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A Critical Role of Fc Receptor-Mediated Antibody-Dependent Phagocytosis in the Host Resistance to Blood-Stage Plasmodium berghei XAT Infection

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Plasmodium berghei XAT is an irradiation-induced attenuated variant derived from the lethal strain P. berghei NK65, and its blood-stage parasites are spontaneously cleared in immune competent mice. In the present study, we studied the mechanism of host resistance to blood-stage malaria infection using P. berghei XAT. Infection enhanced Ab-dependent phagocytosis of PRBC by splenic macrophages in wild-type C57BL/6 mice. In contrast, FcR-gamma-chain knockout (FcRγ−/−) mice, which lack the ability to mediate Ab-dependent phagocytosis and Ab-dependent cell-mediated cytotoxicity through FcγRI, FcγRII, and FcγRIII, could not induce Ab-dependent phagocytic activity. These FcRγ−/− mice showed increased susceptibility to the P. berghei XAT infection, with eventually fatal results, although they produced comparable amounts of IFN-γ by spleen cells and anti-XAT Abs in serum. In addition, passive transfer of anti-XAT IgG obtained from wild-type mice that had recovered from infection into FcRγ−/− mice could not suppress the increase in parasitemia, and almost all of these mice died after marked parasitemia. In contrast, passive transfer of anti-XAT IgG into control wild-type mice inhibited the increase in parasitemia. IFN-γ−/− mice, which were highly susceptible to the P. berghei XAT infection, failed to induce Ab-dependent phagocytic activity and also showed reduced production of serum anti-XAT IgG2a isotype compared with control wild-type mice. These results suggest that FcR-mediated Ab-dependent phagocytosis, which is located downstream of IFN-γ production, is important as an effector mechanism to eliminate PRBC in blood-stage P. berghei XAT infection.

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3 Abbreviations used in this paper: ADCC, Ab-dependent cell-mediated cytotoxicity; IFA, indirect fluorescent Ab; PRBC, parasitized RBC; y.. yoelii.
berghesi XAT parasites are spontaneously cleared in immune competent mice with two peaks of parasitemia at about 5 and 12 days after the inoculation of parasitized RBC (PRBC), while parasitemia in mice infected with blood-stage *P. berghesi* NK65 increases progressively and all mice die in 2–3 wk (23, 24). In addition, mice that had recovered from the *P. berghesi* XAT infection exhibited a strong resistance to the following challenge with its lethal *P. berghesi* NK65 parasites, indicating that *P. berghesi* XAT is a good model for live vaccine. Therefore, comparison of immune responses induced by these two parasites could help elucidate the mechanism of host resistance to blood-stage malaria infection. We previously demonstrated that IL-12 production in spleen and resultant IFN-γ production by CD4+ T cells play a pivotal role in host resistance to blood-stage *P. berghesi* XAT infection (5, 23, 25, 26). Neither NK cell activation nor NO production was shown to be essential to the resistance as effector mechanisms located downstream of IL-12 and IFN-γ production, but the phagocytic activity of macrophages was suggested to be important for the resistance by experiments using carrageenan (26). To further investigate the effector mechanism to eliminate PRBC, we examined the involvement of FcR-mediated Ab-dependent phagocytosis in host resistance using FcRγ−/− mice and also IFN-γ−/− mice in the present study. We have found that FcR-mediated Ab-dependent phagocytosis, which is located downstream of IFN-γ production, is important as an effector mechanism to eliminate PRBC in blood-stage *P. berghesi* XAT infection. Our results are consistent with those reported in human malaria infection (11–13), in which the host resistance appears to depend on FcR-mediated Ab-dependent phagocytosis. Thus, *P. berghesi* XAT would be a good mouse model to investigate the mechanism of protective immunity against blood-stage malaria infection in humans.

**Materials and Methods**

**Mice**

Female C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan), FcRγ−/− mice (22), CD4 knockout (CD4−/−) mice, and CD4 mutant mice (27) backcrossed six generations onto C57BL/6 mice (22), CD4 knockout (CD4−/−) mice, and CD4 mutant mice (27) backcrossed six generations onto C57BL/6 mice, and IFN-γ knockout (IFN-γ−/−) (28) mice on C57BL/6 background were used. Wild-type C57BL/6 mice were used as controls in all experiments. Mice were used for experiments at 6–10 wk of age.

**Culture media**

RPMI 1640 (JRH Biosciences, Lenexa, KS) supplemented with 10% FCS (Summit Biotechnology, Fort Collins, CO), 5 × 10−3 M 2-ME (Wako Pure Chemical, Osaka, Japan), and kanamycin (100 μg/ml; Meiji Seika, Tokyo, Japan) was used for cell culture. Eagle’s MEM (JRH Biosciences) and PBS were used for cell washing.

**Parasite infection**

For malaria infection, mice were injected i.v. with a RBC suspension containing 1 × 107 PRBC with nonlethal strain *P. berghesi* XAT (23), which is an irradiation-induced attenuated variant of the lethal strain *P. berghesi* NK65. Parasitemia was assessed by the microscopic examination of Giemsa-stained smears of tail blood. The percentage of parasitemia was calculated as follows: parasitemia (percent) = (number of infected RBC)/ (total number of RBC counted)) × 100.

**Assay for phagocytic activity by splenic macrophages**

Infection of CD4−/− mice or CD4 mutant mice (both do not have any functional CD4+ T cells) with blood-stage *P. berghesi* XAT induces a progressive increase in parasitemia with eventually fatal results (26). RBC containing ~30% PRBC were obtained from these mice ~1 mo after the infection, and the cells were washed three times with PBS and incubated at 37°C for 1 h, followed by the Percoll treatment, as described elsewhere (29). The purity of PRBC in the RBC preparation was ~98%, and these PRBC were used for the phagocytic activity assay. Five million splenocytes were incubated at 37°C (5% CO2) for 2 h on 13-mm round cover slides (Matsumani, Tokyo, Japan) in 24-well culture plates, and then nonadherent cells were removed by washing three times with prewarmed MEM. Adherent cells were incubated at 37°C (5% CO2) for 45 min with PRBC that had been incubated with anti-XAT serum for 1 h. To remove noningested PRBC, the adherent cells were briefly incubated in PBS diluted with distilled water (1/5) and then washed with PBS. These adherent cells were stained with FITC-conjugated anti-Mac-1 (rat IgG2b; Pharmingen, San Diego, CA) at room temperature for 30 min after blocking the nonspecific binding by treatment with anti-FcRs (2.4G2, rat IgG2b; Pharmingen). The cells were then fixed with 3.7% Formalin, and Mac-1-positive cells were assessed under a light-illuminating fluorescence microscope (Olympus, Tokyo, Japan). The percentage of phagocytic activity was calculated as follows: phagocytic activity (percent) = ((number of Mac-1-positive cells contained PRBC)/(total number of Mac-1-positive cells counted)) × 100.

**Assay for IFN-γ production by spleen cells**

After the inoculation of mice with PRBC, the spleen was removed from each mouse and spleen cells were cultured at 6 × 106 cells/ml for 48 h without further addition of parasite Ag. The culture supernatants were then assayed for IFN-γ in a sandwich ELISA using two different clones of rat mAbs against mouse IFN-γ (R4-6A2, rat IgG1, and XM1G2, rat IgG1; Pharmingen), according to the manufacturer’s instruction.

**Assay for serum anti-parasite Abs titration**

Anti-XAT total IgGs and their isotypes, IgG1, IgG2a, IgG2b, and IgG3 in serum were titrated by an indirect fluorescent Ab (IFA) test, as described (30), using acetone-fixed parasitized blood smears as Ags and FITC-conjugated rat anti-mouse total IgGs, IgG1, IgG2a (ICN Pharmaceuticals, Costa Mesa, CA), IgG2b (Caltag, South San Francisco, CA), and IgG3 (Pharmingen) as second Abs.

**Passive transfer experiments**

Passive transfer was performed using anti-XAT IgGs prepared from anti-XAT immune serum, as described before (5). The immune serum was obtained from mice that had been infected >6 wk previously and had recovered from the infection. Five mice in each group were transferred by i.v. injection with 0.2 ml of anti-XAT IgGs (211 IFA titers) or equivalent protein amounts of normal mouse IgGs as control for 3 consecutive days starting on the day of inoculation with PRBC.

**Statistical analysis**

Statistical analysis was performed by Student’s t test. A p value of <0.05 was considered to indicate statistical significance.

**Results**

Enhanced Ab-dependent phagocytic activity against PRBC by splenic macrophages in blood-stage *P. berghesi* XAT infection

Normal immune competent mice infected with blood-stage *P. berghesi* XAT show two peaks of parasitemia and recover from the infection within about 3 wk. However, mice spleenectomized 1 wk before the parasite inoculation failed to clear PRBC, the parasitemia increased progressively, and eventually all mice died of the infection (25). Therefore, the spleen is considered to be an indispensable organ to develop host resistance to the infection and to clear PRBC. To investigate the effector mechanism of the development of host resistance, we first examined the phagocytic activity against PRBC by splenic macrophages obtained from wild-type C57BL/6 mice infected with blood-stage *P. berghesi* XAT. PRBC was treated with anti-XAT immune serum obtained from C57BL/6 mice that had been infected >6 wk previously and had recovered from the infection, and then the phagocytic activity against these PRBC by splenic macrophages was measured at various time intervals after the inoculation. The phagocytic activity increased significantly 4 days after the inoculation, peaked at about 7 days, and decreased gradually thereafter (Fig. 1). Without serum treatment, almost no induction of phagocytic activity against PRBC was observed under these conditions. Similar but delayed onset of the phagocytic activity was also observed when serum obtained from each mouse at the indicated time after the inoculation was used. These results suggest that blood-stage *P. berghesi* XAT infection
obtained in two independent experiments.

Enhanced Ab-dependent phagocytic activity against PRBC by splenic macrophages.

Increased susceptibility of FcRγ−/− mice to blood-stage P. berghei XAT infection. After i.v. inoculation of C57BL/6 mice with PRBC, the spleen was removed after various time intervals and spleen adherent cells were prepared. Then the phagocytic activity of splenic macrophages (Mac-1-positive cells) was measured using anti-XAT immune serum obtained from mice that had been infected >6 wk previously and had recovered from the infection, serum obtained from each mice at the indicated time, and nonimmune serum as control. Data are shown as the mean ± SD of three mice. * and **, p < 0.05 and p < 0.01, respectively, compared with day 0. Similar results were obtained in two independent experiments.

Induced Ab-dependent phagocytic activity against PRBC by splenic macrophages.

Increased susceptibility of FcRγ−/− mice to blood-stage P. berghei XAT infection with abrogated Ab-dependent phagocytic activity against PRBC by splenic macrophages

FcRs have been suggested to play an important role in Ab-dependent phagocytosis against various kinds of pathogens (31). To examine the involvement of FcRs in Ab-dependent phagocytosis in the infection with P. berghei XAT, we next used FcRγ−/− mice (22), which lack the ability to mediate Ab-dependent phagocytosis and ADCC through FcγRI, FcγRII, and FcγRIII. First of all, we measured the phagocytic activity of splenic macrophages against PRBC treated with anti-XAT immune serum obtained from mice that had been infected >6 wk previously and had recovered from the infection. As expected, the phagocytic activity by splenic macrophages obtained from FcRγ−/− mice hardly increased after the inoculation of PRBC, although that from control wild-type mice significantly increased (Fig. 2), suggesting the inability of FcRγ−/− mice to induce Ab-dependent phagocytosis against PRBC by splenic macrophages. The susceptibility to infection was then compared between FcRγ−/− mice and control mice (Fig. 3). The parasitemia of FcRγ−/− mice increased as much as that of control mice before the second peak of parasitemia. After the second peak, the parasitemia of FcRγ−/− mice failed to decrease and continued to increase progressively, and all of these mice died eventually, although all parasites were cleared in control mice after the second peak. These results suggest that FcR-mediated Ab-dependent phagocytosis is important for host resistance to blood-stage P. berghei XAT infection.

To confirm that the increased susceptibility of FcRγ−/− mice to the infection is due to the inability of FcRγ−/− mice to induce FcR-mediated Ab-dependent phagocytosis, we next compared IFN-γ production by spleen cells in vitro and serum anti-XAT Ab titers, both of which are critical in host resistance, between FcRγ−/− mice and control mice. No significant difference in the IFN-γ production was observed in terms of the amounts and time kinetics between them after the inoculation of PRBC (Fig. 4A). In addition, the serum titer of anti-XAT IgG2a isotype, which was previously demonstrated to be important for host resistance by passive transfer experiments (5), in FcRγ−/− mice increased similarly to that in control mice. No difference was also observed in the serum titers of anti-XAT IgG1, IgG2b and IgG3 isotypes, and total IgGs (Fig. 4B). These results support that the increased susceptibility of FcRγ−/− mice to the infection is due to the inability of FcRγ−/− mice to induce the FcR-mediated Ab-dependent phagocytosis against PRBC.

Impaired passive protective transfer of anti-XAT IgG in FcRγ−/− mice to blood-stage P. berghei XAT infection

We previously demonstrated that passive transfer of anti-XAT IgG obtained from mice recovered from blood-stage P. berghei XAT infection into naïve mice inhibits the increase in parasitemia (5). To further examine the involvement of FcRs in the protective immunity, we next compared the susceptibility between FcRγ−/− mice and control wild-type mice to infection after passive transfer of anti-XAT IgG. As reported previously (5), passive transfer of anti-XAT IgG into control wild-type mice inhibited the increase in parasitemia, especially the second peak of parasitemia (Fig. 5A). In contrast, passive transfer of anti-XAT IgG into FcRγ−/− mice failed to suppress the increase in parasitemia as that of control IgG into FcRγ−/− mice did, and almost of all of these mice died after inoculation of PRBC.
Increased susceptibility of IFN-γ−/− mice to blood-stage P. berghei XAT infection. After i.v. inoculation of FcRγ−/− mice and control wild-type mice with PRBC, the spleen was removed after various time intervals and the cells were cultured in vitro without further addition of parasite Ag for 48 h. The culture supernatants were assayed for measurement of IFN-γ in ELISA (A). Serum was also collected from each mouse, and the anti-XAT Abs were titrated by IFA using anti-mouse Ig isotype Abs (B). Data are shown as the mean ± SD of three mice. Similar results were obtained in two independent experiments.

Discussion
In the present study, we examined the involvement of FcR-mediated Ab-dependent phagocytosis in host resistance by using FcRγ−/− and IFN-γ−/− mice. We found that FcR-mediated Ab-dependent phagocytosis, which is located downstream of IFN-γ production, is important as an effector mechanism to eliminate PRBC in blood-stage P. berghei XAT infection. During the early course of the infection, IL-12 production in spleen and resultant IFN-γ production by CD4+ T cells, which are critical for host resistance, were observed at about the first peak of parasitemia (25). In addition, NK cells are activated coincident with the first peak, and NO production is seen at about 7 days after the inoculation. However, neither NK cell activation nor NO production is essential to host resistance as effector mechanisms located downstream of IL-12 and IFN-γ production. These were demonstrated...
by using depleted mAb against NK1.1 and cytokine-inducible NO synthase knockout mice, respectively (26). In contrast, phagocytic activity of splenic macrophages was demonstrated to be important for the resistance by using its inhibitor, carrageenan (26). Anti-parasite Ab titers in serum started to increase at about day 7 after the first peak of parasitemia, and all parasites were eventually cleared by FcR-mediated Ab-dependent phagocytosis, which controls the second peak of parasitemia (Figs. 3 and 5). Among anti-parasite Abs, the IgG2a isotype was previously shown to efficiently participate in the clearance of parasites potentially through interaction with its high-affinity receptor, FcγRI, on phagocytic cells (5). Therefore, the protection against blood-stage \emph{P. berghei} XAT infection is highly dependent on IFN-γ because it activates macrophages to induce both phagocytic activity against PRBC (Fig. 7A) and FcγRI expression (32) and also induces anti-XAT IgG2a isotype production by B cells (Fig. 7B) (33). Furthermore, we are presuming the presence of another early protective mechanism induced by IFN-γ at about the first peak, which remains to be elucidated. This is because the first peak of parasitemia was hardly seen in IFN-γ−/− mice, but was seen in FcγRγ−/− mice and control wild-type mice, as shown in Figs. 6 and 3, respectively. During this early period, degenerating parasites are observed inside RBC in blood (34), which may be caused by IFN-γ. Further studies are necessary to fully understand the protective mechanism against blood-stage \emph{P. berghei} XAT infection.

In the infection with \emph{P. y. yoelii} 17XL, protection appears to be directly mediated by Abs and not to require the participation of FcRs (21). Passive transfer of immune serum against the major merozoite surface protein into Fcγγ−/− mice was demonstrated to induce as strong protection as in control mice. In contrast, Fcγγ−/− mice showed increased susceptibility to the \emph{P. berghei} XAT infection with eventually fatal results (Fig. 3). In addition, passive transfer of anti-XAT IgG obtained from mice recovered from the infection into Fcγγ−/− mice failed to suppress the increase in parasitemia, and almost all of these mice died after marked parasitemia, although passive transfer of anti-XAT IgG into control wild-type mice inhibited the increase in parasitemia (Fig. 5B), although usually all five Fcγγ−/− mice died after marked parasitemia (Fig. 3). In humans, Abs appear to control parasitemia through interaction with FcRs on monocytes, and the two cytotoxic isotypes, IgG1 and IgG3, predominate in protected subjects with lower parasitemia (11–13). Thus, \emph{P. berghei} XAT would be a good mouse model for human malaria, to investigate the mechanism of protective immunity against blood-stage malaria infection.

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blood stages do not on their own inhibit parasite growth and invasion in vitro, but act in cooperation with monocytes. J. Exp. Med. 172:1633.


