Altered Immune Response of Interferon Regulatory Factor 1-Deficient Mice against Plasmodium berghei Blood-Stage Malaria Infection

ROSEMARY SOK-PIN TAN,1 CHIGUANG FENG,2 YOSHIHIRO ASANO,2 AND ANNA URSULA KARA1*

Molecular Parasitology Laboratory, Department of Biological Sciences, National University of Singapore, Singapore 119260, Singapore,1 and Department of Microbiology and Immunology, Ehime University School of Medicine, Onsen-gun, Ehime 791-0295, Japan.

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Nitric oxide (NO) is a short-lived biological mediator which can be induced in various cell types and is able to cause many metabolic changes in target cells. Inhibition of tumor cell growth and antimicrobial activity has been attributed to the stimulation of NO production by transcriptional upregulation of inducible nitric oxide synthase. In the present study, we used mice devoid of functional interferon regulatory factor 1 by targeted gene disruption (IRF-1−/−) to investigate the role of NO in the host immune response against blood-stage Plasmodium berghei ANKA infection. IRF-1−/− mice survived longer with a later onset of and a lower peak parasitemia despite the inability to produce appreciable levels of NO. The administration of exogenous interleukin-12 (IL-12) was able to prolong survival in the wild-type mice with an upregulation in the expression of both gamma interferon (IFN-γ) and NO. However, the administration of IL-12 did not improve the survival of IRF-1−/− mice. These studies indicate that while IL-12 is able to mediate protection via an IFN-γ- and NO-dependent pathway in the wild-type mice, such a protective mechanism may not be functional in the IRF-1−/− mice. Our results suggest that NO may not be essential for host immunity to the parasite and that IRF-1−/− mice are able to induce an IFN-γ- and NO-independent mechanism against P. berghei infection.

Nitric oxide (NO) plays an important role in many biological functions, ranging from antiparasitic activity (21), antiparasitic activity (8, 32), and antibacterial activity (15) to other physiological effects (29). NO is generated from L-arginine by the enzyme NO synthase (NOS) and, at low concentrations, acts as a signal in many physiological processes including neurotransmission and blood flow regulation (reviewed in reference 28). When produced in large quantities by the activation of cytokine-inducible NOS (iNOS), NO can have host defense, tumor cell growth and antimicrobial activity, and immune regulatory effects (24). Its role as an antioncogene in human leukemia has also been ascertained (12, 46).

In the present study, we infected IRF-1−/− and wild-type littermate mice with a lethal strain of the agent of murine malaria, Plasmodium berghei ANKA (20, 47), to examine the role of NO in host immunity against this blood-stage malaria infection. In addition, we examined the effects resulting from the administration of recombinant murine interleukin-12 (rIL-12) in both IRF-1−/− and wild-type mice.

IL-12 is a cytokine which is able to exert regulatory effects on...
both T lymphocytes and natural killer (NK) cells and promote the induction of a Th1 response (45). IL-12 has the ability to stimulate endogenous IFN-γ production and can therefore enhance the development of a protective cell-mediated immunity in vivo via an NO-dependent mechanism (37). It has been shown that the administration of exogenous rIL-12 during blood-stage infection with Plasmodium chabaudi AS significantly reduced the peak parasitemia level and enhanced the survival of susceptible A/J mice (37). The protective effects of rIL-12 treatment have previously been demonstrated in Plasmodium yoelii sporozoite-induced malaria infection in BALB/c mice (35) and Plasmodium cynomolgi sporozoite-induced malaria in monkeys (11). We were therefore interested to see the effects that the administration of rIL-12 had during Plasmodium berghei blood-stage infections in both IRF-1−/− and wild-type mice.

In summary, in the following study we have analyzed the immunological differences which occur in two types of mice, wild type and IRF-1−/−, subjected to murine malaria infections and have determined the roles of IL-12, IFN-γ, and NO production during this murine malaria infection.

MATERIALS AND METHODS

Mice. Mice with a targeted disruption in the gene coding for IRF-1 were generated as previously described (24) and were maintained by backcrossing to C57BL6 mice. Six- to 8-week-old IRF-1−/− and wild-type mice were used in this study. These mice were bred under approved conditions at the Laboratory Animal Center at Ehime University School of Medicine, Ehime, Japan.

Parasites. P. berghei ANKA (20, 47) was maintained by serial blood passage. Mice were infected intraperitoneally (i.p.) with 107 parasitized erythrocytes (PRBC). Parasitemia was monitored by Giemsa-stained thin-blood films of tail bleeds.

Cytokine for immunization. rIL-12 was a generous gift from the Genetics Institute (Cambridge, Mass.). Mice were treated i.p. with 100 ng of rIL-12 diluted in 1% normal mouse serum (NMS)-saline to give doses of 0.1 ml. Doses were given on days −3, −2, −1, 0, 2, 4, and 6, with day 0 being the day of infection. Control mice were treated with 1% NMS-saline.

mRNA detection by RT-PCR. Organs (brain, liver, spleen, and kidneys) were removed at indicated times (day 0, 4, 8, 11, and 15), and total RNA was isolated by using the RNeasy kit from Qiagen, Santa Clarita, Calif., following the manufacturer's instructions. Equal amounts of RNA (1 μg) were reverse transcribed with 1 μl of reverse transcriptase (RT) (Rho-2, Takara Shuzu Co., Otsu, Shiga, Japan), 2.5 mM deoxynucleotide triphosphate, and 300 ng of random primers (Takara Shuzu Co.) in a total volume of 20 μl. Reverse transcription was carried out at 42°C for 60 min, and 30 μl of RNase-free Tris-EDTA buffer was added to each sample. PCR amplification was carried out in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, Conn.) with 1 μl of the reverse-transcribed product and 1 μl of Taq polymerase (Takara Shuzu Co.) in a final volume of 10 μl. The reaction condition was as follows: DNA denaturation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 53°C for 1 min, and 72°C for 2 min, and DNA denaturation at 72°C for 10 min. The following oligonucleotide primers were used: IFN-γ, 5′-GAA AGC CTA GAA AGT CTG AAT AAC T-3′ and 5′-GGT CAG AAG GAC TCC TTT TCC GCT T-3′; IFN-β, 5′-ACC AAG GCT CAG CTC CAG GAA GGT GGG-3′, and 5′-ATA ACC TGG CAG CTC CAG GAA GGT GGG-3′; and β-actin, 5′-ATG CCT GAG CAC TCT CAG GAA GGT GGG-3′, and 5′-CCC ACC AAG GAA GGA GCC TGG-3′.

The predicted sizes of amplified products for IFN-γ, IFN-β, and β-actin were 388, 950, and 665 bp, respectively. Aliquots of 5 μl of the reaction mixture were analyzed on a 1.5% agarose gel in Tris-acetate-EDTA buffer. The PCR products were visualized, photographed, and recorded with Fujifilm Digital Image File DF-20 and the Sony CCD Video Camera Module XC-75/75CE (Tokyo, Japan). Intensities of the bands were determined by using a computer program (Adobe Photoshop 2.51 and NIH Image).

Administration of rIL-12 in wild-type and IRF-1−/− mice. As P. berghei is lethal in mice with a C57BL6 background, all mice were expected to die. However, treatment of mice with rIL-12 was able to significantly prolong the survival of wild-type mice. In preliminary studies, we had observed that treatment with different doses (30, 60, or 100 ng or 1 μg) of rIL-12 was able to induce partial protection in wild-type and inbred C57BL6 mice (data not shown). We established a route for the effective administration of 100 ng of rIL-12 per day per mouse on days −3, −2, −1, 0, 2, 4, and 6, with day 0 being the day of infection. As shown in Fig. 1, 2 of the 10 wild-type mice treated with rIL-12 died on day 20 postinfection while the remaining mice survived beyond 23 days postinfection. Time of death was significantly different from that of the untreated wild-type mice. These observations suggest that the IRF-1−/− mice are more resistant to P. berghei ANKA infection than the wild-type mice, with a significant difference in time of death.

RESULTS

Prolonged survival of IRF-1−/− mice during blood-stage P. berghei ANKA infection. IRF-1−/− mice were infected with P. berghei ANKA to determine if the absence of the IRF-1 gene affects the infectivity profile of the parasite and the immune response to the parasite in these mice. All mice were challenged i.p. with 106 PRBC on day 0. The survival of the mice was monitored daily. Parasitemia levels were examined by microscopic examination of Giemsa-stained thin-blood slides from tail bleeds obtained from day 4 to day 23 postinfection. IRF-1−/− mice were more resistant to infection and survived significantly longer than their wild-type counterparts (Fig. 1). All 10 wild-type mice developed parasitemia which increased exponentially, and they died between 9 and 13 days postinfection (Fig. 1A). In contrast, both of the eight IRF-1−/− mice died on day 19 postinfection, with the remaining mice surviving beyond day 23 (Fig. 1A). IRF-1−/− mice had a slower onset of parasitemia and significantly lower peak parasitemia levels (Fig. 1B) than the wild-type mice. These observations suggest that the IRF-1−/− mice are more resistant to P. berghei ANKA infection than the wild-type mice, with a significant difference in time of death.

Determination of nitrite concentration. The nitrite concentrations in spleen cell supernatants and sera were determined by the Griess reaction (7). Briefly, 100 μl of spleen cell supernatants stimulated by ConA for 24 h, nitrate standards (range, 10 nM to 50 μM; Sigma Chemical Co.), and medium only were mixed with 100 μl of Griess reagent, which consists of equal volumes of 1% sulfanilamide (Sigma Chemical Co.) and 0.1% naphthylethylene diamine dihydrochloride (Sigma Chemical Co.) in 2.5% H3PO4 in a 96-well microplate. The A540 was measured 10 min later by using a microplate reader (Fujirebio, Tokyo, Japan). Concentrations were determined by referring to a standard curve. The nitrite concentration in the serum was determined essentially similarly to the above protocol except that 50 μl of serum was used.

Cytokine ELISA. A sandwich ELISA was performed with sera and spleen cell supernatants to quantify the amount of IFN-γ. Both monoclonal IFN-γ antibodies and biotinylated anti-IFN-γ were purchased from Pharmingen, San Diego, Calif. A standard curve was constructed by using mouse recombinant IFN-γ (Genzyme). The sensitivity of this assay was 0.07 ng/ml.

Statistical analysis. The statistical significance of differences in survival and NO and IFN-γ levels between groups of mice were analyzed by Student's t test. A P value of <0.05 was considered significant.

Administration of rIL-12 in wild-type and IRF-1−/− mice.
treated IRF-1\(^{-/-}\) mice. However, iNOS mRNA expression was evident in both rIL-12-treated and untreated infected wild-type mice. The priming of rIL-12 in wild-type mice was able to induce a higher level of iNOS mRNA expression in the liver, spleen, and kidneys on day 0 compared to that in untreated wild-type mice. The treatment of rIL-12 in wild-type mice enabled an upregulation of iNOS mRNA expression in the brain on day 11 and a gradual increase of iNOS expression in both the liver and spleen. Livers from untreated mice also had an increase in iNOS expression, albeit with lower levels than the livers from treated mice. Peak expression was on day 4, but the transcript was undetectable after day 8. In the spleen of rIL-12-treated mice, iNOS mRNA expression was significantly upregulated, with a peak at day 11. However, iNOS expression in the spleens of untreated wild-type mice was either very low or undetectable. Expression of iNOS appeared to be lower in the kidneys where the peak of expression occurred on day 4 and then slowly decreased.

In order to assess the production of NO, nitrite concentrations were determined in the supernatants of spleen cell cultures stimulated with IFN-γ and LPS (data not shown) or ConA (Fig. 2B) and in sera (Fig. 2C) collected at indicated times. The concentrations of nitrite in both the spleen cell supernatants and sera of rIL-12-treated or untreated IRF-1\(^{-/-}\) mice were either very low or totally undetectable. In contrast, administration of rIL-12 to the wild-type mice induced a significantly higher production of NO, with an increase in nitrite concentration of approximately 10 times over that of the untreated wild-type mice on day 8 postinfection in the spleen cell supernatants and in the serum on day 11 postinfection. Untreated wild-type mice were able to induce NO production throughout the infection, but the levels were much lower than in treated mice.

**Differential IFN-γ mRNA expression and IFN-γ production during *P. berghei* infection.** All mice were infected with 10⁶ PRBC on day 0 and on indicated days (days 0, 4, 8, 11, and 15 postinfection), they were sacrificed, and pieces of their organs (brain, liver, spleen, and kidneys) were removed for total RNA isolation. RT-PCR results with IFN-γ primers indicated differential expression of IFN-γ in the organs of IRF-1\(^{-/-}\) and wild-type mice. As shown in Fig. 3A, treatment of both wild-type and IRF-1\(^{-/-}\) mice with rIL-12 was able to upregulate IFN-γ levels in all organs. The priming effect of the rIL-12 treatment was already apparent on day 0, the day of infection, and 3 days after the first rIL-12 treatment, as levels of IFN-γ were upregulated in the liver, spleen, and kidneys. There seemed to be no detectable upregulation of IFN-γ in the brain for all mice except for the rIL-12-treated IRF-1\(^{-/-}\) mice, since mRNA expression of IFN-γ on both day 4 and day 8 in the brains of rIL-12-treated IRF-1\(^{-/-}\) mice was significantly higher than in the rIL-12-treated wild-type mice. Untreated IRF-1\(^{-/-}\) mice were not able to upregulate their IFN-γ levels to the same amount as the wild-type mice, as mRNA expression of IFN-γ in all organs was very low. The patterns of induction of IFN-γ mRNA by rIL-12 in both wild-type and IRF-1\(^{-/-}\) mice were similar in all organs, except for the brain. The rIL-12-treated IRF-1\(^{-/-}\) mice showed very high levels of IFN-γ. Induction of IFN-γ appeared to be stronger in the livers and kidneys of the rIL-12-treated wild-type mice, where the mRNA expression of IFN-γ was still detectable at day 11 postinfection, which is 5 days after the last rIL-12 administration.

Expression of IFN-γ protein in spleen cell supernatants was detected by sandwich ELISA. As shown in Fig. 3B, spleen cells from rIL-12-treated *P. berghei* ANKA-infected wild-type and IRF-1\(^{-/-}\) mice stimulated with ConA for 24 h produced significantly higher IFN-γ levels in vitro compared to that of untreated infected mice. Expressions of IFN-γ protein in the sera of rIL-12-treated *P. berghei* ANKA-infected wild-type and IRF-1\(^{-/-}\) mice were similar to that observed for spleen cell supernatants (data not shown).
FIG. 2. Effect of rIL-12 on expression of iNOS mRNA and NO production in *P. berghei* ANKA-infected wild-type and IRF-1−/− littermates. (A) Kinetics of iNOS mRNA levels in the brains, livers, spleens, and kidneys of both infected wild-type and IRF-1−/− mice treated with and without IL-12. Total RNA was purified from the organs isolated from two mice per time point, and levels of iNOS mRNA in individual organs were determined as described in Materials and Methods. The data shown represent the means ± standard deviations and are representative of two replicate experiments. (B) The Griess reaction test for the determination of nitrite concentration was performed with supernatants from spleen cells prepared from two mice per time point and stimulated with ConA for 24 h. (C) The Griess reaction test was performed with sera obtained from two mice per time point. Data represent means ± standard deviations and are representative of two replicate experiments. N.A., not available for analysis; O.D., optical density.
DISCUSSION

Our present study demonstrates for the first time that NO production by iNOS may not be essential in the protection against blood-stage *P. berghei* ANKA infection. Mice with a targeted disruption of the IRF-1 gene (IRF-1−/−) were more resistant to *P. berghei* ANKA infection than their wild-type littermates despite the inability of the IRF-1−/− mice to produce appreciable levels of NO (15, 34). Many reports have suggested that increased NO production correlates with resistance against pathogens (2, 13, 14, 36, 37). However, previous studies using IRF-1−/− or iNOS-deficient mice exposed to a variety of infections already suggested that NO production by iNOS may not play a universal role in protection against pathogens. Infection of IRF-1−/− mice with several viral pathogens has shown conflicting results. While IRF-1−/− mice were more susceptible to encephalomyocarditis virus infection than were wild-type mice, the absence of IRF-1 did not apparently affect the replication of vesicular stomatitis virus and herpes simplex virus (18). *Mycobacterium bovis* BCG bacterial infection was found to be more extensive and severe
in IRF-1−/− mice than in wild-type mice, and the elimination of *M. bovis* was impaired in IRF-1−/− mice (15). IRF-1−/− mice infected with the *Leishmania major* parasite showed reduced IL-12 and IFN-γ production, leading to a failure in the development of a Th1 response, and the mice rapidly succumbed to the infection (22). On the other hand, infection of IRF-1−/− mice with another intracellular pathogen, *Toxoplasma gondii*, revealed that NO production may not be essential for protection (17).

IRF-1 is a transcription factor which is responsible for a wide range of effects (19). Proinflammatory cytokines like tumor necrosis factor alpha and IL-1 are able to induce activation of IRF-1 (6, 16), suggesting a possible role for IRF-1 in inflammation. In addition, the role of IRF-1 in the induction of apoptosis (40, 43, 44) suggests that the parasite-mediated induction of apoptotic and inflammatory processes, which may contribute to the pathology of *Plasmodium* infection, are affected and thus may occur at reduced levels.

Our study analyzed the immunological differences in IRF-1−/− mice during blood-stage *P. berghei* malaria infection. *P. berghei* is lethal in mice with a C57BL/6 background, and the infected wild-type mice died as expected within 9 to 13 days postinfection. Administration of 100 ng of rIL-12 was able to prolong the survival of these mice for an additional 10 days. It is conceivable that the introduction of rIL-12 was able to induce an IFN-γ-dependent pathway leading to increased NO production. This is in accordance with many studies done on mice and monkeys in both blood-stage and sporozoite-induced malaria infections (11, 13, 14, 37). In contrast to the wild-type mice, all rIL-12-treated IRF-1−/− mice died within 8 days postinfection, suggesting that the introduction of rIL-12 into *P. berghei* ANKA-infected IRF-1−/− mice might have triggered a cascade of events that is toxic and lethal to the mice. Administration of rIL-12 into uninfected mice did not cause any lethal effects (data not shown). In our study, the administration of 0.1 or 1 μg of rIL-12 per IRF-1−/− mouse did not enhance survival at all, which is in contrast to the results reported by Khan et al. (17), who found that protection was enhanced by the administration of exogenous rIL-12 (0.33 μg/mouse) into IRF-1−/− mice infected with the parasite *T. gondii*. IFN-γ mRNA and protein expression of treated IRF-1−/− mice were significantly enhanced, with no corresponding increase in iNOS expression and NO production. Especially worth noting is the high expression of IFN-γ mRNA in the brains of these mice. We observed that even though there was an induction of IFN-γ mRNA expression in the liver, spleen, and kidneys, as well as an increase in IFN-γ levels in the serum and spleen cell supernatants of both rIL-12-treated wild-type and IRF-1−/− mice, there was no apparent induction of IFN-γ mRNA in the brains of rIL-12-treated wild-type mice. The consequences of the high expression of IFN-γ in the brains of the rIL-12-treated IRF-1−/− mice remain to be investigated. Some clues to explain these results may be obtained from the analysis of possible pathological damage that may have occurred in the brain. Yet, we did not observe any significant differences in the pathological sections of liver, kidney, or spleen between IRF-1−/− and wild-type mice (41a), and thus it is not very likely that brain pathology will account for the early death of the rIL-12-treated IRF-1−/− mice.

It was previously shown that both macrophages and CD4+ T cells in the IRF-1−/− mice were impaired in Th1 differentiation (39). Expression of IFN-γ mRNA in all organs of the untreated IRF-1−/− mice was significantly lower than in untreated wild-type mice throughout the course of infection. Mice with a targeted disruption of the *IRF-1* gene are not able to produce iNOS mRNA and NO, as IRF-1 is needed to bind to the promoter of the iNOS gene (15). Despite the inability to induce appreciable amounts of IFN-γ and NO, IRF-1−/− mice were able to survive longer than the wild-type mice, with a later onset of and a lower peak parasitemia. This observation suggests that the IRF-1−/− mice are able to induce a protective immune host response to *P. berghei* ANKA infection through a pathway other than by induction of IFN-γ and NO production.

For the wild-type mice, expression of iNOS mRNA was upregulated and nitrite concentrations were significantly increased by the administration of rIL-12. Previous reports have indicated that the ability to produce high amounts of NO early during infection may correlate with resistance to blood-stage malaria infections (14, 31, 37), and NO production in the spleen appears to be critical to this resistance (13). Our results on IL-12-mediated protection in wild-type mice confirmed this hypothesis. We observed a differential regulation of iNOS mRNA expression in the spleens of rIL-12-treated and untreated wild-type mice. While there was high expression of iNOS in the spleens of the rIL-12-treated wild-type mice, iNOS expression was not detectable in the spleens of the untreated wild-type mice. The protection in the rIL-12-treated wild-type mice could be due to the increased production of IFN-γ and NO, which probably occurred via an IFN-γ- and NO-dependent pathway.

In view of the fact that the IRF-1−/− mice were able to survive longer than their wild-type littermates, we suggest that NO cannot play a part in the protection against this blood-stage malaria infection. To the contrary, NO is absolutely essential for the protection of wild-type mice against this infection, as it boosts the survival rate, which is correlated with an increase in the level of NO. This result is consistent with those of numerous studies cited previously in the text. We postulate that such a protective mechanism by NO is redundant or nonfunctional in the IRF-1−/− mice and that an alternative pathway exists that could lead to a protection equivalent to that observed in rIL-12-treated wild-type mice. In addition, we do not exclude the possibility that IRF-1−/− mice have defects other than NO production (24, 39, 41, 43, 44).

Taken together, our results indicate the presence of an altered immune response in IRF-1−/− mice during blood-stage *P. berghei* ANKA infection. Prolonged survival with a later onset of and a lower peak parasitemia with reduced productions of IFN-γ and NO indicate that an alternative protective mechanism independent of IFN-γ and NO plays an important role in this murine malaria infection.

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