSA per dose. After the mice were intravenously injected, the infection was monitored by giemsa staining of tail vein thin blood smears from day 3 to day 21 post sporozoite infection.

2.7. Statistical analysis

Statistical analysis was performed by One-Way Analysis-of-Variance (ANOVA) and student t-test through Graph Pad prism 5. Data were presented as mean ± S.D. of at least triplicate determinations. P-values of <0.05 were considered as statistically significant. *p < 0.05, ** p < 0.01, ***p < 0.001.

3. Results

3.1. Phylogenetic analysis of nucleoside transporters for parasitic protozoa

The phylogenetic tree of all NTs in parasitic protozoa was generated using evolutionary genetic analysis software (MEGA) on sequences obtained from PlasmoDB, TriTrypDB, TrichDB, CryptoDB and ToxoDB (Figure 1). NT family protein ID numbers are listed in supplementary information. Unannotated proteins were symbolized using star in phylogenetic tree. Phylogenetic analysis reveals that parasitic protozoa have conserved members of ENT family, and that *Plasmodium* species has distinct four NTs (Figure 1). While NT1 and NT2 are highly conserved in all plasmodial parasites and are more closely related to each other, NT3 is only present in human malaria parasite species and NT4 is conserved in all malaria parasites but somewhat distant from NT1 and NT2, and closely related to NT3 (Figure 1).

3.2. Conservation of nucleoside transporter gene 4 among all Plasmodium species

Amino acid identity percentages of NT4 in *Plasmodium* spp. and *Hepatocytis* sp. were determined using Clustal Omega alignment online tool (Figure 2). Domains were identified in the PlasmoDB database. The alignment results show that *P. berghei* NT4 protein shares more than 50% amino acid identity with the human malaria parasites and has more than 80% amino acid identity with other rodent malaria parasites (Figure 2).

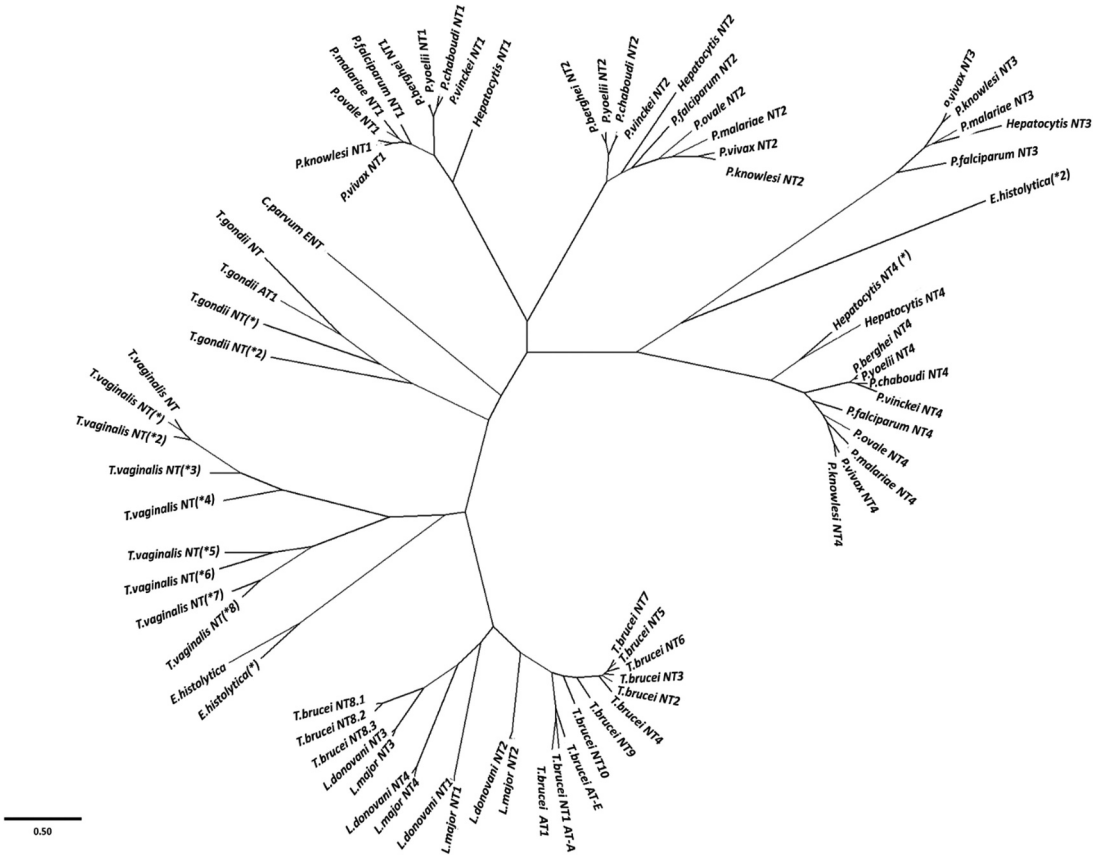

Figure 1. **Phylogenetic analysis of Nucleoside Transporter Gene Family for parasitic protozoas.** Phylogenetic tree was analyzed using Molecular Evolutionary Genetic Analysis (MEGA). Unannotated genes were symbolized using star (*) and gene ID was shown in supplementary information.

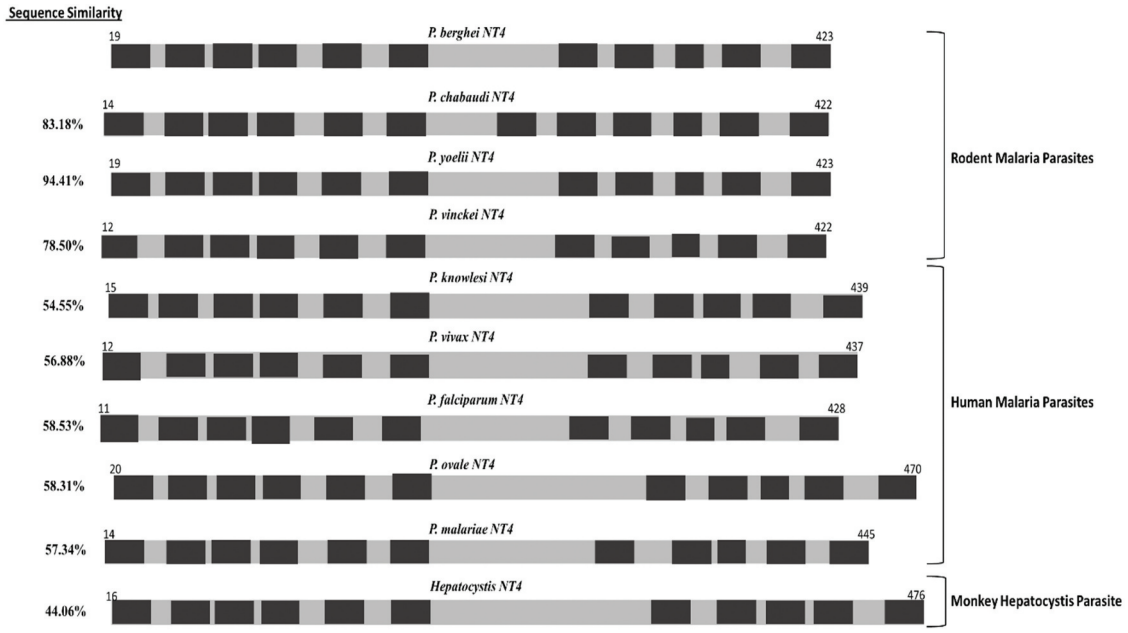

Figure 2. Schematic representation of amino acid identity and the transmembrane domains of *NT4* in human and rodent *Plasmodium* species and *Hepatocytis*. The transmembrane domains are shown as dark gray boxes. Amino acid idenetity percentages are shown on the left side.

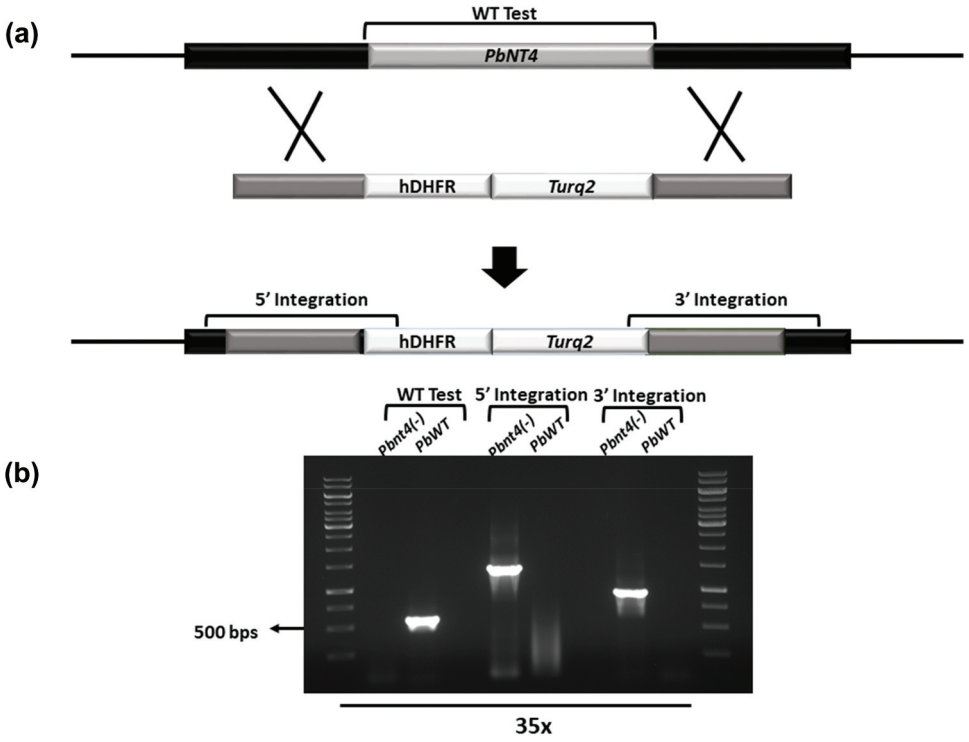

Figure 3. Generation of *P.berghei Pbnt4(-)* parasites by homologous recombination: (a) Schematic representation of the gene deletion strategy to generate *Pbnt4(-)* parasites. The donor plasmids containing the 5′ and 3′ UTR sequences of *Pb*NT4 flanking the hDHFR and Turquoise2 cassettes was used to replace the endogenous *Pb*NT4 by double crossover homologous recombination. **(b)** Genomic integration was confirmed by diagnostic PCR. Gene specific primer pairs that can amplify from the 5′ and 3′ integration sites only in recombinant loci were used to confirm the integration of recombinant locus.

3.3. NT4-deficient parasites display normal blood stage growth in mice

To determine the role of NT4 in the parasite life cycle, a genetic approach was used to generate NT4 deficient parasites in *P. berghei* ANKA. The *Pb*NT4 deficient parasites were generated using double crossover homologous recombination strategy. A donor plasmid pAA40 basic backbone containing the mTurquoise2 fluorescence protein cassette and the human *DHFR* drug selection marker cassette was used to clone the 5' and 3' UTR regions of NT4 gene. To confirm the genomic integration of *Pbnt4(-)* construct in the parasite genome and parasite cloning, diagnostic PCR was performed. The diagnostic PCR using gene specific primer that amplifies only from 5' and 3' integration sites of knock-out construct confirms the integration of knock-out construct into chromosomal *loci* and generation of *Pbnt4(-)* parasite strain (Figure 3).

To determine the role of NT4 gene in the asexual erythrocytic stage development of *Pb*WT, we compared the blood stage parasitemia of *Pbnt4(-)* parasites with *Pb*WT parasites in mice. A group of CD1 mice (n = 4 mice per genotype) was intravenously injected with 20,000 *Pbnt4(-)* and 20,000 *Pb*WT blood stage parasites. Parasitemia was recorded daily by Giemsa-stained thin blood smears for 2 weeks post infection. No significant difference was observed between the asexual growth of *Pbnt4(-)* and *Pb*WT injected parasites, which confirms that *Pb*NT4 is not essential for blood stage infection of *P. berghei* ANKA in mice (Figure 4(a)).

3.4. PbNT4 is not essential for gametocytogenesis, male gamete exflagellation, and ookinete development of P. berghei

The normal blood stage growth of *Pbnt4(-)* parasites in mice made it possible to identify the role of NT4 in gametocyte formation, maturation, and gametogenesis. We also checked the role of NT4 in the ookinete development of *Pb*WT. For this phenotypic characterization, groups of mice (n = 3 for each genotype) were intravenously injected with the 1×10^7 *Pbnt4(-)* and 1×10^7 *Pb*WT parasites. Third day post infection percentage of male and female gametocytes in both genotypes was recorded in giemsa-stained thin blood smears. The male gamete exflagellation events were counted by diluting blood from the same mice with incomplete ookinete medium (containing RPMI, Sodium bicarbonate, Hypoxanthine, and Xanthurenic acid). We did not observe any significant difference in

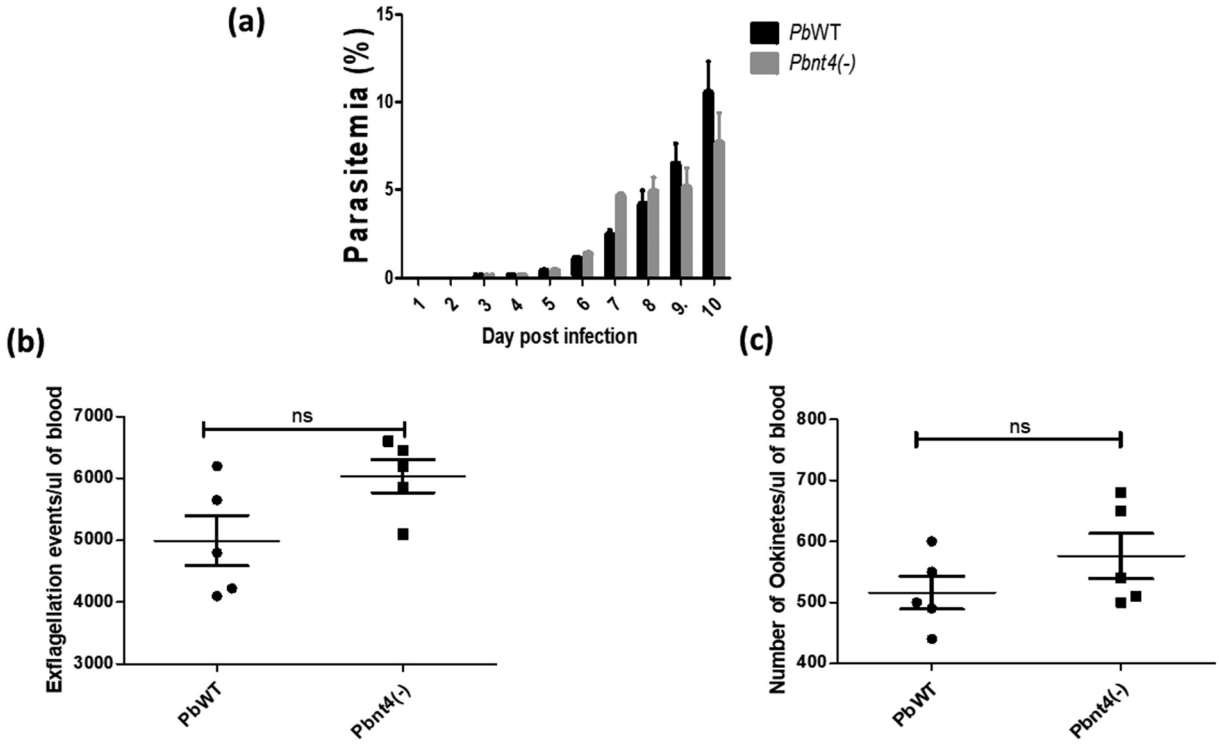

Figure 4. **Determination of blood stage development, male gamete exflagellation events and ookinete development for *Pbnt4(-)* and *Pb*WT. (a)** 20,000 parasitized erythrocytes were IV injected per mouse in groups of 4 mice . No significant difference was observed between both genotypes. **(b)** Number of male gamete exflagellation events per µL of mouse blood counted by a hemocytometer (1:50 dilution) for *Pbnt4(-)* and *Pb*WT. **(c)** Ookinete numbers were determined in in vitro culture for *Pbnt4(-)* and *Pb*WT parasite after 20 hours incubation. Statistical analysis was performed by one-way ANOVA and statistical significance was set at a P value of 0.05.

gametocytogenesis (data not shown) and male gamete exflagellation between *Pbnt4(-)* and *Pb*WT parasites, which confirms that NT4 is not essential for gametocytogenesis and male gamete formation of *P. berghei* (Figure 4(b)).

To check the effect of NT4 deletion on ookinete development, mice were bled, and the blood stage parasites were cultured in a complete ookinete medium for 24 hours at 21°C. Fully developed ookinetes were extracted and counted using a hemocytometer. No significant difference in ookinete number was observed between NT4-deficient parasites and WT parasites (Figure 4(c)). This confirms that NT4 is not essential for ookinete development of *P. berghei* ANKA.

3.5 PbNT4 plays significant roles in sporozoite oocyst egress and colonization of the sporozoites into the salivary glands

To examine the importance of NT4 in mosquito stage development (oocyst, oocyst sporozoites and salivary gland sporozoites) of *P. berghei,* female *Anopheles stephensi* mosquitoes were blood fed on WT and knock-out parasite infected mice which displayed the highest exflagellation. Oocyst numbers were counted on 10th, 14th, 16th, 18th, and 21st day post mosquito feeding. The results showed that number of oocysts per mosquito midgut were almost similar in WT and knock-out on days 10, 14 and 16 post blood feeding, however there was a significant difference in oocyst number between WT and knock-out on day 18 and 21, where number of oocysts decreased in WT parasites

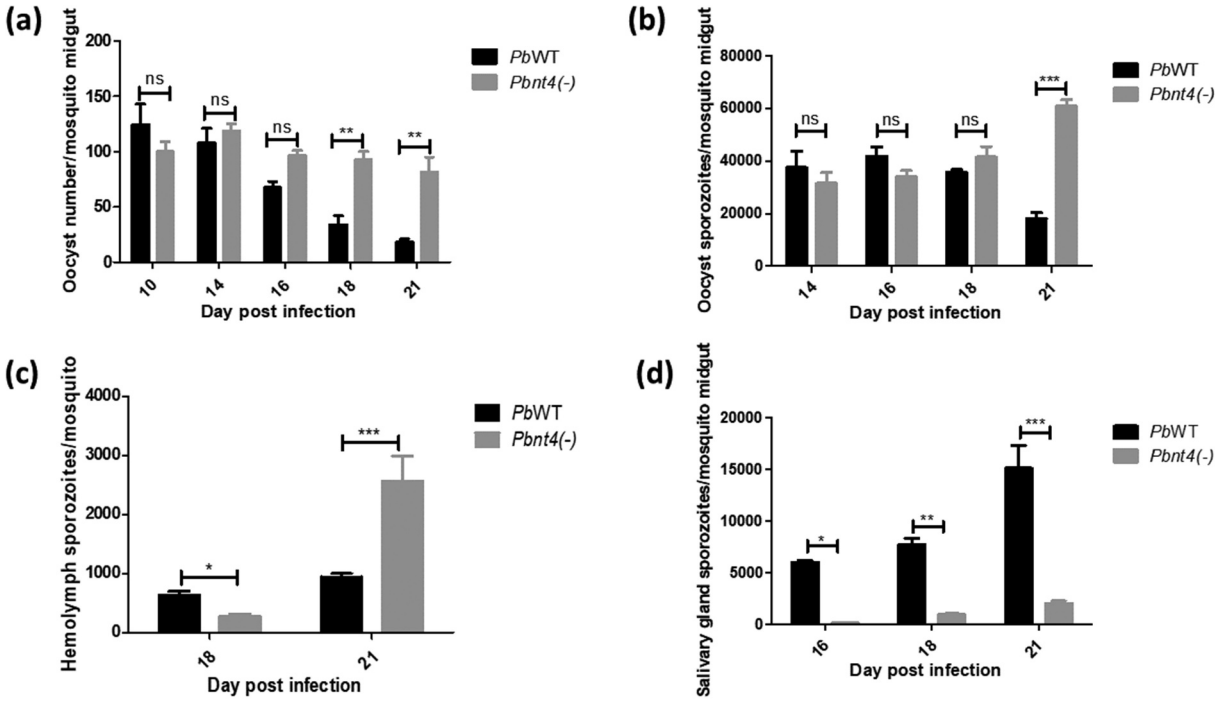

Figure 5. **Normal development of *Pbnt4(-)* oocysts and oocysts sporozoites but significant decrease in sporozoite egress from oocysts and diminished salivary gland invasion of hemolymph sporozoites, compared to *Pb*WT. (a)** Number of oocysts had no significant difference until day 18 post infection. **(b)** significant difference in the number of oocyst sporozoites in infected midguts is only detected at day 21 post infection. **(c)** Despite *Pb*WT oocyst sporozoites egressed more from oocysts as shown in **(b)**, they didn't accumulate much in the hemolymph as shown in **(c)** but they accumulated inside the salivary glands compared to *Pbnt4(-)* as shown in **(d)**.

Table 1. Determination of the role of NT4 in sporozoite infectivity in mice with salivary gland sporozoites (sgspz) and hemolymph (hmspz) sporozoites of *Pbnt4(-)* and *Pb*WT parasites.

Group (n)	Injection Route	Infection Dose	Blood stage infection	Prepatent period
Day 18 sgspz *Pbnt4(-)* (3)	Intravenous	10,000	0/3	-
Day 18 sgspz *Pb*WT (3)	Intravenous	10,000	3/3	4
Day 21 hmspz *Pbnt4(-)* (3)	Intravenous	100,000	0/3	-
Day 21 hmspz *Pb*WT (3)	Intravenous	100,000	3/3	7
Day 21 sgspz *Pbnt4(-)* (3)	Intravenous	10,000	0/3	-
Day 21 sgspz *Pb*WT (3)	Intravenous	10,000	3/3	4

compared to knock-out parasites (Figure 5(a)). The number of oocysts sporozoites were also counted on day 14, 16, 18 and 21 post feeding. The results did not show any significant difference in oocyst sporozoite numbers for day 14, 16 and 18 however day 21 shows a significant difference in oocyst sporozoite numbers where knock-out parasites exhibit higher oocyst sporozoite numbers in comparison to WT parasites (Figure 5(b)). To identify the role of NT4 in sporozoite migration from mosquito midgut to the salivary gland, the number of hemolymph sporozoites were counted on day 18 and 21 post feeding. The results of hemolymph sporozoites demonstrate a significantly high number on day 18 in wild type parasite infected mosquito as compared to knock-out parasites. Interestingly, on day 21, the hemolymph sporozoites were higher in numbers in knock-out parasites compared to WT parasites (Figure 5(c)). To investigate the importance of NT4 in salivary gland sporozoite invasion, the mosquito salivary glands were dissected on day 16, 18 and 21 post feeding and number of salivary gland sporozoites were estimated. The results of salivary gland sporozoites showed a significant difference in the numbers between WT and knock-out parasites at each time point. Furthermore, the number of salivary gland sporozoites increased significantly from day 16 to 18 to 21 in WT parasites infected mosquito, whereas a slight increase was observed in number of salivary gland sporozoites between days 16, 18 and 21 in knock-out parasites infected mosquitoes (Figure 5(d)). The exponential increase in numbers of hemolymph sporozoites from day 18 to 21 in the knock-out parasites with a very slight increase in the numbers of salivary gland sporozoites suggests, that NT4 also plays an important role in sporozoite colonization of the salivary glands. This colonization defect could be due a defect in migration and attachment or a direct defect on invasion into the ducts of the salivary glands. Altogether, the mosquito stage studies showed that NT4 is not involved in oocyst and sporozoite development but plays important roles in sporozoite egress from oocyst and colonization of salivary glands.

3.6. NT4 is essential for sporozoite infectivity in the mammalian host

To examine the role of NT4 in sporozoite infectivity in the mammalian host, mice were intravenously injected with the hemolymph and salivary gland sporozoites. On the 18th day post mosquito blood feeding, salivary gland sporozoites for both *Pbnt4(-)* and *Pb*WT, were collected from infected mosquitoes and intravenously injected into mice. On 21st day post mosquito blood feeding, hemolymph and salivary gland sporozoites were collected for both genotypes and intravenously injected into groups of 3 mice for each genotype. Sporozoite infectivity was determined by evaluating the blood stage parasitemia by giemsa staining of thin blood smears starting on day 3 till day 21 post sporozoite infection. The mice injected with *Pb*WT sporozoites showed a prepatent period of 4 days and 7 days for salivary glands sporozoites and hemolymph sporozoites, respectively, and they did not survive due to high parasitemia beyond days 16 and 18 post sporozoite injection, respectively. (Table 1). However, all mice injected with knockout sporozoites did not develop any blood stage parasitemia till day 21 post sporozoite injection. These results confirm that NT4 play an important role in the efficient infectivity of sporozoites in mammalian host.

4. Discussion

The malaria parasites are purine auxotrophic protozoan parasites, which lack *de novo* purine synthesis pathways [13]. Because the parasites salvage these nutrients from host cells, the detailed information of the molecules that involved in the salvage from the host will be of great interest to develop purine based antimalarial drugs. The malaria parasites are known to get these nutrients from host cells with the help of a group of proteins known as NT proteins. Four NT proteins are identified in the malaria parasites (NT1-NT4) [14,15]. Previous studies show one of these NTs transporter NT1 seems to be essential for the parasite growth at physiological purine concentration. Gene deletion studies targeting NT1 in *Plasmodium* parasites show the parasites without NT1 are severely growth attenuated [10,16,17]. In contrast to the human malaria parasites, rodent malaria parasites have NT1, NT2, and NT4, however, NT3 has not been reported in any of the rodent or bird malaria parasites.

Earlier studies recognized NT4 as an important determining factor of pharmacokinetics where it plays major role in absorption and distribution of drugs and treatment of leishmaniasis [6]. Interestingly, one study has used codon-optimized synthetic NT4 sequences from *P. falciparum* and *P. vivax* to be expressed in *Xenopus laevis* oocytes and it showed that NT4 can transport Adenine and 2′-Deoxyadenosine [13]. Later, it was confirmed that NT4 in *Leishmania major* is involved in Adenine salvage [18]. The membrane transport capacity of NT4 makes it a crucial drug target. For example, Allopurinol is a purine analog which enters in cells through purine transporter, causing cell death to treat leishmaniasis. Meglumine antimoniate is another purine analog, which is now the first choice drug used to treat Leishmaniasis patients [19,20]. Moreover, the knockdown of NT4 using RNAi in *L. major* results in reduced infection in BALB/c mice [18].

The gene targeting study of the *Pb*NT4 confirms that this gene is not essential for the blood stage growth of *P. berghei*, where *Pbnt4(-)* shows normal blood stage growth as *Pb*WT. This is presumably due

to the fact that NT1 is the main NT for blood stages, and therefore NT4 deletion does not have a significant effect on blood stage growth of the parasites [16,21]. The NT4 gne deletion does not have any significant effect on male gamete exflagellation, ookinete or oocyst development. In our previous study of NT1 deletion in *P. yoelii*, The mosquito transmission of NT1 deficient parasites was completely abolished [10]. However, our current study showed that NT4 deletion does not affect parasite transmission to the mosquito as the oocyst development was similar between both *Pbnt4(-)* and *Pb*WT parasites.

The evaluation of salivary gland sporozoites showed a significant difference in sporozoite numbers between *Pbnt4(-)* and *Pb*WT, where *Pbnt4(-)* deficient parasites show very less salivary gland sporozoites. The number of *Pb*WT sporozoites increased significantly from day 16 to 21, however a very marginal increase in number was observed in NT4 deficient parasites. The low number of salivary gland sporozoites in NT4 deficient parasites is also reported by Kenthirapalan and coworkers [22]. The accumulation of hemolymph sporozoites in significantly higher numbers than salivary gland sporozoites provided clear evidence that sporozoite colonization of the salivary glands is diminished in sporozoites lacking NT4. The knockout sporozoites diminished colonization of the salivary glands, could be due to either defects in migration of sporozoites to and attachment to the salivary glands or a defect in direct invasion of the acinar cells or due to both. Therefore, it is expected that sporozoite infectivity functions in the mammalian host would be similarly impacted as the same infectivity mechanisms are used both in the mosquito and mammalian host [23]. Indeed, the results of the sporozoite infectivity study of NT4 knockout parasites in CD1 mice confirmed essential roles in sporozoite infectivity functions.

In conclusion, here in this study, we showed that NT4 is not essential for blood stage growth of the parasite and is easily accessible for genetic modification. We conclude that NT4 is crucial for infectivity sporozoite functions. The results presented here in this study could be helpful in designing of future studies to evaluate the importance of NT4 in transportation of nutrients across the plasma membrane and develop it as a potential anti-plasmodial drug target. It will also be interesting to explore the cross talk between NT4 and other important nucleoside/nucleobase salvage pathways that will help in designing of more precise strategies in the development of anti-parasitic chemotherapy.

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Author's contribution

Conceptualization: A.S.I.A.; Methodology: G.D., M.K. and A.S.I.A, U.Y.K; Data analysis: G.D., M.K. and A.S.I.A.; Supervision and Funding acquisition: A.S.I.A and B.A.T; Writing and editing of the manuscript: G.D, M.K., A.S.I.A.

Disclosure statement

No conflict of interest was reported by the author(s).

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