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Attenuated *Plasmodium yoelii* lacking purine nucleoside phosphorylase confer protective immunity

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Abstract

Malaria continues to devastate sub-Saharan Africa owing to the emergence of drug resistance to established antimalarials and to the lack of an efficacious vaccine. *Plasmodium* species have a unique streamlined purine pathway in which the dual specificity enzyme purine nucleoside phosphorylase (PNP) functions in both purine recycling and purine salvage^{1–4}. To evaluate the importance of PNP in an *in vivo* model of malaria, we disrupted *PyPNP*, the gene encoding PNP in the lethal *Plasmodium yoelii* YM strain. *P. yoelii* parasites lacking PNP were attenuated and cleared in mice. Although able to form gametocytes, PNP-deficient parasites did not form oocysts in mosquito midguts and were not transmitted from mosquitoes to mice. Mice given PNP-deficient parasites were immune to subsequent challenge to a lethal inoculum of *P. yoelii* YM and to challenge from *P. yoelii* 17XNL, another strain. These *in vivo* studies with PNP-deficient parasites support purine salvage as a target for antimalarials. They also suggest a strategy for the development of attenuated nontransmissible metabolic mutants as blood-stage malaria vaccine strains.

Malaria is caused by a protozoan parasite of the genus *Plasmodium* and is the most deadly vector-borne disease in the world. The World Health Organization estimates that there are 300–500 million clinical cases annually, resulting in approximately 1.5–2.7 million deaths⁵. Because there is no effective vaccine, and drug-resistant parasites are widespread, development of alternative chemotherapeutic targets and new vaccine strategies is crucial.

Unlike their mammalian hosts, malaria parasites cannot synthesize purines *de novo* and depend exclusively on purine salvage and recycling for RNA and DNA synthesis. The purine salvage pathway is streamlined in *Plasmodium*, composed of only adenosine deaminase (ADA), PNP and hypoxanthine-guanine-xanthine phosphoribosyl-transferase^{1–4}. Hypoxanthine is a precursor for all purines and a central metabolite for nucleic acid synthesis in *P. falciparum*⁶. The dual action of *P. falciparum* ADA and PNP for purines and methylthiopurines allows the parasite to form hypoxanthine from host purine pools and to recycle hypoxanthine from methylthio-adenosine, a product of polyamine synthesis. *P.*

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AUTHOR CONTRIBUTIONS

L.-M.T., M.G. and A.C. performed experiments. L.-M.T., P.S. and K.K. planned experiments and analyzed data. L.-M.T. and K.K. wrote the manuscript.

falciparum ADA and PNP function at the intersection of the polyamine metabolism⁷⁻⁹ and purine salvage pathways, essential pathways for viability of *Plasmodium* in its human host.

In this study, we use genetic approaches to investigate the importance of *Plasmodium* PNP *in vivo* in the rodent malaria *P. yoelii* (lethal strain YM). *P. yoelii* is a favored model for vaccine development because of its high infectivity of laboratory mice, particularly in sporozoite infection of hepatocytes¹⁰⁻¹⁴. *P. yoelii* YM, like *P. falciparum*, infects both reticulocytes and mature erythrocytes, whereas nonlethal *P. yoelii* 17XNL and another rodent malaria, *Plasmodium berghei*, infect only reticulocytes. Because rodent and human erythrocytes have ample PNP and purine salvage activity, erythrocytes may provide sufficient purines to sustain *Plasmodium* parasites in infected animals.

The PNP genes from all *Plasmodium* species are highly conserved, with *P. falciparum* PNP being 78% identical at the amino acid level to *P. yoelii* PNP (PyPNP; Supplementary Fig. 1 and Supplementary Table 1 online). Detailed biochemical analysis showed that recombinant PyPNP has substrate specificity and kinetic properties similar to *P. falciparum* PNP (Table 1).

The gene encoding PyPNP was disrupted by a single-crossover insertion strategy^{15,16} (Supplementary Fig. 2 and Supplementary Methods online). The viability of PNP-disruptant *P. yoelii* clones (PyPNP-INT) was compared to a recombinant parasite control with insertion into the PNP-encoding locus without disruption (PyPNP-WT). When PyPNP-INT parasite clones were passaged in mice without drug selection, wild-type revertants developed and rapidly over-grew the PyPNP-INT population (data not shown), providing initial genetic evidence that parasites lacking PNP were less fit than wild-type parasites. To prevent reversion, we adopted a PCR approach¹⁷ to generate PyPNP-knockout clones with the genes encoding the selectable markers human dihydrofolate reductase (hDHFR; Δ PyPNP clones) or hDHFR-gfp, a fusion protein of hDHFR and green fluorescence protein¹⁸ (Δ PyPNP-GFP clones) replacing PyPNP by double crossover homologous recombination (Supplementary Fig. 3 and Supplementary Methods online). In parallel, we created a control strain lacking the gene encoding the circumsporozoite protein or CSP (Δ PyCSP), a gene that is not essential in erythrocytic stages¹⁹.

The virulence of PNP-deficient clones was compared to recombinant controls by infecting BALB/c mice and monitoring parasitemia for 30 days (Fig. 1). The growth rate of PyPNP-INT parasites was slower than that of the wild-type *P. yoelii* YM strain and of PyPNP-WT (Fig. 1a). All WT-infected and PyPNP-WT-infected mice developed high parasitemias and died within 10 d of challenge (Fig. 1a,b). Similarly, Δ PyPNP and Δ PyPNP-GFP parasites had no detectable impairment of intraerythrocytic development, but growth of these lines was impaired compared to that of WT or Δ PyCSP parasites (Fig. 1c,d). None of the parasite lines lacking PNP (PyPNP-INT, Δ PyPNP and Δ PyPNP-GFP) were lethal to mice, and infected mice recovered from infection with no detectable parasitemia (Fig. 1b,d). The PyPNP-INT, Δ PyPNP and Δ PyPNP-GFP clones were also attenuated in Swiss Webster mice (data not shown). Thus PNP is crucial for optimal replication of malaria parasites during the erythrocytic cycle, despite the considerable levels of PNP activity present in mammalian erythrocytes.

There are other points in the life cycle at which parasites have substantial purine requirements. The sexual-stage gametocytes are taken up by mosquitoes from mammalian blood. In the mosquito midgut, male gametocytes undergo exflagellation, a process that involves four rapid nuclear divisions and results in formation of eight microgametes within 15 min. Ookinetes develop from the fertilized zygote and migrate across the mosquito midgut, where they form oocysts. Thousands of sporozoites are made within each oocyst.

These sporozoites invade mosquito salivary glands and are injected into the mammalian host. After sporozoite invasion of hepatocytes, the parasites develop into exoerythrocytic forms that undergo extensive multiplication, eventually releasing tens of thousands of merozoites into the circulation.

We analyzed oocyst and sporozoite development in *Anopheles stephensi* mosquitoes fed on mice infected with wild-type, *PyPNP*-WT, *PyPNP*-INT or Δ *PyPNP* parasite clones bearing similar numbers of mature gametocytes. At day 8 after feeding, *PyPNP*-INT-infected mosquitoes had markedly fewer numbers of oocysts compared to WT- or *PyPNP*-WT-infected mosquitoes, and we detected no Δ *PyPNP* oocysts (Table 2). On day 15 after feeding, *PyPNP*-INT- or Δ *PyPNP*-infected mosquitoes had no detectable sporozoites (Table 2). The numbers of Δ *PyCSP* oocysts were normal, but infected mosquitoes had no sporozoites in their salivary glands, confirming that CSP is essential for development of *P. yoelii* sporozoites in mosquitoes, as previously described for *P. berghei*¹⁹. Thus PNP activity is key in blood stage development and crucial for mosquito stage development.

Because attenuated microorganisms are frequently used as vaccines, we tested whether *PNP*-knockout parasites protected against subsequent malaria infection (Table 3). Naive mice challenged with *PyPNP*-INT, Δ *PyPNP* or Δ *PyPNP*-GFP survived and remained parasite-free. (Table 3). These mice were rechallenged 6–8 weeks later with a lethal dose of wild-type *P. yoelii* YM parasites (2×10^5 infected erythrocytes). Immunized mice were completely protected, with none developing detectable parasitemia (Table 3).

The protection conferred by infection with attenuated PNP-deficient parasites was long lasting and also provided protection from the nonlethal 17XNL strain of *P. yoelii*. Twenty Δ *PyPNP*-immunized mice surviving a lethal challenge with YM (Experiment 3; Table 3) had no detectable parasitemia after challenge with *P. yoelii* 17XNL erythrocytic stages 5 months after initial exposure to Δ *PyPNP*. Age-matched control mice (five mice) developed parasitemias of up to 50% and then cleared the infection (data not shown).

Mice were also protected from 17XNL sporozoite challenge (Supplementary Fig. 4 online). Five BALB/c mice inoculated with 2.0×10^4 *P. yoelii* YM Δ *PyPNP* were exposed to the bites of 35 *A. stephensi* mosquitoes infected with *P. yoelii* 17XNL. Four of these mice developed low level parasitemia on days 3–5 that resolved within 3 d, with peak parasitemia of 0.05%. The naive mice all developed high parasitemias (up to 80%) but then cleared the infection (Supplementary Fig. 4).

Attenuated whole-parasite vaccines, particularly irradiated attenuated sporozoites, have been proposed as alternatives to subunit vaccines^{20–22}. Whereas the implementation of such vaccines poses considerable logistical difficulties, better protection has been elicited with attenuated sporozoites than with candidate subunit vaccines²². More recently, genetically attenuated sporozoites have been developed²³. Whole-parasite vaccines may circumvent the problems resulting from heterogeneous host responses to individual antigens by providing an array of conserved immunogenic proteins less subject to immune pressure than current subunit vaccine components²⁴.

A small study in humans has suggested that low levels of malaria infection may be protective in humans²⁵. This study was performed by drug-curing infected individuals and suggests that repeated low-level malaria infection may be protective²⁵. Routine administration of chemotherapy is constrained by the widespread resistance to commonly used antimalarials and has the potential to enhance the rate of selection of drug-resistant organisms. An alternative approach might be vaccination with a highly attenuated asexual stage parasite that clears spontaneously.

Development of a live attenuated vaccine strain requires a balance between the level of attenuation and the immunogenicity of the vaccine to optimize safety and efficacy. Some level of parasite replication will probably be required to mount an effective immune response^{26,27}. The parasitemias detected after infection with *PNP*-deficient parasites are higher than are likely to be tolerated in humans, but our studies illustrate that development of attenuated erythrocytic stage metabolic mutants is feasible. Although attenuated sporozoite vaccines are promising, their success is dependent upon complete protection, as a single successful sporozoite infection with development of merozoites within hepatocytes could lead to symptomatic malaria²⁸. In contrast, even a partially effective erythrocytic stage malaria vaccine could have considerable clinical impact on morbidity and mortality by decreasing the incidence of severe disease, such as cerebral malaria.

Ideally, genetically engineered *Plasmodium* vaccine strains will not revert to wild-type virulence but will be able to establish a self-limited, subclinical and protective course of infection. Because disruption of the gene encoding *P. falciparum* PfNT1 (ref. 29), a purine nucleoside transporter, results in parasites that cannot survive at physiological concentrations of purines, parasite strains with multiple purine-salvage deletions will probably be more highly attenuated than single-deletion strains. Parasite strains lacking PNP can still make gametocytes and may have activity as transmission-blocking vaccines while not being transmissible. Although we favor nontransmissible vaccines as being safer, it is possible that attenuated vaccine strains that can infect mosquitoes will promote protective immunity beyond the vaccinee and therefore be more beneficial to the general population. Finally, our studies establish the importance of *Plasmodium* purine-salvage enzymes for *in vivo* survival and support continued exploration of purine salvage as a chemotherapeutic target.

METHODS

Cloning and expression of *P. yoelii* PNP

PyPNP was identified as a protein of 244 amino acids and 26.943 kDa by protein sequence homology to *P. falciparum* PNP (gene PY04622; <http://www.PlasmoDB.org/>). We amplified the PyPNP open reading frame sequence by PCR from genomic DNA of *P. yoelii* strain YM with primers PyPNP-F (5'-GATGAGGAACAAAGACATATAAAGC-3') and PyPNP-R (5'-ATATTTTCTGATAATCTTGCACAA-3'). We ligated the 729-base pair PCR product into a pTrcHis2-TOPO vector (Invitrogen) and expressed it in *E. coli* strain Top 10. We induced expression of recombinant PyPNP with a C-terminal 6 × His tag and the c-MYC epitope with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 10 h and purified recombinant protein by nickel chromatography. The purified PyPNP was >95% homogeneous, as indicated by denaturing PAGE and staining with Coomassie blue. We measured protein concentration by Bradford protein assay (Bio-Rad).

Enzymatic assays and determination of kinetic constants

We performed all phosphorylase activity assays with purified enzyme in 50 mM KPO₄ pH 7.4 as previously described¹. Briefly, we measured phosphorylysis of inosine, 2'-deoxyinosine, and 5'-methylthioinosine in a coupled assay with 115 milli-units ml⁻¹ xanthine oxidase to convert hypoxanthine into uric acid. We followed uric acid formation by spectrophotometric measurement of absorbance at 293 nm ($\epsilon_{293} = 12.9 \text{ mM}^{-1} \text{ cm}^{-1}$). We monitored phosphorylysis of guanosine to guanine by measuring the disappearance of guanosine at an absorbance of 258 nm ($\epsilon_{258} = 5.2 \text{ mM}^{-1} \text{ cm}^{-1}$). We measured adenosine and 5'-methylthioadenosine phosphorylase activities by following the disappearance of the substrate at an absorbance of 265 nm ($\epsilon_{265} = 1.9 \text{ mM}^{-1} \text{ cm}^{-1}$). We measured uridine phosphorylase activity by following the conversion of uridine to uracil at an absorbance of

272 nm ($\epsilon_{272} = 2.9 \text{ mM}^{-1} \text{ cm}^{-1}$). We determined the Michaelis constant K_m as the substrate concentration at one-half the maximum velocity. We determined the catalytic constant k_{cat} for each substrate by fits of substrate saturation data to the Michaelis-Menten equation. Values for k_{cat} assume one catalytic site per subunit and are expressed as moles of product per second per mole of subunit.

Phenotypic analysis of parasite growth and infectivity in mice

To determine whether the disruption of *PyPNP* had an effect on parasite growth *in vivo*, we injected parasite inocula (2×10^4 or 2×10^5 blood-stage parasites) intravenously into groups of BALB/c mice (female, 8 weeks of age). We prepared a thin blood smear daily from each infected mouse and stained it with Giemsa reagent (Sigma). For each sample, we examined at least 1,000 red blood cells by microscopy and calculated the percentage of infected erythrocytes (parasitemia). All experiments with mice were conducted in Association for Assessment and Accreditation of Laboratory Animal Care–approved facilities after review and approval of experimental protocols by the Institutional Animal Care and Use Committees of Albert Einstein College of Medicine and New York University School of Medicine.

Phenotypic analysis of parasite development in mosquitoes

We infected *A. stephensi* mosquitoes with *P. yoelii* parasites by blood-feeding on two sequential days for 15 min on infected Swiss Webster mice, and we subsequently maintained them under 80% humidity at 24 °C. We monitored the presence of gametocytes on Giemsa-stained blood smears before mosquito blood meal. We dissected infected mosquitoes at days 8 and 15 after the first infectious blood meal to evaluate the development of parasites by counting the numbers of oocysts per midgut and the numbers of sporozoites per salivary gland. We determined the number of sporozoites per salivary gland by mixing the salivary glands of 20 infected mosquitoes in 200 μl of PBS and counting the numbers of sporozoites in duplicate in a hemocytometer.

Statistical analyses

We analyzed statistical significance by analysis of variance with the GraphPad Prism software package.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

P. yoelii lacking PNP are attenuated. (a–d) Parasitemia (percentage of infected erythrocytes $\pm$ s.d.; a,c) and survival curves (b,d) from groups of five female BALB/c mice (age 8–10 weeks) infected with the indicated parasite lines. Blood smears were made daily, stained with Giemsa and counted. For experiments with single crossover integrants (a,b), mice were infected with 2×10^5 infected erythrocytes from wild-type (wild-type YM), *PyPNP-WT* clone 1 (*PNP* control integrant), *PyPNP-INT* clone 1-3 (*PNP* disruptant) or *PyPNP-INT* clone 2-3 (*PNP* disruptant). For experiments with double-crossover knockout lines (c,d), mice were infected with 2×10^4 wild-type (wild-type YM), Δ *PyCSP* clone 2-2 (*CSP* knockout), Δ *PyPNP* (*PNP* knockouts made with hDHFR cassette; clones 3-4, 4-2 and 4-5), Δ *PyPNP-GFP* clone 8 (*PNP* knockout made with hDHFR-GFP cassette). All mice were infected with parasites on day 0 by tail intravenous injection. For each parasite line, parasitemia was checked daily for 30 d (at least 7 d after parasitemia was no longer detectable).

Table 1

Kinetic constants of *Plasmodium* PNP

Substrate	<i>P. yoelii</i> PNP			<i>P. falciparum</i> PNP		
	K_m (μ M)	k_{cat} (s^{-1})	k_{cat} / K_m ($M^{-1} s^{-1}$)	K_m (μ M)	k_{cat} (s^{-1})	k_{cat} / K_m ($M^{-1} s^{-1}$)
Inosine	8.6 ± 1.5	3.5 ± 0.1	4.0×10^5	4.7 ± 0.9	1.1 ± 0.2	2.3×10^5
Methylthioinosine	5.0 ± 0.4	3.4 ± 0.1	6.8×10^5	10.8 ± 0.9	2.6 ± 0.8	2.4×10^5
Guanosine	8.9 ± 1.6	3.8 ± 0.2	4.2×10^5	9.4 ± 1.2	2.6 ± 0.5	2.8×10^5
2'-deoxyinosine	79 ± 10	1.2 ± 0.1	1.5×10^4	91 ± 35	0.9 ± 0.3	9.8×10^3

Parasite development in mosquitoes

Table 2

Experiment	Parasite clone	No. of midgut oocysts per mosquito ^a	No. of salivary gland sporozoites per mosquito ^b	No. of infected / No. of mice bitten by mosquitoes	Pre-patent period (d) ^c ; genotype ^d
1	WT clone	47.7 ± 10.2	11,875	2/2	3; WT
	<i>PγPNP</i> -WT C1	45.4 ± 6.5	11,000	2/2	3; <i>PγPNP</i> -WT
	<i>PγPNP</i> -INT C1-3	2.8 ± 0.6	0	0/2	— ^e
2	WT clone	42.8 ± 7.5	7,750	2/2	3; WT
	<i>PγPNP</i> -WT C2	40.6 ± 7.4	8,000	2/2	3; <i>PγPNP</i> -WT
	<i>PγPNP</i> -INT C1-4	1.5 ± 0.5	0	1/2	4; WT ^e
3	WT clone	46.8 ± 7.9	8,875	2/2	3; WT
	$\Delta P\gamma CSP$ C2-2	41.4 ± 10.1	0	0/2	— ^e
	$\Delta P\gamma PNP$ C4-5	0	0	0/2	— ^e
	$\Delta P\gamma PNP$ -GFP C8	0	0	0/2	— ^e

^aMean number of oocysts per midgut was determined by dissecting at least 20 mosquitoes at day 8. All three experiments show statistically significant differences by analysis of variance; $P < 0.0001$.

^bMean number of sporozoites per salivary gland was determined by dissecting 20 mosquitoes at day 15.

^cNumber of days after mosquito bite until detectable blood stage parasites by microscopic examination of blood smear.

^dGenotype of blood stage parasites recovered after sporozoite infection by mosquitoes was analyzed by PCR with genomic DNA as template. *PγPNP*-WT is the control recombinant, whereas WT indicates the original wild-type *PNP* genotype.

^eWild-type parasites were recovered from a mouse bitten by mosquitoes infected with *PγPNP*-INT, suggesting that wild-type revertants are able to develop into infectious sporozoites. Similar reversion was described during characterization of *P. berghei* TRAP (thrombospondin-related anonymous protein) single-crossover disruptants³⁰.

Table 3Protection of immunized mice against challenge with wild-type *P. yoelii*

Experiment ^a	Mouse strain	Immunization with parasite clone; no. of parasites	No protected / challenged (% protected)	Detection of parasitemia ^b
1	BALB/c	<i>PyPNP</i> -INT C1-4; 2.0×10^5	10/10 (100)	Not detectable
2	BALB/c	<i>PyPNP</i> -INT C1-3; 2.0×10^5	5/5 (100)	Not detectable
	BALB/c	<i>PyPNP</i> -INT C2-3; 2.0×10^5	5/5 (100)	Not detectable
	BALB/c	None	0/5 (0)	Day 2
3	BALB/c	Δ <i>PyPNP</i> ^c ; 2.0×10^4	20/20 (100)	Not detectable
	BALB/c	None	0/5 (0)	Day 2
4	Swiss Webster	<i>PyPNP</i> -INT C1-4; 1.0×10^7	3/3 (100)	Not detectable
5	Swiss Webster	<i>PyPNP</i> -INT C1-3; 1.0×10^7	5/5 (100)	Not detectable
	Swiss Webster	None	0/5 (0)	Day 2
6	Swiss Webster	Δ <i>PyPNP</i> C4-5; 1.0×10^7	3/3 (100)	Not detectable
	Swiss Webster	Δ <i>PyPNP</i> -GFP C8; 1.0×10^7	3/3 (100)	Not detectable
	Swiss Webster	None	0/3 (0)	Day 2

^a Age-matched mice were used as naive controls. All mice were challenged with an intravenous inoculum of 2.0×10^5 *P. yoelii* YM-infected erythrocytes.

^b Blood smears were monitored daily for 14 d. Number of days indicates when parasitemia was detectable after mice were challenged with wild-type *P. yoelii* YM.

^c A total of 20 female BALB/c mice were infected with Δ *PyPNP* clones C3-4, C4-2, C4-5 or Δ *PyPNP*-GFP C8 (five mice per clone). These mice were later rechallenged with 2.0×10^5 *P. yoelii* 17XNL at 7 months of age and did not develop detectable parasitemia.