

Immunological Responses of Neonates and Infants to DNA Vaccines

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Summary

In some parts of sub-Saharan Africa, it is believed that most of the deaths attributed to malaria occur in infants. For this and other logistical reasons, if a malaria vaccine is developed and licensed, it will have to be administered to neonates or young infants, when they have maternally acquired antibodies against malaria parasite proteins. Pre-erythrocytic malaria vaccines in development rely on CD8⁺ T cells as immune effectors, yet some studies indicate that neonates do not mount optimal CD8⁺ T-cell responses. We report that BALB/c mice first immunized as neonates (7 d) with a *Plasmodium yoelii* circumsporozoite protein (PyCSP) DNA vaccine mixed with a plasmid expressing murine granulocyte macrophage-colony stimulating factor (DG) and boosted at 28 d with pox virus expressing PyCSP were protected (93%) as well as mice immunized entirely as adults (70%). Like adults, protection was dependent on CD8⁺ T cells and accompanied by excellent anti-PyCSP interferon- γ and cytotoxic T-lymphocyte responses. Mice born of immune mothers (previously exposed to *P. yoelii* parasites or immunized with the same vaccine given to the neonates) were also protected and had excellent T-cell responses. These data support assessment of this immunization strategy in neonates/young infants in areas where malaria exacts the greatest toll.

Key Words: Rodent; CD8⁺ T-cell responses; malaria; maternal antibodies; DNA vaccination.

1. Introduction

For many infectious diseases, immunizations are first administered to neonates (children less than 30 d of age) or 5- to 8-wk-old infants (children less than 12 mo of age). This is because infants, even in the first half of the first year of life are highly susceptible to many infectious diseases. Despite the fact that hundreds of millions of infants and even neonates have been successfully immunized with vaccines (e.g., against diphtheria [*Corynebacterium diphtheriae*], pertussis [*Bordetella pertussis*], tetanus [*Clostridium tetani*], polio [poliovirus], influenza [*Haemophilus influenza*], hepatitis B [hepatitis B

virus], some vaccines such as the measles vaccine cannot be successfully administered until late in infancy, because of inadequate responses in younger infants. Unconjugated polysaccharide vaccines, such as the original *H. influenza* vaccines cannot be administered at all to infants for the same reasons.

It is generally thought that in the first few months of life, infants are protected against developing serious malaria by transplacentally transferred antibodies from their mothers. This maternally acquired immunity wanes by 4 mo of age, and in some parts of the world more than 50% of all deaths from malaria occur in the first 8–10 mo of life ([1,2](#)). For this reason and logistical reasons, it is thought by many that if a malaria vaccine is developed and licensed, it will have to be administered in the Expanded Programme for Immunization of the World Health Organization, which starts immunizations at 6 wk of life ([1](#)). However, it is known that transplacentally acquired maternal antibodies and immaturity of the immune system of neonates and infants can adversely affect protective immune responses to vaccines ([3–6](#)). Furthermore, it is generally thought that an effective pre-erythrocytic stage malaria vaccine will have to elicit protective CD8⁺ T-cell responses, and there has been concern that neonates and young infants do not generate effective CD8⁺ T-cell responses. Finally, there has been at least one report indicating that immunization of neonatal mice (2-d-old mice) leads to immune tolerance ([7](#)).

DNA vaccines are one of the modern, molecular, subunit approaches to vaccine development. There are a number of potential advantages to DNA vaccines over other approaches to modern, molecular, subunit vaccine development that include purified recombinant proteins, recombinant live vectors, and synthetic peptides. However, the current generation of DNA vaccine strategies on their own are not as effective as are “so-called,” prime-boost or sequential immunization approaches to vaccine development, which include priming with a DNA vaccine and boosting with a recombinant virus or recombinant protein encoding the same protein as the DNA vaccine.

We have been working to develop a DNA-based malaria vaccine for a number of years ([8](#)) and began clinical trials in 1997 ([9,10](#)). However, because one of our primary aims is the development of a vaccine to be administered initially at 6 wk of age to human infants, we explored the responses of such an immunization approach in young mice. It has now been demonstrated that although the stage of immune maturation is significantly less advanced at birth in mice than in humans, a postnatal maturation period of at least 7 d allows pups to reach an immune maturation capacity comparable to that of human neonates ([11](#)). Interestingly, there have been some reports indicating that DNA vaccines may offer some important advantages over other conventional vaccines when it comes to immunizing neonates with pre-existing immunity ([12,13](#)).

Our most effective strategy for immunizing adult mice is an approach that we have called DG-V. Mice received a first immunization with a plasmid expressing a *Plasmodium* sp. pre-erythrocytic stage protein (D) mixed with a plasmid expressing murine granulocyte macrophage-colony stimulating factor (GM-CSF) (G). Several weeks later they are boosted with a recombinant attenuated vaccinia virus expressing the same *Plasmodium* sp. protein (V). This regimen reproducibly protects >80% of mice against challenge with approximately ten ID₅₀s (50% infectious doses) of *Plasmodium yoelii* sporozoites (14). We used the *P. yoelii* mouse model to address the question of whether neonates (7- to 10-d old pups) born to naïve or immune mothers can be immunized against malaria by immunization with the DG-V approach. We found that BALB/c mice first immunized as neonates with a *P. yoelii* circumsporozoite protein (PyCSP) DNA vaccine mixed with a plasmid expressing murine GM-CSF (DG) and boosted at 28 d with pox virus expressing PyCSP were protected as well as mice immunized as adults. Protection was dependent on CD8⁺ T cells, and mice had excellent anti-PyCSP interferon gamma and cytotoxic T-lymphocyte responses. Mice born of mothers previously exposed to *P. yoelii* parasites or immunized with the DNA vaccine (the same vaccine that was later given to the neonates) were protected and had excellent T-cell responses. In contrast to the CD8⁺ T-cell responses, the antibody responses to the target antigen, *P. yoelii* sporozoites, were inversely associated with pre-existing maternally transferred antibody levels at the time of immunization. Although antibody levels produced after vaccinating neonates born of naïve mothers were generally lower than those produced in adults, there was no evidence of tolerance in any of these neonates after active immunization.

2. Materials

1. *P. yoelii* 17XNL nonlethal strain, clone 1.1 sporozoites obtained by dissection of infected *Anopheles stephensi* salivary glands.
2. 8-wk-old BALB/cByJ mice for breeding.
3. Plasmid DNA constructs encoding the *P. yoelii* circumsporozoite protein (pPyCSP, designated as D) diluted in 1X PBS and stored at -20°C until use.
4. Plasmid DNA constructs encoding the murine GM-CSF plasmid (pGM-CSF, designated as G) diluted in 1X PBS and stored at -20°C until use.
5. Recombinant attenuated vaccinia virus (COPAK) encoding the PyCSP, designated as V.
6. 0.3-mL Insulin syringe fitted with 29.5-gauge needle for plasmid injections.
7. 1.0-mL Insulin syringe fitted with 26-gauge needle for recombinant attenuated vaccinia virus injections and sporozoite injections.

3. Methods

The methods described next include (1) production of neonates born to mothers with different immunological backgrounds; (2) injection of neonates; (3) immunization with different regimens; (4) measurement of humoral and cellular immune responses after immunization; (5) preparation of *P. yoelii* parasites for infection and challenge of mice; (6) evaluation of protection after immunization; and (7) characterization of immune mechanisms in protected mice.

3.1. Parasites and Infections

P. yoelii 17XNL nonlethal strain, clone 1.1 sporozoites were obtained by dissection of infected *A. stephensi* salivary glands as previously described (15).

To produce malaria-recovered mothers, adult females were infected by IV injection of 10,000 sporozoites. These mice were then evaluated to ascertain that they had *P. yoelii* parasitemia (positive blood smear) on d 7, and were negative for *P. yoelii* parasitemia (negative blood smear indicating self-cure) on d 21. To evaluate vaccinated pups for vaccine efficacy, immunized mice were challenged intravenously (iv) with 50 *P. yoelii* sporozoites after immunization. Protection was defined as absence of patent parasitaemia during 14 d after challenge with *P. yoelii* sporozoites.

3.2. Immunogens

The construction of the plasmid DNA encoding the *P. yoelii* circumsporozoite protein (pPyCSP) designated as (D), the murine GM-CSF plasmid (pGM-CSF), designated as (G), the recombinant attenuated vaccinia virus (COPAK) construct encoding the PyCSP, designated as (V), control empty plasmid, and control virus have been described (15,16). The doses of vaccines used were D (100 µg), G (30 µg), and V (2×10^7 plaque forming units [pfus]).

3.3. Production of Neonates

The generation of pups with three different types of immunological backgrounds is described in Subheadings 3.3.1.–3.3.3. These include production of neonates born to naïve mothers, neonates born to malaria immune mothers that had recovered from *P. yoelii* infection, and neonates born to mothers immunized with the PyCSP DNA vaccine.

3.3.1. Neonates Born to Naïve Mothers

Female naïve mice (mothers) were mated with naïve males to produce pups. Generally, pups were born between 3 and 9 wk after initiation of mating. The gestation period is 3 wk. We referred to these pups as (Naïve/Mother) (17) (see Note 1).

3.3.2. Neonates Born to Malaria-Immune Mothers After Natural Recovery From *P. yoelii* Infection

Prior to mating, adult female mice (mothers) were infected with 10,000 *P. yoelii* sporozoites and demonstrated to have cleared the parasites without intervention by d 21–24. These females were then mated with naïve males. We referred to their pups as (Recovered/Mother) ([17](#)) (see **Note 2**).

3.3.3. Neonates Born to Mothers Immunized With the PyCSP DNA-Based Vaccine

Three weeks prior to mating, adult female mice (mothers) were immunized with the PyCSP DNA-based malaria vaccine. The first set of mothers were primed with a mixture of two plasmids, (D) plus (G) and boosted 3 wk later with (V). These females were then mated with naïve males. The pups born to these mothers were designated as (DGV/Mother) (see **Note 3**). The second set of mothers was immunized with two doses of the PyCSP DNA vaccine (D). These mothers were primed and boosted 3 wk later with the same plasmid (D), and these females were then mated with naïve males. Pups born to these mothers were referred to as (DD/Mother) ([17](#)).

3.4. Immunization of Neonates

All immunization of neonates began at 7 d after birth (see **Note 4**). For each mouse, two immunizations were administered at 3-wk intervals (d 7 and 28 after birth).

3.4.1. Handling of Pups for Injections

The handling of pups was as previously described ([13](#)) (see **Note 4**).

3.4.2. Immunization Regimen

For priming, D or DG (a mixture of D and G) was injected IM in two 25- μ L injections, one in each tibialis anterior muscle using a 29-gauge needle. V was injected intraperitoneally (IP) in 100 μ L PBS using a 26.5-gauge needle. Pups either received DG as the first dose and V as the second dose (DGV), or D as the first and second doses (DD).

3.5. Blood Collection and Antibody Assays

To obtain sera from 1-wk-old pups, pups were euthanized and blood was collected by cardiac puncture. For older mice, repeated bleeds were done by tail bleed on the same mice at designated times. Parasite-specific antibodies in sera were measured by immunofluorescent assay against air-dried *P. yoelii* parasites (native protein) (see **Note 5**).

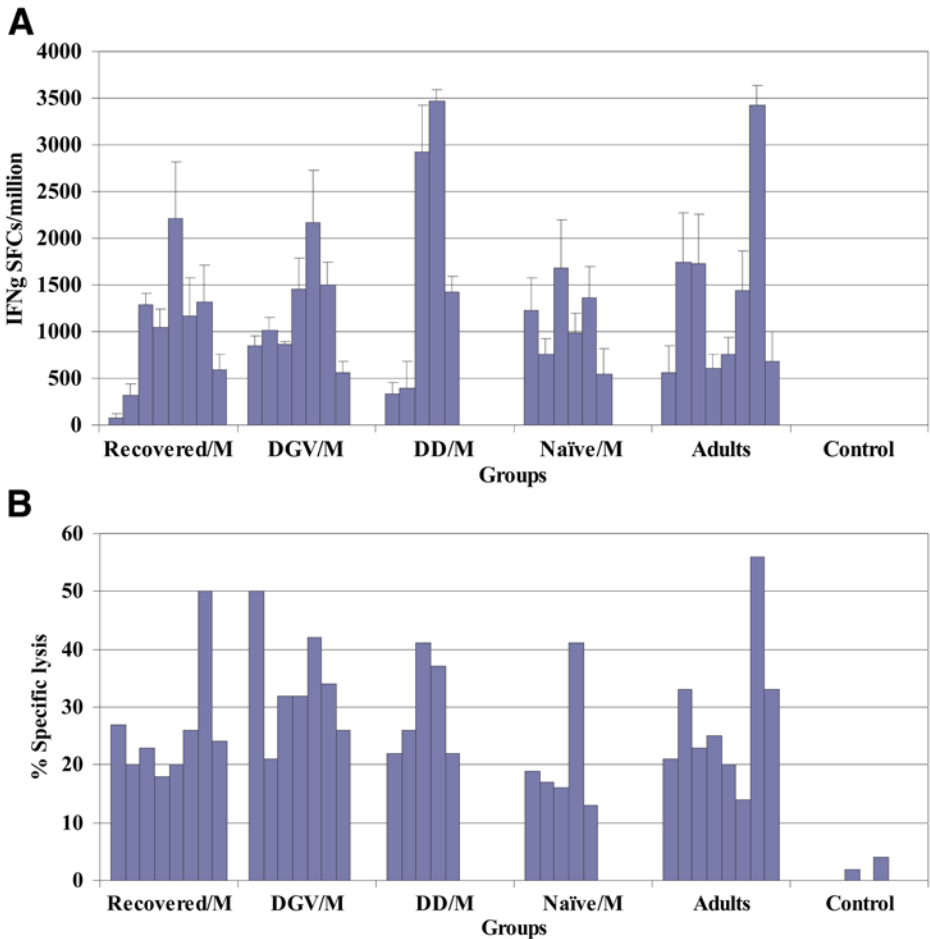


Fig. 1. CD8⁺ T-cell responses in DGV-immunized mice. **(A)** IFN γ producing cells in ELISPOT. Mice born of mothers never exposed to *Plasmodium yoelii* or immunized with a *P. yoelii* vaccine (Naïve Mother), or from mothers previously exposed (Recovered Mother), or immunized (DGV Mother or DD Mother) were immunized on d 7 with D or DG and boosted with V on d 28. Immunized adult mice (adults) were the positive controls, and unimmunized mice were the negative controls (control). Spleen cells from individual mice, five to eight immunized mice per group were obtained 2 wk after the recombinant poxvirus boost (V). The number of antigen specific interferon (IFN)- γ producing cells was determined and expressed as the number of spot forming cells per million spleen cells. (The error bars indicate the standard deviation of the mean counts.) **(B)** Ex vivo CTL assay. Spleen cells from same mice used for the ELISPOT assay were used directly as effectors in a chromium release ex vivo CTL assay. Data are presented as net percent specific lysis at 150:1 effector:target ratio. For both the IFN- γ ELISPOT and ex vivo CTL assays, the mean spot forming cells per million and the mean net

3.6. IFN γ Secreting CD8-Cell Detection by ELISPOT Assay

Fourteen days after the second immunization on d 42 after birth, spleens were removed, single cell suspensions of spleen cells made, and interferon (IFN)- γ responses measured by ELISPOT (see **Note 6**). The results of the assays (see **Fig. 1A**) showed that antigen-specific IFN- γ responses were similar in mice first immunized as adults and those first immunized as neonates.

3.7. Ex Vivo CTL Assays

Spleen cells from the same mice used for the ELISPOT assay were used directly as effectors in a chromium release ex vivo CTL assay. These assays were conducted directly without any restimulation of spleen cells in vitro as is usually done in cytotoxic T-cell (CTL) assays (see **Note 7**). The assays (see **Fig. 1B**) showed that ex vivo CTL responses were similar in mice first immunized as adults and those first immunized as neonates.

3.8. Mechanism of Protection

After it was established that the immunization regimen protected the mice, a new set of mice was immunized and depleted of their CD8 $^{+}$ T cells in vivo just prior to challenge with infective sporozoites to determine if CD8 $^{+}$ T depletion would abrogate protection (see **Note 8**). This treatment eliminated the vaccine-induced protection (**Table 1**), indicating that the protection was dependent on CD8 $^{+}$ T cells, as in adults (**14**).

3.9. Effect of Maternal Immunity on Response to Immunization Vaccination

The data showed that T-cell responses, induced in neonates born to mothers previously exposed to *P. yoelii* infection or immunized with PyCSP DNA did not differ from those induced in pups from naïve mothers. However, there were differences in antibody responses.

3.9.1. Effect on CD8 $^{+}$ T-Cell Responses

ELISPOT and a direct ex vivo CTL assays were used to measure T-cell responses after second immunization because our previous studies showed that results of these assays were associated with protection against sporozoite challenge after immunization (**14**). Immunized adults were tested simultaneously

Fig. 1. (continued) specific lysis for each of the six groups were compared in a one-way analysis of variance (with Scheffe *post-hoc* test). The only significant differences seen were between the control group and each of the other five immunized groups, *p* values ranging between 0.003 and <0.0009.

Table 1
DGV-Induced Protection Dependent on CD8⁺ T-Cells

Mice	Immunization	Protected/challenged (%)
Experiment A		
Neonates from Mother: Naïve	DGV	13/14 (93)
Neonates from Mother: Naïve	Control(V)	1/7 (14)
Adults	DGV	7/10 (70)
5-wk-old naïve mice	Not immunized	0/10 (0)
Experiment B		
Neonates from Mother: Naïve	DGV and treated with anti CD8 antibody	1/11 (9)
Neonates from Mother: Naïve	DGV and treated with control antibody	8/9 (89)
5-wk-old naïve mice	Not immunized	0/10 (0)
Experiment C		
Neonates from Mother: Recovered	DGV	10/16 (63)
Neonates from Mother: Recovered	Control	0/8 (0)
Neonates from Mother: DGV	DGV	13/16 (81)
Neonates from Mother: DGV	Control	0/15 (0)
Neonates from Mother: DD	DGV	7/8 (88)
Neonates from Mother: DD	Control	0/8 (0)

In Experiment A, 7-d-old pups born to naïve mothers, and 7-wk-old adult mice were primed with a mixture of pPyCSP(D) and pGM-CSF(G), boosted with recombinant attenuated vaccinia virus expressing PyCSP (V) 3 wk later (DGV), and challenged 2 wk later with 50 *Py* sporozoites. In Experiment B, 7-d-old pups born to naïve mothers were immunized with DGV regimen and, treated with monoclonal antibodies to CD8⁺ T-cells or control antibodies prior to challenge with sporozoites. In Experiment C, 7-d-old pups born to different immune mothers, Mother:Recovered, Mother:DGV, or Mother:DD were immunized with the DGV immunization regimen and challenged two weeks later with 50 *Py* sporozoites. Seven-day-old pups from different litters from mothers with similar immunological background received control immunizations which consisted of priming with control plasmids followed by boosting with control virus (no *Py*CSP insert). Ten naïve infectivity control age-matched mice were also challenged (17).

in order to compare their responses to those obtained in pups from four different groups (Recovered/Mother; DGV/Mother; DD/Mother; and Naïve/Mother) that were primed when they were only 7 d old. The measured IFN γ and ex vivo CTL responses were similar in adults and those first immunized as neonates, regardless of the immune status of the mother. An example of the T-cell responses in neonates born to mothers with different immunological status and first immunized as neonates is shown in **Fig. 1A,B**.

3.9.2. Effect on Antibody Responses

Our data demonstrated that mice first immunized at 7 d with plasmid DNA and boosted 3 wk later with a recombinant poxvirus mounted protective immune responses comparable to those induced in mice first immunized when they were adults. Although our previous studies (**14**) showed that the best correlates of protection against sporozoite challenge conferred by either plasmid DNA or by DNA prime/poxvirus boost vaccination were IFN γ and ex vivo CTL activity specific for a CD8⁺ T-cell epitope (which was unaffected in the different pups studied), we have reported an association between protection and the levels of anti-PyCSP or sporozoite antibodies (**14**). Furthermore, the production of high levels of antibodies is a critical effector mechanism for other types of malaria vaccines that are based on erythrocytic stage proteins. We therefore measured antibody titers to sporozoites by the indirect fluorescent antibody test in mice 2 wk after boost in the different groups of neonates studied. Our aim was to determine the effect the pre-existing maternal antibodies had on the induction of antibodies to the vaccine. Antibody responses in the mice born of immune mothers were significantly lower than antibody responses in mice born of naïve mothers (**17**).

4. Notes

1. Studies in which 12- to 24-h-old pups were used (**12,13**) reported no evidence of tolerance and only one study utilizing 2-d-old pups reported tolerance (**7**). We did all of our initial immunizations in 7-d-old pups. It is believed that a post-natal maturation period of at least 7 d allows pups to reach an immune maturation capacity comparable to that of human neonates (**11**).
2. After infection with *P. yoelii* sporozoites, blood stage parasites become detectable in the blood at 4 d postinfection and by 24 d after sporozoite exposure, parasites are cleared in all mice. Mice that have recovered from infection with *P. yoelii* are protected against blood stage infection and that protection is mediated primarily by antibodies against erythrocytic stages parasites because these mice are capable of developing mature liver stage parasites (Sedegah, unpublished). In

Table 2
Maternal Antibodies in Pups Born to *P. yoelii*-Recovered Mothers

Mice		Weeks after birth											
		1 wk ^a		2 wk ^a		3 wk		5 wk		8 wk		10 wk	
Pup no.	spz	prbc	spz	prbc	spz	prbc	spz	prbc	spz	prbc	spz	prbc	spz
Mother	320	20,480	2560	20,480	2560	20,480	1280	40,960	1280	81,920	640	40,960	
1	640	40,960	2560	40,960	640	20,480	160	5120	320	5120	40	2,560	
2	640	40,960	5120	40,960	640	20,480	160	10,240	320	5120	160	640	
3	640	40,960	5120	40,960	640	20,480	160	20,480	20	2560	80	2560	
4	640	40,960	2560	40,960	640	20,480	320	5120	20	2560	40	1280	
5	320	40,960	2560	40,960	640	20,480	80	10,240	40	1280	160	2560	
6	10,240	20,480	5120	40,960	640	20,480	160	10,240	80	5120	80	1280	
7	10,240	20,480	5120	40,960	640	20,480	160	20,480	40	5120	20	5120	
8	2560	20,480	2560	40,960	640	20,480	160	40,960	20	2560	20	5120	
GM	1396	31,584	3620	40,960	640	20,480	160	12,177	57	3320	57	2153	

^aSera were prepared from mice from two different litters for wk-1 and wk-2 bleeds, because these mice were euthanized to obtain serum. Week-3, week-5, week-8, and week-10 bleeds were done on mice from the same litter. Individual (pups and mother) and geometric mean (pups) IFA titers against air-dried *P. yoelii* sporozoites (spz) and asexual stage parasitized erythrocytes (prbc) are shown (17).

our experiments, we followed maternally transferred antibodies in the pups for up to 10 wk. We found that antibody titers in the pups were highest at 2 wk and declined with time. However, significant levels were still detected 10 wk after birth (**Table 2**).

3. Antisporozoite antibodies were maternally transferred to pups born to mothers previously immunized by priming with a mixture of pPyCSP (D) plus pGM-CSF (G) (d 0) and boosted with recombinant attenuated vaccinia virus expressing PyCSP (V) 3 wk later. The maternally transferred antibodies reached their highest level at 2 wk after birth and levels began to wane with time (**Table 3**).
4. The handling of neonates for injections was done essentially as described (**13**). We however, were able to conduct our immunizations without keeping pups anesthetized. First, we removed the mother very gently from the cage and placed her in a separate clean cage while the pups were being handled. All pups were from the nest and placed in a temporary clean cage. One pup was gently picked up from the nest and draped it over the index finger (of the opposite hand that you will inject with). The thumb and middle finger are used to restrain the hind legs. The needle was inserted into the lateral thigh muscle so that the point ends in the quadriceps muscle mass (posterior to anterior direction). We slowly injected the total DNA vaccine dose in 25 μ L per each leg. The pups were placed back in their nest and the mother returned on top of her litter.
5. Antibodies against *P. yoelii* sporozoites and erythrocytic stages in sera were assayed by immunofluorescent staining of air-dried *P. yoelii* sporozoites or infected erythrocytes by previously described methods (**15**). Briefly, diluted sera were reacted on air-dried sporozoites or infected erythrocytes and antibodies were detected using FITC labeled rabbit anti-mouse Ig.
6. Using previously described methods (**15**), spleen cells from mice that 14 d earlier had received their second and last immunization were used in these determinations. Briefly, freshly isolated spleen cells were incubated for 36 h with irradiated antigen presenting cells (P815 cells pulsed with 1 μ M of the PyCSP CTL epitope, PyCSP (280-288), sequence SYVPSAEQI in 96-well nitrocellulose plates (Millipore), previously coated with purified rat anti-mouse IFN- γ Ab (PharMingen). Spleen cells were also incubated with unpulsed irradiated P815 target cells to serve as controls. The number of antigen specific spots (number of spots with peptide pulsed targets minus the number of spots with unpulsed targets) corresponding to IFN γ producing cells in wells was enumerated using the Zeiss Elispot reader and the results were expressed as the number of IFN- γ secreting cells per 10^6 spleen cells.
7. Spleen cells from same mice used for the ELISPOT assay were used directly as effectors in a chromium release ex vivo CTL assay. In this method, varying ratios of freshly isolated spleen cells were added to 5000 peptide labeled P815 cells with medium containing 2% rat T-cell stim (Collaborative Biomedical Products Inc., Bedford, MA) in 96-well U-bottom plates. For experimental targets, PyCSP CTL epitope, PyCSP (280-288) (SYVPSAEQI), was used to pulse P815 cells at 0.025 μ M. Control targets did not receive any peptide. A 12 h chromium release

Table 3
Maternal Antibodies in Pups Born to Mothers Immunized With DGV

Mice		Weeks after birth			
Pup no.	1 wk ^a	2 wk ^a	3 wk	5 wk	10 wk
Mother	nd	nd	5120	5120	5120
1	640	5120	1280	640	160
2	80	5120	1280	320	160
3	320	5120	1280	640	320
4	640	5120	1280	640	320
5	1280	10,240	640	640	640
6	1280	20,480	640	640	160
7	1280	20,480	640	640	640
8	640	20,480	640	640	320
9	640	20,480	1280	1280	160
GM	593	10,240	941	640	274

^aSera were prepared from mice from three different litters because mice bled for the 1- and 2-wk time points were euthanized at the time of acquisition of serum. Sera for the wk-3, wk-5, and wk-10 bleeds came from mice from the same litter. Individual (pups and mother) and geometric mean (pups) IFA titers against air dried *P. yoelii* sporozoites are shown (17).

assay was conducted and net percent specific lysis was calculated at varying effector to target ratios.

8. Neonates were primed with DG and boosted with V 3 wk later, and depleted of CD8⁺ T cells in vivo prior to challenge with infective sporozoites 2 wk after boost. Mice received single daily IP injections of 0.5 mg of MAb 2.43 for 3 consecutive days (14) and then were challenged with sporozoites. Control mice received control rat Ig. The treatment with anti CD8 antibodies eliminated protection (Table 1), and indicated that the mechanism of protection induced in the neonates is similar to that induced in adults.

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