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The Complexity of Protective Immunity Against Liver-Stage Malaria^{1,2}

Denise L. Doolan^{3*†} and Stephen L. Hoffman*

Sterile protective immunity against challenge with *Plasmodium* spp. sporozoites can be induced in multiple model systems and humans by immunization with radiation-attenuated *Plasmodium* spp. sporozoites. The infected hepatocyte has been established as the primary target of this protection, but the underlying mechanisms have not been completely defined. Abs, CD8⁺ T cells, CD4⁺ T cells, cytokines (including IFN- γ and IL-12), and NO have all been implicated as critical effectors. Here, we have investigated the mechanisms of protective immunity induced by immunization with different vaccine delivery systems (irradiated sporozoites, plasmid DNA, synthetic peptide/adjuvant, and multiple Ag peptide) in genetically distinct inbred strains, genetically modified mice, and outbred mice. We establish that there is a marked diversity of T cell-dependent immune responses that mediate sterile protective immunity against liver-stage malaria. Furthermore, we demonstrate that distinct mechanisms of protection are induced in different strains of inbred mice by a single method of immunization, and in the same strain by different methods of immunization. These data underscore the complexity of the murine host response to a parasitic infection and suggest that an outbred human population may behave similarly. Data nevertheless suggest that a pre-erythrocytic-stage vaccine should be designed to induce CD8⁺ T cell- and IFN- γ -mediated immune responses and that IFN- γ responses may represent an *in vitro* correlate of pre-erythrocytic-stage protective immunity. *The Journal of Immunology*, 2000, 165: 1453–1462.

Parasitic pathogens are extremely complex, with a molecular structure comprising thousands of genes and complicated life cycles encompassing discrete developmental stages during which the parasite may be sequestered from protective host immune responses. The *Plasmodium* spp. parasite is the etiologic agent of malaria. Malaria is the most important parasitic disease in humans, affecting 40% of the world's population with an estimated toll of 1.5–2.7 million deaths and 300–500 million clinical cases per year (1). The development of insecticide resistance in the vector and drug resistance in the parasite is becoming increasingly widespread, and an effective vaccine is still not available despite intense research for many decades. Contributing to this is the complexity of the parasite and the fact that the mechanisms responsible for protective immunity have not been fully elucidated and specific markers of protection have not been defined.

Sterile protective immunity against malaria can be induced by immunization with radiation-attenuated sporozoites in every model studied, including rodents, nonhuman primates, and humans (reviewed in Refs. 2 and 3). This protection is effective against challenge with massive doses of infectious sporozoites, is species-specific but not strain-specific, is efficacious in outbred and inbred mouse strains differing in genetic background as well as MHC-

diverse humans, and persists for at least 9 mo in humans. It is now well established that the infected hepatocyte is the primary target of this sporozoite-induced protection and that CD8⁺ and CD4⁺ T cells can recognize parasite-derived peptides presented by class I or class II molecules, respectively, on the surface of infected hepatocytes. However, the underlying mechanisms responsible for this protection have not been completely defined, and vaccine delivery systems that induce protection comparable to that achieved by immunization with irradiated sporozoites have not been developed. *In vivo* and *in vitro* data have implicated each of Abs, CD8⁺ T cells, CD4⁺ T cells, cytokines (including IFN- γ and IL-12), and other factors (including NO) as critical effectors in protection against pre-erythrocytic (sporozoite/liver-stage) malaria (reviewed in Ref. 3).

The study reported here was designed to systematically elucidate the mechanism of sporozoite-induced protection in mice of diverse genetic backgrounds and to define the requirement for CD8⁺ T cells, CD4⁺ T cells, NK cells, IFN- γ , IL-12, and NO in the protective immunity induced by immunization with distinct vaccine delivery systems. Working with genetically different inbred and congenic strains, gene-knockout mice, genetically deficient mice, outbred mice, and *in vivo* depletion methods, we have defined six distinct T cell-dependent immune mechanisms that can confer sterile protection against *Plasmodium yoelii* sporozoite challenge, depending on the host and the vaccine delivery system.

Materials and Methods

Mice

Female 3- to 5-wk-old inbred BALB/cByJ (JR001026), C57BL/6J (JR000664), B6.129 (JR101045), A/J (JR000646), B10.BR/SgSnJ (JR000465), B10.D2/nSnJ (JR000463), and B10.Q/SgJ (JR002024) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female 3- to 5-wk-old outbred CD-1 mice were obtained from Charles River Lab-

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oratories (Wilmington, MA). Perforin pore-forming protein (ppo)⁴-deficient (C57BL/6-*Pfp*^{tm1Scz}) mice homozygous for the *Pfp*^{tm1Scz} targeted mutation were purchased from The Jackson Laboratory (JR002407) at 3–8 wk of age and have been described previously (4). Ppo^{-/-} controls were on the C57BL/6 background (JR000664). Granzyme B (Gzmb)-deficient (B6,129-Gzmb^{tm1Ley}) mice homozygous for the Gzmb^{tm1Ley} targeted mutation were purchased from The Jackson Laboratory (JR002247) at 3–8 wk of age and have been described previously (5). Gzmb controls were provided from the B6129F₂/J colony (JR101045). Generalized lymphoproliferative disease (gld) (C3H/HeJ-*Fas*^{gld}) mice homozygous for a spontaneous mutation of the Fas Ag ligand (FasL) were purchased from The Jackson Laboratory (JR000784) at 3–5 wk of age and have been described previously (6). Gld controls were on the C3H/HeJ background (JR000659). Inducible NO synthase (iNOS; NOS2)-deficient (C57BL/6-*Nos2*^{tm1Lau}) mice homozygous for the *Nos2*^{tm1Lau} targeted mutation were purchased from The Jackson Laboratory (JR002609) at 3–8 wk of age and have been described previously (7). NOS2 controls were on the C57BL/6J background (JR000664). All mice were female, and age-matched controls were used in all experiments. Studies were approved by the Naval Medical Research Institute's Animal Use Committee, and the experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences Press, 1996.

Parasites

P. yoelii (17XNL nonlethal strain, clone 1.1) was maintained by alternating passage of the parasites in *Anopheles stephensi* mosquitoes and CD-1 mice.

Irradiated sporozoite immunizations

P. yoelii 17XNL-infected mosquitoes taken 14 days after an infectious blood meal were subjected to 10,000 rad of gamma radiation from a ¹³⁷Cs source, and sporozoites were isolated using a modification of the technique described by Ozaki et al. (8), as described previously (9). Briefly, infected mosquitoes were anesthetized at -20°C, washed in 80% ethanol, Medium 199 (M199) (Quality Biologicals, Gaithersburg, MD) containing Fungizone (2.5 µg/ml), and M199 containing penicillin (100 U/ml) and streptomycin (100 µg/ml) and then placed on a glass slide. The thorax of each mosquito was cut immediately anterior to the wings, the proboscis separated from the upper body, and both segments from groups of 50 mosquitoes were suspended in a volume of 50 µl M199 and centrifuged through a sterile column of glass wool in a microcentrifuge tube for 2 min at 10,000 rpm. Each column was rinsed with 50 µl M199. Sporozoite pellets were harvested after each centrifugation and combined. Irradiated sporozoites were counted using a hemocytometer and diluted to a final concentration of 100,000 sporozoites per 0.2 ml volume in M199 containing penicillin (100 U/ml) and streptomycin (100 µg/ml) without serum. Mice were immunized three times (BALB/c, B10.BR, A/J, B10.D2, and CD-1 strains) or four times (C57BL/6 and B6,129 strains) at 3-wk intervals i.v. in the tail vein with 100,000 irradiated sporozoites in a total volume of 200 µl M199 without serum.

DNA immunizations

The DNA vaccines encoding the *P. yoelii* circumsporozoite protein (PyCSP) and *P. yoelii* 17-kDa hepatocyte erythrocyte protein (PyHEP17) genes have been described previously (10–12). Briefly, the full-length PyCSP or PyHEP17 genes were cloned into the VR1012 vector, with expression of the encoded gene being driven by a CMV immediate/early gene promoter. Expression was confirmed *in vitro* by transient transfection of UM449 cells and subsequent analysis by Western blot as previously described (13), using the PyCSP-specific mAb NYS1 (14) or the PyHEP17-specific mAb NYLS3 (15). Mice were immunized three times at 3-wk intervals i.m. in each tibialis anterior muscle with 50 µg of each plasmid DNA construct, or unmodified VR1012 plasmid, in a total volume of 50 µl.

Linear peptide immunizations

An 18-aa linear synthetic peptide consisting of three copies of NPNEPS, the major repeat of *P. yoelii* sporozoite surface protein 2 (PySSP2) (16), was dissolved in PBS and emulsified with the nonionic block copolymer TiterMax (CytRx Corporation, Norcross, GA) at a 1:1 ratio. Mice were

immunized three times at 3-wk intervals s.c. at the base of the tail with 25 µg linear peptide in TiterMax, or with TiterMax alone (adjuvant control), in a total volume of 50 µl, as described previously (17).

Multiple Ag peptide (MAP) immunizations

The MAP was composed of a central glycine-lysine core and four branched chains, each consisting of a 20-aa linear peptide from the PyCSP (residues 280–299; SYVPSAEQILEFVKQISSL) containing the previously defined H-2K^d-restricted CTL epitope (residues 280–288; SYVPSAEQI) (18) co-linearly synthesized with two promiscuous Th epitopes from tetanus toxin, P2 (QYIKANSKFIGITEL) and P30 (FNNFTVSVFLWRVPKVSASHLE) (19). This MAP has been described previously (20). A MAP consisting of a glycine-lysine core and four branched chains of P2P30 was used as a negative control. Mice were immunized three times at 3-wk intervals s.c. at the base of the tail with a mixture of 16 µg MAP in PBS and 15 µl lipofectin (Life Technologies, Gaithersburg, MD) in a total volume of 50 µl.

Parasite challenge

Two weeks after the last immunization, mice were challenged by tail-vein injection of infectious sporozoites. It has been established previously that infection with as few as one or two sporozoites of *P. yoelii* 17XNL will result in patent infection of 50% of BALB/c mice (ID₅₀). For challenge of irradiated sporozoite-immunized mice, sporozoites were harvested from nonirradiated *P. yoelii* 17XNL-infected mosquitoes 14 days after an infectious blood meal using the modified Ozaki technique described above, and diluted to a final concentration of 5000 infectious sporozoites (BALB/c, B10.Q, and CD-1 mice) or 1000 infectious sporozoites (B10.BR, C57BL/6, B6,129, B10.D2, and A/J mice) per 0.2 ml volume in M199 containing 5% FCS. For challenge of DNA-immunized mice, peptide-immunized mice, or MAP-immunized mice, sporozoites were obtained 14 days after an infectious blood meal by hand dissection of *P. yoelii* 17XNL-infected mosquito glands in M199 medium containing 5% FCS and diluted to a final concentration of 50 infectious sporozoites (DNA-immunized BALB/c and B10.BR mice; MAP-immunized BALB/c mice), 100 infectious sporozoites (DNA-immunized A/J mice), or 200 infectious sporozoites (peptide-immunized A/J mice) per 0.2 ml volume in M199 containing 5% FCS. In experiments where mice were rechallenged, 2% normal mouse serum was substituted for 5% FCS in the challenge inoculum. Challenge doses were selected so as to ensure that all naive control mice were infected but that vaccine-induced sterile protection was not completely overwhelmed. In preliminary experiments where mice immunized with a given vaccine delivery system were challenged with different doses of infectious sporozoites (for example, DNA-immunized BALB/c or CD-1 mice challenged with 50, 100, or 200 sporozoites; or irradiated sporozoite-immunized BALB/c mice challenged with 1,000, 5,000, or 10,000 sporozoites), there was no apparent effect of challenge inoculum on the type of immune mechanism mediating the protection (data not shown). Giemsa-stained thin blood films were examined on days 5–14 postchallenge, up to 50 oil immersion fields being examined for parasites. Protection was defined as the complete absence of blood-stage parasitemia.

Antibodies

Purified control rat Ig was purchased from Rockland (Gilbertsville, PA). The anti-CD4⁺ mAb GK1.5 (rat IgG2a) (21) was obtained from American Type Culture Collection (TIB207; Manassas, VA). The anti-CD8⁺ mAb 2.43 (mouse IgG2a) (22) was also obtained from American Type Culture Collection (TIB210). The anti-IFN-γ mAb XMG-6 (rat IgG1) (23) was provided by Dr. F. Finkelman (University of Cincinnati College of Medicine, Cincinnati, OH). The anti-IL-12 mAb C17.8 (rat IgG2a) (24) was kindly provided by Drs. M. Wysocka and G. Trinchieri (The Wistar Institute, Philadelphia, PA). All Igs were purified from ascites (Harlan Bioproducts for Science, Indianapolis, IN) by 50% ammonium sulfate precipitation, and final Ab concentrations were determined by OD. Anti-asialo G_{M1} antiserum (rabbit) was purchased from Wako Bioproducts (Richmond, VA).

In vivo depletions

In vivo depletion regimes were designed so as to ensure that the treatments were effective and reproducible (data not presented). Immunized mice were treated as follows: undepleted, on days -7, -6, -5, -4, -3, -2, and 0 (relative to challenge with *P. yoelii* sporozoites on day 0), mice received a single i.p. dose of 1.0 mg purified rat Ig; CD4⁺ T cell depletion, on days -7, -6, -5, -4, -3, -2, 0, and +2, mice received a single i.p. dose of 1.0 mg of the anti-CD4⁺ mAb GK1.5; CD8⁺ T cell depletion, on days -5, -4, -3, -2, and 0, mice received a single i.p. dose of 0.5 mg of

⁴ Abbreviations used in this paper: ppo, perforin pore-forming protein; Gzmb, granzyme B; gld, generalized lymphoproliferative disease; FasL, Fas ligand; M199, Medium 199; iNOS, inducible NO synthase; PyCSP, *P. yoelii* circumsporozoite protein; PyHEP17, *P. yoelii* 17-kDa hepatocyte erythrocyte protein; PySSP2, *P. yoelii* sporozoite surface protein 2; MAP, multiple Ag peptide.

the anti-CD8⁺ mAb 2.43; IFN- γ depletion, on days -3, -2, -1, and +2, mice received a single i.p. dose of 1.0 mg of the anti-IFN- γ mAb XMG-6; IL-12 depletion, at 12 h before and 3 h after challenge, mice received a single i.p. dose of 1.0 mg of the anti-IL-12 mAb C17.8; NO depletion, twice daily, commencing 24 h before sporozoite challenge and for 72 h postchallenge, mice were administered 50 mg aminoguanidine (Sigma, St. Louis, MO) per kg body weight in 0.5 ml PBS via gastric lavage; NK cell depletion, on days -2, 0, +2, and +4, mice received a single i.v. dose of 200 μ l of anti-asialo G_{M1} antiserum diluted 1:8 in 0.5 \times PBS (25 μ l stock; \sim 675 μ g purified Ab).

FACS analysis

The efficiency of anti-CD4⁺ and anti-CD8⁺ Ab depletion in vivo was determined by performing single-color FACS using the FACScan (FAX 4000 Royal, Becton Dickinson Immunocytometry Systems, San Jose, CA). Spleen cells and/or PBMCs from Ab-treated and untreated mice were examined either at the time of challenge or when parasites were first detected in the peripheral blood. Approximately 1×10^6 cells of the population to be analyzed were stained with either anti-CD8⁺ FITC or anti-CD4⁺ FITC (PharMingen, San Diego, CA) for 1 h at 4°C. Unstained and FITC controls were included for each sample. Stained cells were washed three times, resuspended in paraformaldehyde (0.5% v/v), and stored at 4°C before analysis.

Statistical analysis

Statistical analysis was performed using the χ^2 test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five) (Epi Info Version 6.04b; Centers for Disease Control, Atlanta, GA).

Results and Discussion

Irradiated sporozoite vaccination induces distinct mechanisms of protection in different hosts

Recently (9), we established that the protection induced in BALB/c mice by immunization with irradiated sporozoites or plasmid DNA is mediated by a novel mechanism of adaptive immunity that we believe is initiated by CD8⁺ T cells following recognition of parasite-derived peptide-MHC complexes on the surface of infected hepatocytes, requires NK cells, and is dependent on IFN- γ , IL-12, and NO. We proposed that induction of IFN- γ is a direct consequence of the CD8⁺ T cell activation, that IFN- γ production precedes and initiates production of IL-12, and that the IL-12 in turn induces IFN- γ production by NK cells in a positive feedback loop that represents an important amplifying mechanism. The IFN- γ then activates NO synthase and induces the L-arginine-dependent NO pathway, subsequently eliminating the infected hepatocyte or the intrahepatic schizont. We further demonstrated that, in BALB/c mice, parasite-specific CD8⁺ CTL are not sufficient and that CD4⁺ T cells are not sufficient or required for the initial triggering of the effector mechanism, nor for the feedback induction of IFN- γ .

Despite that genetically distinct inbred mouse strains as well as MHC-diverse humans can be protected against *Plasmodium* spp. sporozoite challenge by irradiated sporozoite immunization, the protective immunity induced by immunization with radiation-attenuated sporozoites (25), synthetic peptide/adjuvant (17, 26), or plasmid DNA (11) is genetically restricted, the elimination of *P. yoelii* (27)- or *Plasmodium berghei* (28, 29)-infected hepatocytes from in vitro culture is also genetically restricted, and CD8⁺ T cell responses, CD4⁺ T cell responses, and B cell responses to defined *P. yoelii*, *P. berghei*, *Plasmodium falciparum*, and *Plasmodium vivax* epitopes are regulated by both MHC and non-MHC genes (reviewed in Ref. 30). Indeed, such restriction of protective CD8⁺ and/or CD4⁺ T cell responses is expected, because the TCR can only recognize its target peptide when complexed with a class I or II MHC molecule, respectively, and there are specific amino acid restrictions on the formation of that complex (reviewed in Ref. 31). Accordingly, we questioned whether the protective mechanism induced by immunization with radiation-attenuated sporozoites in

the BALB/c model could be extrapolated to other systems. We elected to define the requirement for CD8⁺ T cells, CD4⁺ T cells, NK cells, IFN- γ , IL-12, and NO in the sporozoite-induced protection in mice of diverse genetic backgrounds.

Seven inbred strains (BALB/c, H-2^d; B10.BR, H-2^k; A/J, H-2^a; C57BL/6, H-2^b; B6,129, H-2^b; B10.D2, H-2^d; and B10.Q, H-2^q) as well as outbred CD-1 mice were studied. Mice were immunized with radiation-attenuated *P. yoelii* sporozoites and depleted in vivo of CD8⁺ T cells (by treatment with anti-CD8⁺ mAb 2.43), CD4⁺ T cells (anti-CD4⁺ mAb GK1.5), NK cells (anti-asialo G_{M1} antiserum), IFN- γ (anti-IFN- γ mAb XMG-6), IL-12 (anti-IL-12 mAb C17.8), NO (aminoguanidine), or left undepleted (no Ab and/or control rat Ig), before challenge with infectious sporozoites. Protection was defined as the complete absence of blood-stage parasitemia.

Data are summarized in Tables I and II. A total of five distinct mechanisms of protective immunity were identified. In all of seven inbred strains and outbred mice, protection was absolutely dependent on CD8⁺ T cells because in vivo depletion of CD8⁺ T cells completely eliminated protective immunity. In four of seven inbred strains (BALB/c, H-2^d; B10.BR, H-2^k; A/J, H-2^a; B10.Q, H-2^q) as well as outbred CD-1 mice, CD4⁺ T cells were not sufficient nor required for the effector mechanism. Other studies have demonstrated a similar dependence on CD8⁺ but not CD4⁺ T cells in BALB/c mice immunized with irradiated *P. yoelii* (9, 25) or *P. berghei* (32) sporozoites, or A/J mice immunized with irradiated *P. berghei* sporozoites (33). However, in our studies, an absolute requirement for CD4⁺ T cells was demonstrated in C57BL/6 (H-2^b), B6,129 (H-2^b), and B10.D2 (H-2^d) strains (Tables I and II). This was unexpected but not surprising because it has been established that CD4⁺ T cells can recognize *Plasmodium* parasite-derived peptides presented on the surface of the infected hepatocyte in association with class II MHC (34, 35) and that CD4⁺ T cells can eliminate *P. yoelii*-infected hepatocytes from in vitro culture in an MHC-restricted manner (35) and protect against *P. yoelii* sporozoite challenge in adoptive transfer (35) and active immunization (17, 26) experiments. Indeed, it is curious that a CD4⁺ T cell protective immune response is required in only three of seven strains. The CD4⁺ T cell-mediated protection of A/J mice following peptide immunization (17) demonstrates that the lack of requirement for CD4⁺ T cells following immunization with either *P. yoelii* or *P. berghei* sporozoites is not due to an intrinsic inability of the mice to mount such a response. Furthermore, data suggest that this mechanism may not be under Ir gene control because BALB/c and B10.D2 mice both possess the H-2^d haplotype.

In an earlier study (25), it was reported that only two (BALB/c and B10.Q) of ten congenic strains of mice immunized with irradiated sporozoites were highly protected against challenge with *P. yoelii* sporozoites. On the BALB background, only H-2^d mice were protected, and this protection was dependent on CD8⁺ T cells. In contrast, on the B10 background, only H-2^q mice were protected, and this protection was not dependent on CD8⁺ T cells. In that study, the effect of CD4⁺ T cell depletion was not assessed, and it is likely that the reported lack of requirement for CD8⁺ T cells in the B10.Q strain may have reflected inadequate in vivo depletion. Furthermore, Weiss and colleagues used an uncloned line of *P. yoelii* 17XNL known to contain two distinct clones (designated 1.1 and 1.2) that differ in their pattern of Ag expression as assessed by immunoblot of circumsporozoite protein; *P. yoelii* 17XNL clone 1.1 was used in all studies reported here. In a parallel study, consistent with our data, it was shown (36) that all of nine B10 congenic strains could be protected against *P. berghei* sporozoite challenge in that study neither the CD8⁺ dependence nor mechanism of protection were determined.

Table I. Protective immune mechanisms induced by irradiated sporozoite immunization

	H-2	Treatment ^a (% protection (no. protected/no. challenged))							
		None	Control	CD8 ⁺ (p) ^b	CD4 ⁺ (p)	IFN- γ (p)	IL-12 (p)	iNOS (p)	NK cells (p)
BALB/c	d	100 (16/16)	94 (15/16)	0 (0/16) 0.0000000	100 (16/16) –	0 (0/10) 0.0000002	0 (0/10) 0.0000002	0 (0/16) 0.0000000	50 (5/10) 0.0038
B10.BR	k	56 (5/9)	56 (5/9)	0 (0/9) 0.029	50 (5/10) –	0 (0/9) 0.029	44 (4/9) ^c –	0 (0/9) 0.029	50 (5/10) –
A/J ^d	a (k/d)	30 (3/10)	19 (6/31)	0 (0/18) 0.045	45 (8/18) –	0 (0/18) 0.045	20 (6/30) –	21 (5/24) –	6 (1/18) 0.154
C57BL/6	b	60 (6/10)	67 (6/9)	0 (0/9) 0.011	0 (0/9) 0.011	0 (0/10) 0.005	0 (0/9) 0.011	0 (0/9) 0.011	0 (0/10) 0.005
B6,129	b	56 (5/9)	56 (5/9)	0 (0/9) 0.029	0 (0/9) 0.029	0 (0/10) 0.011	0 (0/10) 0.011	10 (1/10) 0.057	ND
B10.D2	d	ND	46 (5/11)	0 (0/10) 0.035	0 (0/10) 0.035	0 (0/9) 0.038	0 (0/10) 0.035	9 (1/11) 0.149	ND
B10.Q	q	100 (16/16)	100 (16/16)	0 (0/10) 0.0000002	86 (6/7) –	100 (10/10) –	94 (15/16) –	ND ^e	ND
CD-1	Outbred	100 (9/9)	100 (9/9)	0 (0/9) 0.00004	89 (8/9) –	56 (5/9) 0.082	56 (5/9) 0.082	67 (6/9) 0.206	56 (5/9) 0.082

^a Mice were immunized with irradiated sporozoites and immune mice were treated before challenge with 5000 (BALB/c, B10.Q, and CD-1) or 1000 (B10.BR; C57BL/6; B6,129; B10.D2; and A/J) infectious sporozoites, as indicated: control, control rat Ig; CD8⁺, anti-CD8⁺ mAb 2.43; CD4⁺, anti-CD4⁺ mAb GK1.5; IFN- γ , anti-IFN- γ mAb XMG-6; IL-12, anti-IL-12 mAb C17.8; iNOS, aminoguanidine; NK cells, anti-asialo G_{M1} antiserum. All naive mice of all strains ($n = 9-10$) developed patent blood-stage parasitemia.

^b Values of p were calculated by comparing protection with test treatment versus control, for each strain, using the χ^2 test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five).

^c Protected B10.BR mice were randomized into two groups and treated with either control Ab or anti-IL-12 mAb administered at a higher dose and more intense schedule and were rechallenged. A total of 11/11 (100%) of control Ab-treated and 12/12 (100%) of anti-IL-12-treated mice were protected. All naive controls developed patent blood-stage parasitemia.

^d Data with the A/J strain are the accumulated results of three separate experiments. Values of p are calculated relative to total number of mice either treated with control Ig or not treated (9/41 mice).

^e Protected B10.Q mice were randomized into three groups and treated with control Ab, anti-CD4⁺ mAb, or aminoguanidine and were rechallenged. A total of 3/6 (50%) control Ab-treated, 5/7 (71%) anti-CD4⁺ mAb-treated, and 3/6 (50%) aminoguanidine-treated mice were protected. All naive controls developed patent blood-stage parasitemia.

For six of the seven inbred strains, we were able to demonstrate an absolute requirement for IFN- γ in the *P. yoelii* sporozoite-induced protection (Tables I and II), as previously established in the *P. yoelii*-BALB/c (9), *P. berghei*-BALB/c (32), and *P. berghei*-A/J (33) systems. Consistent with our proposal (9) that IFN- γ activates NO synthase, subsequently inducing the L-arginine-dependent NO pathway, an absolute requirement for NO was also demonstrated for five of the six IFN- γ -dependent strains (Tables I and II). However, in A/J mice, data indicated that elimination of the infected hepatocyte or intrahepatic schizonts may occur via an alternative mediator, perhaps free radicals. Previously, Seguin et al. (32) noted that iNOS activity was induced in the liver of naive BALB/c mice following i.v. challenge with a massive dose ($>10^6$) of viable sporozoites (presumably the result of NK cell production of IFN- γ), but these mice were not protected, suggesting that induc-

tion of NO activity is necessary but not sufficient for protection against sporozoites. Consistent with a putative role for an alternate mediator to NO, we here demonstrated partial protection in NOS2^{-/-} mice that lack iNOS (Table III). Similar results have recently been reported with blood-stage *P. berghei* (XAT) infection (37). In that study, infected iNOS^{-/-} splenocytes did not produce detectable levels of NO but iNOS^{-/-} mice were able to clear blood-stage *P. berghei* parasitemia in an IFN- γ -dependent manner, leading the authors to speculate on the existence of an alternative pathway of pathogen clearance in iNOS^{-/-} mice.

We have also proposed (9) that IFN- γ initiates production of IL-12, and that the IL-12 in turn induces IFN- γ production by NK cells in a positive feedback loop. Consistent with this, an absolute requirement for IL-12 was demonstrated in C57BL/6, B6,129, and B10.D2 mice, and for NK cells in C57BL/6 mice (B6,129 and

Table II. Summary of protective immune mechanisms induced by irradiated sporozoite immunization

	H-2	Treatment ^a						Mechanism
		CD8 ⁺	CD4 ⁺	IFN- γ	IL-12	iNOS	NK cells	
BALB/c	d	+	–	+	+	+	+	1
B10.BR	k	+	–	+	–	+	–	2
A/J	a (k/d)	+	–	+	–	–	+	3
C57BL/6	b	+	+	+	+	+	+	4
B6,129	b	+	+	+	+	+	ND	4
B10.D2	d	+	+	+	+	+	ND	4
B10.Q	q	+	–	–	–	(–)	ND	5
CD-1	Outbred	+	–	+/-	+/-	+/-	+/-	

^a Mice were immunized with irradiated sporozoites and immune mice were treated before challenge with 5000 (BALB/c, B10.Q, and CD-1) or 1000 (C57BL/6; B6,129; B10.D2; and A/J) infectious sporozoites, as indicated: CD8⁺, anti-CD8⁺ mAb 2.43; CD4⁺, anti-CD4⁺ mAb GK1.5; IFN- γ , anti-IFN- γ mAb XMG-6; IL-12, anti-IL-12 mAb C17.8; iNOS, aminoguanidine; NK cells, anti-asialo G_{M1} antiserum. +, Dependent; –, not dependent; +/-, partially dependent.

Table III. *NOS2^{-/-} mice are partially protected against challenge following sporozoite immunization*

Strain	Treatment ^a	No. Protected/No. Challenged	% Protection	<i>p</i> Value ^b
C57BL/6	wt	14/15	93	
C57BL/6	NOS2 ^{-/-}	8/12	67	0.139

^a All naive wild-type (wt) mice (*n* = 10) developed patent blood-stage parasitemia.

^b The value of *p* was calculated by comparing protection in deficient vs wt mice using the Fisher's exact test (two-tailed).

B10.D2 mice not tested) (Tables I and II). However, in B10.BR mice, neither IL-12 nor NK cells were required. Because it could be argued that these results may reflect inadequate depletion *in vivo*, B10.BR mice that were protected against challenge in the first experiment were randomized and subjected to a more rigorous depletion regime with anti-IL-12 mAb or control Ab. This treatment had no effect on protection (11/11 control Ab-treated vs 12/12 anti-IL-12-treated mice were protected), indicating that the IL-12/NK cell feedback loop is not required in B10.BR mice. In this strain, localized IFN- γ secreted by T cells appears to be adequate.

Curiously, in A/J mice, there was an absolute requirement for NK cells but not for IL-12 (Tables I and II), suggesting that the IL-12-induced amplification of IFN- γ via NK cells does not occur and that the IFN- γ produced initially as a direct consequence of the CD8⁺ T cell activation and subsequently by NK cells is sufficient for protection. Consistent with this, the degree of protective immunity induced in A/J mice by immunization with irradiated sporozoites was not as profound as in those strains where a role for both IL-12 and NK cells was demonstrated (Table I).

Data above demonstrate that in six of seven inbred strains of mice with diverse genetic backgrounds, protective immunity induced by immunization with radiation-attenuated sporozoites is mediated by a cytokine cascade initiated by parasite-specific CD8⁺ T cells following recognition of defined peptide-MHC complexes on the surface of the infected hepatocyte. IFN- γ , which is produced as a direct consequence of the CD8⁺ T cell activation and the production of which may be up-regulated by a feedback loop involving IL-12 and NK cells, depending on the host, is a necessary component of this effector mechanism. IFN- γ , via signal transducers associated with transcription, then activates NO synthase and induces the L-arginine-dependent NO pathway, subsequently eliminating the infected hepatocytes or the hepatic schizonts within those cells. However, in at least one strain (A/J), NO is not required. Furthermore, in some strains, CD4⁺ T cells are not required or sufficient for the initial triggering of the effector mechanism nor for the feedback induction of IFN- γ , but, in other strains, there is an absolute requirement for CD4⁺ T cells. Finally, in at least one strain (B10.Q), sporozoite-induced protective immunity may be mediated via classical CD8⁺ CTL because the effector mechanism requires CD8⁺ T cells, but not CD4⁺ T cells, NK cells, IFN- γ , IL-12, or NO (Tables I and II). However, confirmation of this is difficult, because perforin or granzyme B gene-deficient mice are not available on the H-2^q background, and H-2q-restricted CTL epitopes have not yet been defined for any *P. yoelii* Ags. Alternatively, it is possible that the B10.Q strain studied here may have some as yet unreported immune defect that may affect the production of cytokines or other immune mediators but that is independent of the genetic haplotype.

Consistent with the diversity of immune responses observed in genetically distinct inbred strains, sporozoite-induced protection in outbred CD-1 mice appears to be mediated by a variety of effector

Table IV. *Perforin^{-/-} mice, Gzmb^{-/-} mice, and FasL-deficient mice are protected against challenge following sporozoite immunization^a*

Expt.	Strain	Knockout	No. Protected/No. Challenged	% Protection	<i>p</i> Value ^b
1	C57BL/6	wt	10/17	59	
	C57BL/6	Perforin ^{-/-}	19/20	95 ^c	0.014
2	B6,129	wt	4/10	40	
	B6,129	Gzmb ^{-/-}	4/8	50	1.000
3	C3H/HeJ	wt	12/12	100	
	C3H/HeJ	gld (FasL)	13/13	100	–

^a All naive mice (Expt. 1, *n* = 16; Expt. 2, *n* = 8; Expt. 3, *n* = 10) developed patent blood-stage parasitemia.

^b Values of *p* were calculated by comparing protection in deficient vs wt mice, for each experiment, using the Fisher's exact test (two-tailed).

^c Perforin^{-/-} mice remained protected when rechallenged in subsequent experiments (Table V).

mechanisms, all of which have an absolute requirement for CD8⁺ T cells (Tables I and II).

It is apparent from these data that a single vaccine delivery system, namely immunization with irradiated sporozoites, can induce multiple different mechanisms of protection, depending on the host.

Perforin, granzyme B, and FasL are not required for irradiated sporozoite-induced protection

For many years, the potent protective immunity of mice and humans induced by immunization with radiation-attenuated sporozoites, directed primarily against the *Plasmodium* spp. parasite developing within the host hepatocyte, has been presumed to be mediated directly by CD8⁺ CTL (reviewed in Refs. 3 and 38). Our data, here and elsewhere (9), demonstrate that the protection induced by immunization with irradiated sporozoites in most inbred mice as well as in outbred CD-1 mice is mediated indirectly via a CD8⁺ T cell-initiated cytokine cascade, rather than directly via CD8⁺ CTL. Indeed, we have shown in BALB/c mice that parasite-specific CD8⁺ CTL are not sufficient for protection, because IFN- γ gene knockout mice were not protected against sporozoite challenge despite the presence of high levels of CTL (9). However, those studies could not establish that CD8⁺ CTL are not required because the requirement for perforin, granzyme B, or FasL could not be assessed on the BALB/c background.

Two principal mechanisms whereby CTL can kill their target cells have been defined (reviewed in Ref. 39). One mechanism is mediated by perforin and granzyme B (formerly called CTL-associated protein-1, Ctla1), where CD8⁺ T cells release perforin and granzyme B in response to recognition of a target cell expressing MHC class I/peptide complexes. The second mechanism is mediated by the interaction of the Fas receptor (CD95) on the target cell and its ligand FasL (CD95L) on the activated effector T cell. Ppo^{-/-} mice (C57BL/6-*Pfp^{tm1Sdz}*) homozygous for the *Pfp^{tm1Sdz}* targeted mutation have normal numbers of CD8⁺ T cells and NK cells but lack a functioning perforin gene; hence the cytotoxicity mediated by CD8⁺ T cells and NK cells is greatly impaired. Gzmb^{-/-} mice (B6,129-Gzmb^{tm1Ley}) are homozygous for the *Gzmb^{tm1Ley}* targeted mutation, and activated CTL from Gzmb^{-/-} mice are deficient in the early induction of DNA fragmentation and apoptosis in allogenic target cells. Gld mice (C3H/HeJ-*FasL^{gld}*) are homozygous for a spontaneous point mutation in FasL, a TNF-related type II membrane protein that binds to Fas and mediates apoptosis.

Accordingly, having established that a cytokine-mediated effector mechanism operates in the respective wild-type strains, we next

Table V. Sporozoite-induced protection in perforin^{-/-} mice is CD8⁺ T cell dependent and IFN- γ independent^a

Expt.	Knockout	Treatment	Treatment Regimen ^b	No. Protected/No. Challenged	% Protection	<i>p</i> Value ^c
1	Perforin ^{-/-}	Control Ab		9/10	90	0.0003
	Perforin ^{-/-}	Anti-CD8 ⁺ mAb		1/10	10	
2	Perforin ^{-/-}	Control Ab		6/6	100	0.455
	Perforin ^{-/-}	Anti-IFN- γ mAb	1.0 mg on day -2, -1, 0, and +2	4/5	80	
3	wt	None		9/9	100	-
	Perforin ^{-/-}	Control Ab		5/5	100	
	Perforin ^{-/-}	Anti-IFN- γ mAb	2.0 mg on day -2, -1, 0, and +2	5/5	100	
4	Perforin ^{-/-}	Control Ab		5/5	100	-
	Perforin ^{-/-}	Anti-IFN- γ mAb	2.0 mg on day -2, -1, 0, +1, +2, and +3	5/5	100	

^a All naive mice in all experiments (Expt. 1, *n* = 6; Expt. 2, *n* = 6; Expt. 3, *n* = 10; Expt. 4, *n* = 10) developed patent blood-stage parasitemia.

^b In Expt. 2, immunized mice were treated with 1.0 mg of anti-IFN- γ mAb on day -2, -1, 0, and +2 relative to challenge with 1000 sporozoites on day 0. In Expt. 3, mice were treated with 2.0 mg of anti-IFN- γ mAb via IP injection on day -2, -1, 0, and +2. In Expt. 4, mice were treated with 2.0 mg on day -2, -1, 0, +1, +2, and +3. In all experiments, immunized mice were treated with control Ab in parallel.

^c Values of *p* were calculated by comparing protection in deficient vs wt mice, for each experiment, using the χ^2 test (Expt. 1) or Fisher's exact test (two-tailed) (Expt. 2).

assessed the requirement for CTL in sporozoite-induced protective immunity by studying ppo^{-/-} mice, Gzmb^{-/-} mice, and FasL-deficient mice. In our studies, ppo^{-/-} mice, Gzmb^{-/-} mice and FasL-deficient mice were protected by immunization with irradiated sporozoites (Table IV). Therefore, data establish that parasite-specific CTL are neither sufficient nor required for the protective immunity induced in mice by immunization with radiation-attenuated *P. yoelii* sporozoites. Similar results have been reported with perforin and FasL-deficient mice in the *P. berghei* system (40).

Curiously, in our studies, ppo^{-/-} mice were better protected than wild-type mice (Table IV). This protection was absolutely dependent on CD8⁺ T cells (Table V), as observed for the wild-type mice (Table I). However, we were unable to eliminate protection by in vivo depletion of IFN- γ , despite three different depletion regimes (Table V). This requirement for CD8⁺ T cells but not IFN- γ nor perforin in the effector mechanism is at odds with each of the five mechanisms defined in the eight strains studied here. It is possible that this anomaly may reflect the up-regulation

Table VI. Protective immune mechanisms induced by different vaccine delivery systems

Vaccine ^b	Strain ^c	H-2	Treatment ^a (% protection (no. protected/no. challenged))						
			Control	CD8 ⁺ (<i>p</i>) ^d	CD4 ⁺ (<i>p</i>)	IFN- γ (<i>p</i>)	IL-12 (<i>p</i>)	iNOS (<i>p</i>)	NK cells (<i>p</i>)
SPZ	BALB/c ^e	d	94 (15/16)	0 (0/16)	100 (16/16)	0 (0/10)	0 (0/10)	0 (0/16)	50 (4/8)
SPZ	B10.BR ^e	k	56 (5/9)	0 (0/9)	50 (5/10)	0 (0/9)	44 (4/9)	0 (0/9)	50 (5/10)
SPZ	A/J ^e	k/d	19 (6/31)	0 (0/18)	45 (8/18)	0 (0/18)	20 (6/30)	21 (5/24)	6 (1/18)
DNA	BALB/c ^f	d	28 (20/72)	+ ^g	- ^g	0 (0/39)	0 (0/32)	0 (0/37)	0 (0/30)
						0.0003	0.0009	0.0004	0.0012
DNA	B10.BR ^h	k	90 (9/10)	+ ^g	- ^g	0 (0/10) ^g	ND	0 (0/10) ^g	ND ⁱ
						0.0001		0.0001	
DNA	A/J ^j	k/d	28 (5/18)	+ ^g	- ^g	0 (0/10)	0 (0/8)	0 (0/10)	ND
						0.128	0.281	0.128	
Peptide	A/J	k/d	80 (16/20)	- ^k	+ ^k	0 (0/7)	91 (10/11)	86 (6/7)	ND
						0.0004	0.631	1.0000	
MAP	BALB/c	d	13 (6/46)	0 (0/30)	ND	11 (3/27) ^l	25 (4/16)	12 (3/25) ^l	ND
				0.075		1.0000	0.433	1.0000	

^a Immune were mice treated before challenge, as indicated: control, control rat Ig; CD8⁺, anti-CD8⁺ mAb 2.43; CD4⁺, anti-CD4⁺ mAb GK1.5; IFN- γ , anti-IFN- γ mAb XMG-6; IL-12, anti-IL-12 mAb C17.8; iNOS, aminoguanidine; NK cells, anti-asialo G_{M1} antiserum.

^b Sporozoite (SPZ)-immunized mice were challenged with 5000 (BALB/c) or 1000 (B10.BR and A/J) infectious sporozoites. DNA-immunized mice were challenged with 50 (BALB/c) or 100 (B10.BR and A/J) infectious sporozoites. Peptide-immunized mice (A/J) were challenged with 100 infectious sporozoites. MAP-immunized mice (BALB/c) were challenged with 50 infectious sporozoites.

^c All naive mice of all strains (*n* = 9–10) developed patent blood-stage parasitemia. All control mice immunized with empty plasmid and treated with control Ab (BALB/c, *n* = 46; B10.BR, *n* = 10; A/J, *n* = 10) developed patent blood-stage parasitemia. All control mice immunized with adjuvant and treated with control Ab (A/J, *n* = 16) or anti-IL-12 mAb (A/J, *n* = 16) developed patent blood-stage parasitemia. All control mice immunized with P2P30 control MAP and treated with control Ab (BALB/c, *n* = 16) or anti-IL-12 mAb (BALB/c, *n* = 16) developed patent blood-stage parasitemia.

^d Values of *p* were calculated by comparing protection with test treatment versus control, for each strain in each experiment, using the χ^2 test (uncorrected) or Fisher's exact test (two-tailed) (if the expected cell value was less than five).

^e Summarized from Table I.

^f BALB/c mice were immunized with PyCSP DNA or a mixture of PyCSP plus PyHEP17 DNA. Data are the accumulated results of four separate experiments.

^g Summarized from Doolan et al. (Ref. 11).

^h B10.BR mice were immunized with a mixture of PyCSP plus PyHEP17 DNA.

ⁱ In a separate experiment, immune B10.BR mice were randomized into three groups and treated with control Ab or anti-asialo G_{M1} Abs (anti-NK cells) and were challenged. A total of 2/12 (17%) of control Ab-treated and 2/11 (18%) of anti-asialo G_{M1}-treated mice were protected. All naive controls developed patent blood-stage parasitemia.

^j A/J mice were immunized with PyHEP17 DNA.

^k Summarized from Wang et al. (Ref. 17).

^l In these experiments with the MAP vaccine, protection was defined as a 3- to 4-day delay in patency; in all other experiments, protection was defined as the complete absence of blood-stage parasitemia.

Table VII. Summary of protective immune mechanisms induced by different vaccine delivery systems

Vaccine	Strain	Treatment ^a							
		H-2	Control	CD8 ⁺	CD4 ⁺	IFN- γ	IL-12	iNOS	NK cells
SPZ	BALB/c	d	94 (15/16)	+	-	+	+	+	+
SPZ	B10.BR	k	56 (5/9)	+	-	+	-	+	-
SPZ	A/J	k/d	19 (6/31)	+	-	+	-	-	+
DNA	BALB/c	d	28 (20/72)	+	-	+	+	+	+
DNA	B10.BR	k	90 (9/10)	+	-	+	ND	+	ND
DNA	A/J	k/d	28 (5/18)	+	-	+	+	+	ND
Peptide	A/J	k/d	80 (16/20)	-	+	+	-	-	ND
MAP	BALB/c	d	13 (6/46)	+	ND	-	-	-	ND

^a Immune mice were treated before challenge, as indicated: control, control rat Ig; CD8⁺, anti-CD8⁺ mAb 2.43; CD4⁺, anti-CD4⁺ mAb GK1.5; IFN- γ , anti-IFN- γ mAb XMG-6; IL-12, anti-IL-12 mAb C17.8; iNOS, aminoguanidine; NK cells, anti-asialo G_{M1} antiserum. +, Dependent; -, not dependent.

of compensatory immune mechanisms in gene knockout mice and that, in this strain, protection may be mediated via the Fas/FasL receptor/ligand system. In other systems, it has shown that cytolytic destruction of the target cell may be mediated via either perforin- or Fas-dependent processes where, for instance, perforin-mediated cytotoxicity may be the preferred mode but a Fas-based mechanism could serve as an alternative mechanism (41–44). Our data does not exclude this possibility, and in that case double depletion of perforin and Fas would be necessary to reveal cytolytic involvement. Alternatively, it is possible that the depletion regimes used here in the ppo^{-/-} mice may have been inadequate to completely deplete a potentially enhanced pool of effector molecules. Perforin has been implicated recently with a role in immune regulation (44, 45), with increased numbers of activated CD8⁺ T cells (44) and enhanced cellular and humoral immune responses (46) noted in perforin-deficient mice.

Distinct mechanisms of protection induced by irradiated sporozoite vaccination are not associated with host MHC

Here, we have defined five distinct T cell-mediated mechanisms of protective immunity induced following immunization with radiation-attenuated sporozoites (Table II). However, data suggest that these effector mechanisms are not under Ir gene control, because distinct mechanisms were established for each of the three congenics on the B10 background (B10.BR, B10.D2, and B10.Q). Congenic strains represent inbred strains that were derived from their origin by selective matings such that they differ from the originating strain at only one independently segregating genetic loci, namely their H-2 complex, but not in any other loci. Furthermore, distinct mechanisms were also identified for each of the H-2^d haplotypes on the B10 (B10.D2) and BALB (BALB/c) back-

grounds (Table II) and for each of BALB/c (H-2^d), B10.BR (H-2^k), and A/J (H-2^a = H-2^{k/d}) mice (Table II). However, we cannot exclude the possibility that some of the anomalous behavior of the inbred strains studied may result from previously unidentified defects in immune mechanisms in the mice strains studied, rather than genetic variation.

A single vaccine delivery system induces the same mechanism of protection in different hosts

Previously (11), we reported that the protective immunity induced by immunization of BALB/c, B10.BR, and A/J mice with plasmid DNA encoding the pre-erythrocytic Ags PyCSP or PyHEP17, or the combination, is dependent on CD8⁺ T cells but not CD4⁺ T cells. In B10.BR mice, we established an additional requirement for IFN- γ and NO (11). We now extend those observations to demonstrate the same requirement for IFN- γ and NO in BALB/c mice (Ref. 9; Tables VI and VII) and A/J mice (Tables VI and VII). We further establish an absolute requirement for IL-12 and NK cells in the effector mechanism of DNA-induced protection in all of three genetically different strains, where tested (Tables VI and VII). These data illustrate that the protective immunity conferred by one vaccine delivery system, plasmid DNA, may be mediated by a single effector mechanism, independent of the host (Table VIII). However, it is likely that the nature of the protective immune response will be influenced by the Ag as well as the delivery system.

Different vaccine delivery systems induce the same mechanism of protection in the same host

In BALB/c mice, protection induced by immunization with either irradiated sporozoites or plasmid DNA was absolutely dependent

Table VIII. Summary of protective immune mechanisms induced by different vaccine delivery systems in the same strain

Vaccine	Strain	Treatment ^a						
		H-2	CD8 ⁺	CD4 ⁺	IFN- γ	IL-12	iNOS	NK cells
SPZ	BALB/c	d	+	-	+	+	+	+
DNA	BALB/c	d	+	-	+	+	+	+
MAP	BALB/c	d	+	ND	-	-	-	ND
SPZ	B10.BR	k	+	-	+	-	+	-
DNA	B10.BR	k	+	-	+	ND	+	ND
SPZ	A/J	k/d	+	-	+	-	-	+
DNA	A/J	k/d	+	-	+	+	+	ND
Peptide	A/J	k/d	-	+	+	-	-	ND

^a Immune mice were treated before challenge, as indicated: CD8⁺, anti-CD8⁺ mAb 2.43; CD4⁺, anti-CD4⁺ mAb GK1.5; IFN- γ , anti-IFN- γ mAb XMG-6; IL-12, anti-IL-12 mAb C17.8; iNOS, aminoguanidine; NK cells, anti-asialo G_{M1} antiserum. +, Dependent; -, not dependent.

Table IX. Linear peptide (NPNEPS)-induced protection (A/J mice) is CD4⁺ T cell and IFN- γ dependent but IL-12 and NO independent

Expt.	Treatment ^a	No. Protected/No. Challenged	% Protection	<i>p</i> Value ^b
1	Control Ab	11/13	85	1.000
	Anti-IL-12 mAb	10/11	91	
2	Control Ab	5/7	71	0.021
	Anti-IFN- γ mAb	0/7	0	
	Anti-iNOS	6/7	86	

^a All adjuvant control mice treated with control Ab ($n = 16$) or anti-IL-12 mAb ($n = 16$) developed patent blood-stage parasitemia.

^b Values of *p* were calculated by comparing protection with test treatment versus control, in each experiment, using Fisher's exact test (two-tailed).

on CD8⁺ T cells, IFN- γ , IL-12, and NO and partially dependent on NK cells (Tables VII and VIII). Likewise, in B10.BR mice, the effector mechanism induced by immunization with irradiated sporozoites or with plasmid DNA showed the same dependence on CD8⁺ T cells, IFN- γ , and NO (Tables VII and VIII). These data illustrate that the protective immunity conferred by different vaccine delivery systems may be mediated by the same effector mechanism in the one host.

Different vaccine delivery systems induce distinct mechanisms of protection in the same host

We have previously reported that immunization of A/J mice with a linear synthetic peptide from PySSP2 (17) or PyHEP17 (26) in adjuvant induces a high degree of protection that is dependent upon CD4⁺ T cells and IFN- γ , but not CD8⁺ T cells. Here, we extend those studies to demonstrate that the PySSP2 peptide-induced IFN- γ -dependent immunity is not dependent on IL-12 nor NO (Table IX). This IFN- γ -dependent but IL-12- and NO-independent protective mechanism resembles that induced in the same strain by immunization with irradiated sporozoites, with one exception. Specifically, the effector mechanism is mediated by CD4⁺ T cells following peptide immunization and by CD8⁺ T cells following sporozoite-immunization. It is not apparent why CD8⁺ T cells are not adequate or required for peptide-induced protection and why CD4⁺ T cells are not adequate or required for sporozoite-induced protection. However, CD4⁺ T cells are a more potent cellular source of IFN- γ , which may perhaps explain why the CD4⁺ T cell-mediated peptide-induced protection is more solid (80% vs 19%) and can withstand a much higher sporozoite challenge (our unpublished observations) compared with sporozoite-induced protection.

Our data (Tables VI–VIII) demonstrate that protection in A/J mice induced by immunization with each of three distinct vaccine

delivery systems, namely irradiated sporozoites, synthetic peptide, or plasmid DNA, is absolutely dependent on IFN- γ . However, DNA-induced protection is dependent on both IL-12 and NO, but sporozoite-induced and peptide-induced protection are independent of both IL-12 and NO. The abrogation of DNA-induced protection by in vivo depletion of IL-12 and NO shows that the treatment regimes used are effective in A/J mice, so the lack of a demonstrated role for IL-12 and NO in peptide-induced and sporozoite-induced effector mechanisms does not reflect inadequate depletion. Because A/J mice are intrinsically capable of mounting IL-12- and NO-mediated immune responses, it is not clear why responses that presumably would increase protective efficacy are not invoked by all vaccine delivery systems.

This complexity of protective immunity is reinforced in the BALB/c system (Tables VI–VIII). In BALB/c mice, protection induced by two vaccine delivery systems, namely irradiated sporozoites and plasmid DNA, appears to be mediated by the same mechanism. In contrast, however, the effector mechanism induced by immunization with a MAP construct of the immunodominant PyCSP CD8⁺ CTL epitope (20) is independent of IFN- γ , NO, and IL-12 and presumably mediated by classical CD8⁺ CTL.

These data establish that different vaccine delivery systems can induce distinct mechanisms of protection in the same host.

Surrogate markers of protective immunity

Herein, we have identified a total of six distinct T cell-mediated effector mechanisms of protection against liver-stage malaria. Data establish that a single vaccine delivery system may induce distinct mechanisms of protective immunity in different hosts and that a single vaccine delivery system may induce the same mechanism of protection in different hosts. Furthermore, different vaccine delivery systems may induce the same mechanism of protection in the same host, and different vaccine delivery systems may induce distinct mechanisms of protection in the same host. This complexity of protective immunity in the mouse model is likely to reflect a similar diversity in humans, in response to parasitic infection. Such complexity may pose serious obstacles to the development of an efficacious vaccine and to the identification of surrogate markers of protective immunity.

However, despite this apparent complexity, we noted an absolute requirement for both CD8⁺ T cells and IFN- γ in five of the six effector mechanisms (Table X). Furthermore, our data indicate that IFN- γ may be a suitable correlate of protective immunity. IFN- γ was implicated in both CD8⁺ and CD4⁺ T cell-mediated protection. Indeed, the only mechanism that was independent of IFN- γ was that induced in B10.Q mice by immunization with irradiated sporozoites and in BALB/c mice by immunization with a CTL

Table X. In vitro markers of protection against Plasmodium sporozoite challenge

Mechanism	Vaccine	Strain	Treatment ^a					
			CD8 ⁺	CD4 ⁺	IFN- γ	IL-12	iNOS	NK cells
1	Sporozoite DNA	BALB/c	+	–	+	+	+	+
		BALB/c; B10.BR; A/J	+	–	+	–	+	–
2	SPZ	B10.BR	+	–	+	–	+	–
3	SPZ	A/J	+	–	+	–	–	+
4	SPZ	C57BL/6; B6.129; B10.D2	+	+	+	+	+	+
5	SPZ	B10.Q	+	–	–	–	–	ND
6	MAP Peptide	BALB/c	–	+	+	–	–	ND
		A/J	–	+	+	–	–	ND
Representative			5/6	2/6	5/6	2/6	3/6	

^a Immune mice were treated before challenge, as indicated: CD8⁺, anti-CD8⁺ mAb 2.43; CD4⁺, anti-CD4⁺ mAb GK1.5; IFN- γ , anti-IFN- γ mAb XMG-6; IL-12, anti-IL-12 mAb C17.8; iNOS, aminoguanidine; NK cells, anti-asialo G_{M1} antiserum. +, Dependent; –, not dependent.

MAP, where protection is presumably mediated directly by CD8⁺ CTL. Because IFN- γ would be released as a consequence of CD8⁺ T cell activation, even in these systems, IFN- γ would be predicted to be a surrogate marker of activation and therefore of protection.

Conclusion

Herein, working with irradiated sporozoite, DNA, and synthetic peptide/adjuvant immunizations, we have demonstrated that distinct T cell-dependent immune responses mediate sterile protective immunity in one mouse strain, depending upon the method of immunization. Furthermore, we have shown that the mechanism of protection induced by a single method of immunization may vary among different strains.

The data presented here underscore the complexity of the murine host response to a parasitic infection and suggest that an outbred human population may behave similarly. Data nevertheless suggest that a vaccine that induces the requisite immune responses should be feasible provided that the correct vaccine delivery system is used, and a vaccine designed to protect humans against *Plasmodium* spp. sporozoite challenge should induce IFN- γ -mediated immune responses.

Small animal models have been extremely helpful throughout the history of vaccine development for a wide variety of infectious diseases. To study protective immunity against malaria, the foundation for vaccine development, a model of *Plasmodium* spp. infection is required. Results obtained in the *P. yoelii* rodent model by many investigators indicate that this is a good model for human malaria, and, where testable, most results can be extrapolated to the species of *Plasmodium* that causes disease in humans. For example, both mice and humans can be protected from challenge with *Plasmodium* sporozoite challenge by immunization with radiation-attenuated *P. yoelii* or *P. falciparum* sporozoites, respectively; the targets of protective immunity in the *P. yoelii* system appear to be homologous to the targets of immune responses in humans immunized with irradiated *P. falciparum* sporozoites, and the immune mediators and mechanisms that act in the *P. yoelii* system have been shown to be active in human volunteers immunized with radiation-attenuated *P. falciparum* sporozoites (3). Specifically, mice immunized and protected against *P. yoelii* sporozoite challenge produce CD8⁺ T cells specific for a peptide derived from the PyCSP (9, 27), and human volunteers protected by immunization with radiation attenuated *P. falciparum* sporozoites also produce CD8⁺ CTL directed at an epitope in the *P. falciparum* circumsporozoite protein (3, 38). Similarly, mice can be protected by adoptive transfer of a T cell clone specific for PySSP2, and protected sporozoite-immunized volunteers produce a T cell response against *P. falciparum* sporozoite surface protein 2 (3, 38). Indeed, the *P. yoelii* system has accurately predicted the success or failure of every approach to malaria vaccination that has been tested in humans. Thus, the choice of malaria vaccination strategies for induction of protective immunity against malaria in humans should be considered in the context of the findings reported herein. These studies have important implications for the development of vaccines designed to protect a heterogeneous human population against pathogen infection and of assays that predict protective immunity.

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