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# Subpatent infection with nucleoside transporter 1-deficient *Plasmodium* blood stage parasites confers sterile protection against lethal malaria in mice

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## Summary

**Repeated immunizations with whole *Plasmodium* blood stage parasites and concomitant drug cure of infection confer protective immunity against parasite challenge in mice, monkeys and humans. Moreover, it was recently shown that infections with genetically modified rodent malaria blood stage parasites conferred sterile protection against lethal blood stage challenge. However, in these models vaccination resulted in high parasitemias and, in consequence, carries risk of vaccine-induced pathology and death. Herein, we generated a novel, completely blood stage-attenuated *P. yoelii* rodent malaria strain by targeted deletion of parasite nucleoside transporter 1 (NT1). Immunization of inbred and outbred mouse strains with a single low dose of *Pynt1*$^{-}$ blood stages did not induce any patent infections and conferred complete sterile protection against lethal heterologous blood stage and sporozoite challenges. Partial protection was observed against lethal challenges with another parasite species, *P. berghei*. Importantly, subcutaneous immunization with *Pynt1*$^{-}$ conferred sterile protection against lethal blood stage challenges. We show that cellular and humoral immune responses are both essential for sterile protection. The study demonstrates that genetic manipulation provides a platform for the designed, complete attenuation of malaria parasite blood stages and suggests testing the safety and efficacy of *P. falciparum NT1* knockout strains in humans.**

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## Introduction

Malaria remains a major global cause of disease and death and disproportionately affects children and pregnant women in developing, resource-poor regions of the globe (Greenwood *et al.*, 2005; Snow *et al.*, 2005). Therefore, a vaccine that is safe and confers sterile protection against the pathogenic blood forms of the malaria parasites is urgently needed. However, current blood stage (BS) subunit vaccine candidates have not yielded any protection in field trials (Ogutu *et al.*, 2009; Sagara *et al.*, 2009). An alternative to subunit vaccine approaches is the use of whole pathogens as the immunogen; however, organisms need to be either killed or completely attenuated for use as vaccines. One strategy for achieving attenuation is the inactivation of critical pathogen metabolic pathways.

A major metabolic difference between malaria parasites and their human host is the avenue by which they synthesize purine nucleotides. Mammalian cells harbour the enzymatic machinery necessary for the synthesis of the purine ring *de novo*, whereas *Plasmodia* lack such enzymes and rely solely on the uptake of host purines for the synthesis of the genomes of their daughter cells (Downie *et al.*, 2008). The first step in purine acquisition by *Plasmodium falciparum,* the causative agent of the most severe form of human malaria, is the translocation of host purines into the parasite. This process is mainly mediated by the plasma membrane permease *Pf*NT1 (Carter *et al.*, 2000; Parker *et al.*, 2000; Rager *et al.*, 2001). Genetic studies demonstrated that *Pf*NT1 plays an essential role in parasite development and replication within human erythrocytes (El Bissati *et al.*, 2006; 2008). Parasites lacking *Pf*NT1 are conditionally lethal, growing only when purines are provided at supra-physiological concentrations (El Bissati *et al.*, 2006). This unique property of *PfNT1*-deficient parasites suggested that they could potentially be used as attenuated malaria vaccine strains.

## Results

### *NT1 is a conserved Apicomplexan protein with a constitutive expression pattern in* Plasmodium *life cycle stages*

In order to test the validity of the aforementioned concept, we identified the orthologue of *PfNT1* in the rodent malaria model *Plasmodium yoelii.* Similar to *Pf*NT1, the putative *Py*NT1 has nine conserved transmembrane domains distributed throughout the protein (Fig. 1A). Moreover, *Py*NT1 shares ~60% amino acid sequence identity with *Pf*NT1 and ~25% amino acid identity with the *Toxoplasma gondii* NT1 (Fig. 1A). Transcriptional profiling of *PyNT1* revealed a constitutive expression pattern in multiple life cycle stages of *P. yoelii* (Fig. 1B).

### *Targeted deletion of* PyNT1

We employed targeted deletion to generate nucleoside transporter-deficient parasites (*Pynt1*$^-$) in the *P. yoelii* XNL (nonlethal strain) genetic background (Fig. 2A). We confirmed the integration of the gene replacement construct and the deletion of the *Py*NT1 open reading frame by genomic PCR in two independent enriched populations (from two independent transfection experiments) (Fig. 2B). Reverse transcription (RT)-PCR analysis confirmed the absence of *PyNT1* transcripts in *Pynt1*$^-$ BS (Fig. 2C). Initial observations of the asexual parasitemia of two parental *Pynt1*$^-$ lines showed defective and slow replication of BS parasites in mice. We were unable to clone *Pynt1*$^-$ parasites by limited dilution using 1, 5, 10 and 50 parasites per mouse. Only after starting with 100 *Pynt1*$^-$ parasites as the intravenous inoculum, 15–20% of the recipient mice became patent 11–14 days post inoculation (p.i.).

### Pynt1$^-$ *blood stages are severely growth-attenuated*

To quantitatively evaluate the BS replication deficiency of *Pynt1*$^-$ in comparison to wild-type parasites, we intravenously infected BALB/c mice with 50 and 5000 BS parasites (Fig. 3A and B). Mice infected with 50 *Pynt1*$^-$ did not show detectable parasitemia by thin blood smears up to 30 days p.i. (Fig. 3A). Furthermore, mice infected with 5000 *Pynt1*$^-$ became patent at day 8 p.i. with a peak average parasitemia of 2.4 % at day 12 p.i. (Fig. 3B). In marked contrast, mice infected with 50 and 5000 wild-type BS parasites (isolated from the same transfection experiment) became patent at days 4 and 2 p.i., respectively, and showed average peak parasitemias of 30.2% and 36.5% at days 12 and 11 p.i., respectively (Fig. 3A and B). The results demonstrate that *Pynt1*$^-$ parasites are severely attenuated during BS replication, representing the most severe genetically engineered growth defect of BS parasites identified to date in the murine malaria model.

### Pynt1$^-$ *parasites fail to complete mosquito stage development*

Since *PyNT1* transcripts were detected in other life cycle stages of wild-type parasites (Fig. 1B), we tested if *Pynt1*$^-$ parasites can be transmitted and develop normally in the mosquito. Initial mosquito feeding experiments with *Pynt1*$^-$ failed to detect any oocysts whereas wild-type parasites, isolated from the same transfection experiment, transmitted normally to the mosquito and generated oocyst and salivary gland sporozoites (data not shown). Parasite transmission to mosquitoes is dependent on the initial parasite inoculation density in the mammalian host (Sinden *et al.*, 2007). In order to maximize the likelihood

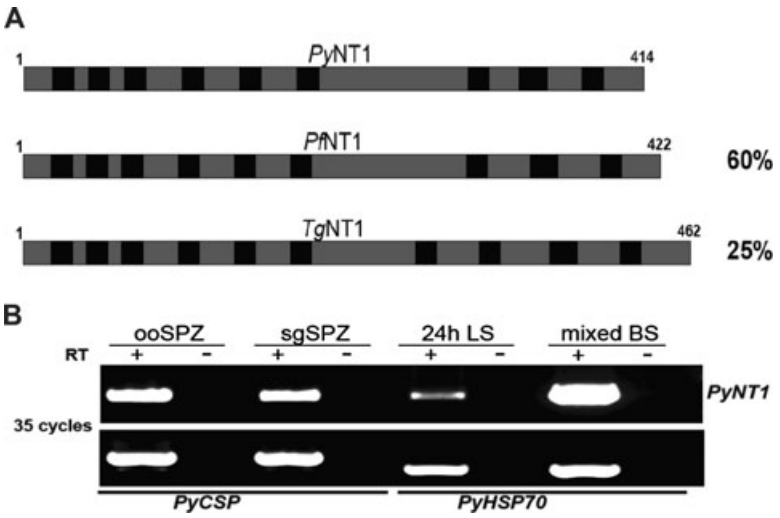


**Fig. 1.** NT1 protein structure, conservation among apicomplexan species and transcriptional profiling.
A. Schematic representation of the putative *Py*NT1 protein organization and alignment with *Pf*NT1 and *Tg*NT1. The transmembrane domains are shown as dark grey boxes. Total amino acid sequence identities to *Py*NT1 are shown to the right.
B. RT-PCR analysis of RNA isolated from *P. yoelii* life cycle stages shows constitutive expression of *PyNT1* in ooSPZ (oocyst sporozoites), sgSPZ (salivary gland sporozoites), mixed blood stages (mixed BS) and 24 h liver stages (24 h LS). *PyCSP* (circumsporozoite protein) is a positive RT-PCR control for sporozoite transcript expression and *PyHSP70* is a positive RT-PCR control for mixed blood stages and 24 h liver stages.

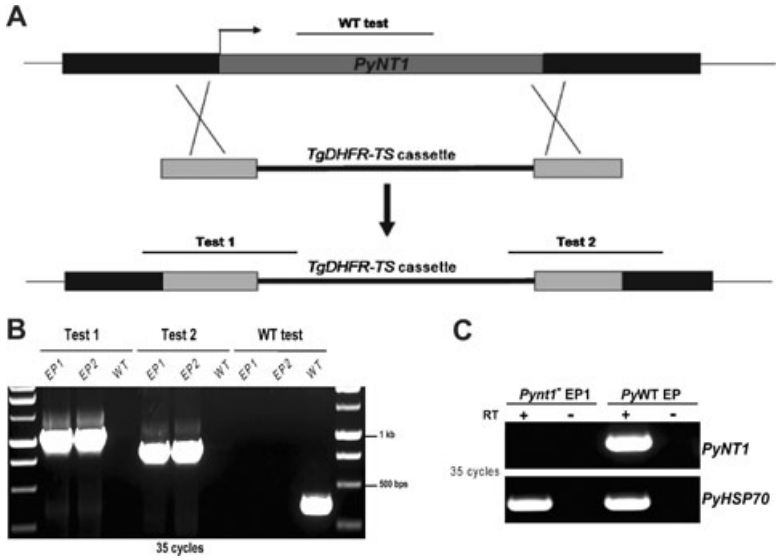


**Fig. 2.** Targeted deletion of *PyNT1*.
A. Schematic representation of the replacement strategy to generate *Pynt1*⁻ parasites. The endogenous *PyNT1* genomic locus is targeted with a replacement fragment containing the 5′ and 3′ *PyNT1* UTRs sequences flanking the *T. gondii DHFR-TS* positive selection marker. Diagnostic wild-type-specific or integration-specific test amplicons are indicated by lines.
B. PCR genotyping shows the gene-replacement using oligonucleotide primer combinations that can only amplify from the recombinant locus (Test 1 and Test 2). The wild-type-specific PCR reaction (WT) confirms the absence of wild-type parasites in the enriched populations of *Pynt1*⁻ parasites.
C. RT-PCR analysis (35 cycles) shows the loss of *PyNT1* transcripts in RNA isolated from *Pynt1*⁻ mixed blood stages. The *PyNT1*-specific amplicon used for the analysis is shown above in panel A as the wild-type (WT) test. *PyHSP70* was used as a positive control.

of a productive mosquito infection, we intravenously infected SW mice with 1 million *Pynt1*⁻ BS or wild-type parasite BS. We monitored the mice for 8 days for parasitemia, gametocytemia and male gamete exflagellation and fed mosquitoes on the infected mice for 5 days (days 3 and 4, days 5 and 6, and day 8) to test for mosquito infection. Interestingly, inefficient micro- and macrogametocytogenesis and male gamete exflagellation occurred with significant delay in *Pynt1*⁻ when compared with wild-type parasites (data not shown). However, even with a low and late occurrence of male gamete exflagellation at days 5, 6 and day 8 (with rates that would allow

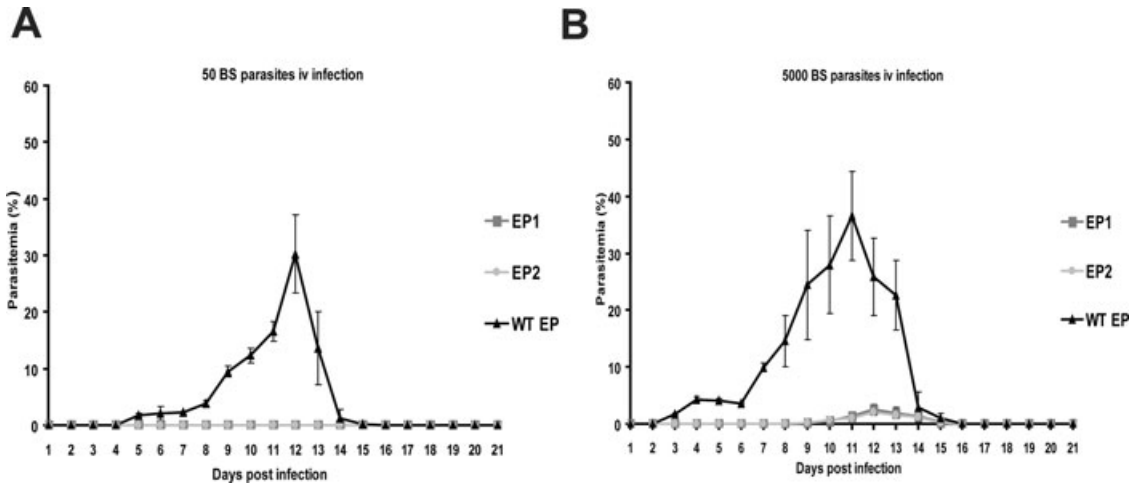


**Fig. 3.** *Pynt1*⁻ BS parasites are severely growth attenuated. Asexual BS parasitemia (percentage of infected erythrocytes) from groups of BALB/c mice (3 mice per genotype for each experiment) after intravenous injection of (A) 50 or (B) 5000 BS parasites of *Pynt1*⁻ (enriched population 1) EP1, EP2 and WT EP. Parasitemia was checked daily for all mice by Giemsa stained thin blood smears for at least 21 days post infection. Note that no parasitemia developed for mice infected with 50 *Pynt1*⁻ EP1 and EP2.

**Table 1.** *Pynt1⁻* parasites are unable to develop into mosquito stages oocysts and generate sporozoites.

| Parasite | Feeding days post infection[a] | Midgut infectivity %[b] | Oocyst sporozoites number/mosquito[b] |
|---|---|---|---|
| *Pynt1⁻* EP1 | 3 and 4 | 0 | 0 |
| *Pynt1⁻* EP1 | 5 and 6 | 0 | 0 |
| *Pynt1⁻* EP1 | 8 | 0 | 0 |
| WT EP | 3 and 4 | 100% | 110 500 |
| WT EP | 5 and 6 | 76% | 48 000 |

**a.** Infection dose was one million BS parasites injected intravenously into each mouse. $N = 4$ mice per genotype.
**b.** Midgut infectivity and oocyst sporozoites numbers/mosquito were determined at day 10 post mosquito feeding from at least 25 mosquitoes per cage for each experiment.

for the development of poor mosquito stage development in case of wild-type), we could not detect any *Pynt1⁻* oocysts on the mosquito midgut (Table 1). Although the lack of transmission could be sexual stage density dependent, the results presented here might also indicate an essential function of NT1 during the development of mosquito stages.

### *Immunization of inbred and outbred mouse strains with a single, low dose of* Pynt1⁻ *BS confers complete protection against lethal malaria challenge*

Although mice inoculated with high doses of *Pynt1⁻* parasites developed low-grade transient parasitemias, inoculation with 50 *Pynt1⁻* parasites never resulted in any detectable BS infection as evaluated by thin blood smears. Therefore, we tested if *Pynt1⁻* immunized mice are protected against a lethal *P. yoelii* challenge. Inbred and outbred mice were immunized with a single dose of 50 *Pynt1⁻* BS. All immunized mice were completely protected against intravenous lethal challenge with 1000 *P. yoelii* YM BS parasites 30 days after immunization (Table 2). Furthermore, separate cohorts of mice immunized with a single dose of 100 *Pynt1⁻* parasites received a lethal challenge of up to 100 000 *P. yoelii* YM BS 3 months after immunization. All mice showed complete protection. Protection was observed in inbred mouse strains as well as outbred mice (Table 2). Mice were also protected against sporozoite challenge (Table 2). Importantly, partial sterile protection was observed when *Pynt1⁻*-immunized mice were challenged with 1000 or 100 000 lethal *P. berghei* ANKA BS parasites 3 months after immunization (Table 2). *Pynt1⁻*-immunized mice, which were not completely protected against the heterologous challenge with *P. berghei* ANKA showed 3–5 days delay in onset of patency, indicating significant but not sterile protection. Strikingly, mice injected subcutaneously with 10 000 *Pynt1⁻* parasites never developed patent infections and were completely protected against a lethal challenge dose of 1000 YM strain BS parasites (Table 2). All groups of immunized mice had strain and age-matched parallel control naïve groups that all developed lethal high parasitemia after challenge. Together, our data show that a genetically engineered BS parasite, which is deficient in an essential nucleoside uptake pathway, is highly attenuated *in vivo* and completely protects against lethal parasite challenge.

### *Cellular and humoral immune responses are critical for sterile,* Pynt1⁻ *BS-induced immunity*

In order to investigate which type of immune responses are mediating sterile protective immunity we used alpha–beta T cell-deficient mice (Tcra(tm1Mom)/J), which lack the alpha–beta T-cell receptor, and mature B cell-deficient mice [Igh-6(tm1Cgn)/J] in *Pynt1⁻* immunizations. Both transgenic mouse strains are in the C57BL/6 genetic background. We immunized all mouse groups with a single dose of 100 *Pynt1⁻* BS (Fig. 4). No BS parasitemia developed in any of the immunized mice, showing that the degree of severe attenuation is an intrinsic feature of the *Pynt1⁻* parasites and independent of the presence of an intact immune system. We next challenged all mouse strains with 10 000 *P. yoelii* YM BS parasites. Both transgenic mouse strains were not protected and developed lethal parasitemias, which were first detected in blood smears at day 4 after challenge. WT C57BL/6 control mice showed complete protection (Fig. 4). Naïve control groups all developed lethal parasitemia after challenge. Thus, we conclude that both alpha–beta T-cell responses and B-cell responses are essential for the development of sterile immunity against BS malaria parasites. Previous studies using whole BS immunizations have identified a critical role for cell-mediated immune responses in long-lasting protective immunity against BS challenge (Pombo *et al.*, 2002; Elliott *et al.*, 2005). Our data confirm this but also indicate that humoral responses are critical for protection.

## Discussion

Previously, a number of studies showed that repeated low-dose immunizations with non-attenuated BS parasites in mice, monkeys and humans under anti-malarial drug treatment (infection treatment vaccination, ITV)

**Table 2.** Sterile protection of outbred and inbred mice against lethal malaria challenge after immunization with subpatent *Pynt1*$^{-}$ BS.

| Immunization dose | Immunization route | Immunized mice | BS patency[a] | Intravenous challenge dose | Challenge period[b] | Protected[c]/ challenged[d] |
|---|---|---|---|---|---|---|
| 10 000 *Pynt1*$^{-}$ EP1 | Subcutaneous | 5 BALB/c | No | 1 000 *P. yoelii* YM BS | 1 month | 5/5 |
| 10 000 *Pynt1*$^{-}$ EP1 FS[e] | Intraperitoneal | 5 SW | No | 1 000 *P. yoelii* YM BS | 1 month | 5/5 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 5 BALB/c | No | 25 000 *P. yoelii* YM sporozoites | 1 month | 5/5 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 5 SW | No | 10 000 000 *P. yoelii* XNL BS | 1 month | 5/5 |
| 100 *Pynt1*$^{-}$EP1 | Intravenous | 4 BALB/c | No | 1 000 *P. yoelii* YM BS | 1 month | 4/4 |
| 50 *Pynt1*$^{-}$ EP1 | Intravenous | 5 BALB/c | No | 1 000 *P. yoelii* YM BS | 1 month | 5/5 |
| 50 *Pynt1*$^{-}$ EP2 | Intravenous | 5 BALB/c | No | 1 000 *P. yoelii* YM BS | 1 month | 5/5 |
| 50 *Pynt1*$^{-}$ EP1 | Intravenous | 5 SW | No | 1 000 *P. yoelii* YM BS | 1 month | 5/5 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 4 BALB/c | No | 1 000 *P. yoelii* YM sporozoites | 3 months | 4/4 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 5 C57BL/6 | No | 25 000 *P. yoelii* YM sporozoites | 3 months | 5/5 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 4 SW | No | 25 000 *P. yoelii* YM sporozoites | 3 months | 4/4 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 4 BALB/c | No | 1 000 *P. yoelii* YM BS | 3 months | 4/4 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 4 BALB/c | No | 100 000 *P. yoelii* YM BS | 3 months | 4/4 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 4 SW | No | 1 000 *P. yoelii* YM BS | 3 months | 4/4 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 5 C57BL/6 | No | 1 000 *P. yoelii* YM BS | 3 months | 5/5 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 4 BALB/c | No | 1 000 *P. berghei* ANKA BS | 3 months | 2/4 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 5 C57BL/6 | No | 1 000 *P. berghei* ANKA BS | 3 months | 2/5 |
| 100 *Pynt1*$^{-}$ EP1 | Intravenous | 5 C57BL/6 | No | 100 000 *P. berghei* ANKA BS | 3 months | 1/5 |

**a.** Determined by the daily examination of > 50 whole microscopic fields (1000× magnification) of Giemsa stained thin blood smears for up to 3 weeks p.i., plus parasitemia examination of > 20 microscopic whole fields at days 25 and 30 p.i..
**b.** The period after the initial BS immunization with *Pynt1*$^{-}$ parasites.
**c.** Protection is defined as the complete absence of BS parasites in challenged mice determined by the daily examination of > 50 whole microscopic fields (1000× magnification) of giemsa stained thin blood smears for up to 2 weeks p.i., plus parasitemia examination of > 20 microscopic whole fields at days 25 and 30 p.i..
**d.** All groups of immunized mice had strain and age-matched parallel control Naïve groups that all developed lethal high parasitemia after challenge.
**e.** Frozen stocks were made by the addition of freezing solution (5% glycerol in Alsever's solution) with 2:1 ratio to BS parasites.

 

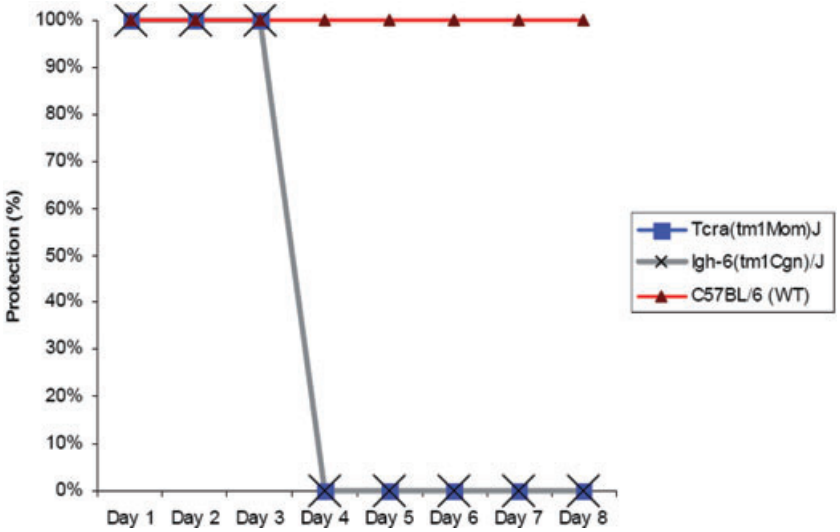


**Fig. 4.** Sterile protection induced by *Pynt1*⁻ BS immunization is dependent on cellular and humoral immune responses. Groups of alpha–beta T cell-deficient mice (Tcra(tm1Mom)/J), mature B cell-deficient mice [Igh-6(tm1Cgn)/J] and C57BL/6 WT control mice (8 mice per group for the transgenic mice and 4 mice for the control mice) were immunized intravenously with a single dose of 100 *Pynt1*⁻ EP1 BS parasites. Graph shows the percentage of mice protected after intravenous challenge with 10 000 *P. yoelii* YM BS parasites. Only C57BL/6 WT mice are completely protected against lethal BS challenge. Protection here is defined as the complete absence of BS parasites in challenged mice determined by the daily examination of > 50 whole microscopic fields (1000× magnification) of giemsa stained thin blood smears for up to 2 weeks p.i., plus parasitemia examination of > 20 microscopic whole fields at days 25 and 30 p.i.. A parallel challenge control naïve mouse group (4 mice per group), with the same genotypes described above, developed lethal parasitemia after intravenous challenge with 10 000 *P. yoelii* YM BS parasites (data not shown).

conferred sterile protection against homologous parasite strain challenge (Collins and Jeffery, 1999a,b; Jones *et al*., 2000; Pombo *et al*., 2002; Elliott *et al*., 2005). The results imply that protective vaccination with whole BS parasites is not model specific. However, the ITV approach is impractical and unlikely to be a feasible path for malaria vaccine development. Furthermore, malaria parasites develop drug resistance against the most effective drugs available (Greenwood *et al*., 2005; Snow *et al*., 2005). Thus, the development of genetically engineered, BS-attenuated parasite strains might provide a significant step forward on a potential path for whole-cell vaccination against malaria. However, the attenuation of the parasite to a degree that results in absence of detectable parasitemia had heretofore not been accomplished.

A recent genetic deletion study of a purine nucleoside phosphorylase (PNP) in *P. yoelii* YM (lethal strain) showed a significant reduction in parasitemia that allowed mice to survive a normally lethal infection (Ting *et al*., 2008). However, the YM strain infects mature RBCs and reticulocytes equally and kills infected mice by excessive parasitemia (> 80%). Indeed, *Pypnp*⁻ parasites were still growing to up to ~30% average parasitemia (Ting *et al*., 2008). Moreover, a more recent study showed that *P. falciparum Pfpnp*⁻ develop *in vitro* parasitemias that are only slightly reduced in comparison to wild-type, without growing under supra-physiological concentrations of exogenous purines (Madrid *et al*., 2008). Therefore, a *Pfpnp*⁻ strain cannot be considered for testing as a live attenuated malaria vaccine candidate in humans. In contrast, *Pynt1*⁻ parasites are attenuated to a degree where low-dose immunizations never resulted in patent BS infection and high dose inoculations only resulted in transient low-grade parasitemias. Yet, a single low-dose immunization conferred protracted sterile protection against lethal BS and sporozoite challenge. Remarkably, protection was also achieved against challenge with a different rodent malaria parasite species. In addition, the lack of NT1 also renders the parasite non-transmissible to the mosquito vector.

Achieving severe growth attenuation of BS parasites in a rodent malaria model *in vivo* by gene deletion as shown here constitutes a critical advance that makes it conceivable to develop and test NT1-deficient *P. falciparum* parasites as an experimental live attenuated malaria vaccine. *In vivo* data using mouse models of malaria constitute an important proof-of-concept step for vaccine candidates before moving on to nonhuman primate testing and human clinical trails. As an important example, radiation-attenuated sporozoite immunizations were first demonstrated to confer sterile protection in mice. Subsequent studies with radiation-attenuated *P. falciparum* sporozoites in humans confirmed the validity of the mouse model data (Nussenzweig *et al*., 1967; Clyde *et al*., 1973). Importantly, the immune responses that develop following BS immunization in mice were shown to be highly similar

to those that are elicited in humans and are associated with protecting humans against subsequent BS challenges (Wykes and Good, 2009; Good, 2009; Wipasa *et al*., 2009, McCarthy and Good, 2010).

*Plasmodium falciparum* parasites lacking *PfNT1* are growth-arrested, but growth can be rescued by the addition of supra-physiological exogenous purines *in vitro* (El Bissati *et al*., 2006). This allows for the production of the attenuated strain in the cell culture system. However, if *Pfnt1*$^{-}$ parasites were to be injected into humans, they would be deprived of the supra-physiological exogenous purines required for their growth, and therefore would likely arrest in their growth and replication and be cleared by the immune system. Furthermore, the fact that subcutaneous immunizations with *Pynt1*$^{-}$ parasites in mice do not induce any patent infections and confer sterile protection against lethal malaria challenge suggests that *PfNT1* could be administered subcutaneously without losing potency. However, considering the aforementioned results from human ITV studies, it is unlikely that a single dose would be sufficient to achieve protection in monkeys or humans (Jones *et al.* 2000; Pombo *et al*., 2002; Collins and Jeffery, 1999a,b).

Lastly, *Pfnt1*$^{-}$ parasites must be produced in human RBC culture using human blood products, thus raising potential safety issues because these products would be part of the inoculum. However, rigorous screening of blood products for extraneous agents might alleviate these concerns. In this context it is of interest to note that the establishment of a human BS challenge model is underway (Moorthy *et al*., 2009). Thus, although many obstacles remain, our data provide a foundation from which to further explore the use of whole BS parasites as a vaccine to protect against malaria.

## Experimental procedures

### *Experimental rodents, mosquitoes and parasites*

Six- to eight-week-old female BALB/c, Swiss Webster (SW) and C57BL/6 mice were purchased from Harlan (Indianapolis, Indiana). Transgenic mice [B6.129S2-Tcra(tm1Mom)J] and [B6.129S2-Igh-6(tm1Cgn)/J] and their WT C57BL/6 control were purchased from Jackson laboratories (Bar Harbor, Maine). Animal handling was conducted according to Institutional Animal Care and Use Committee approved protocols. Mice were infected with either cryo-preserved stocks or by syringe passage of wild-type *P. yoelii* 17XNL (non-lethal strain) clone 1.1 (Landau and Chabaud, 1965; Weinbaum *et al*., 1976; Weiss *et al*., 1989), *Pynt1*$^{-}$ EP1 (enriched population 1) and EP2, *Py*WT EP, *P. yoelii* lethal strain YM (Yoeli *et al*., 1975) or *P. berghei* lethal ANKA strain. *Anopheles stephensi* mosquito blood meal feedings were carried out on SW mice infected intravenously with 1 million parasites of WT EP or *Pynt1*$^{-}$ EP1. Mosquitoes were allowed to feed on *Pynt1*$^{-}$ EP1 or WT EP infected mice (4 mice for each genotype) on days 3 and 4 p.i. (cages A and B respectively) or days 5 and 6 p.i. (cages C and D respectively). Wild-type EP infected mice were sacrificed on day 7 p.i. due to high morbidity. On day 8 p.i. mosquitoes were fed only on *Pynt1*$^{-}$ EP1 (cage E). Mosquito cages had a maximum of ~100 mosquitoes/cage and feeding was allowed on each cage for 5 min/day. Infected mosquitoes were maintained on sugar water at 24°C and 70% humidity. Whole mosquito midguts and oocyst sporozoites were extracted from infected mosquitoes (25 mosquitoes/genotype/experiment) at day 10 post blood meal for evaluation of midgut oocyst infectivity and oocyst sporozoite numbers per mosquito midgut.

### *Generation and cloning of* Pynt1$^{-}$ *parasites*

Targeted deletion of *PyNT1* was achieved by double cross-over homologous recombination with a replacement plasmid in the b3D.DT.H Db. The primer sequences used for constructing the replacement plasmid and testing for integration and *NT1* transcript depletion are listed in Table S1. The transfection procedures and the genomic and RT-PCR conditions are as described in a previous study (Aly *et al*., 2008). We enriched the *Pynt1*$^{-}$ recombinant genotype in the parental transfected parasite population after multiple repeated drug selection treatments until no wild-type genotype was detectable by genomic PCR. Cloning experiments by limited dilution with 1, 10 or 50 drug-resistant *Pynt1*$^{-}$ parasites, using limited dilution, failed to give rise to any detectable BS infections in mice. Some 15–20% of the recipient mice became patent 11–14 days p.i. only after inoculation with 100 *Pynt1*$^{-}$ parasites. Two *Pynt1*$^{-}$-enriched populations (EP1 and EP2) derived from independent transfection experiments were isolated in two different cloning experiments with 100 parasites from each independent parental population. A wild-type enriched population (WT EP) was obtained by limited dilution of 100 parasites from a resistant parental population, the same parental population from which *Pynt1*$^{-}$ EP1 was isolated.

### *Mouse infections and Immunizations with blood stages*

All mouse infections, immunizations and challenges with BS of different genotypes were done intravenously or subcutaneously (through the injection into a dorsal skin-fold) or intraperitoneally. Numbers of asexual BS parasites per microlitre of donor mice-infected blood were determined by giemsa stained thin blood smears (to determine the parasitemia %) and haemocytometer counts of donor mouse erythrocytes. Limited dilution of donor mouse blood, when the parasitemia of donor mice were between 0.1% and 1%, was used to aliquot the specific number of parasites to be injected into each mouse into separate 1.5 ml eppendorf tubes. In immunization experiments, challenged mice were checked daily for BS patency by thin blood smears. All challenged mice were monitored until day 30 for infection. If mice did not develop any BS patency for 30 days after challenge they were recorded as completely protected.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Sequences of primers used in this study, restriction endo-nucleases sites are underlined.

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