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The Role of IL-18 in Blood-Stage Immunity Against Murine Malaria Plasmodium yoelii 265 and Plasmodium berghei ANKA

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A possible protective role of IL-18 in host defense against blood-stage murine malarial infection was studied in BALB/c mice using a nonlethal strain, Plasmodium yoelii 265, and a lethal strain, Plasmodium berghei ANKA. Infection induced an increase in mRNA expression of IL-18, IL-12p40, IFN-γ, and TNF-α in the case of P. yoelii 265 and an increase of IL-18, IL-12p40, and IFN-γ in the case of P. berghei ANKA. The timing of mRNA expression of IL-18 in both cases was consistent with a role in the induction of IFN-γ protein expression. Histological examination of spleen and liver tissues from infected controls treated with PBS showed a very cellular inflammatory reaction, massive necrosis, a large number of infected parasitized RBCs, and severe deposition of hemozoin pigment. In contrast, IL-18-treated infected mice showed massive infiltration of inflammatory cells consisting of mononuclear cells and Kupffer cells, decreased necrosis, and decreased deposition of the pigment hemozoin. Treatment with rIL-18 increased serum IFN-γ levels in mice infected with both parasites, delayed onset of parasitemia, conferred a protective effective, and thus increased survival rate of infected mice. Administration of neutralizing anti-IL-18 Ab exacerbated infection, impaired host resistance and shortened the mean survival of mice infected with P. berghei ANKA. Furthermore, IL-18 knockout mice were more susceptible to P. berghei ANKA than were wild-type C57BL/6 mice. These data suggest that IL-18 plays a protective role in host defense by enhancing IFN-γ production during blood-stage infection by murine malaria. The Journal of Immunology, 2002, 168: 4674–4681.
to *P. berghei* ANKA infection. Finally, to explore the role of endogenous IL-18, we investigated the effect of neutralizing anti-IL-18 Ab in mice infected with *P. berghei* ANKA.

**Materials and Methods**

**Mice and parasite infection**

Six- to 8-wk-old female BALB/c (H-2b) pathogen-free mice were obtained from the animal breeding facility of the National Institute of Nutrition (Hyderabad, India). The C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). IL-18 knockout mice were bred in the animal center of the Advanced Biomedical Sciences Center, Hyogo College of Medicine (Nishinomiya, Japan). The animals used in this study were used in strict accordance with the guidelines set forth by the National Institutes of Health (NIH) Animal Care and Use Committee. All animal procedures were performed in accordance with the guidelines set forth by the National Institutes of Health (NIH) Animal Care and Use Committee. All animal procedures were performed in accordance with the guidelines set forth by the National Institutes of Health (NIH) Animal Care and Use Committee. All animal procedures were performed in accordance with the guidelines set forth by the National Institutes of Health (NIH) Animal Care and Use Committee.

**Measurement of cytokine proteins in serum and culture supernatant**

For analysis of production of different cytokines, livers and spleens of mice infected with *P. yoelii* 265 and *P. berghei* ANKA were removed aseptically at indicated times. Tissue samples were dissociated by trituration and mincing. Primary cell cultures were established by scraping intact liver and spleen specimens, washing in RPMI 1640 medium, and filtering. Cell suspensions were prepared in RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FCS (Life Technologies). Isolated liver and spleen cells were cultured by seeding at 2 × 10^6 cells/well in 100 μl of the above medium in 24-well tissue culture plates (Costar, Cambridge, MA) and incubated for 48 h at 37°C in a humidified 5% CO2 incubator to monitor cytokine production. For polyclonal sera, mice within each group were bled from the retro-orbital plexus at the indicated times, and sera within each group were pooled, allowing to clot for 30 min at 4°C, and centrifuged at 12,000 × g for 10 min. Liver and spleen cell supernatants and sera were stored at −80°C for subsequent ELISA analysis for cytokine proteins. The production of IFN-γ (sensitivity, 15 pg/ml), IL-4 (sensitivity, 10 pg/ml) (BD PharMingen, San Diego, CA), and IL-2 (sensitivity, 10 pg/ml) (Genzyme Diagnostics, Cambridge, MA) were measured in triplicate from serum (1/50 dilutions) and cell-free culture supernatant with commercial ELISA kits following the manufacturer’s instructions. The quantitation was achieved using standard curves obtained for individual cytokines (provided by the manufacturer).

**Histological examination**

Liver and spleen specimens were fixed in 10% phosphate-buffered formalin, processed, and embedded in paraffin. Sections were cut, stained with H&E, and examined under a light microscope. Analyses of several characteristics, each by a corresponding scale, were performed as follows: evaluation of architecture: maintained (0), partial loss (1), moderate loss (2), total loss (3); pigment deposition: nil (0), mild (1), moderate (2), intense (3); number of necrotic foci: nil (0), very few foci (1), moderate (2), extensive (3); extent of fatty degeneration: nil (0), very little (1), moderate (2), severe (3); inflammation: nil (0), mild (1), moderate (2), extensive (3).

**Statistical analysis**

Statistical analysis was performed by Student’s *t* test, except for survival. Survival was evaluated by generation of Kaplan-Meier plots, 6 graphs, and log rank analysis. A *p* value < 0.05 was considered statistically significant.

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**Abbreviation used in this paper:** PRBC, parasitized RBC.
Results

In vivo induction of IL-18 gene expression in livers and spleens of mice infected with blood-stage P. yoelii 265 and P. berghei ANKA parasites

To investigate whether the infection of BALB/c mice with the blood-stage P. yoelii 265 parasite induced IL-18 expression, we examined mRNA expression of IL-18 and other related cytokines in the livers and spleens of infected mice. Mice were injected i.p. with 1 × 10⁷ PRBCs, and livers and spleens from these mice were excised and analyzed as described in Materials and Methods at different time points starting 2 days after the infection. Messenger RNA expression was examined by RT-PCR. In the livers of mice infected with P. yoelii, IL-18 mRNA expression was seen as early as 3 days postinfection and was observable at least up to 13 days postinfection (Fig. 1A). Interestingly, there was no detectable expression of IL-18 mRNA in the spleens of P. yoelii-infected mice (Fig. 1B). In contrast, expression of IL-12p40 mRNA was observed from 3 days postinfection and lasted up to 17 days postinfection, in both liver and spleen of these mice. Similarly, IFN-γ mRNA was also present in both the liver and spleen of P. yoelii-infected mice, starting 3 days postinfection (Fig. 1A). The intensity of IFN-γ mRNA expression was considerably higher in spleens than in livers of infected mice, particularly from days 7 to 11 postinfection (Fig. 1B). The control G3PDH housekeeping gene was expressed constitutively at similar levels in both control and infected mice and in spleen. In the case of P. berghei infection, IL-18 mRNA expression in the liver was first observed on day 5, reached peak levels on day 7, and thereafter declined, vanishing by day 11 postinfection (Fig. 1C). Also in these mice, IL-18 mRNA gene expression in the spleen started earlier, being detectable as early as day 3, but was transient, disappearing by day 7 (Fig. 1D). IL-12p40 mRNA was expressed in both liver and spleen with the same kinetics, starting from day 3 and lasting up to 11 days postinfection. IFN-γ mRNA expression began on day 3 and lasted up to day 11 postinfection in liver, whereas in spleen it was first seen on day 3 and lasted up to day 7 postinfection. Control G3PDH mRNA (followed at regular 2-day intervals) was constitutively expressed in both liver and spleen (Fig. 1, C and D).

Southern and sequence analysis

Results of Southern analysis confirmed that IL-18 mRNA expression occurred in the livers of P. yoelii 265-infected mice. Similar to our results from RT-PCR, there was a signal of equal intensity on days 3, 5, 7, 9, and 11 postinfection. There was no detectable signal after day 13 postinfection, suggesting no long-term expression of IL-18. G3PDH was constitutively expressed in all groups, signals of equal intensity being observed up to 21 days postinfection (Fig. 1E). The sequencing of IL-18 cDNA further confirmed IL-18 gene sequences.

Measurement of cytokine proteins in sera of infected mice

The production of IFN-γ, IL-2, and IL-4 was analyzed at the protein level by ELISA. After i.p. infection with 1 × 10⁷ PRBCs, serum was separated at 2-day intervals postinfection from P. yoelii- and P. berghei-infected mice and was kept at −80°C. There were marked increases in the levels of IFN-γ and IL-2 production in both cases, although with different kinetics (Fig. 2). In P. yoelii infection, IFN-γ levels increased from day 7 and reached a sharp peak by day 9 postinfection (Fig. 2). This increase was transient and demonstrated a lag time of ~7 days, followed by a rapid increase between 7 and 9 days postinfection. Levels declined after the rest of the measurement period, remaining elevated above control levels past 21 days. In contrast, in the case of P. berghei infection, amounts of secreted IFN-γ increased more rapidly from day 3, raised to a peak level on day 7, and decreased rapidly to the control levels by 9 days postinfection (Fig. 2). The presence of enhanced IL-2 levels was also observed in both infections. In P. yoelii infection, increased levels of IL-2 were observed from day 7, reaching a peak on day 9, with a steady decline lasting up to day 15 postinfection (Fig. 2). In contrast, in the case of P. berghei, the IL-2 secretion followed a similar pattern as in the case of IFN-γ, with the IL-2 levels increasing from day 5, reaching a peak level by day 7, and declining rapidly thereafter (Fig. 2). There was only a marginal increase in the amount of circulating IL-4, starting from day 5 and lasting until day 9 postinfection, in the sera of P. berghei- or P. yoelii-infected mice. These results indicate that infection with blood-stage P. yoelii and P. berghei parasites in mice

![FIGURE 1. Cytokine mRNA gene expression in liver (A) and spleen (B) of P. yoelii 265-infected mice and in liver (C) and spleen (D) of P. berghei ANKA-infected mice at different time kinetics. After i.p. inoculation with 1 × 10⁷ PRBCs, liver and spleen were obtained at various time intervals, and total RNA was extracted and subjected to semiquantitative RT-PCR analysis. The amplified products were size-fractionated by 2% agarose gel electrophoresis. G3PDH was constitutively expressed in all groups, and the expression levels were similar. E, Southern blot analysis of IL-18 mRNA in P. yoelii 265-infected mouse liver. Similar results were obtained in two repeated experiments.](http://www.jimmunol.org/ by guest on January 21, 2018)
Treatment with 5000 ng of IL-18 significantly lowered parasitemia, whereas mice treated with different doses of rIL-18 showed noticeable lowering of parasitemia, whereas mice treated with 1000 and 5000 ng of IL-18 delayed the onset of parasitemia for a week in P. berghei ANKA-infected mice (Figs. 3B and 4B). Treatment with 5000 ng of IL-18 significantly increased the survival rate of mice (BALB/c) infected with both strains of Plasmodium. Control mice died earlier than IL-18-treated mice (Fig. 4).

Production of serum IFN-γ in BALB/c mice treated with rIL-18 and infected with P. yoelii 265 and P. berghei ANKA

Groups of mice were treated with either rIL-18 or PBS and then were injected with an infective dose of P. yoelii 265 or P. berghei ANKA. After 7 days postinfection, serum concentrations of IFN-γ were measured by ELISA. As shown in Fig. 5, serum concentrations of IFN-γ were significantly higher in rIL-18-treated mice than in the PBS control group, suggesting that the presence of exogenous IL-18 can lead to enhanced levels of IFN-γ, which may in part be responsible for the observed IL-18-induced protection against P. yoelii 265 and P. berghei ANKA infections. It is noteworthy that considerably higher levels of secreted IFN-γ were observed in the case of P. yoelii compared with P. berghei infection. At 500- and 1000-ng doses, the amount of IFN-γ in the serum was almost double in the rIL-18-treated, P. yoelii-infected group compared with the corresponding P. berghei-infected group (Fig. 5). These results suggest that IL-18 plays an important role in host resistance against infection.

Measurement of cytokines in P. berghei ANKA-infected mice

To further validate the specificity of production of circulatory IL-18, resistant C57BL/6 mice were infected with P. berghei ANKA, and circulatory IL-18 was measured by ELISA. As shown in Fig. 6A, serum IL-18 levels were significantly increased on day 6 and were slightly lower on day 8, but they remained higher than in the controls. Exogenous treatment of C57BL/6 mice with rIL-18 also significantly increased the survival rate of mice infected with P. berghei ANKA (Fig. 6B). RT-PCR further demonstrates IL-18 mRNA gene expression on days 2, 4, and 6 postinfection (Fig. 6C). IL-12p40 mRNA gene expression was also detected on day 6 postinfection. A housekeeping gene, β-actin, was constitutively expressed at a constant level.
Endogenous IL-18 is involved in host defense to P. berghei ANKA infection

To determine the role of endogenous IL-18, BALB/c mice infected with P. berghei ANKA were injected with rabbit anti-mouse IL-18 neutralizing Ab at 400/mg/day/mouse from day 0 to day 5. Administration of neutralizing anti-IL-18 Ab exacerbated the infection, severely impaired the host resistance, and finally shortened the mean survival, suggesting the protective role of endogenous IL-18 (Fig. 7A).

IL-18 knockout mice are more susceptible to infection

To further confirm the role of IL-18 in host defense against P. berghei ANKA infection, IL-18 knockout mice (n = 5) were infected with P. berghei ANKA. As shown in Fig. 7B, targeted disruption of the IL-18 gene increased susceptibility to infection in these knockout mice over that in wild-type mice. Thus, knockout mice died earlier than control mice. These data provide further evidence confirming the role of IL-18 in host resistance to infection.

Histopathology

Histological examination of liver tissues from PBS-treated control mice infected with P. yoelii 265 revealed extensive hepatocyte necrosis, heavy pigment deposition in Kupffer cells, and sparse inflammatory cell infiltration. In contrast, infiltration of inflammatory cells

FIGURE 4. Effect of IL-18 on the survival rate of mice infected with P. yoelii 265 (A) and P. berghei ANKA (B). Mice received i.p. injections of PBS (n = 5, 500 ng of rIL-18; n = 5, 1000 ng of rIL-18; n = 5, 5000 ng of rIL-18) for −1, 0, 1, and 3 days postinfection of 1 × 10⁷ PRBCs. The mice were monitored for survival daily.

FIGURE 5. Effect of IL-18 on serum IFN-γ. Mice received i.p. injections of IL-18 in three different doses (500, 1000, and 5000 ng) at −1, 0, 1, and 3 days postinfection of PRBCs (1 × 10⁷) infected with P. yoelii 265 and P. berghei ANKA. The serum IFN-γ was measured 1 wk after infection by ELISA. Each column represents the means ± SD of five mice; *, p < 0.01 by Student’s t test.

FIGURE 6. Induction of serum IL-18 in P. berghei ANKA-infected C57BL/6 mice. A, Circulatory serum IL-18 level was measured by ELISA in P. berghei ANKA-infected mice at 0, 2, 4, 6, and 8 days postinfection. Protective effect of IL-18 in C57BL/6 mice infected with P. berghei ANKA. Intraperitoneal injections of murine rIL-18 were administrated at 5 µg/mouse/day from day 0 to day 5. B, Mice were monitored for survival daily. C, Induction of mRNA gene expression of IL-18 and IL-12 were analyzed by RT-PCR in P. berghei ANKA-infected mouse spleen cells. A housekeeping gene, β-actin, was constitutively expressed, and the expression level was similar. Experiments were repeated twice with similar results.
(mononuclear cells and Kupffer cells), reduced deposition of hemozoin pigment, minimal fatty changes, and no necrosis of hepatocytes were present in IL-18-treated mice infected with *P. yoelii* 265 (Fig. 8).

Similar results were observed in liver tissues from mice treated with IL-18 before *P. berghei ANKA* infection. Strikingly, whereas splenic tissue taken from mice in the control (PBS-treated and *P. berghei ANKA*-infected) group showed extensive necrosis and moderate pigmentation, reduced pigmentation and almost no necrosis were seen in IL-18-treated mice after infections with either the lethal or the non-lethal murine malaria strains used in this study (Fig. 8).

**Discussion**

The effector mechanisms whereby CD4+ Th cells mediate control and resolution of the blood-stage malaria parasite remain unclear. CD4+ T cells might possibly achieve elimination of the blood-stage parasites by activating other effector cells, and Th1 and Th2 cytokines play decisive roles in the outcome of malarial infections (8–15, 18). IL-12, which regulates the development of Th1 cells, plays an important role in host immune responses to malarial infections (16, 17, 35). It is not yet known whether IL-18, which is functionally similar to IL-12 but has no structural similarity to IL-12 and belongs to the IL-1 family of cytokines (47, 48), has any role in the development of immunity to blood-stage murine malaria parasites. To investigate this, we infected BALB/c mice with blood parasites of *P. yoelii* 265 and *P. berghei ANKA* strains and then followed the course of infection with respect to production of IL-18 and related cytokines. We found mRNA expression of IL-18 and TNF-α in the livers but, surprisingly, not in the spleens of *P. yoelii* 265-infected mice. In contrast, mRNA expression of IFN-γ and IL-12p40 was detected in cells from the liver as well as from the spleens of these mice. In the case of *P. berghei ANKA*, mRNA expression of IL-18, IL-12p40, and IFN-γ was detected in both liver and spleen cells.

A comparison of the time course of IL-18 mRNA expression in the livers of infected mice showed that mRNA expression started somewhat earlier in the case of *P. yoelii* 265, and the level of IL-18 mRNA expression was noticeably higher in the case of *P. berghei ANKA* infections. The basis for the observed differences between levels of mRNA expression of IL-18 and related cytokines in response to the two different strains of malaria parasite remain undefined. It is possible that the activation of IL-1β converting enzyme, which cleaves precursor IL-18 and processes it into mature IL-18, could be a critical factor in controlling amounts of...
biologically active IL-18 (49). The different levels of IL-18 mRNA expression nevertheless indicate that after activation, mature IL-18 protein levels may be higher in P. berghei ANKA- than in P. yoelii 265-infected mice. The production of Th1 cytokines IL-2 and IFN-γ was significantly increased in infected mice compared with the control mice, which is consistent with the observed IL-18 and IL-12p40 mRNA expression.

Earlier studies have shown the importance of IFN-γ in the regulation of acquired immunity to blood-stage malaria parasites. For example, it was shown that during the first 14 days after Plasmodium chabaudi AS infection, IL-2- and IFN-γ-producing Th1 cells predominated, whereas later in the infection, Th2 cells predominate (50). Although the exact effector mechanisms involved in the clearing of the parasites remain unclear, at least one study has suggested a role for IFN-γ in activated macrophages (51). Acquired immunity to blood-stage Plasmodium vinckei vinckei has also been shown to be mediated by CD4+ T cells (52). In this study, IFN-γ release occurred as early as day 1 postinfection, suggesting that immunity to P. vinckei vinckei was predominantly a Th1 cell-mediated response. Nevertheless, the exact role of IFN-γ in the pathology and death associated with this infection remains unclear (52). It has previously been shown that mice infected with P. yoelii nonlethal strains produced high levels of IFN-γ compared with those infected with lethal strains (10). T cell lines and clones generated in response to crude P. yoelii blood-stage Ag were CD4−, produced IFN-γ in response to malaria Ags in vitro, and transferred protection to immunodeficient mice (53). In the present study, Th1 responses again appeared to dominate and, consequently, increased levels of IFN-γ and IL-2 were observed. Despite this, some differences were exhibited between the two murine infections. For example, although the pattern of IL-2 secretion was similar in the two cases, the peak level of IL-2 was reached somewhat earlier in the case of P. berghei ANKA infections. Similarly, levels of IFN-γ also peaked earlier in case of P. berghei infection and subsided rapidly, whereas significantly higher levels were maintained in P. yoelii 265-infected mice for several days after reaching a peak level on day 9 postinfection. An earlier study showed that in mice susceptible to both lethal and nonlethal variants of P. yoelii, IFN-γ was produced only in response to the nonlethal variant (10). Based on our results showing lower peak IFN-γ levels and a rapid declining expression pattern after the peak in response to infection by the lethal strain P. berghei, compared with sustained high levels of this cytokine in the case of a nonlethal P. yoelii infection, it is tempting to speculate that a role may exist for IFN-γ in determining the outcome in terms of lethality of murine malaria infections.

In contrast with the observed increases in Th1 cytokines, there was no significant increase in the production of the Th2 cytokines such as IL-4 in the two experimental infected groups compared with the control group in our study. This result is consistent with those of White et al. (54), who found that immunization with P. berghei sporozoites induced IL-2- and IFN-γ, but not IL-4 production. Also, with P. vinckei vinckei infection, Th1-type responses were found to dominate, with very few cells producing IL-4 (52). Recently, a dual signaling mechanism consisting of IL-18-induced NF-κB activation and TCR/CD3-mediated NFAT activation has been elucidated for IFN-γ production by IL-18 in murine Th1 cells (55). There have also been reports indicating that IL-18R is not expressed on Th2 cells and that IL-18 stimulated only Th1 cells to produce IFN-γ (31, 56). It has also been shown that IL-18 can stimulate IFN-γ production in an IL-12-independent manner in KG-1 cells by up-regulating ICAM-1 (CD54) expression (57). It remains unclear how IL-18 cooperates with IL-12 in inducing IFN-γ production and protecting animals against infection. Recent studies have demonstrated that IL-18 induces the production of IFN-γ by NK and T cells (23, 36). Nonetheless, there is also evidence suggesting that IL-18 is an important factor involved in IFN-γ production and that IL-18 deficiency cannot be compensated for by IL-12 or other cytokines (40). These results are consistent with the notion that IL-18, through the Th1-type pathways, plays a key role in the development of the cellular immune response to malaria infection. Enhanced levels of serum IFN-γ were observed in the IL-18-treated mice, suggesting the possible involvement of IFN-γ in the development of immunity during the blood-stage of malaria infection.

We found that mice that received rIL-18 before infection showed delayed onset of parasitemia and lower peak parasitemia compared with control mice, indicating a possible role for this cytokine in protection. Increased IFN-γ production has been observed in infected mice treated with IL-18, in response to various disease conditions including infection by L. major (23, 32, 34, 36, 37). Furthermore, several investigators have recently reported the important role of this cytokine in host defense against infection using neutralizing Ab or IL-18 knockout mice (58–60). Administration of neutralizing anti-IL-18 Ab exacerbated the infection, severely impaired the host resistance, and finally shortened the mean survival, suggesting the role of endogenous IL-18. IL-18 knockout mice seemed more susceptible to infection than wild type, and they died much earlier than controls, further confirming a role of IL-18 in host resistance to infection. Taken together, the above results indicate that administration of rIL-18 can delay the onset of parasitemia, lower the peak parasitemia, and thereby induce a protective effect in mice, possibly through the involvement of IFN-γ. Although a direct effect of IL-18 on the protection of liver and spleen tissues remains unclear, the histopathological changes in IL-18-treated mouse liver and spleen tissues can suggest accumulation of inflammatory cells indirectly, through production of IFN-γ, which in turn may result in protection of mice against infection of P. yoelii 265 and P. berghei ANKA. In conclusion, the results of this study suggest that IL-18 plays an important role in host defense in mouse models of malaria, possibly through Th1 stimulation and, consequently, enhanced IFN-γ production.

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