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Interleukin 12 induction of interferon γ-dependent protection against malaria

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ABSTRACT **Intraperitoneal injection of recombinant Interleukin 12 (rIL-12) at 30 ng/day for 5 days beginning 1 to 2 days before sporozoite challenge or administration of a single dose of 150 ng of rIL-12 2 days before challenge protected 100% of BALB/c mice against challenge with 10^2 *Plasmodium yoelii* sporozoites. rIL-12-induced protection was eliminated in all mice by administration of a monoclonal antibody against interferon γ and in 50% of mice by administration of N^G-monomethyl-L-arginine, a competitive inhibitor of nitric oxide synthase. rIL-12 protected BALB/c mice treated with cytotoxic anti-CD4 and anti-CD8 monoclonal antibodies, as well as T-cell- and B-cell-deficient severe combined immunodeficiency mice. These data suggest that rIL-12 stimulates non-B, non-T cells to produce interferon γ that kills intrahepatic parasites by stimulating nitric oxide production. If rIL-12 proves to be well tolerated by humans, our findings support consideration of rIL-12 as an immunoprophylactic against malaria.**

Malaria is one of the most important infectious diseases in the world (1) and during the past 30 yr, many of the drugs most useful against malaria have lost their efficacy due to drug resistance. There have therefore been considerable efforts to develop new drugs and malaria vaccines targeted at the various developmental stages of the parasite (2). After inoculation by mosquitoes, *Plasmodium* sp. sporozoites rapidly enter hepatocytes, where they develop for several days. This hepatic stage of the life cycle is not associated with any clinical or pathologic manifestations, and one approach to malaria drug and vaccine development has been to attack parasites as they develop within hepatocytes (3). Interferon γ (IFN-γ) has considerable activity *in vitro* and *in vivo* against *Plasmodium* sp.-infected hepatocytes (4–6), presumably by inducing the infected hepatocytes to produce nitric oxide that kills the parasites (7). However, exogenous IFN-γ, which has a short *in vivo* half-life, does not consistently provide complete protection against sporozoite-induced malaria and has never been shown to protect against *Plasmodium yoelii*, a highly infectious rodent malaria. Interleukin 12 stimulates endogenous IFN-γ production by T lymphocytes and natural killer (NK) cells, and treatment with recombinant IL-12 (rIL-12) can maintain considerable IFN-γ levels *in vivo* (8–11). Recombinant IL-12 (rIL-12) prolonged survival in severe combined immunodeficiency (SCID) mice infected with an avirulent strain of the intracellular protozoa *Toxoplasma gondii* (12) and provided partial protection and reduction of parasite burden in BALB/c mice infected with *Leishmania major* (13). The current studies were undertaken to determine whether rIL-12 would protect against *P. yoelii* sporozoite-induced malaria.

MATERIALS AND METHODS

Experimental Animals. Four- to 8-week-old BALB/cByJ female mice from The Jackson Laboratory and CB-17 SCID mice from Harlan Bioproducts (Indianapolis) were used.

Infection with *P. yoelii*. *P. yoelii* (17XNL) clone 1.1 sporozoites for challenges were obtained by hand dissection of infected mosquito glands in M199 medium/5% normal mouse serum. Erythrocytic-stage parasites were obtained from heparinized blood of a previously infected mouse. Parasites were injected i.v. into the tail vein, and blood films were examined for blood-stage parasites on days 5, 7, 11, and 14 after inoculation. Intravenous administration to BALB/c mice of 2–8 *P. yoelii* (17XNL) clone 1.1 sporozoites routinely infects at least 50% of recipient animals (14). Mice were considered protected if all blood films were negative and were considered infected if any blood smear was positive for this nonlethal parasite.

rIL-12. Murine rIL-12 was provided by Hoffman–La Roche. It was diluted in sterile 1% normal mouse serum (NMS)/phosphate-buffered saline (PBS), pH 7.2, to give the required doses of rIL-12 in 0.25 ml.

Neutralization of IFN-γ Activity. To neutralize IFN-γ, a rat IgG1 monoclonal antibody (mAb) against IFN-γ, XMG-6 (15, 16), or rat IgG1 control mAb GL113 against *Escherichia coli* β-galactosidase (prepared from a hybridoma provided by J. Abrams, DNAX Research Institute, Palo Alto, CA) purified from ascites was injected into mice treated with rIL-12. mAbs were diluted in sterile PBS, pH 7.2 to give 1 mg in 0.2 ml. Experimental and control mice were injected i.v. with 1 mg of the mAbs on days −3, 0, and +2 relative to sporozoite injection on day 0.

Depletion of CD8+ and CD4+ T Lymphocytes. mAb 2.43 (mouse IgG2a, anti-CD8) and mAb GK1.5 (rat IgG2a, anti-CD4) were used for depleting BALB/c mice of either CD8+, CD4+, or both T-cell subsets (17). Mice received single daily i.p. injections of either 0.5 mg of mAb 2.43 or 1 mg of mAb GK1.5 for 3 consecutive days. The double depletion groups received both mAbs. Control mice received 1 mg of control rat immunoglobulin. Spleen cells were prepared from two mice in each group, and microfluorometric analysis (fluorescence-activated cell sorting) was done to determine the effectiveness of the depletions 3 days after the last injection (17).

Depletion of NK Cells. Rabbit anti-asialoganglioside GM1 antibodies (Wako Chemicals USA, Richmond, VA) were reconstituted by adding 1 ml of sterile distilled water to the lyophylized product and further diluted four times with PBS, pH 7.2. Mice were injected i.v. with 0.2 ml of the diluted antibodies (equivalent to 1.4 mg) on days −3, −2, 0, and +2, relative to sporozoite challenge on day 0.

Inhibition of Nitric Oxide Production. Groups of mice were treated by gastric instillation with 0.5 ml of 25 mM N^G-monomethyl-L-arginine (N^GMMLA) (Calbiochem) on days −1, 0, +1, and +2, relative to sporozoite injection on day 0.

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Abbreviations: rIL-12, recombinant interleukin 12; mAb, monoclonal antibody; IFN-γ, interferon γ; SCID, severe combined immunodeficiency; NK, natural killer; N^GMMLA, N^G-monomethyl-L-arginine; NMS, normal mouse serum.

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RESULTS AND DISCUSSION

Protection After Administration of rIL-12. Eight daily doses of 10, 100, or 1000 ng of rIL-12 were injected i.p. into BALB/c mice beginning on day −3 relative to injection with 10^2 *P. yoelii* sporozoites on day 0. Fifty, 100, and 83%, respectively, of the mice were completely protected. In a second experiment we established that injection of 10 or 30 ng of rIL-12 i.p. for 8 days (days −3 through day 0, and days +1, +3, +5, and +7) protected 100% (six/six) of mice and that administration of the same doses daily for 7 days (days −3 through +3) protected 83% (five/six; 10-ng dose) and 100% (six/six) (30-ng dose) of mice. In these experiments administration of NMS/PBS, pH 7.2, as a control had no effect on sporozoite infectivity. Subsequent experiments showed that 100% protection could be obtained with a minimum of five 30-ng doses administered from day −1 through day 3 or day −2 through day 2 (Table 1). However, 100% protection was also obtained when a single dose of 150 ng of rIL-12 was administered 2 days before sporozoite injection on day 0 (Table 3).

rIL-12 had no protective effects against sporozoite challenge if the injections were begun at the same time as sporozoites were injected (Table 1), or if they were begun 30 hr after sporozoite injection and continued through the time when parasitemia developed (data not shown), suggesting that the effect was on sporozoites or infected hepatocytes, not on infected erythrocytes. This result was confirmed by administering 100 ng of rIL-12 from days −3 to +6 (10 doses) to six mice and challenging them with 10^4 erythrocytes infected with *P. yoelii* on day 0. All six mice became infected at the same time as control mice.

Fifty percent of naive mice can generally be infected by i.v. injection of 2–8 sporozoites of the *P. yoelii* 17XNL clone 1.1 used in these experiments (14). Thus, the 10^2 sporozoites used in all experiments for challenge represent an ID_{50} of 10–100. To further determine the potency of the protection, mice were challenged with increased doses of sporozoites. Five injections of 30 ng of rIL-12 protected two of six mice against challenge with 5×10^3 sporozoites.

Reversal of Protection by Anti-IFN-γ. To determine whether induction of IFN-γ was responsible for the protection associated with rIL-12 administration, mice treated with rIL-12 were injected with XMG-6, a mAb to IFN-γ (15, 16). This treatment completely reversed the protective activity of rIL-12 (Table 2).

Reversal of Protection by Inhibition of Nitric Oxide Production. Our previous work had established that IFN-γ activity against infected hepatocytes depends on L-arginine, indicating that IFN-γ induces infected hepatocytes to produce nitric oxide that kills the parasites within hepatocytes (7, 18). Because IFN-γ is required for the protection found after administration of rIL-12, it seemed logical that the IFN-γ induced the infected hepatocytes to produce nitric oxide that killed the intracellular parasites. This hypothesis is supported by experiments in which N^GMMLA, an inhibitor of nitrogen oxidation of L-arginine, was administered to mice that received a single 150-ng dose of rIL-12. This treatment reversed the protective effects of rIL-12 in 50% of the mice (Table 3). Interestingly, this level of reversal of protection could be achieved even when N^GMMLA was administered 1 day after sporozoite inoculation and 3 days after administration of the single dose of rIL-12. This result suggests that the IFN-γ-induced nitric oxide production must be sustained throughout the 48-hr *P. yoelii* liver cycle for it to be inhibitory. It should be noted that in a number of experiments we have not been able to reverse by >50% rIL-12-induced protection (data not shown). Perhaps nitric oxide is not responsible for all antiparasite activity after rIL-12 administration.

CD4+ T Cells, CD8+ T Cells, and NK Cells in IL-12-Induced Protection. Having established that IFN-γ and nitric oxide were involved in the antiparasitic effects found after IL-12 administration, we turned our attention to the cells that produced the IFN-γ. IL-12 induces T cells and NK cells to produce IFN-γ (8). To determine whether T cells were required for the protective effects noted, mice were depleted of their CD4+ T cells, CD8+ T cells, or both (17) and challenged. Mice treated with the anti-CD8 mAb were 93% depleted of CD8+ T cells, and mice treated with the anti-CD4 mAb were 95% depleted of CD4+ T cells. Mice treated with both mAbs were 87% and 93% depleted of the CD8+ and CD4+ T cells, respectively. All mice were still protected after administration of rIL-12 (data not shown).

T lymphocytes play a fundamental role in protection against the liver stages of *Plasmodium* sp. (3). It is possible that the depletions described above were inadequate to demonstrate a requirement for T cells in the rIL-12-induced protection. To determine whether T lymphocytes were required for the protection induced by administration of rIL-12, we treated SCID mice that do not have B or T lymphocytes with seven doses of 100 ng of rIL-12 and challenged them with

Table 1. Complete protection of mice against sporozoite challenge by administration of five doses of rIL-12

Group	Days rIL-12 administered	Doses of rIL-12, no.	Infected/total (% protection)
A	−2 to +3	6	0/6 (100)
B	−2 to +2	5	0/6 (100)
C	−1 to +3	5	0/6 (100)
D	−2 to +1	4	1/6 (83)
E	−1 to +2	4	2/6 (67)
F	0 to +3	4	6/6 (0)

Groups of six mice were injected with 4–6 doses of 30 ng of rIL-12 and challenged by i.v. injection of 10^2 sporozoites on day 0.

Table 2. Elimination of rIL-12-induced protection by administration of mAb against IFN-γ

Treatment	mAb	Infected/total (% protected)
rIL-12	None	0/6 (100)
rIL-12	Anti-IFN-γ	6/6 (0)
rIL-12	Control mAb	0/6 (100)
NMS	Anti-IFN-γ	6/6 (0)
None	None	6/6 (0)

Groups of six BALB/c mice were injected i.p. with a daily dose of 30 ng of rIL-12 from day −3 through day +3 relative to i.v. challenge with 10^2 sporozoites on day 0. Mice also received i.v. injections with mAb XMG-6 against IFN-γ or control mAb GL113 on days −3, 0, and +2.

Table 3. Effect of N^GMMLA on protection induced by administration of rIL-12

Treatment	Inhibitor	Infected/total (% protected)
rIL-12	Untreated	0/6 (100)
rIL-12	N^GMMLA on day −1	3/6 (50)
rIL-12	N^GMMLA on day +1	3/6 (50)
rIL-12	N^GMMLA on day 0, +1	3/6 (50)
NMS	N^GMMLA on day −1	5/6 (17)
NMS	N^GMMLA on day +1	6/6 (0)
NMS	N^GMMLA on day 0, +1	6/6 (0)
None	None	6/6 (0)

Groups of six BALB/c mice were injected with a single dose of 150 ng of rIL-12 on day −2 before sporozoite challenge on day 0. Mice were then treated by gastric instillation of either a single dose (on day −1 or day +1) or two doses (day 0 and day +1) of 0.5 ml of 25 mM N^GMMLA.

Table 4. Elimination of rIL-12-induced protection of SCID mice by treatment with mAb against IFN-γ

Treatment	mAb	Infected/total (% protection)
rIL-12	Anti-IFN-γ	5/5 (0)
rIL-12	Control mAb	1/5 (80)
NMS	Anti-IFN-γ	5/5 (0)
None	None	5/5 (0)

Groups of five SCID mice were injected with seven doses of 100 ng of rIL-12 from day −3 through day +3 relative to i.v. challenge with 10^2 sporozoites on day 0. Mice also received i.v. injections of mAb against IFN-γ or control mAb on days −3, 0, and +2.

10^2 *P. yoelii* sporozoites. Only one of the six SCID mice treated with rIL-12 developed parasitemia, whereas all six SCID mice treated with NMS became infected. In an additional experiment, we demonstrated that, as with normal BALB/c mice, the protection was reversed by treatment with a mAb against IFN-γ (Table 4).

Knowing that T lymphocytes were not required for rIL-12-induced protection of BALB/c or SCID mice and that IFN-γ was required for this protection, we hypothesized that the IFN-γ was produced by NK cells. To assess if NK cells played a role in this protection, we treated naive BALB/c mice with rabbit anti-asialoganglioside GM1 antibodies in an effort to deplete their NK cells and injected them with five doses of 30 ng of rIL-12. The quantity of anti-asialoganglioside GM1 used in our studies has inhibited NK cell activity in other systems (12), but it had no apparent effect on rIL-12-induced protection in BALB/c mice. We recognized that NK cells could have produced the IFN-γ in the T-cell-depleted mice and that T cells could have produced the IFN-γ in the NK-cell-depleted mice, so we studied the effect of anti-asialoganglioside GM1 antibodies in SCID mice. Eight of eight SCID mice treated with seven doses of 100 ng of rIL-12 were protected, whereas only six of the eight treated with rIL-12 and anti-asialoganglioside GM1 were protected, suggesting, but not proving, that NK cells may be involved in rIL-12-induced protection. The NK activity may not have been completely eliminated in the SCID mice, or an as-yet-unidentified cell may have produced the inhibitory IFN-γ.

Administration of 30 ng of rIL-12 for as few as 5 days beginning 1 day before exposure to infective *P. yoelii* sporozoites provides complete protection against challenge with 10–100 ID_{50}s of *P. yoelii* sporozoites. Furthermore, 100% protection was obtained with a single dose of 150 ng administered 2 days before sporozoite injection (Table 3). Our data indicate that IL-12 induces cells, presumably NK cells and T cells and perhaps other cells, to produce IFN-γ and that the IFN-γ induces the infected hepatocytes to produce nitric oxide that kills the parasite.

This work was primarily undertaken to determine the role IL-12 may play in vaccine-induced protection against *Plasmodium* sp.-infected hepatocytes (3, 19). However, chemoprophylaxis of malaria is accomplished by taking drugs that must be taken for several weeks before and for 4–6 weeks after travel to a malarious area (20). These drugs are associated with significant side effects and in many parts of the world provide inadequate protection against malaria. rIL-12 is only beginning to be studied in clinical trials, and thus side effects of the cytokine have not been defined. If IL-12 proves to be well tolerated in humans, the data presented in this paper raise the possibility that administration of low doses of rIL-12 several days before exposure to infected mosquitoes and during the period that an individual is exposed to malaria could provide excellent, short-term prophylaxis against malaria.

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