Brief Definitive Report

Induction of Nitric Oxide Synthase Protects against Malaria in Mice Exposed to Irradiated *Plasmodium berghei* Infected Mosquitoes: Involvement of Interferon γ and CD8$^+$ T Cells

By Matthew C. Seguin, Francis W. Klotz, Imogene Schneider, Jerry P. Weir,* Marlyn Goodbary,§ Mike Slayter,§ Jennifer J. Raney,‡ Jacinta U. Aniagolu,‡ and Shawn J. Green‡

*From the Departments of Immunology and Entomology, Division of Communicable Disease and Immunology, Walter Reed Army Institute of Research, Washington, DC 20307; the *Henry M. Jackson Foundation for the Advancement of Military Medicine, and ‡EntreMed, Inc., Rockville, Maryland 20850; and §Armed Forces Institute of Pathology, Washington, DC 20306*

Summary

Exposure of BALB/c mice to mosquitoes infected with irradiated *Plasmodium berghei* confers protective immunity against subsequent sporozoite challenge. Immunized mice challenged with viable sporozoites develop parasitemia when treated orally with substrate inhibitors of nitric oxide synthase (NOS). This suggests that the production of nitric oxide (NO) prevents the development of exoerythrocytic stages of malaria in liver. Liver tissue from immunized mice expressed maximal levels of mRNA for inducible NOS (iNOS) between 12 and 24 h after challenge with sporozoites. Intraperitoneal injection of neutralizing monoclonal antibody against interferon γ (IFN-γ) or in vivo depletion of CD8$^+$ T cells, but not CD4$^+$ T cells, at the time of challenge blocked expression of iNOS mRNA and ablated protection in immunized mice. These results show that both CD8$^+$ T cells and IFN-γ are important components in the regulation of iNOS in liver which contributes to the protective response of mice immunized with irradiated malaria sporozoites. IFN-γ, likely provided by malaria-specific CD8$^+$ T cells, induces liver cells, hepatocytes and/or Kupffer cells, to produce NO for the destruction of infected hepatocytes or the parasite within these cells.

Within minutes after an infected *Anopheles* mosquito bites the vertebrate host, malaria sporozoites migrate to the liver and invade hepatocytes. There, the parasite matures, and after several days the infected hepatocytes lyse, releasing thousands of merozoites. Once in circulation, the parasite infects erythrocytes causing parasitemia. Prior exposure to irradiated sporozoites confers protective immunity (1, 2). This immunity is directed against liver stage malaria, and does not protect against the blood stage malaria.

CD8$^+$ T cells and IFN-γ are required for protective immunity to sporozoite challenge. In vivo depletion of CD8$^+$ T cells or neutralization of IFN-γ blocks induction of effector activity at the hepatic stage, resulting in parasitaemia (3–5). In vitro studies show that IFN-γ kills parasites by stimulating malaria-infected hepatocytes to produce nitric oxide (NO), and the addition of monomethyl-L-arginine (N^GMMLA), a substrate inhibitor of nitric oxide synthase (NOS), to primary cultures of mouse hepatocytes reversed the antiparasitic effects of IFN-γ (6, 7). Human hepatocytes also respond to IFN-γ for NO production (8). As to whether human hepatocytes exhibit antimalaria activity when stimulated to produce NO, remains to be examined.

At present, the antimalaria effector mechanism triggered by sporozoites in immunized animals is not fully understood. Presumably malaria-specific CD8$^+$ T cells act directly against infected hepatocytes by recognizing malaria antigen on the cell surface (i.e., induction of CTLs) or malaria-specific lymphocytes release cytokines, such as IFN-γ, upon parasite stimulation, which induces an antimalarial response (3–5, 9–12). The relationship between CD8$^+$ T cells, IFN-γ, and NO-mediated protection in sporozoite-immunized mice was explored by examining the effects of mAbs to IFN-γ and to CD8$^+$ T cells on induction of inducible NOS (iNOS) in liver and protection against sporozoite challenge. Our results show that NO production is required for protection in irradiated sporozoite–immunized mice, and induction of iNOS in liver depends on the presence of CD8$^+$ T cells and IFN-γ.

Materials and Methods

Immunization and Challenge Protocol. Female, 4–6-wk-old, BALB/CBYJ (The Jackson Laboratory, Bar Harbor, ME) mice were immunized by mosquito bite with irradiation-attenuated ANKA

strain *Plasmodium berghei* sporozoites (15,000 rad) from infected *Anopheles stephensi* mosquitoes. Sedated mice were placed on a nylon mesh screened container containing infected mosquitoes. Each mouse was removed after a minimum of five observed mosquito feedings over 5 min. This was done on week 0 and week 2. The same procedure was repeated on day of challenge except the mosquitoes were not irradiated. Parasitemia or patency of experimental animals was determined on Giemsa-stained thin blood films. Individual mice were screened by daily blood film beginning on day 4 through day 15 after challenge or until a positive parasitemia was observed (2).

L-Arginine Analogues. Mice were treated by gastric instillation with either L-arginine, aminoguanidine, N^G-nitro-L-arginine, ammonium acetate (Sigma Chemical Co., St. Louis, MO), or N^GMMLA (Calbiochem-Novabiochem Corp., San Diego, CA) in sterile water. The treatments were initiated 24 h before viable sporozoite challenge and administered as indicated for 72 h.

mRNA Analysis. The presence of iNOS mRNA in liver was analyzed by reverse transcription (RT) of total RNA using oligo dT followed by PCR amplification of the cDNA. Frozen livers were disrupted with a Stomacher Lab 80 blender (Seward, London, England) in 6 ml of guanidinium isothiocyanate solution. After addition of CsCl to 0.33 g/ml, the RNA was pelleted through a cushion of 5.7 M CsCl at 155,000 *g* for 16 h (Beckman Instruments, Inc., Palo Alto, CA). The RNA pellet was resuspended in water and precipitated in the presence of 2 M sodium acetate, pH 5.2, and ethanol. The precipitate was resuspended and extracted with phenol/chloroform/isoamyl alcohol, followed by precipitation with ethanol. cDNA was prepared by incubation of 5 μg of RNA (in 10 μl) at 65°C for 3 min followed by the addition of 15 μl of reverse transcriptase reaction mixture (Promega Corp., Madison, WI), and 400 U of reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and incubated at 41°C for 1 h. Sense and antisense primers at final concentration of 0.8 mM were added to 2 μl of the cDNA reaction in a reaction volume of 50 μl and amplified with Amplitaq (Perkin Elmer Cetus, Norwalk, CT). The sense and antisense oligonucleotides for iNOS, obtained from the nucleotide sequence of the pMAC-NOS, were as follows: 5′-ATG GCT TGC CCC TGG AAG TTT CTC-3′; 5′-CAG CTT CCA GCC TGG CCA GAT G-3′. The predicted PCR product was 486 bp (13, 14). The primers for β-actin were: 5′-CGT GGG CCG CCC TAG GCA CCA GGG-3′; 5′-CGG AGG AAG AGG ATG CGG CAG TGG-3′. The predicted β-actin PCR product was 605 bp. The cycle conditions were 1.5 min at 94°C, 1.5 min at 55°C, and 2 min at 72°C for 40 cycles (iNOS) or 25 cycles (actin). The PCR products were analyzed by electrophoresis on 1.5% agarose gels. The identity of the specific PCR product was verified by Southern blot analysis and hybridization with oligonucleotide probes specific for iNOS or β-actin genes. The hybridization for iNOS was ACT GGG GCA GTG GAG AGA TT, and the hybridization probe for β-actin was GTA GCC ATC CAG GCT GCT GTG CT. Verification that the amplified product originated from RNA rather than DNA was determined by preparing duplicate cDNA samples without reverse transcriptase, followed by PCR amplification.

Neutralization of IFN-γ. Mice were injected intraperitoneally 1 d before sporozoite challenge with mAb to IFN-γ (H22) or isotype IgG control Ab (Organon Teknika Corp., West Chester, PA) in sterile PBS. H22 was generously provided by Robert D. Schreiber (Washington University, St. Louis, MO) (15). Three mice were killed at various times and livers removed for RNA analysis. The remaining mice were evaluated for parasitemia.

Table 1. *Effects of Substrate Inhibitors for NOS on Sporozoite Immunity*

	Immune status	Infected/total	Protected	Patency
			%	*d*
Experiment No. 1				
	Naive*	15/15‡	0‡	5–7§
	+ L-arginine	15/15	0	6–7
	+ Ammonium acetate	15/15	0	5–6
	+ N^GMMLA	15/15	0	5–6
	Immunized	1/15	93	6
	+ L-arginine	2/10	81	6–7
	+ Ammonium acetate	1/10	90	5
	+ N^GMMLA	15/15	0	5–6
Experiment No. 2				
	Naive	8/8	0	6–7
	Immunized:	1/8	88	5
	+ N^G-nitro-L-arginine	2/8	75	5–6
	+ Aminoguanidine	8/8	0	4–6

* Naive and immunized BALB/c mice were exposed to *P. berghei*-infected mosquitoes as described in Materials and Methods. In experiment No. 1, 0.5 ml of a 50 mM solution of L-arginine, ammonium acetate, or N^GMMLA was orally administered twice daily. In experiment No. 2, 0.5 ml of 25 mM solution, N^G-nitro-L-arginine or aminoguanidine was administered once daily.

‡ Number of mice that had infected erthrocytes by day 15 was defined as infected (2).

§ Patency is the days or range of days in which all of the infected mice exhibited parasitemia.

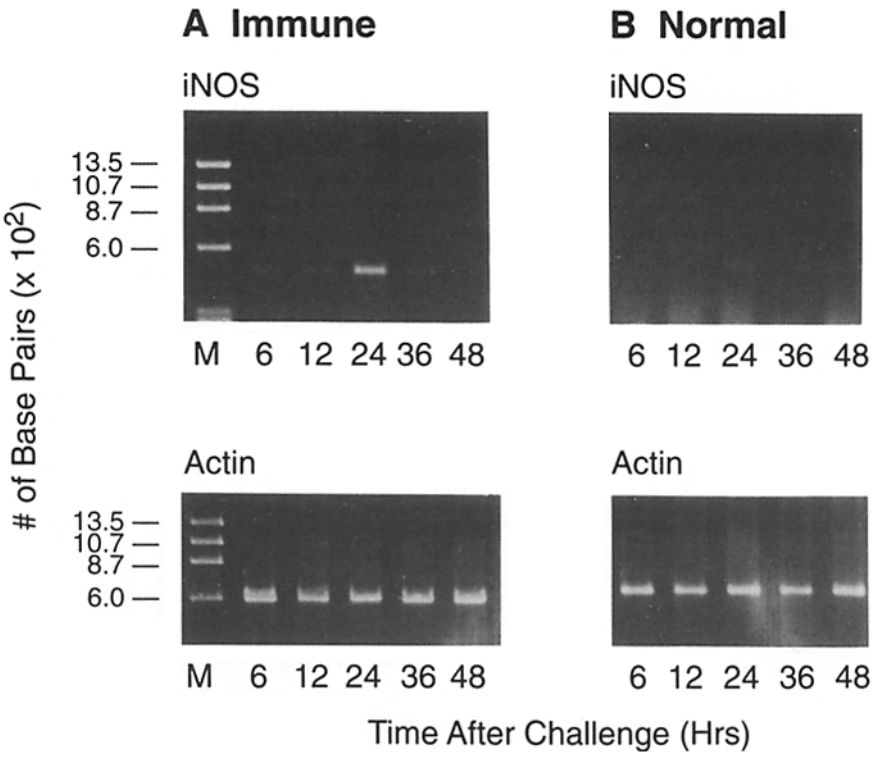

Figure 1. RT-PCR analysis of liver mRNAs from mice exposed to malaria sporozoites. cDNAs were generated by reverse transcription of RNAs isolated from the liver of immune (*A*) and normal (*B*) mice at the indicated times after exposure to mosquitoes infected with *P. berghei*. cDNA were amplified by PCR with primers specific for iNOS or β-actin (*Actin*) genes as described in Materials and Methods. (*M*) Base pair markers.

Depletion of CD8⁺ and CD4⁺ T Cells. 3 d before challenge, immunized mice were depleted of either $CD8^+$ or $CD4^+$ T cells by three daily intraperitoneal doses of 1 mg of either mAb 2.43 (mouse IgG2a, anti-$CD8^+$) or GK1.5 (rat IgG2a, anti-L3T4, anti-$CD4^+$) and two mice from each group also received 1 mg control Ab (mAb NSV3; anti-*P. vivax*) or rat Ig obtained from pooled normal rat serum. These purified antibodies were a gracious gift from Stephen L. Hoffman (Naval Medical Research Institute, Bethesda, MD). On day of challenge, a mouse from each group was killed and microfluorometric analysis was done on the spleen cells. Spleen cell preparations were 97% depleted of each T cell population.

Results and Discussion

In Vivo Effects of N^{ω}-substituted L-Arginine Analogues on Sporozoite Immunity. To determine if NO participates in the protective response to malaria, immunized mice were treated orally with L-arginine analogues before sporozoite challenge. Synthesized by NOS, NO is derived from L-arginine (16). Substrate inhibitors for NOS, aminoguanidine, and N^{ω}-substituted L-arginine analogues, such as N^GMMLA and N^G-nitro-L-arginine, are shown to suppress NO synthesis and NO-mediated events both in vitro and in vivo (16). BALB/c mice were immunized twice with irradiated sporozoites by the natural vector, *A. stephensi* mosquitoes, and treated orally with N^GMMLA, N^G-nitro-L-arginine, aminoguanidine, or control compounds (i.e., L-arginine, ammonium acetate) (Table 1). Mice were then challenged with viable sporozoites to determine the animals' state of protection. Oral administration of NOS inhibitors or controls began on the day of challenge and the regimen was continued for 4 d. All nonimmunized mice that were challenged with sporozoites developed patent blood infection. In contrast, >90% of immunized mice were protected from challenge. Oral administration of L-arginine or ammonium acetate had no effect on protective immunity. However, all immunized mice that were given N^GMMLA displayed parasitemia within 5–7 d. A recent report showed a similar in vivo effect with N^GMMLA (17).

Since iNOS is believed to be a principal mechanism for cytotoxicity against intracellular parasites, the enzyme source of NO involved in this protective response was addressed. In vitro studies earlier demonstrated that N^GMMLA and aminoguanidine are both potent inhibitors of iNOS, whereas N^G-nitro-L-arginine blocks the constitutive NOS and is relatively ineffective as an inhibitor of the inducible isoform (18, 19). Immunized mice were given either N^G-nitro-L-arginine or aminoguanidine (Table 1). N^G-nitro-L-arginine had minimal effect on protective immunity. In contrast, all immunized mice treated orally with aminoguanidine became infected. Collectively, these data show that immunized mice that received substrate inhibitors for iNOS (i.e., aminoguanidine or N^GMMLA) became susceptible to sporozoite challenge, whereas immunized mice that received N^G-nitro-L-arginine, a compound more selective for the constitutive enzyme, remained protected. Thus, interference with the cytokine-inducible NO pathway ablates a critical component in protective immunity against malaria.

Expression of iNOS mRNA in Livers from Immunized Mice. To determine whether iNOS is present in the liver of protected mice, RNA was isolated from the livers of either normal or immune mice at different times after sporozoite

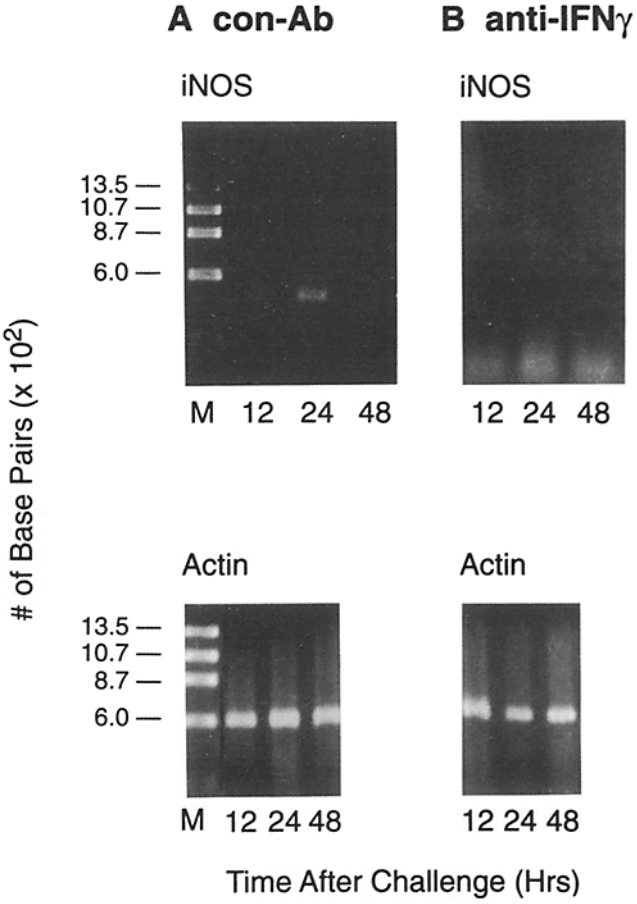

Figure 2. RT-PCR analysis of mouse liver mRNAs for iNOS and actin after exposure to malaria sporozoites and neutralizing mAb to IFN-γ. As detailed in the Materials and Methods, RNA was isolated from the liver of immune mice at the indicated times after exposure to mosquitoes infected with *P. berghei* and injected intraperitoneally with control Ab (*A*) and IFN-γ (*B*).

challenge and analyzed for the presence of iNOS mRNA by RT-PCR. iNOS was not detectable in the livers of either immune or normal mice (data not shown), whereas iNOS was induced in the livers of immune mice within 12 h after sporozoite challenge (Fig. 1). The induction of iNOS was transient since maximal levels of iNOS mRNA were detected between 12 and 24 h after challenge, but were not detectable by 48 h after challenge. In normal mice, faintly detectable levels of iNOS mRNA were evident after sporozoite challenge. Thus, iNOS mRNA is produced in the liver of mice upon sporozoite challenge and this may contribute to protective immunity.

This observation, together with earlier reports demonstrating that induction of NO by IFN-γ–stimulated hepatocytes prevents the development of *P. berghei* in mouse hepatocytes, suggests that iNOS might be produced in the liver of immune mice when challenged with sporozoites (7, 8). The administration of neutralizing mAbs against IFN-γ to immune mice can reverse immunity to sporozoite challenge (3, 4). However, the effector mechanism induced by IFN-γ in vivo has not clearly been demonstrated.

Table 2. *Effects of Neutralizing Antibody to IFN-γ on Sporozoite Immunity*

Immune status	Infected/total	Protected	Patency
		%	*d*
Naive*	5/5‡	0‡	5–6§
Immunized:	0/5	100	–
+ Control IgG Ab	0/5	100	–
+ Anti-IFN-γ mAb	5/5	0	7

* Immunized BALB/c mice were exposed to *P. berghei*–infected mosquitoes and injected intraperitoneally (100 μl) with 200 μg/mouse of either isotype control IgG Ab or mAb to IFN-γ (15).
‡ Number of mice that had infected erthrocytes by day 15 was defined as infected (2).
§ The range of days in which all of the infected mice exhibited parasitemia.

Effects of mAb to IFN-γ on Protection and Expression of iNOS mRNA in Liver of Immunized Mice. To assess the relationship between IFN-γ, protection against sporozoite challenge, and induction of liver iNOS, immunized mice were challenged and treated with mAb against IFN-γ. As expected, immunized mice which received anti-IFN-γ mAb at the time of chal-

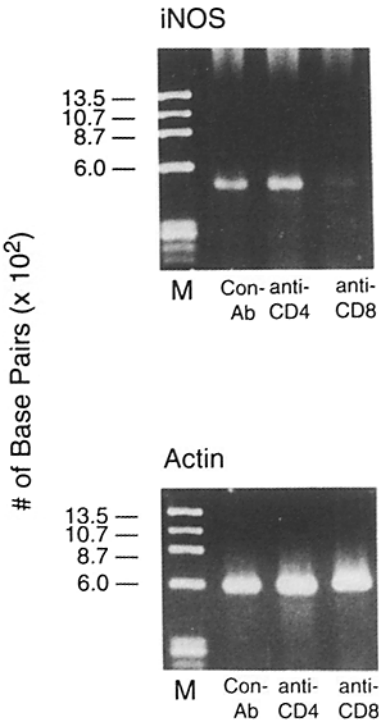

Figure 3. RT-PCR analysis of mouse liver mRNAs for iNOS and actin after exposure to malaria sporozoites and depletion of CD8⁺ and CD4⁺ cells. RNA was isolated from the liver of immune mice at 24 h after exposure to mosquitoes infected with *P. berghei* and injected intraperitoneally with control antibody (*Con-Ab*) or mAbs to CD4⁺ (*anti-CD4*) or CD8⁺ (*anti-CD8*) cells, as described in Materials and Methods.

Table 3. *Effects of Anti-CD4 and Anti-CD8 mAb on Immunity*

Immune status	Infected/total	Protected
Naive*	5/5‡	0§
Immunized:		
+ control Ig	0/5	100
+ anti-CD4+	0/5	100
+ anti-CD8+	5/5	0

* Immunized BALB/c mice were exposed to *P. berghei*–infected mosquitoes and injected intraperitoneally with indicated Abs 3 d before challenge as described in Materials and Methods.
‡ Number of mice that had infected erthrocytes by day 15 was defined as infected (2).

lenge displayed parasitemia (Table 2). In contrast, immunized mice that were challenged with viable sporozoites and treated with isotype control IgG were protected. When RNA was isolated from the liver of challenged mice, iNOS mRNA was easily detectable by 12–24 h after challenge in the control IgG treated animals (Fig. 2). However, we were unable to detect iNOS in the liver of challenged, IFN-γ mAb treated mice. Such observations support the idea that viable sporozoites trigger malaria-specific T cells to release IFN-γ, which in turn, stimulates the iNOS pathway in infected hepatocytes and neighboring Kupffer cells.

Effects of CD8+ T Cell Depletion on Protective Immunity and Expression of iNOS. Both CD8+ and CD4+ T cells are a rich source of IFN-γ. Infiltrates containing predominately CD8+ and to a lesser extent CD4+, are found in the livers of mice immunized with irradiated sporozoites and challenged with viable sporozoites, whereas substantially less cellular infiltrates were found in nonimmunized mice that were challenged (12). In agreement with earlier reports (3, 5), in vivo depletion of CD8+ T cells with mAbs after immunization and before challenge results in parasitemia (Table 3). Anti-CD8 treatment completely reversed protection, whereas anti-CD4 mAb had no effect. It is interesting to note that by depleting CD8+ T cells, expression of iNOS mRNA was inhibited in livers taken from immunized mice 24 h after challenge (Fig. 3). In contrast, iNOS mRNA was present in the livers of immunized mice depleted of CD4+ T cells (or treated with an irrelevant Ab) 24 h after challenge. These data support the hypothesis that both IFN-γ and CD8+ T cells, not CD4+ T cells, are required for induction of NO and protection against liver stage malaria. As to whether CD8+ T cells influence hepatic iNOS in other rodent malaria models remains to be investigated, because depletion of CD8+ T cells did not reverse sporozoite protection against *P. yoeli* in some immunized B10 congenic mouse strains (2) or against *P. berghei* strain NK65 in immunized BALB/c mice (20). Aside from using a different rodent malaria (*P. berghei* ANKA clone), our immunization and challenge protocol was distinctively different from those of other reports (2, 20). Here, we immunized and challenged via the bite of (irradiated and nonirradiated) infected mosquitoes. Introducing the sporozoite "naturally" through the bite of a mosquito is clearly different from injecting large amounts of isolated sporozoites intravenously. Such differences (i.e., frequency of immunizations, number of sporozoites, routes of exposure, strains of malaria and mice) may influence the host response and outcome of the disease process.

Taylor-Robinson et al. (21) reported that induction of NO by Th1 CD4+ cells controls blood stage malaria. Whereas Weiss et al. (22) have recently shown that CD4+ T cells are required at the time of immunization for protection against sporozoites, CD4+ T cells are involved in the development of protective immunity against liver stage malaria, and are not essential for protection after animals have been immunized. In contrast, CD8+ T cells are involved in the effector response or induction of effector activity (2, 3, 5, 9, 10). A recent report (17) suggests that a T cell–independent (i.e., NK cells) source for IFN-γ mediates protective immunity against liver stage malaria, however, protection was never assessed in immunized animals depleted of NK cells. Investigators found elevated NOS activity upon infection with malaria. It is interesting to note that we also found that intravenous injection of a large excess ($>10^6$) of viable sporozoites into naive mice induces iNOS activity in liver, yet these mice develop parasitemia and ultimately die (data not shown). In response to high concentrations of sporozoites, the NO produced under these conditions is likely the result of NK cells producing IFN-γ during acute hepatic inflammation. In a similar fashion, NOS expression in liver tissue can be induced by injecting bacterial products causing hepatic inflammation (23). However, this vigorous immune response was insufficient to render protection. Therefore, induction of NO activity is necessary, but not sufficient for protection against sporozoites.

Here, we report that CD8+ T cells directly regulate NO production in infected livers of immunized mice. Therefore, CD8+ T cells in immunized mice may participate as cytotoxic T cells, and facilitate the protective response by providing a source of IFN-γ. This study provides evidence that IFN-γ induction of the L-arginine–dependent NO pathway in vivo is a necessary component for effector activity in sporozoite-immunized animals, and the first to report that CD8+ T cells are required in the regulation of liver cells to produce NO for the elimination of infected hepatocytes or the malaria schizonts within these cells.

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Address correspondence to Dr. S. J. Green, EntreMed, Inc., 9610 Medical Center Drive, Suite 200, Rockville, MD 20850.

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