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Early Gamma Interferon Responses in Lethal and Nonlethal Murine Blood-Stage Malaria

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This study was undertaken to explore early differences in cytokine production during nonlethal and lethal blood-stage murine malaria infections. Cytokine analysis of spleens during these infections showed that the principal difference between two nonlethal and two lethal *Plasmodium* species was the production of gamma interferon 24 h after infection with nonlethal parasites. In contrast, no increases in interleukin-4 production were observed in the first 24 h and tumor necrosis factor alpha levels increased equally in both nonlethal and lethal infections. During the later phase of infection with nonlethal parasites, both gamma interferon and interleukin-4 levels increased markedly a few days before parasite clearance. Early increases in gamma interferon production in nonlethal infections of *Plasmodium yoelii* and *Plasmodium chabaudi* were dose related and increased significantly with the size of the inoculum. Studies with the nonlethal *P. yoelii* suggest that the early gamma interferon response is mediated by T cells and natural killer cells, as it was reduced in athymic mice and in mice depleted of their natural killer cells by treatment with specific antiserum. Infecting mice with increasing numbers of lethal *P. yoelii* and *Plasmodium berghei* parasites did not increase the amount of gamma interferon, interleukin-4, and tumor necrosis factor alpha produced in a dose-dependent fashion. We conclude that one consequence of the early production of gamma interferon and tumor necrosis factor-alpha, particularly after nonlethal *P. yoelii* infection, may be to adjust the balance of T-helper cell subset activation, and probably that of other immune responses, so as to enhance the mechanisms that are essential for elimination of the parasites. This suggests that a successful vaccine should contain antigens capable of inducing such responses.

It is now clear that T helper (Th) cell subset activation plays a vital role in the outcome of asexual blood-stage malaria infections in mice. These subset responses vary according to the type of parasite species concerned. In self-resolving infections of *Plasmodium chabaudi* a sequential Th1-Th2 response is required for elimination of parasitemia (17, 31). In mice infected with attenuated nonlethal *Plasmodium berghei* (37) or with *Plasmodium vinckei* (22), Th1 subset activity appears to be crucial for parasite elimination. During a lethal *Plasmodium yoelii* infection there is a failure in activation of both Th1 and Th2 cell subsets; however, this can be overcome by vaccination which induces cytokine production from both subsets that peaks shortly before resolution of parasitemia (5, 6).

We noted that significantly higher gamma interferon (IFN-γ) and interleukin-4 (IL-4) responses occurred as early as 24 h after challenge in mice vaccinated with our strongest vaccine preparations compared with unvaccinated controls (8a). This early cytokine response was reminiscent of the increased lymphocyte accumulation that occurs in the spleens and livers of vaccinated mice 24 h after challenge, which we attributed to T-cell activation and which may be linked to the subsequent rapid control of parasitemia (23). To see if the early IFN-γ response was a distinguishing feature of self-resolving infections in general, we measured levels of this cytokine and of IL-4 and tumor necrosis factor alpha (TNF-α) 24 h after infection with nonlethal *P. yoelii* (NLPY) and *P. chabaudi* parasites and compared them with those in infections with lethal *P. yoelii* (LPY) and *P. berghei* parasites. Our data suggest that an increased 24-h IFN-γ response may be a hallmark of self-resolving infection, particularly with *P. yoelii*, and that it is a product of both T cells and natural killer (NK) cells.

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MATERIALS AND METHODS

Mice. (BALB/c × C57BL/6) F_1 mice were bred at University College London Medical School, and mice of both sexes were used at 10 to 12 weeks of age. Eight-week-old female BALB/c nude (Nu/Nu) mice and littermates (Nu/+) were obtained from Harlan Olac (Shaws Farm, Essex, United Kingdom).

Parasites. Mice were infected intravenously (i.v.) with 10^4 parasitized erythrocytes, and parasitemias were estimated from Giemsa-stained blood films from day 3 onwards. In our mice, infections with NLPY (from N. Wedderburn, Royal College of Surgeons, London, United Kingdom) were resolved within 3 weeks, and with *P. chabaudi* AS (from K. N. Brown, National Institute for Medical Research, Mill Hill, United Kingdom), parasitemias were cleared from the blood in 2 weeks; we did not see a second wave of infection. The lethal YM strain of LPY (from D. Walliker, University of Edinburgh, Edinburgh, United Kingdom) kills nonimmune mice by day 8, while *P. berghei* Anka (also from N. Wedderburn) kills them in 18 to 22 days (Fig. 1).

Depletion of NK cells. Mice were injected i.v. with 50 μl of rabbit anti-asialo GM1 antiserum (Wako Chemicals GmbH, Neuss, Germany) 2 days before infection with parasites. NK cell-mediated cytotoxicity of tumor cells in vitro is abolished from spleen cells of mice treated with 10 μl of this antibody (20). Control mice were injected i.v. with 50 μl of phosphate-buffered saline. It has been demonstrated that mice treated with this antiserum have significantly reduced levels of NK cells but no reduction of T- or B-cell functions (11).

Cytokine extractions. Endogenous levels of IFN-γ, IL-4, and TNF-α in the spleen were determined by a modification of the method of Nakane et al. (21) as described previously (5). Individual spleens were weighed and homogenized in 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS; Sigma, Dorset, United Kingdom) in a Dounce tissue homogenizer, and 10% (wt/vol) homogenates were prepared. They were left on ice for 1 h, and insoluble debris was then removed by centrifugation at 2,000 × *g* for 20 min. The clear supernatants were stored at −70°C.

Cytokine assays. Standard capture enzyme-linked immunosorbent assays were used with monoclonal antibody pairs and Maxisorp (Nunc, GIBCO, Paisley, United Kingdom) plates for the IFN-γ and IL-4 assays. Primary monoclonal antibodies against IFN-γ (R46A2) and IL-4 (11B11) and secondary biotinylated

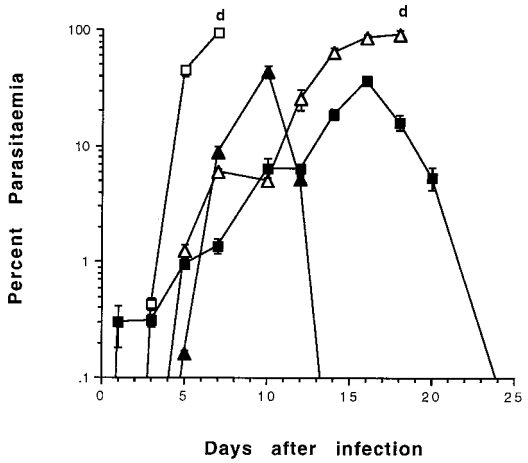

FIG. 1. Course of infection in mice injected i.v. with 10^4 erythrocytes parasitized with NLPY (■), *P. chabaudi* (▲), LPY (□), or *P. berghei* (△). Representative levels of parasitemia (means ± standard errors) from groups of six to eight mice from two separate experiments are shown. d, day of death of mice infected with the lethal parasites LPY and *P. berghei.*

anti-mouse IL-4 (BVD6-24G2) and anti-mouse IFN-γ (XMG1.2) monoclonal antibodies (PharMingen, San Diego, Calif.) were used with streptavidin peroxidase (Dako, Glostrup, Denmark) and *o*-phenylenediamine (Sigma) as a substrate. Recombinant mouse IFN-γ and IL-4 standards came from PharMingen. TNF-α assays were performed as described previously (32) with Maxisorp plates coated with a hamster monoclonal antibody against murine TNF (TN3) (kindly provided by Celltech, Slough, United Kingdom), polyclonal rabbit antibody against murine TNF (Genzyme, Cambridge, United Kingdom) peroxidase-conjugated anti-rabbit immunoglobulin G (Sigma), and *o*-phenylenediamine. A recombinant murine TNF (Genzyme) was used as a standard. Results are expressed as mean nanograms per spleen from at least six mice.

Statistics. Significance levels were determined by Student's *t* test for unpaired observations.

RESULTS

We measured concentrations of IFN-γ, TNF-α, and IL-4 in the spleens of mice at 24 h and thereafter at various times during the course of lethal and self-resolving infections. Spleen weights of normal and nude mice were 0.105 ± 0.01 g (mean ± standard deviation) and 0.11 ± 0.016 g, respectively, and there were no differences in weight 24 h after infection. Spleen weights increased significantly from baseline values on day 3 after all infections, as follows: for NLPY infections, 0.149 ± 0.02 g, $P < 0.0001$; for *P. chabaudi* infections, 0.142 ± 0.04 g, $P < 0.003$; for LPY infections, 0.141 ± 0.02 g, $P < 0.0002$; and for *P. berghei* infections, 0.143 ± 0.03 g, $P < 0.003$. Thereafter, spleen weights in infected mice varied with the parasite used for infection; spleen weights in mice infected with NLPY were threefold higher (1.31 ± 0.13 g) than those in mice infected with the other parasites from day 10 onwards.

IFN-γ production (Fig. 2a). Levels of IFN-γ in uninfected normal mice were 4.2 ± 1.93 ng/spleen. Levels were highest in NLPY infections, initially at 24 h and then from day 7 onwards. IFN-γ was not detected 24 h after *P. chabaudi* infection, but amounts increased from day 7 to day 12, when parasitemia was cleared (Fig. 1). Early IFN-γ production was not seen in either LPY or *P. berghei* infections, but increases were evident in the former on day 5 and in the latter on days 5, 7, and 10, with a decrease in *P. berghei* on day 15.

TNF-α production (Fig. 2b). Levels of TNF-α in uninfected normal mice were 0.53 ± 0.21 ng/spleen. TNF-α was induced and measurable 24 h after infection with the nonlethal parasites NLPY and *P. chabaudi*, with a later peak a few days before parasite clearance from the blood (Fig. 1). Although TNF-α was not induced 24 h after LPY infection, higher levels were seen on days 3, 5, and 7. TNF-α was induced 24 h after *P. berghei* infection, reaching a peak on day 7 and subsequently decreasing on days 10 and 15.

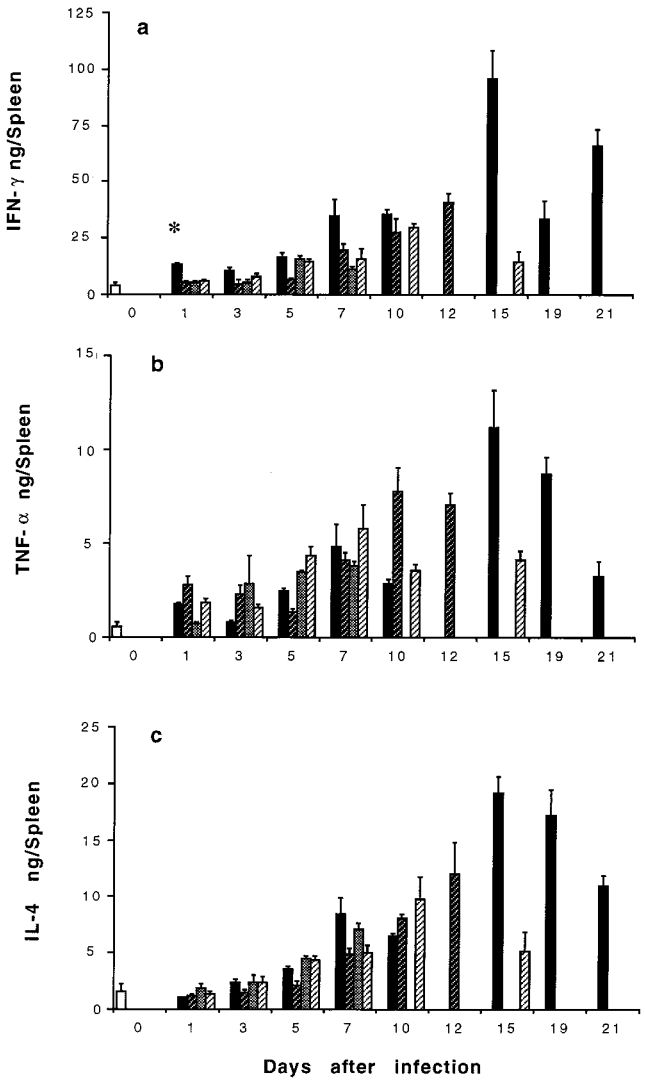

FIG. 2. Cytokine levels in the spleens of mice during malaria infection. Mice were injected i.v. with 10^4 erythrocytes parasitized with NLPY (■), *P. chabaudi* (▨), LPY (▩), or *P. berghei* (▨). IFN-γ (a), TNF-α (b), and IL-4 (c) were assayed at various times after infection. Each bar represents the mean (+ standard error) cytokine concentration of groups of six to eight mice from two separate experiments. *, $P < 0.0001$ compared with mice infected with *P. chabaudi*, LPY, or *P. berghei* or with normal uninfected mice (□).

IL-4 production (Fig. 2c). Levels of IL-4 in uninfected normal mice were 1.31 ± 0.64 ng/spleen. While IL-4 responses in both nonlethal and lethal infections were no higher than those of background controls at 24 h, two- to fourfold higher levels were seen on days 5, 7, and 10. By days 12 to 15, levels in the mice with nonlethal infections had risen 10-fold or more, while those in *P. berghei*-infected mice had decreased.

It appears that in the case of NLPY infections both Th1 and Th2 activities increase shortly before parasites are cleared from the blood.

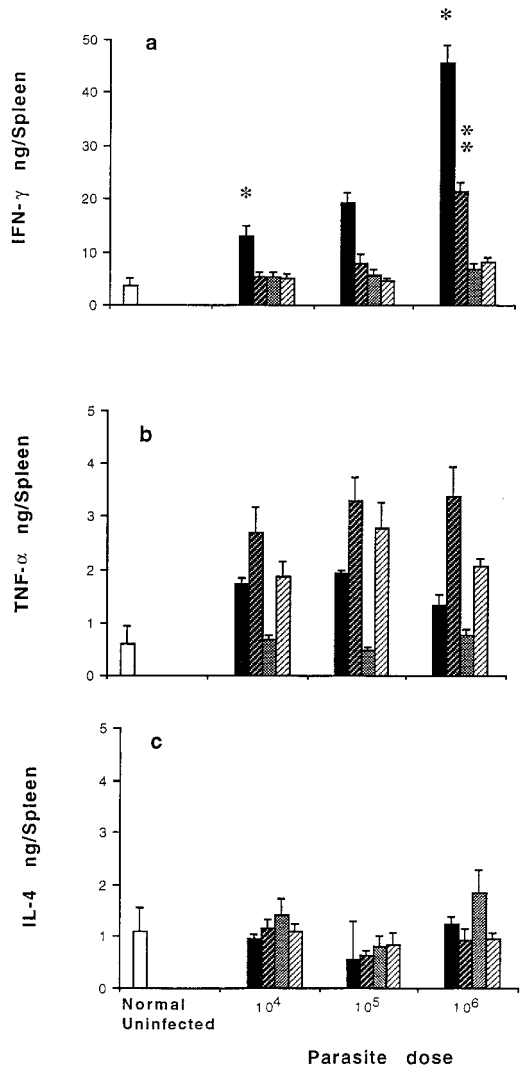

FIG. 3. Cytokine dose responses 24 h after infection. Mice were injected i.v. with 10^4, 10^5, or 10^6 parasitized erythrocytes (details as in the legend to Fig. 1). Twenty-four h later IFN-γ (a), TNF-α (b), and IL-4 (c) levels in the spleens were assayed. Each bar represents the mean (+ standard error) cytokine concentration of groups of 8 to 12 mice from three separate experiments. *, $P < 0.0001$ compared with mice infected with *P. chabaudi*, LPY, or *P. berghei* or with normal uninfected mice. *, $P < 0.0003$ compared with mice infected with LPY or *P. berghei*. Bars are as described for Fig. 2.

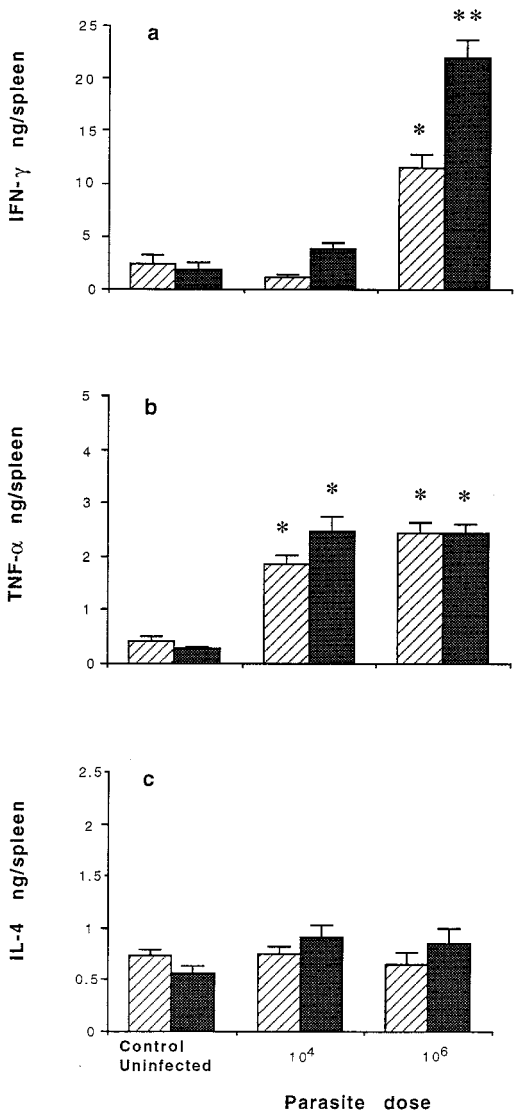

FIG. 4. Cytokine levels in the spleen of athymic mice infected with NLPY. BALB/c nude (▨) and littermate (▩) mice were injected i.v. with 10^4 or 10^6 NLPY-parasitized erythrocytes, and IFN-γ (a), TNF-α (b), and IL-4 (c) responses were assayed 24 h later. Each bar represents the mean (+ standard error) cytokine concentration of groups of 8 to 10 mice from two separate experiments. *, $P < 0.0001$ compared with uninfected mice; **, $P < 0.0002$ compared with athymic nude mice.

Cytokine dose-response characteristics 24 h after infection. Since NLPY infection induced a significantly higher IFN-γ response than infections by the other parasites 24 h after infection ($P < 0.0001$) (Fig. 2a), we investigated whether this early cytokine response was dose dependent and whether it could be triggered by higher doses of the other parasites. Groups of mice were injected i.v. with 10^4, 10^5, or 10^6 erythrocytes parasitized with NLPY, *P. chabaudi*, LPY, or *P. berghei* (Fig. 3). While levels of IFN-γ increased with the dose of the two nonlethal parasites, NLPY and *P. chabaudi* (Fig. 3a), the two lethal parasites, LPY and *P. berghei*, even at the highest parasite concentration, failed to induce any IFN-γ production. TNF-α levels increased after infection with NLPY, *P. chabaudi*, or *P. berghei*, though not with LPY, but there were no dose-related differences (Fig. 3b). IL-4 responses were not activated 24 h after infection with any of the four parasites (Fig. 3c).

Cytokine dose-response characteristics 24 h after NLPY infection in athymic mice. Since NLPY stimulated the strongest IFN-γ response 24 h after infection, we chose this parasite to determine whether cytokine production was T cell mediated. Groups of BALB/c nude mice and their littermates were infected i.v. with 10^4 or 10^6 NLPY parasites, and 24 h later their spleens were taken for cytokine analysis (Fig. 4). IFN-γ levels in both nude mice and littermates increased significantly ($P < 0.0001$) above control levels after infection with the higher dose; the lower dose induced a small increase only in the littermates, which was not significant (Fig. 4a). TNF-α levels were significantly increased ($P < 0.0001$) with both doses of parasites in both nude mice and littermates (Fig. 4b). IL-4 remained at control levels in both types of mice regardless of the size of the parasite inoculum (Fig. 4c).

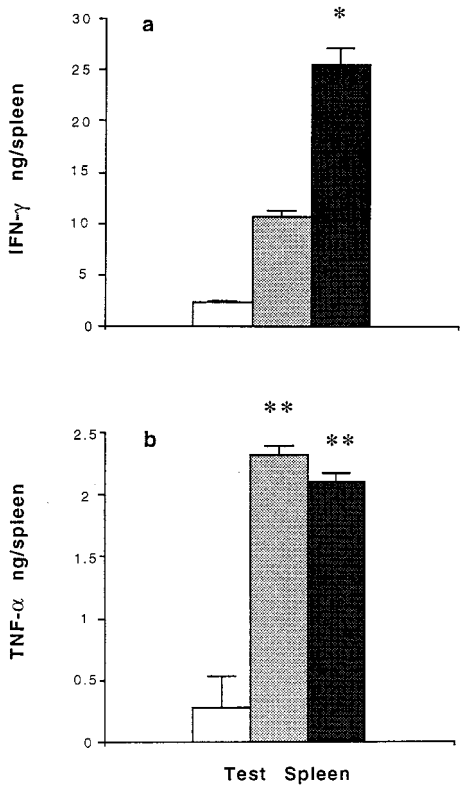

FIG. 5. Effect of NK cell depletion on cytokine production 24 h after NLPY infection. Mice depleted of their NK cells by treatment with rabbit anti-asialo GM1 antibody (▨) and controls treated with phosphate-buffered saline (■) were injected i.v. with 10^6 NLPY-parasitized erythrocytes, and 24 h later splenic IFN-γ (a) and TNF-α (b) levels were assayed. Each bar represents the mean (+ standard error) cytokine concentration of groups of 8 to 10 mice from two separate experiments. *, $P < 0.0001$ compared with normal uninfected and NK cell-depleted mice; **, $P < 0.0001$ compared with normal uninfected mice (□).

While TNF-α levels were similar in both groups of mice, IFN-γ levels in the nude mice were half those of the littermates. This suggests that the early IFN-γ response to NLPY is partly mediated by T cells. Furthermore, nude mice required a higher dose of parasites for the induction of IFN-γ.

Effect of NK cell depletion on cytokine production 24 h after NLPY infection. To see if the early cytokine response was also mediated by NK cells, normal and NK cell-depleted mice were infected i.v. with 10^6 NLPY parasites and their spleens were taken 24 h later for cytokine analysis (Fig. 5). There were significant differences in IFN-γ production between the NK cell-depleted and control groups ($P < 0.0001$): the response was reduced by 58% in the antibody-treated group. Early IFN-γ production thus appears to be mediated by both NK cells and T cells (Fig. 5a). TNF-α levels increased significantly above the background ($P < 0.0001$) and to the same extent in both the NK cell-depleted and control groups (Fig. 5b). As before, IL-4 levels after 24 h were no different from those of the untreated controls (data not shown).

DISCUSSION

These experiments clearly show that one characteristic feature of these two nonlethal malaria infections is their ability to stimulate IFN-γ production 24 h after challenge. This early IFN-γ response to NLPY was reduced significantly, by about 50%, in both nude and NK cell-depleted mice, suggesting that both T cells and NK cells are involved. Nude mice cannot control an NLPY infection (35), and it has been reported that their B cells lack the appropriate T-cell (possibly αβ T cell) cytokine activation signals essential for isotype switching and the immunoglobulin G2a production necessary for resolution of parasitemia (33). However, nude mice do have γδ T cells (18), and these may be involved in the early IFN-γ response after NLPY infection. The protective role of IFN-γ against the sporozoite (27) and liver (19) stages of malaria has been well documented, and a recent study has shown that while IFN-γ receptor knockout mice which have been immunized with a single dose of irradiated NLPY sporozoites are not protected against live sporozoite challenge, multiple doses of vaccine induce additional protective mechanisms that are independent of IFN-γ and its receptor (34). The study also showed that in unimmunized knockout mice, recovery from the blood-stage infection was consistently delayed by a few days. Our data suggest that IFN-γ plays an important role in immunity against both NLPY and *P. chabaudi*, and this agrees with earlier studies where it was shown that the administration of recombinant IFN-γ increased the survival of susceptible mice infected with LPY 17X and that in strains of mice resistant to both NLPY and LPY there was an initial increase in IFN-γ activity (28). It has also been shown that the administration of recombinant IFN-γ delayed the onset of parasitemia in mice infected with *Plasmodium chabaudi adami* (4).

Our data show that TNF-α levels are also increased 24 h after infection with NLPY, *P. chabaudi*, or *P. berghei*. Early TNF-α responses have also been implicated in protective immunity against *P. chabaudi* AS in resistant mouse strains (14), and daily intraperitoneal injections of recombinant human TNF appeared to suppress *P. chabaudi adami* infections in mice (4). Thus, macrophage activation may be the first response to occur after infection, giving rise to the production of TNF-α and possibly of IL-12 (12), both of which stimulate NK cells to produce IFN-γ (2). This in turn would enhance major histocompatibility complex class II expression and finally Th1 cell and B-cell activation, leading to production of subclass-specific antiparasite antibodies. These early cytokine responses could therefore be critical in activating the effector mechanisms necessary for resolution of parasitemia. The protective value of early IFN-γ responses is consistent with the hypothesis of Garside and Mowat (10), who suggest that polarization of Th cell subset responses is determined by nonspecific, inflammatory responses occurring soon after infection.

In the case of *P. berghei* and LPY, failure to induce IFN-γ could theoretically be due to suppression of NK cell activation by parasite-derived molecules. In the case of *P. berghei*, some NK cell activation may occur, since beige mutant mice, deficient in NK cells, have higher parasitemias than parental C57BL/6 mice during the first week of infection (30). Parasite-derived molecules could also block activation of macrophages for IL-12 production (12), resulting in a lack of NK cell activation. Alternatively, the lethality of *P. berghei* and LPY might be explained by the release of parasite-derived molecules which preferentially stimulate mast cells to secrete cytokines which we have not measured, such as IL-3, IL-5, IL-6, or granulocyte-macrophage colony-stimulating factor (13), leading to the activation of Th0 cells and the production of IL-10; this would then actively suppress IFN-γ production by NK cells (16) or by Th precursor and Th1 cells (10), thereby preventing resolution of infection. We are currently investigating this possibility.

An understanding of host-parasite interactions in malaria is an important prerequisite for vaccine design. A knowledge of

the immunological mechanisms evoked, or inhibited, shortly after infection might provide important clues regarding the type of response that needs to be induced by vaccination. One possible approach to this problem is critical analysis of the differences in immune responses in self-resolving and lethal infections. During self-resolving blood-stage infections there is strong T-cell activation, which is absent in lethal infections (15). Our previous studies have shown that there are also clear differences in lymphocyte homing (23): homing to the spleen and liver is markedly less 24 h after infection with the LPY and *P. berghei* parasites than with NLPY and *P. chabaudi*. Reduced homing later on is accompanied by a loss of lymphocytes during lethal infections, associated with the presence of antilymphocyte autoantibodies (7), which do not occur in nonlethal infections (7, 8, 36). Early lymphocyte homing in nonlethal infections may be associated with the early IFN-γ response reported here. Thus, TNF, secreted by splenic macrophages activated by nonlethal parasite antigens (24), could activate NK cells to produce IFN-γ, which in turn might initiate lymphocyte trapping. It appears that both 24-h lymphocyte homing and IFN-γ responses, leading to T-cell activation, distinguish self-resolving from lethal infections.

In general, early NK cell-mediated IFN-γ responses play an important role in innate immunity to infection (reviewed by Bancroft [1]). This has been reported for a number of gram-positive organisms, such as *Listeria monocytogenes* (9), and for gram-negative bacteria, such as *Yersinia enterocolitica* (3). Furthermore, protozoan parasites, such as *Toxoplasma gondii* (29) and *Leishmania major*, trigger NK cells to produce IFN-γ, while strains of inbred mice susceptible to *L. major* fail to develop this early IFN-γ response (26). Resistance to the fungus *Cryptococcus neoformans* is associated with production of IFN-γ and IL-2 by lung-associated lymph node cells and lung cells 24 h after infection (13), and the inhibition of IFN-γ production by monoclonal antibody treatment prevents NK cell activation and increases the susceptibility of mice to infection (25). The data presented here show that an early IFN-γ response is a feature of some nonlethal malaria infections and, together with the reports quoted above, suggest that it may be a general phenomenon in self-resolving infections—bacterial, fungal, and protozoan. Effective vaccines against malaria may depend upon antigens that lead to specific enhancement of Th1 cell subset activation soon after infection.

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