

Suppression of *Plasmodium chabaudi* Parasitemia Is Independent of the Action of Reactive Oxygen Intermediates and/or Nitric Oxide

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The killing of blood-stage malaria parasites *in vivo* has been attributed to reactive intermediates of oxygen (ROI) and of nitrogen (RNI). However, in the case of the latter, this contention is challenged by recent observations that parasitemia was not exacerbated in nitric oxide synthase (NOS) knockout (KO) (NOS2^{-/-} or NOS3^{-/-}) mice or in mice treated with NOS inhibitors. We now report that the time course shows that *Plasmodium chabaudi* parasitemia in NADPH oxidase KO (p47^{phox}^{-/-}) mice also was not exacerbated, suggesting a minimal role for ROI-mediated killing of blood-stage parasites. It is possible that the production of protective antibodies during malaria may mask the function of ROI and/or RNI. However, parasitemia in B-cell-deficient J_H^{-/-} × NOS2^{-/-} or J_H^{-/-} × p47^{phox}^{-/-} mice was not exacerbated. In contrast, the magnitude of peak parasitemia was significantly enhanced in p47^{phox}^{-/-} mice treated with the xanthine oxidase inhibitor allopurinol, but the duration of patent parasitemia was not prolonged. Whereas the time course of parasitemia in NOS2^{-/-} × p47^{phox}^{-/-} mice was nearly identical to that seen in normal control mice, allopurinol treatment of these double-KO mice also enhanced the magnitude of peak parasitemia. Thus, ROI generated via the xanthine oxidase pathway contribute to the control of ascending *P. chabaudi* parasitemia during acute malaria but alone are insufficient to suppress parasitemia to subpatent levels. Together, these results indicate that ROI or RNI can contribute to, but are not essential for, the suppression of parasitemia during blood-stage malaria.

The adaptive immune response to blood-stage malaria parasites is dependent on both cell- and antibody-mediated immune mechanisms. Accumulating evidence indicates that activated CD4⁺ T cells release factors, including gamma interferon, which induce downstream mechanisms to kill parasites (reviewed in references 32, 34, and 52). Observations of fragmented DNA and condensed chromatin suggest that apoptotic and/or cytotoxic mechanisms are responsible for the killing of blood-stage malaria parasites (40). Both reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), especially nitric oxide (NO), have been suggested as possible malaria-parasite-killing agents (9, 50).

Based on observations that different ROI-generating chemicals, such as *tert*-butyl hydroperoxide, alloxan, and phenylhydrazine, and ROI enzyme-producing substrate systems had the ability to kill murine and human malarial parasites, Clarke et al. proposed that ROI function to kill malaria parasites (9, 11). ROI are utilized in the body as oxidative, cytotoxic agents and are produced by phagocytic cells during the oxidative burst induced by infection (44). The majority of ROI, hydrogen peroxide (H₂O₂) or superoxide ions (O₂⁻), are produced via two pathways, involving phagocyte NADPH oxidase or hypoxanthine metabolism (3, 24). In the phagocyte NADPH oxidase pathway, a complex of four structural proteins, flavocytochrome b₅₅₈ (consisting of gp91^{phox} and p22^{phox}), p47^{phox}, p67^{phox}, and the protein encoded by *rac*, is required for the oxidation of O₂ to H₂O₂ and O₂⁻ (1, 24, 31, 33, 38, 45, 55). During hypoxanthine metabolism, xanthine oxidase (XO) cat-

alyzes the oxidation of hypoxanthine and xanthine in the presence of O₂ and Fe³⁺ to produce uric acid, H₂O₂, and O₂⁻ (3, 16). Both H₂O₂ and O₂⁻ can function independently, as cytotoxic agents, or form other toxic molecules, including the hydroxyl radical OH⁻, hypochlorous acid (HOCl), and peroxyinitrite (ONOO⁻) in the presence of NO (4).

Taylor-Robinson et al. (50) initially reported that NO killed blood-stage *Plasmodium chabaudi* parasites. The NO molecule is the product of a reaction involving the NO synthase (NOS) enzyme with L-arginine in the presence of oxygen (36, 37). NO is highly reactive but has a relatively short half-life under physiological conditions. Three isoforms of the NOS enzyme have been characterized based on the tissue localization of the enzyme. They include neuronal NOS, or NOS1, expressed by nerve cells during neurotransmission, inflammatory NOS, or NOS2, produced by phagocytic cells in response to pathogens, and endothelial NOS, or NOS3, produced by vascular endothelial cells to promote vasodilation (reviewed in reference 15). Because of its location in the brain parenchyma, NOS1 in all likelihood does not contribute to parasite killing during blood-stage *Plasmodium* infections, since the replication of these parasites is confined to the blood compartment. In addition, van der Heyde et al. (54) reported that the levels of NO found in serum during malaria could not be attributed to the function of NOS3 but instead were the product of NOS2 activation. Favre et al. (14) were the first to propose that NO does not kill blood-stage malarial parasites. Subsequent studies by others utilizing NOS2 knockout (KO) mice and NOS inhibitors reported that the time courses of parasitemia in wild-type and NOS2-deficient animals were comparable, thereby supporting the contention of Favre et al. that NO had little if any role in the elimination of blood-stage malarial parasites (54, 59).

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Because NO and O_2^- are extremely reactive molecules, they can form ONOO⁻ under normal physiological conditions (18, 22). Unlike NO, which has antioxidant, anti-inflammatory, and tissue-protective properties, ONOO⁻ is capable of damaging lipids (42), proteins (23, 28), and nucleic acids (28) while producing redox-induced apoptosis (56), or ONOO⁻ can protonate to generate OH[•] and nitrogen dioxide radicals ($\cdot NO_2$), both more reactive than the peroxynitrite precursors (4). Peroxynitrite has a critical role in the immune mechanism against numerous pathogens. Not only is ONOO⁻ necessary for the killing of *Mycoplasma pulmonis* and *Rhodococcus equi* (12, 20), but it is also toxic to many different organisms, including *Trypanosoma* and *Salmonella* spp., most of which have generated defense mechanisms to nullify the toxic effect of ONOO⁻ (7, 51). Although the effect of ONOO⁻ on blood-stage malaria parasites is unknown, Pino et al. (41) have recently suggested that ONOO⁻ may play a role in the pathogenesis of cerebral malaria by inducing apoptosis of endothelial cells.

The issue of compensation, i.e., the parasite-killing function of ROI in place of RNI and vice versa, has not been addressed in previous studies which have focused on the activities of either ROI or RNI alone. Likewise, the possibility of protective antibodies masking RNI and/or ROI antiparasitic activity has been only partially addressed in the studies of van der Heyde et al. (54), who reported that the time course of *P. chabaudi* parasitemia was nearly identical in knockout mice deficient in both B cells and NOS2 and in B-cell-deficient control mice. We have now examined the ability of both B-cell-intact and -deficient mice with genetic mutations of the NOS2, p47^{phox}, or both NOS2 and p47^{phox} genes to suppress acute *P. chabaudi adami* infections. We also examined the ability of mice treated with either aminoguanidine hemisulfate (AG) or allopurinol, known NO or XO inhibitors, respectively, to clear acute infection. Our findings indicate that neither ROI, NO, or ONOO⁻ has an essential role in antibody- or cell-mediated immunity resulting in the destruction of blood-stage malaria parasites.

MATERIALS AND METHODS

Mice. The original breeding stock of p47^{phox}^{-/-} mice was a gift from Steven Holland (National Institutes of Health, Bethesda, Md.). The p47^{phox}^{-/-} mouse, lacking the cytosolic p47^{phox} peptide, fails to activate phagocytic NADPH oxidase, preventing the production of ROI in phagocytic cells (25, 26). Breeding pairs of NOS2^{-/-} mice and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). NOS2^{-/-} mice lack the ability to activate inflammatory nitric oxide synthase, which is responsible for the production of NO by cells of the immune system, primarily macrophages and neutrophils (30, 49). The original breeding stock of B-cell-deficient, J_H^{-/-} mice was kindly provided by Dennis Huszar (Gen Pharm International, Mountain View, Calif.). J_H^{-/-} mice have immunoglobulin J_H gene segment deletions which prevent B-cell differentiation beyond the CD43⁺ precursor stage (8). With the exception of J_H^{-/-} mice, which were on a 129/C57BL/6 background, all knockout mice have been backcrossed to C57BL/6 mice for at least 12 generations. Mice of both sexes, 6 to 12 weeks of age, were used in the experiments. All animals were housed, bred, and cared for by the Animal Care Unit at the University of Wisconsin Medical Sciences Center (Madison, Wisc.).

Double-KO mice, lacking both NOS2 and p47^{phox} functional genes, were produced by mating p47^{phox}^{-/-} and NOS2^{-/-} mice. The F₁ progeny were then mated with parental p47^{phox}^{-/-} mice. The F₂ progeny were genotyped by using primers and conditions described previously, and mice homozygous for the mutated p47^{phox} gene and hemizygous for the NOS2 gene were mated (26, 57). The genotype of the resulting F₃ progeny was determined by PCR to obtain mice homozygous for both the p47^{phox} and NOS2 mutant genes (26, 30). Other double-KO mice, J_H^{-/-} × p47^{phox}^{-/-} and J_H^{-/-} × NOS2^{-/-}, were produced

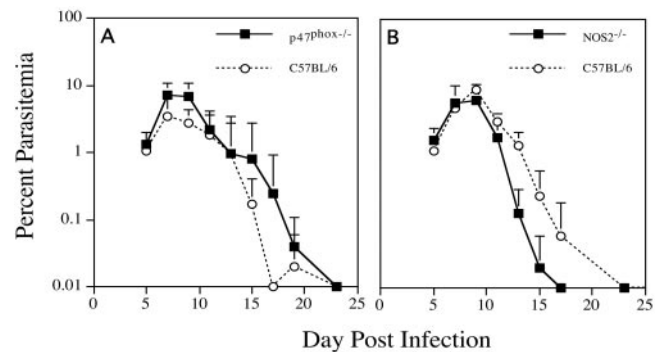


FIG. 1. The time courses of parasitemia in p47^{phox} KO ($n = 8$) and control ($n = 6$) mice (panel A) and in NOS2 KO ($n = 6$) and control ($n = 5$) mice (panel B) infected with 10^6 *P. chabaudi*-parasitized erythrocytes. Data represent the mean (\pm standard deviation [SD]) parasitemia for each time point. These experiments were repeated twice with similar results.

following the same breeding scheme. Genotyping of these mice was achieved by PCR analysis, utilizing primers and conditions described previously (8, 26, 30, 57).

Parasites and infection of mice. The malarial parasite *P. chabaudi adami* 556 KA, referred to as *P. chabaudi*, was maintained as frozen stabilate material and used as previously described (21). Inoculum for the experimental animals was obtained from a BALB/c source mouse that was injected intraperitoneally (i.p.) with thawed stabilate material. Age- and sex-matched experimental and control mice were infected i.p. with 10^6 parasitized erythrocytes or intravenously (i.v.) with 10^5 parasitized erythrocytes as noted. Parasitemia was estimated by enumerating between 200 and 1,000 erythrocytes on Giemsa-stained thin blood films beginning on day 5 postinoculation (PI) and every other day thereafter until the completion of the experiment. The percent parasitemia was calculated as follows: % parasitemia = (number of parasitized erythrocytes/total number of erythrocytes) \times 100.

Allopurinol treatment. To inhibit XO, experimental mice were injected daily via oral gavage with allopurinol (Sigma-Aldrich, St. Louis, Mo.) at a dose of 0.5 mg/kg of body weight suspended in 0.5 ml of dry milk reconstituted with 0.1% (wt/vol) phosphate-buffered saline (PBS), pH 7.4, as previously described by Segal et al. (47). Control mice were administered 0.5 ml of dry milk reconstituted with PBS via oral gavage. The solutions were prepared immediately prior to administration.

NOS inhibition. Mice were injected i.p. with AG (Sigma-Aldrich), a nonspecific NOS inhibitor, to prevent the production of NO. Experimental mice were treated twice daily with 5 mg of AG reconstituted in 0.2 ml of PBS (pH 7.4) per mouse; the solution was prepared immediately before injection (5). Control mice were injected with 0.2 ml of PBS.

Statistical analysis. Statistical analysis was performed with the unpaired, two-tailed, Student's *t* test and GraphPad Prism3 software. A *P* value of <0.05 was considered statistically significant.

RESULTS

Time course of *P. chabaudi* infection in p47^{phox}^{-/-} and NOS2^{-/-} mice. To assess whether ROI produced via the NADPH oxidase pathway are essential in the clearance of *P. chabaudi* blood-stage parasites, we examined the time course of the parasitemia in p47^{phox}^{-/-} mice compared to that in C57BL/6 mice (Fig. 1A). Parasitemia in experimental and control groups followed a similar time course, reaching a peak level on day 7 and declining to subpatent levels at approximately 3 weeks PI. At the peak, the mean parasitemia percentages in p47^{phox}^{-/-} and control mice were similar, being $7.13 \pm 3.90\%$ and $3.45 \pm 3.28\%$, respectively.

To determine whether NO produced by inducible NOS is vital for the clearance of *P. chabaudi* during blood-stage infec-

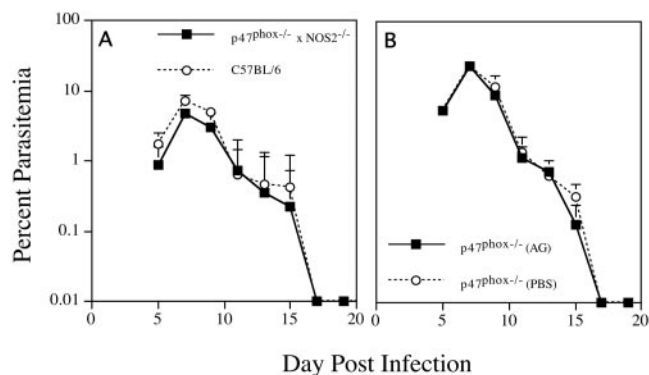


FIG. 2. The time courses of parasitemia in $p47^{phox-/-} \times NOS2^{-/-}$ double-KO ($n = 6$) and control ($n = 4$) mice infected i.p. with 10^6 *P. chabaudi*-infected erythrocytes (panel A) and in $p47^{phox}$ KO mice treated with aminoguanidine ($n = 4$) or phosphate-buffered saline ($n = 3$) and infected i.v. with 10^5 *P. chabaudi*-parasitized erythrocytes (panel B). Data represent the mean (\pm SD) parasitemia for each time point. These experiments were repeated twice with similar results.

tion, the time courses of infection in $NOS2^{-/-}$ and C57BL/6 mice were compared (Fig. 1B). Parasitemia in experimental and control groups peaked on day 9 ($6.32 \pm 4.62\%$ and $8.88 \pm 1.68\%$, respectively) and declined to subpatent levels by day 20 PI; the time course of infection was similar for both groups. Differences in experimental and control mean parasitemia values were statistically significant ($P = 0.003$) only on day 13 PI, when the parasitemia in control mice exceeded that in $NOS2^{-/-}$ mice.

***P. chabaudi* parasitemia in $p47^{phox-/-} \times NOS2^{-/-}$ mice.** To address the possibility that ROI and NO function in a redundant fashion to suppress *P. chabaudi* parasites, the time courses of infections were monitored in groups of double-KO ($p47^{phox-/-} \times NOS2^{-/-}$) and control ($p47^{phox+/-} \times NOS2^{-/-}$) mice (Fig. 2A). Peak parasitemia was achieved on day 7 in both groups of mice, with the mean parasitemia reaching $4.90 \pm 3.87\%$ and $6.42 \pm 0.93\%$ for experimental and control groups, respectively; by 3 weeks PI, parasitemia became undetectable.

Because NO is also produced by other isoforms of the nitric oxide synthase enzyme, $p47^{phox-/-}$ mice were infected i.v. with 10^5 *P. chabaudi* parasites and treated with the nonspecific NOS inhibitor, AG, or PBS (Fig. 2B). Parasitemia in treated and control mice followed a similar time course, reaching a peak on day 7 ($23.59 \pm 1.50\%$ and $24.23 \pm 4.10\%$, respectively) and declining to $<0.01\%$ by 3 weeks PI.

Time course of *P. chabaudi* parasitemia in B-cell-deficient $p47^{phox-/-}$ or $NOS2^{-/-}$ mice. To address the possibility that antibodies mask the putative function of ROI and NO against malaria parasites in wild-type mice, we produced double-KO mice deficient in B cells and either NADPH oxidase or NOS2 as described above. Groups of $J_H^{-/-} \times p47^{phox-/-}$ and control $J_H^{-/-} \times p47^{phox+/-}$ mice were inoculated with 10^6 parasitized erythrocytes, and the subsequent time course of the infection was followed. The results (Fig. 3A) indicate that parasitemia in both the experimental and control groups reached peak values ($8.41 \pm 3.30\%$ and $9.29 \pm 5.63\%$, respectively) ($P = 0.736$) on day 9 PI and declined to $<0.1\%$ by day 19 PI.

The time course of *P. chabaudi* infection was also examined

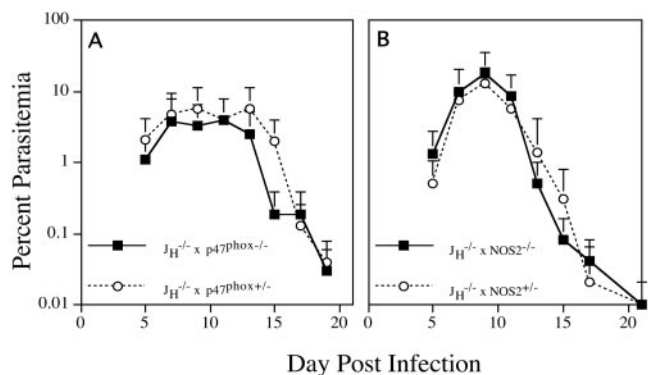


FIG. 3. The time courses of parasitemia in B-cell-deficient $p47^{phox}$ KO ($n = 7$) versus control ($n = 5$) mice (panel A) and B-cell-deficient $NOS2$ KO ($n = 5$) versus control ($n = 5$) mice (panel B) infected i.p. with 10^6 parasitized erythrocytes. Data represent the mean (\pm SD) parasitemia for each time point. These experiments were repeated twice with similar results.

in $J_H^{-/-} \times NOS2^{-/-}$ mice to determine whether NOS2 was essential for the suppression of parasitemia in the absence of B cells. The time courses of infection in $J_H^{-/-} \times NOS2^{-/-}$ and $J_H^{-/-} \times NOS2^{+/-}$ mice were nearly identical (Fig. 3B). Parasitemia in mice from both the double-KO and control groups reached a peak ($17.50 \pm 3.49\%$ and $12.86 \pm 7.87\%$, respectively) ($P = 0.263$) on day 9 PI and declined to undetectable levels by 3 weeks PI.

Time course of *P. chabaudi* parasitemia in $p47^{phox-/-}$ mice treated with allopurinol. To determine whether XO functions in a redundant fashion, producing ROI to suppress parasitemia when the NADPH oxidase pathway is absent, we compared *P. chabaudi* parasitemia in $p47^{phox-/-}$ mice treated with allopurinol in skim milk versus control $p47^{phox-/-}$ mice treated with skim milk alone (Fig. 4A). The peak parasitemia in experimental mice was twofold greater than that observed in control mice, $35.96 \pm 12.79\%$ compared to $14.41 \pm 9.71\%$, on day 7 PI. Subsequently, parasitemia followed an identical time course in both groups, declining to subpatent levels by day 21 PI.

Time course of *P. chabaudi* infection in $NOS2^{-/-}$ mice treated with allopurinol. Because ROI are also produced via the XO pathway, we treated $NOS2^{-/-}$ mice with the XO inhibitor allopurinol to determine whether ROI were produced by XO function in conjunction with NO to suppress parasitemia (Fig. 4B). At the peak, day 7 PI, the $NOS2^{-/-}$ mice treated with allopurinol had a parasitemia percentage ($25.76 \pm 10.79\%$) nearly three times greater than the untreated $NOS2^{-/-}$ control mice ($8.18 \pm 4.33\%$). By day 13 PI, both the experimental and control groups had similar levels of parasitemia; the subsequent suppression of parasitemia in both groups followed a similar time course, with parasitemia becoming undetectable by day 19 PI.

Time course of *P. chabaudi* parasitemia in $p47^{phox-/-} \times NOS2^{-/-}$ mice treated with allopurinol. As indicated above, ROI are produced by two pathways, the XO and NADPH oxidase pathways. To exclude the possibility that these potentially redundant pathways are functioning independently or in conjunction with NO to suppress parasitemia, we examined the time course of *P. chabaudi* in $p47^{phox-/-} \times NOS2^{-/-}$ mice treated with allopurinol versus that in controls treated with

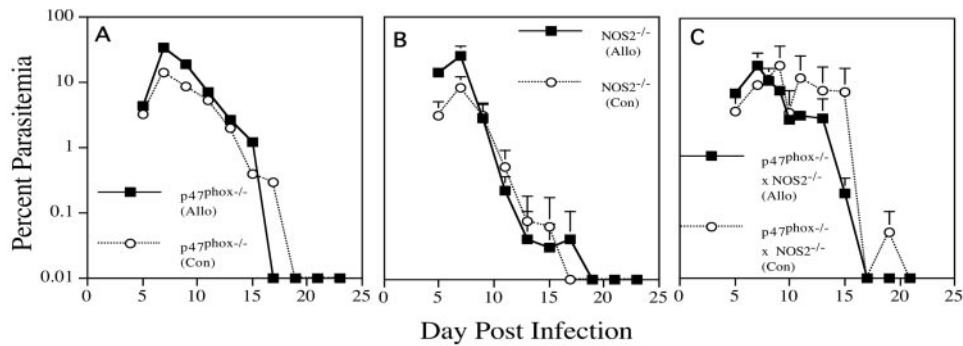


FIG. 4. The time courses of parasitemia in $p47^{phox}$ KO mice treated with allopurinol (Allo) ($n = 3$) versus $p47^{phox}$ KO control mice (Con) ($n = 3$) (panel A), NOS2 KO mice treated with allopurinol ($n = 9$) versus NOS2 KO control mice ($n = 7$) (panel B), and $p47^{phox-/-} \times NOS2^{-/-}$ mice treated with allopurinol ($n = 5$) versus $p47^{phox-/-} \times NOS2^{-/-}$ control mice ($n = 3$) (panel C) infected i.p. with 10^6 parasitized erythrocytes. Data represent the mean (\pm SD) parasitemia for each time point. These experiments were repeated twice with similar results.

skim milk (Fig. 4C). Similar to the results observed in single-KO mice treated with allopurinol, the $p47^{phox-/-} \times NOS2^{-/-}$ double-KO mice treated with allopurinol had a twofold increase in parasitemia ($19.15 \pm 9.68\%$) compared to the control group ($9.27 \pm 3.06\%$) during the peak, but the two groups did not differ in the subsequent course of infection. By day 13 PI, the parasitemia percentages in both groups were similar, and parasites were undetectable by 3 weeks PI.

***P. chabaudi* infection in $J_H^{-/-} \times p47^{phox-/-}$ and $J_H^{-/-} \times NOS2^{-/-}$ mice treated with allopurinol.** To determine whether the possible loss of parasite-killing function in $p47^{phox-/-}$ mice treated with allopurinol was masked by antibodies, we assessed the time course of *P. chabaudi* parasitemia in B-cell-deficient $p47^{phox-/-}$ mice treated with allopurinol (Fig. 5A). During peak infection on day 7 PI, $J_H^{-/-} \times p47^{phox-/-}$ mice treated with allopurinol had nearly four times the number of infected erythrocytes ($30.53 \pm 10.84\%$) compared to the controls treated with skim milk ($8.84 \pm 9.84\%$). However, by day 13 PI, declining parasitemia in both groups was similar, and both

groups followed a similar time course until suppression of the acute infection on day 21 PI.

Similarly, to exclude the possibility that the parasite-suppressive function of ROI produced via XO in conjunction with NO was masked by antibodies, we injected $J_H^{-/-} \times NOS2^{-/-}$ mice with 10^6 parasitized erythrocytes i.p. and treated groups with either allopurinol or skim milk (Fig. 5B). $J_H^{-/-} \times NOS2^{-/-}$ mice had a twofold increase in parasitemia over untreated control animals during the peak of infection on day 7 PI ($34.90 \pm 8.13\%$ versus $17.36 \pm 7.92\%$). By day 13 PI, the declining parasitemia in both groups of mice was similar and remained so through resolution of the acute infection on day 21 PI.

Summary of results. The salient features of the parasitemia data are summarized in Table 1.

DISCUSSION

The immune mechanisms by which the host eliminates blood-stage malaria parasites remain to be elucidated. Although both innate and adaptive mechanisms of immunity appear to be involved, how they function needs further clarification. Among the innate immune mechanisms that have been proposed to kill malaria parasites are those mediated by ROI and RNI, especially NO and ONOO⁻, both generated early during infection prior to the activation of adaptive immune mechanisms and later as components of the effector arm of the adaptive immune response (9, 17, 50). However, data supporting a parasite-killing role for these molecules have often been conflicting, especially when results from in vitro and in vivo studies have been compared (reviewed in reference 34). The current thinking is that these molecules have important immunoregulatory roles and are directly involved in the pathogenesis of malaria (reviewed in reference 34). For example, NO has been reported to down-regulate the production of proinflammatory cytokines, including tumor necrosis factor alpha and interleukin-1, which contribute to the pathology of disease (reviewed in reference 34).

In our initial studies, we examined the course of *P. chabaudi* parasitemia in NOS2^{-/-} and $p47^{phox-/-}$ mice. Our data indicate that the time courses of infection in both KO mice and

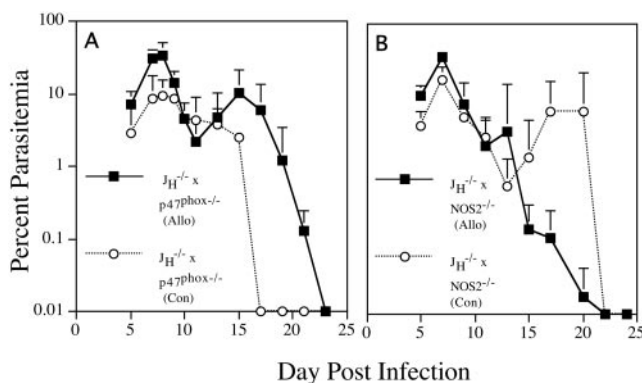


FIG. 5. The time courses of parasitemia in B-cell-deficient $p47^{phox}$ KO mice treated with allopurinol (Allo) ($n = 4$) versus B-cell-deficient $p47^{phox}$ KO control mice (Con) ($n = 3$) (panel A) and B-cell-deficient NOS2 KO mice treated with allopurinol ($n = 14$) versus B-cell-deficient NOS2 KO control mice ($n = 7$) (panel B) infected i.p. with 10^6 parasitized erythrocytes. Data represent the mean (\pm SD) parasitemia for each time point. These experiments were repeated twice with similar results.

TABLE 1. Summary of data on parasitemia in the different mouse models at three time points during infection^a

Mouse group (no. of mice)	Day 7		Day 13		Day 21
	Avg % parasitemia	<i>P</i>	Avg % parasitemia	<i>P</i>	Avg % parasitemia
p47 ^{phox-/-b}					
p47 ^{phox-/-} (8)	7.13 ± 3.90	0.210	0.99 ± 2.43	0.170	Undetectable
C57BL/6 (6)	3.45 ± 3.28		0.97 ± 1.82		Undetectable
NOS2 ^{-/-b}					
NOS2 ^{-/-} (6)	5.69 ± 4.71	0.713	0.13 ± 0.17	0.003	Undetectable
C57BL/6 (5)	4.84 ± 1.73		1.34 ± 0.73		Undetectable
p47 ^{phox-/-} × NOS2 ^{-/-b}					
p47 ^{phox-/-} × NOS2 ^{-/-} (6)	4.90 ± 3.87	0.377	0.35 ± 0.79	0.224	Undetectable
C57BL/6 (4)	6.42 ± 0.93		0.01 ± 0.004		Undetectable
p47 ^{phox-/-} with NOS inhibitor ^c					
p47 ^{phox-/-} AG (4)	23.59 ± 1.50	0.780	0.75 ± 0.31	0.579	Undetectable
p47 ^{phox-/-} PBS (3)	24.23 ± 4.10		0.08 ± 0.11		Undetectable
J _H ^{-/-} × NOS2 ^{-/-b}					
J _H ^{-/-} × NOS2 ^{-/-} (5)	9.87 ± 2.42	0.333	0.49 ± 0.78	0.401	Undetectable
J _H ^{-/-} × NOS2 ^{+/-} (5)	7.22 ± 4.15		1.38 ± 2.64		Undetectable
J _H ^{-/-} × p47 ^{phox-/-b}					
J _H ^{-/-} × p47 ^{phox-/-} (7)	7.77 ± 3.89	0.833	2.23 ± 2.56	0.534	Undetectable
J _H ^{-/-} × p47 ^{phox+/-} (5)	8.27 ± 4.70		3.58 ± 5.67		Undetectable
p47 ^{phox-/-b}					
Allopurinol (3)	17.71 ± 10.62	0.015	0.66 ± 0.89	0.522	Undetectable
Control (3)	7.68 ± 4.17		0.11 ± 0.13		Undetectable
NOS2 ^{-/-b}					
Allopurinol (9)	25.76 ± 10.79	0.001	0.04 ± 0.07	0.421	Undetectable
Control (7)	8.18 ± 4.33		0.08 ± 0.11		Undetectable
p47 ^{phox-/-} × NOS2 ^{-/-b}					
Allopurinol (5)	19.15 ± 9.68	0.150	2.84 ± 2.822	0.329	Undetectable
Control (3)	9.27 ± 3.06		7.69 ± 10.59		Undetectable
J _H ^{-/-} × p47 ^{phox-/-b}					
Allopurinol (4)	30.53 ± 10.84	0.040	4.70 ± 1.62	0.399	0.13 ± 0.12
Control (3)	8.84 ± 9.48		3.76 ± 6.50		Undetectable
J _H ^{-/-} × NOS2 ^{-/-b}					
Allopurinol (14)	34.90 ± 8.13	0.0002	3.34 ± 11.52	0.571	Undetectable
Control (7)	17.36 ± 7.92		0.58 ± 0.80		Undetectable

^a Data represent the mean (±SD) parasitemia percentage for each time point. Differences between the means were compared by an unpaired, two-tailed Student's *t* test, and the significances (*P* values) are noted. Day 7, near or at the peak of infection; day 13, postpeak; day 21, clearance of parasitemia.

^b Animals were inoculated i.p. with 10⁶ *P. chabaudi*-infected erythrocytes.

^c Animals were inoculated i.v. with 10⁵ *P. chabaudi*-infected erythrocytes.

their respective controls were nearly identical, suggesting that neither RNI nor ROI are essential for the elimination of blood-stage malaria parasites. Similar results were obtained when these KO mice were infected with the avirulent 17X strain of *Plasmodium yoelii*, i.e., the time course of parasitemia was nearly identical in KO and control mice (B. M. Gillman, unpublished observations). These observations are in agreement with those of others who reported that *P. chabaudi* parasitemia in NOS2^{-/-} and control mice was nearly identical (14, 54, 59).

To our knowledge, malaria has not been studied previously in the p47^{phox-/-} mouse model. Sanni et al. (46), who followed lethal *Plasmodium berghei* infections in gp91^{phox-/-} mice, which also failed to produce ROI via NADPH oxidase, re-

ported that parasitemia was significantly enhanced in the KO mice compared to that in controls. This observation, in contrast to our findings, suggests that ROI generated via NADPH oxidase does kill blood-stage malaria parasites. A possible explanation for the discrepancies between our findings and those of Sanni et al. is that these *Plasmodium* parasites differ in their susceptibility to the action of ROI, with *P. yoelii* and *P. chabaudi* being more resistant than *P. berghei*. To conclude from our studies and from those of others that RNI and ROI are incapable of killing blood-stage malarial parasites may be premature for several reasons: (i) the in vivo ability to kill malaria parasites may be masked by the antibody response of the infected host, and (ii) the killing mechanisms mediated by these molecules may function in a redundant fashion.

Previously, we reported a plasticity in the adaptive immune responses that eliminate blood-stage malaria parasites (57), i.e., mice can suppress parasitemia by either antibody-mediated immunity or T-cell-dependent cell-mediated immunity. The cell-mediated immune response precedes the antibody response, which subsequently down-regulates the cell-mediated immune response. A plausible explanation for the results seen in our experiments with single-KO mice is that a similar phenomenon was occurring, in that the developing antibody response suppressed parasitemia and obscured our ability to determine whether ROI or RNI were critical for parasite elimination. However, we observed that *P. chabaudi* parasitemia followed a nearly identical time course in B-cell-deficient NOS2^{-/-} or B-cell-deficient p47^{phox}^{-/-} mice and their respective controls. These results exclude the possibility that antibodies were masking the putative function of ROI and/or RNI in the elimination of blood-stage parasites.

Redundancies in the NO-producing machinery of the host might help to explain why parasitemia was unaffected by the deletion of the NOS2 gene in our studies. Of the three NOS isoforms which are known to generate NO, only NOS2 and NOS3 contribute to blood levels of RNI (27). Thus, nitric oxide sufficient to kill blood-stage parasites may have been produced via NOS3 in NOS2^{-/-} mice infected with *P. chabaudi*; however, the findings of van der Heyde et al. (54) argue against this contention. van der Heyde et al. found that *P. chabaudi* parasitemia followed an identical time course in NOS3^{-/-} mice and their controls and concluded from their studies that most of the NO in blood during *P. chabaudi* malaria was the product of NOS2 activity. Our observation that *P. chabaudi* followed the same time course in p47^{phox}^{-/-} mice treated with the nonspecific NOS inhibitor, AG, are in agreement with the concept that other isoforms of NOS do not function to produce parasite-killing levels of RNI in NOS2^{-/-} mice. Similar results were obtained when J_H^{-/-} × NOS2^{-/-} mice were treated with N(G)-monomethyl-L-arginine (Gillman, unpublished).

It is also possible that ROI may compensate for the loss of RNI, or vice versa, during *P. chabaudi* infection. To examine this possible redundancy between reactive intermediate systems, we assessed parasitemia in p47^{phox}^{-/-} × NOS2^{-/-} double-KO mice. When double-KO mice were infected with *P. chabaudi*, the resulting time course of infection was nearly identical to that of control mice. These data suggest that the production of RNI in place of ROI, or ROI in place of RNI, was not responsible for the killing of parasites in single-KO (p47^{phox}^{-/-} or NOS2^{-/-}) mice.

As indicated earlier, ROI are produced via two metabolic pathways, involving either NADPH oxidase or XO. It is possible that a redundancy involving these mechanisms could produce ROI in the absence of either enzyme and explain the ability for p47^{phox}^{-/-} mice to suppress parasitemia in a normal time frame. When p47^{phox}^{-/-} mice were treated with the XO inhibitor allopurinol and infected with *P. chabaudi*, the magnitude of peak parasitemia was more than twice that seen in control mice. When allopurinol was used to treat NOS2^{-/-} mice, we observed similar results, i.e., peak parasitemia increased over twofold compared to controls. These data suggest that ROI produced via the XO enzyme, in contrast to NADPH oxidase, are capable of modulating parasitemia.

Bünger, in the mid-1970s, also reported that infections

induced with different malaria parasites, including *P. chabaudi*, were exacerbated by allopurinol treatment (6). He interpreted his results as being due to an increase in the multiplication of parasites in allopurinol-treated mice. Allopurinol inhibits XO, which normally metabolizes hypoxanthine to xanthine and uric acid, and allows hypoxanthine to accumulate in the blood of the infected host, thereby stimulating parasite growth (60). Subsequently, allopurinol was observed to inhibit the production of ROI by XO, which has been reported to increase in phagocytic cells during the course of malaria (53). Inspection of thin blood films prepared from allopurinol-treated mice and controls failed to reveal differences in parasite life cycle stages between treated and untreated mice (Gillman, unpublished).

When B-cell-deficient p47^{phox}^{-/-} mice or B-cell-deficient NOS2^{-/-} mice were treated with allopurinol and infected with *P. chabaudi*, the peak levels of parasitemia were significantly higher during infection than those in control mice. Parasitemia profiles in *P. chabaudi*-infected p47^{phox}^{-/-} × NOS2^{-/-} mice treated with allopurinol were similar to those reported for other allopurinol-treated mice, i.e., peak levels of parasitemia were two- to threefold greater than those seen in untreated p47^{phox}^{-/-} × NOS2^{-/-} control mice. Collectively, our findings suggest that ROI produced early during *P. chabaudi* infection, via the XO pathway, function to modulate parasitemia but are unable to suppress parasitemia to subpatent levels. Previous observations support the contention that J_H^{-/-} × δ^{-/-} mice, in which ROI-generating mechanisms presumably are intact, lack cell-mediated immunity against blood-stage *P. chabaudi* parasites and consequently develop unremitting parasitemia (59). The different effects on parasitemia observed when the XO pathway versus the NADPH oxidase pathway of ROI production was blocked may be due to differences in the amounts of ROI produced by each pathway. Our findings are in agreement with those of others who reported that molecules capable of causing oxidant stress leading to lipid peroxidation in erythrocytes in vivo down-modulated parasitemia but did not sterilize infection (reviewed in reference 10).

Although both RNI and ROI have been reported to kill *Plasmodium falciparum* as well as murine malaria parasites in vitro (29, 35, 43), it has been difficult to directly assess their function in vivo (19, 39). ROI generated by the action of glucose oxidase or xanthine oxidase on their respective substrates were reported to kill *P. falciparum* and *P. yoelii* in vitro (13, 58). Likewise, RNI induced by various agents were observed to be toxic and/or cytostatic for *P. falciparum* and several murine malaria parasites (2). These cumulative data suggest differences in the susceptibilities to both ROI and RNI between parasite species. In vivo, these radicals are short lived, being scavenged by replenishing molecules of both host and parasite origin, whereas in vitro, the radicals are also short lived but the environment is static. In addition, relatively few target parasites are added to the culture dishes in contrast to the very large number of parasites circulating in the blood of the infected host. Studies in humans have been primarily correlative, suggesting that these reactive intermediates may be involved in the killing of parasites and the pathogenesis of malaria. The use of KO mouse models in our studies has allowed us to test directly whether RNI and/or ROI play an essential role in the suppression of parasitemia.

Based on the similarities of parasitemia curves in p47^{phox}^{-/-}

and NOS2^{-/-} mice and their respective controls, we conclude that ROI and RNI, including NO and ONOO⁻, produced via the activity of the enzymes are not essential for the elimination of blood-stage *P. chabaudi* parasites. We speculate that ROI represent an early response to blood-stage parasites that is aimed at slowing the rate of ascending parasitemia as other elements of innate immunity are being activated. For example, it is now established that NK cells are also activated early during infection by parasite products via pattern recognition mechanisms (48). These cells secrete gamma interferon, which stimulates phagocytic cells to produce ROI, RNI, proinflammatory cytokines, and chemokines, elements that contribute to the activation and regulation of the adaptive immune responses which sterilize infection (48).

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