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IL-12 Is Required for Antibody-Mediated Protective Immunity Against Blood-Stage *Plasmodium chabaudi* AS Malaria Infection in Mice

Zhong Su and Mary M. Stevenson

In this study, we investigated the role of endogenous IL-12 in protective immunity against blood-stage *P. chabaudi* AS malaria using IL-12 p40 gene knockout (KO) and wild-type (WT) C57BL/6 mice. Following infection, KO mice developed significantly higher levels of primary parasitemia than WT mice and were unable to rapidly resolve primary infection and control challenge infection. Infected KO mice had severely impaired IFN-γ production in vivo and in vitro by NK cells and splenocytes compared with WT mice. Production of TNF-α and IL-4 was not compromised in infected KO mice. KO mice produced significantly lower levels of Th1-dependent IgG2a and IgG3 but a higher level of Th2-dependent IgG1 than WT mice during primary and challenge infections. Treatment of KO mice with murine rIL-12 during the early stage of primary infection corrected the altered IgG2a, IgG3, and IgG1 responses and restored the ability to rapidly resolve primary and control challenge infections. Transfer of immune serum from WT mice to *P. chabaudi* AS-infected susceptible A/J mice completely protected the recipients, whereas immune serum from KO mice did not, as evidenced by high levels of parasitemia and 100% mortality in recipient mice. Furthermore, depletion of IgG2a from WT immune serum significantly reduced the protective effect of the serum while IgG1 depletion had no significant effect. Taken together, these results demonstrate the protective role of a Th1-immune response during both acute and chronic phases of blood-stage malaria and extend the immunoregulatory role of IL-12 to Ab-mediated immunity against *Plasmodium* parasites. *The Journal of Immunology*, 2002, 168: 1348–1355.
control a challenge infection (26). Although Th2-type cytokines, in particular IL-4, induce production of IgG1 and IgE Abs and promote Ab-mediated immunity against extracellular pathogens such as helminth parasites (27), the type 1 cytokine IL-12 can preferentially induce Abs of IgG2a, IgG2b, and IgG3 subclasses in mice (1, 28, 29). We have observed that treatment of susceptible A/J mice with rIL-12 during the first 5 days of *P. chabaudi* AS infection not only suppresses primary parasitemia but also results in mice with rIL-12 during the first 5 days of *P. chabaudi* AS infection. We also observed that IL-12 had profound effects on the Ab response during *P. chabaudi* AS infection. Ab subclass depletion and transfer experiments further demonstrated the functional association between IL-12 and Ab-mediated immunity for control of chronic and challenge infections.

In this study, we investigated the role of endogenous IL-12 in protective immunity against blood-stage *P. chabaudi* AS infection using IL-12 p40 gene KO and C57BL/6 WT control mice. KO mice developed impaired immunity against both primary and challenge infections. We also observed that IL-12 had profound effects on the Ab response during *P. chabaudi* AS infection. Ab subclass depletion and serum transfer experiments further demonstrated the functional association between IL-12 and Ab-mediated immunity against blood-stage *P. chabaudi* AS infection.

**Materials and Methods**

*Mice, parasite, and experimental infections*

Breeding pairs of IL-12 p40 gene KO mice and A/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The KO mice, on the C57BL/6 background, were generated by targeted disruption of the IL-12 p40 gene and do not produce biologically active IL-12 (32). WT control C57BL/6 mice were purchased from Charles River Breeding Laboratories (St. Constant, Quebec, Canada). Mice were maintained in the animal facility of the Montreal General Hospital Research Institute (Montreal, Quebec, Canada) under specific pathogen-free conditions. WT and KO mice, 8–12 wk old, were age- and sex-matched in all experiments. *P. chabaudi* AS was maintained in our laboratory as previously described (33). Infections were initiated by i.p. injection of 10^6 *P. chabaudi* AS parasitized RBC (pRBC). In some experiments, KO mice were treated i.p. with 0.1 μg of murine rIL-12 (kindly provided by Genetics Institute, Cambridge, MA) diluted in pyrogen-free saline beginning on the day of infection and daily for 5 days (15). Parasitemia of individual mice was monitored on blood smears stained with Diff-Quik (American Scientific Products, McGraw Park, IL). Anemia was assessed by measuring blood hematocrit levels using standard hematological procedures. Mice were sacrificed at the indicated times and blood was obtained by cardiac puncture. Sera were collected and stored at −20°C for determination of cytokine and Ab levels.

*Spleen cell cultures*

Spleens from normal and infected mice were removed aseptically and single cell suspensions were prepared in RPMI 1640 medium (Life Technologies, Burlington, Ontario, Canada) supplemented with 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT), 25 mM HEPES (Life Technologies), 0.12% gentamicin (Schering-Plough, Montreal, Quebec, Canada), and 2 mM glutamine (Life Technologies) (complete medium). RBCs were lysed with 0.175 M NH₄Cl and membrane debris was removed by filtering the cell suspensions through sterile gauze. The viability of the cells was determined by trypan blue exclusion and was always >95%. Aliquots of 1 ml of cell suspension (5 × 10⁶ cells/ml) were plated in triplicate in 24-well tissue culture plates in the presence of pRBC and incubated for 48 h at 37°C in a humidified CO₂ incubator. Supernatants were collected and stored at −20°C until they were assayed for cytokine levels.

**NK cell enrichment and culture**

Splenic NK cells were enriched by positive selection using magnetic beads conjugated to a mAb against DX5, a mouse pan-NK cell marker (Miltenyi Biotec, Auburn, CA), according to the manufacturer’s instructions. The resulting NK cells were >83% positive for DX5, as determined by flow cytometry. The enriched NK cells were adjusted to 5 × 10⁶ cell/ml in complete RPMI 1640 and 100 μl of the cell suspensions were plated in triplicate in 96-well tissue culture plates in the presence of 80 U/ml IL-2. The plate was incubated at 37°C for 72 h and supernatants were harvested for measuring spontaneous IFN-γ release.

**NK cell cytotoxicity assay**

NK cell cytotoxic activity in total spleen cells was measured against 51Cr-labeled YAC-1 target cells in a standard 4-h chromium release assay as described previously (16). Cytotoxicity assays were performed with various E:T ratios (200:1 to 25:1), and the results from the optimum E:T ratio of 100:1 were presented in this study. Cytotoxicity was expressed as the percentage of specific lysis of target cells according to the formula as described previously (16).

**Cytokine ELISAs**

Levels of IFN-γ, TNF-α, IL-4, and IL-10 in sera for control supernatants were measured by sandwich ELISA using paired capture and detection Abs as previously described (13, 15, 19). Reactivity was revealed using ABTS substrate (Boehringer Mannheim, Laval, Quebec, Canada) and OD values were read in a microplate reader at 405 nm with a reference wavelength of 492 nm. The concentrations of cytokine in samples were calculated against the standard curve generated using recombinant cytokines (BD PharMingen, Mississauga, Ontario, Canada).

**Serum *P. chabaudi* AS-specific Ab titers**

Serum levels of *P. chabaudi* AS-specific Ab isotypes were determined by ELISA. *P. chabaudi* AS Ag was prepared as described previously (34). Immulon II plates (Dynatech Laboratories, Chantilly, VA) were coated with parasite Ag overnight at 4°C and subsequently blocked with 1% BSA in PBS for 1 h. Individual serum samples were serially diluted 2-fold, and 50 μl of each dilution were added to the plate and incubated at room temperature for 3 h. After extensive washing, HRP-conjugated goat anti-mouse isotype/subclass Abs (Southern Biotechnology Associates, Birmingham, AL) were added and incubated at room temperature for 2 h. Reactivity was visualized using ABTS substrate and OD values were read in a microplate reader with a reference wavelength of 492 nm. Ab levels in serum are expressed as endpoint titers, the reciprocal of lowest dilution that yields the background OD.

**Serum NO₃ determination**

To determine NO production, serum NO₃− concentrations were measured as previously described (15). NaN₃, diluted in serum from uninfected WT mice and dialyzed against PBS for 24 h, was used as a standard to calculate serum NO₃− levels.

**Ab subclass depletion and transfer of immune serum**

Immune sera were collected from WT and KO mice 10 days after challenge infection with 10⁶ pRBC and pooled for each group of mice. Ig in the serum was precipitated with saturated ammonium sulfate. The precipitates were resuspended in PBS, dialyzed against PBS, and adjusted to the original volume of serum. Groups of A/J mice were injected i.v. with three doses of 0.2 ml of the immune sera on the day of infection with 10⁶ pRBC and daily for 2 days. Control A/J mice were treated with either PBS or normal serum from uninfected WT mice as a control for immune serum. In some experiments, immune sera were collected from WT and KO mice 17 days after primary infection. The sera were processed as described above and transferred (0.3 ml per mouse, i.v.) to KO mice 14 days after infection.

To determine the protective effects of Ab subclasses, immune sera collected from WT mice after challenge infection were depleted of IgG1 or IgG2a by immunofluinity gel before they were transferred to the recipients. Briefly, streptavidin-agarose conjugate (Pierce, Rockford, IL) was incubated with biotinylated polyclonal goat anti-mouse IgG1, IgG2a, or anti-

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human Ig (as control) Ab (Caluq Laboratories, Burlingame, CA) at room temperature for 3 h with rotation. The resulting affinity gels were washed five times with PBS to remove preservatives. The gel was then mixed with serum and incubated, with rotation, at 4°C overnight. The gel-serum mixture was briefly spun and the serum was collected. Preliminary experiments were performed to establish the optimum ratio of agarose, anti-subclass Ab, and serum. Affinity gel obtained from a mixture of one part streptavidin-agarose (50% suspension) and two parts biotinylated Ab (0.4 mg/ml) depleted >94% IgG1 and >87% IgG2a in 0.4-part serum, and the concentrations of other isotype or subclasses were not changed, as determined by ELISA (data not shown). Groups of A/J mice were injected with control or subclass-depleted sera during the first 3 days of infection with 10⁶ pRBC as described above.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance of differences in cytokine and Ab levels between experimental groups was analyzed by Student’s t test. Repeated measures ANOVA was performed to test the significance of differences in overall parasitemia levels between mouse groups. All analyses were performed using SAS/STAT software (SAS Institute, Cary, NC) and a value of p < 0.05 was considered significant.

Results

Course of P. chabaudi AS infection in WT and KO mice

To determine the effect of endogenous IL-12 on protective immunity against blood-stage P. chabaudi AS infection, the course of infection was monitored in WT C57BL/6 and IL-12 p40 KO mice. Because previous studies in our laboratory (13) and by others (25, 26) demonstrated significant gender difference in the parasitemia and mortality following blood-stage malaria infection, we included both male and female mice in this study. Following blood-stage infection, female and male WT mice developed moderate levels of primary parasitemia which peaked during days 8–10 postinfection (Fig. 1). Female WT mice also had a small recrudescence parasitemia of 2% at day 19. Female and male WT mice cleared the primary infection by days 25 and 28, respectively. In contrast, female and male KO mice developed significantly higher levels of primary parasitemia (from days 4 to 11) than their WT counterparts (p < 0.01 by ANOVA, for both genders), and 40% of male KO mice died by day 14 postinfection. In addition, female and the surviving male KO mice had two recrudescences during days 15–19 and 28–30 (Fig. 1). After the second recrudescence, KO mice had low levels (0.5–1%) of parasitemia up to 35 days after primary infection. To determine whether the infection was completely resolved, we collected 0.1 ml of blood (~10⁹ RBC) from female WT and KO mice 40 days after infection and transferred i.p. to susceptible A/J mice. All A/J mice receiving blood from WT did not develop parasitemia up to 14 days after blood transfer, while three of four A/J mice receiving blood from KO mice developed parasitemia (data not shown). These results demonstrate that KO mice have impaired protective immunity both to control acute phase infection and to resolve the chronic phase of blood-stage P. chabaudi AS infection.

Both WT and KO mice demonstrated a gender difference in resistance to P. chabaudi AS infection. Male WT and KO mice developed significantly higher levels of primary parasitemia than did their female counterparts (p < 0.05 for both WT and KO), and male KO suffered 40% mortality while all WT and female KO mice survived the infection. Due to the severe mortality in the male KO mice, female WT and KO mice were used for all subsequent experiments.

To evaluate the pathology associated with P. chabaudi AS infection, we examined anemia and body weight change. Noninfected female WT and KO mice had similar hematocrit levels (44–45%). Following infection, hematocrit levels in KO mice fell to 20.8 ± 1.4 and 8.7 ± 1.6% on days 8 and 10, respectively, compared with 45 ± 1.9 and 37.7 ± 3.4% in WT mice (both p < 0.01), suggesting that anemia occurred earlier and was more severe in KO than in WT mice. P. chabaudi AS infection resulted in body weight loss which was more severe in WT than in KO mice. WT mice lost 11.5 ± 0.9 and 15.8 ± 1.9% of their original body weight at 8 and 10 days postinfection, respectively, compared with losses of 5.0 ± 2.1 (p < 0.01) and 8.9 ± 1.7% (p < 0.05) in KO mice. P. chabaudi AS infection induced marked splenomegaly in both WT and KO mice, but there were no significant differences between the groups (data not shown).

Cytokine production in vivo and in vitro and NK cell function

Because one of the major biological functions of IL-12 in immune responses is to induce IFN-γ production by NK and CD4⁺ T cells, we analyzed IFN-γ response in WT and KO mice following P. chabaudi AS infection. WT mice had an increased level of IFN-γ in serum, which peaked at day 6 postinfection and then declined to a low level by day 14 (Fig. 2A). In contrast, KO mice produced low levels of IFN-γ in serum and a small peak (p < 0.05 vs day 0) of IFN-γ was observed at day 8 postinfection. As an indication of macrophage activation by IFN-γ, WT mice produced a strong NO response which peaked at 140 ± 7.5 μM on day 8, while the NO response was low in KO mice in which a peak level of 40 ± 9.2 μM (p < 0.01 vs WT) was observed at day 9 postinfection.

Next, we analyzed the function of NK cells, a primary responder cell to IL-12 and an early source of IFN-γ during blood-stage malaria infection (16, 35). At 3 and 6 days postinfection, splenic NK cells from WT mice produced a higher level (p < 0.01) of IFN-γ in comparison with cells from noninfected WT mice (Fig. 2B). Splenic NK cells from infected KO mice also showed increased IFN-γ production, but the level of IFN-γ produced was significantly lower (p < 0.01) on day 6 than the level detected in WT mice (Fig. 2B). NK cell cytotoxic activity, measured in total spleen cells against YAC-1 target cells, was significantly increased in WT mice 6 days postinfection (32 ± 3%) compared with the basal level in uninfected mice (13 ± 2%, p < 0.01). However, in

![FIGURE 1. Course of blood-stage P. chabaudi AS infection in female (A) and male (B) IL-12 p40 KO and C57BL/6 WT control mice. Mice were infected i.p. with 10⁶ pRBC and course of parasitemia was determined. Data are presented as the mean of 10 mice for KO and five mice for WT per group from one of three replicate experiments. SDs (not shown) are between 10 and 20% of mean parasitemias. †, Time and number of death of male KO mice.](image-url)
In contrast, spleen cells from infected KO mice produced a significantly lower (significantly) response during the later stage of infection (Fig. 2C). The levels of IFN-γ in serum and supernatants were quantitated by ELISA. Data are presented as mean ± SEM of four mice per group from one of two replicate experiments. **, p < 0.01 for comparison between infected and uninfected mice. ##, p < 0.001 for comparison between WT and KO mice.

To further understand the immunoregulatory role of IL-12 during P. chabaudi AS infection, we analyzed IL-10, TNF-α, and IL-4 production in vitro by spleen cells from WT and KO mice during the course of primary infection. Seven days after infection, spleen cells from WT and KO mice produced more IL-10 than cells from the uninfected controls, but the IL-10 level was significantly higher in KO mice (p < 0.05) than in WT mice (Fig. 2C). In contrast, spleen cells from infected KO mice produced more IL-10 than spleen cells from infected WT mice.

To understand the immunoregulatory role of IL-12 during P. chabaudi AS infection, we analyzed IL-10, TNF-α, and IL-4 production in vitro by spleen cells from WT and KO mice during the course of primary infection. Seven days after infection, spleen cells from WT and KO mice produced more IL-10 than cells from the uninfected controls, but the IL-10 level was significantly higher in KO mice (p < 0.05) than in WT mice (Fig. 2C). In comparison with uninfected control mice, TNF-α production by spleen cells was significantly (p < 0.01) increased in both WT and KO mice at day 7 postinfection, and the levels declined through day 21 (Fig. 3B). No significant difference in TNF-α production was detected between WT and KO mice. During the late stage of infection (day 21), high levels of IL-4 production by spleen cells were detected in both WT and KO mice (Fig. 3C). However, IL-4 production was comparable between WT and KO mice in both noninfected and infected groups.

**Effect of IL-12 on malaria-specific Ab production**

The parasitemia data described above showed that KO mice not only were unable to control acute stage infection but also had an impaired ability to resolve the chronic stage of P. chabaudi AS infection. Because Ab-mediated immunity has been shown to be required for clearance of late-stage chronic infection, it is possible that IL-12 is important for induction of protective Abs. To address this question, we examined parasite-specific Ab responses during the course of primary infection in WT and KO mice and in a group of KO mice that had been treated with recombinant murine IL-12 for the first 5 days of infection. WT and KO mice developed courses of primary infection similar to those shown in Fig. 1 (Fig. 4). In comparison with nontreated KO mice, short-term IL-12 treatment of KO mice during the early stage of infection significantly reduced the levels of primary parasitemia (p < 0.05, by ANOVA) which were comparable to those observed in WT mice (p > 0.05). In addition, the IL-12-treated KO mice had a lower level of recrudescent parasitemia than the nontreated KO mice between days 14 and 18 (p < 0.05) and cleared the infection by day 28 postinfection (Fig. 4).
FIGURE 4. Course of blood-stage *P. chabaudi* AS infection in female WT, untreated KO, and IL-12-treated KO mice. One group of KO mice was treated i.p. with murine rIL-12 (0.1 μg/mouse) on the day of infection and daily for 5 days. Mice in all groups were infected with 10⁶ pRBC and course of parasitemia was determined. Data are presented as mean ± SEM of four mice per group from one of two replicate experiments.

Ten days after infection, only low levels of *P. chabaudi* AS-specific total Ig and IgM were detected in sera from WT and KO mice (data not shown). Analysis of sera collected on days 15 and 21 postinfection, the time points before and after the first recrudescence parasitemia, respectively, showed different Ab profiles between WT and KO mice (Fig. 5). KO mice produced significantly lower levels of total Ig (p < 0.05), IgM, and IgG2a (both p < 0.01) and a higher level of IgG1 (p < 0.05) than WT mice. Interestingly, short-term IL-12 treatment of KO mice during the early stage of infection had a strong influence on Ab production in the later stage of infection (Fig. 5). IL-12 treatment of KO mice increased the levels of parasite-specific total Ig, IgM, and IgG2a but suppressed IgG1 Ab in comparison with nontreated KO mice. At later time points (days 28 and 36), the Ab levels declined in WT and IL-12-treated KO mice but remained high in the nontreated KO mice (data not shown). In the WT and IL-12-treated KO mice, the decline in Ab levels during the late stage of infection coincided with clearance of the parasites.

To further determine the effect of IL-12 on Ab response, WT, KO, and IL-12-treated KO mice were given a challenge infection 8 wk after primary infection. WT and IL-12-treated KO mice completely controlled the challenge infection. In contrast, KO mice developed low levels (1–2%) of parasitemia between days 6 and 9, and the parasite was cleared by day 10.

In comparison to primary infection, challenge infection induced increased production of total and IgG subclass Abs, but not IgM, in all three groups of mice (Fig. 6). Importantly, WT and KO mice showed remarkable differences in the level of IgG subclases. KO mice produced significantly lower levels of IgG2a (p < 0.001) and IgG3 (p < 0.05) and a higher level of IgG1 (p < 0.01) than WT mice 7 days after challenge infection. The level of IgG2b was not significantly different between WT and KO mice. In comparison with KO mice, IL-12-treated KO mice produced higher levels of IgG2a and IgG3 and less IgG1 (Fig. 6).

Uninfected KO mice had a higher level of serum total IgE than WT mice. However, after primary and challenge infection IgE levels were not increased and comparable levels were detected in WT and KO mice (data not shown). Parasite-specific IgE was not detectable in serum from infected WT and KO mice.

**Passive transfer of immunity by immune serum**

The results described above demonstrate that endogenous IL-12 strongly influences the pattern of IgG subclass response to *P. chabaudi* AS infection. To determine whether the reduced Ab response in KO mice is responsible for the delayed clearance of primary *P. chabaudi* AS infection and impaired ability to control challenge infection, a serum transfer experiment was performed to test the protective ability of immune sera from WT and KO mice. Groups of A/J mice were treated with immune serum from infected WT or KO mice, PBS, or normal mouse serum as control. A/J mice were used as recipients because this mouse strain is highly susceptible to blood-stage *P. chabaudi* AS infection (36). Control
mice treated with PBS or normal mouse serum developed high levels of parasitemia and all mice in these two groups died by day 9 postinfection (Fig. 7A). The development of parasitemia in A/J mice treated with immune serum from KO mice was delayed in comparison with control groups (p < 0.01, ANOVA) but occurred earlier than in mice treated with immune serum from WT mice (p < 0.01) (Fig. 7A). Both groups of mice treated with either KO or WT immune serum reached peak parasitemia at day 11, but the peak level was significantly higher in mice treated with KO immune serum than in those treated with WT immune serum (p < 0.05). All mice treated with KO immune serum were ill and died by day 13 postinfection, while the mice treated with WT immune serum survived the infection and fully recovered (Fig. 7A).

To further determine whether Ab-mediated immunity in KO mice is defective as well as whether this defect can be corrected by immune serum from WT mice, we collected immune serum from WT and KO mice 17 days post primary infection and transferred these sera, along with PBS as control, to three groups of KO mice 14 days after infection. These time points were chosen for collection and transfer of immune serum because WT and KO mice start to show differences in Ab response and recrudescent parasitemia during this period of time (see Figs. 1 and 5). Before injection of PBS or immune serum, the three groups of KO mice showed similar courses of parasitemia. The KO mice treated with PBS or KO immune serum at day 14 developed similar high levels of recrudescent parasitemia with peak levels of 21–22% at day 16. In contrast, treatment of KO mice with WT immune serum significantly (p < 0.01, by ANOVA) suppressed the level of recrudescence parasitemia in comparison with mice treated with PBS or KO immune serum.

The finding that IgG2a production was significantly reduced but IgG1 was increased in KO mice suggested that Th1-dependent, but not Th2-dependent, Ab mediates protection against *P. chabaudi* AS infection. To investigate this possibility, we depleted IgG2a or IgG1 Ab from WT mice immune serum and transferred the subclass-depleted and control sera to groups of *P. chabaudi* AS-infected A/J mice. As described above, A/J mice treated with control WT immune serum had significantly delayed and lower levels of parasitemia in comparison with PBS control group, and all mice survived infection (Fig. 7B). A/J mice treated with IgG1-depleted immune serum had a similar course and outcome of *P. chabaudi* AS infection as mice treated with control WT immune serum (p > 0.05). However, A/J mice treated with IgG2a-depleted immune serum developed significantly higher levels of parasitemia than the group treated with control immune serum (p < 0.05), and two of three mice died by day 11 postinfection (Fig. 7B). These results demonstrate that the Th1-driven IgG2a is the major Ab isotype contributing to Ab-mediated protective immunity against blood-stage *P. chabaudi* AS infection in mice.

**Discussion**

It has been proposed that different immune effector mechanisms operate to control and resolve primary blood-stage *P. chabaudi* AS infection in mice. Activation of innate and adaptive cell-mediated immunity is thought to be critical for limiting primary parasitemia and survival of the host during the acute phase of the infection. Ab-mediated immunity, involving both B and CD4+ T cells, is considered to play a major role in the resolution of the chronic stage of infection (21, 22, 33, 34, 37). The results presented in this study demonstrate that the Th1-associated cytokine, IL-12, which is produced in resistant C57BL/6 mice early during infection (13, 19, 20), is required not only for activation of innate and cell-mediated immune mechanisms to control acute primary infection, but also for development of efficient Ab-dependent immunity to resolve the chronic phase of primary infection and to control challenge infection.

Previous studies in our laboratory and others using various approaches demonstrate an important role for IFN-γ in the protective immune response to blood-stage *P. chabaudi* AS infection in mice (9–11, 35). Recent studies using IFN-γ or its receptor gene KO mice provide conclusive evidence for the pivotal role for this cytokine in the control of acute parasitemia and survival of the host during primary infection (13, 14, 38). Studies in human malaria also show that IFN-γ production is associated with protection (39, 40). In this study, we demonstrated that, in the absence of endogenous IL-12, IFN-γ production during the early stage (before peak parasitemia) of infection was significantly reduced in comparison with WT control mice. NO production, a hallmark of macrophage activation, was also markedly decreased in KO mice. The impaired IFN-γ response in KO mice resulted in more severe blood-stage *P. chabaudi* AS infection as evidenced by higher levels of parasitemia and more severe anemia in female and male KO mice and high mortality in male KO mice. These results support the conclusions of our previous studies (15), using IL-12 treatment or Ab neutralization in vivo, that an early IL-12 response and the downstream effector response involving IFN-γ and TNF-α are critical for protection against blood-stage *P. chabaudi* AS infection.

In addition to their inability to control acute infection, KO mice also developed significant recrudescence parasitemias during the chronic stage of infection and a delayed clearance of the parasites. Because Ab-mediated protective immunity is thought to play an
important role in the resolution of chronic *P. chabaudi* AS infection (21, 22, 34), we analyzed Ab responses in WT and KO mice during the late stage of primary infection as well as during challenge infection. KO mice produced significantly lower levels of total parasite-specific Ab and showed an altered IgG subclass profile, that is, lower levels of IgG2a and IgG3 and a higher level of IgG1 than WT mice. These results indicate that IL-12 strongly influences the quantity and quality of the Ab response to blood-stage *P. chabaudi* AS infection. The reduced total Ab production and altered IgG subclass distribution may be responsible for the impaired ability of KO mice to resolve primary infection and control challenge infection. In a separate study, we also observed that IL-12 is required for immunization-induced acquired immunity to *P. chabaudi* AS infection. *P. chabaudi* AS Ag-immunized WT mice developed significantly delayed and reduced parasitemia following infection in comparison with nonimmunized WT mice. Immunization of KO mice failed to induce protective immunity, and both immunized and nonimmunized KO mice developed similar high levels of parasitemia following infection (our unpublished observations).

To investigate whether the reduced quantity and the altered IgG subclass response in KO mice are the major reasons for the reduced protective immunity of these mice, we prepared immune sera from WT and KO mice, and evaluated the protective ability of these sera in susceptible A/J mice. Immune serum from WT mice provided strong protection to recipient A/J mice as evidenced by a significantly delayed course of infection, a lower level of peak parasitemia, and survival in comparison with PBS or normal serum control groups. Although immune serum from KO mice slightly delayed the course of infection in comparison with the control groups, the mice in this group developed significantly higher levels of parasitemia than those treated with immune serum from WT mice and, similar to control mice, all these mice died, albeit 3–4 days later. Furthermore, transfer of immune serum collected from WT mice 17 days post primary infection to KO mice at day 14 of infection significantly suppressed the levels of recrudescence parasitemia in recipient mice, but serum from KO mice had no effect. These results clearly demonstrate that Abs produced in KO mice had a reduced protective effect against blood-stage *P. chabaudi* AS infection in comparison with the Abs in WT mice. Also, the defective Ab-mediated immunity in KO mice can be at least partially corrected by immune serum from WT mice.

CD4+ T cells have been shown to be essential for development of Ab-mediated immunity to blood-stage malaria because athymic nude mice (37) and CD4+ T cell-depleted mice (33) are unable to resolve *P. chabaudi* AS infection. Cytokines and costimulatory signals produced by CD4+ T cells are required for B cell activation and clonal expansion. Ig gene rearrangement, somatic hypermutation (41), and Ab class switching (42). IL-4 promotes switching from IgM to IgG1 and IgE, while IFN-γ induces switching to IgG2a and IgG3. As observed in C57BL/6 mice in our earlier study (11) and in NIH mice by others (21), the WT mice in this study developed a strong Th1 cytokine response during the early stage of *P. chabaudi* AS infection, while a Th2-type response predominated in the late stage of infection. The long-standing paradigm regarding immune mechanisms to *P. chabaudi* AS is that the Th2 cytokine response during the late stage of infection is important for induction of Ab-mediated protective immunity (23, 43). However, evidence from studies using IL-4 gene KO mice does not support this contention (14, 24, 25). In the present study, Ag-stimulated spleen cells from KO mice produced similar levels of IL-4 as cells from WT mice during chronic infection and infected KO mice produced even higher levels of IgG1 than WT mice. Despite these strong Th2-associated responses, KO mice showed increased susceptibility during both primary and challenge infections. Instead, KO mice produced significantly reduced levels of IgG2a and IgG3 during primary and challenge infections. These results suggest that the Th1-dependent, but not Th2-dependent, Ab response contributes to protective immunity against blood-stage *P. chabaudi* AS.

To critically evaluate the role of IgG1 and IgG2a Abs in protective immunity to this parasite, we depleted IgG2a or IgG1 from WT immune serum and transferred the subclass-depleted sera to recipient A/J mice. Depletion of the Th1-dependent IgG2a Ab dramatically reduced the protective efficacy of the immune serum, while removal of the Th2-dependent IgG1 subclass did not significantly alter its protective ability. These results indicate that Th1-associated IgG2a is one of the major protective Ab subclasses in Ab-mediated immunity against *P. chabaudi* AS infection and that the significantly lower levels of IgG2a Ab produced in KO mice might be the major reason for the reduced protective immunity in these mice. It should be pointed out that the immune serum from WT mice contains a higher titer of parasite-specific IgG2a (1/5300) than IgG1 (1/1700) (Fig. 6). The differential protective effects of IgG2a- and IgG1-depleted immune serum observed in this study may be due to the quantitative difference of these two Ab subclasses in the serum. These results do not directly reveal whether the two Ab subclasses are qualitatively different in protection which can only be assessed by transfer of equal amounts of parasite-specific IgG2a and IgG1 Abs. It has been reported that transfer of *P. yoelii* merozoite surface protein 1-specific IgG3 mAb confers strong protection in recipient mice (44, 45). In the present study, KO mice also produced a significantly lower level of IgG3 during *P. chabaudi* AS infection, suggesting that this IgG subclass may also contribute to protection against this parasite. The protective effect of this Th1-dependent Ab subclass was not addressed in the present study.

Resistant WT mice produced a strong IL-12 response early during *P. chabaudi* AS infection (13, 19, 20) which, in turn, induced production of IFN-γ by NK and CD4+ T cells. This in vivo Th1-dominant cytokine milieu during the early stage of infection may modulate Ab isotype switching, resulting in production of the protective Ab subclass IgG2a and possibly IgG3. Indeed, treatment of KO mice with rIL-12 during the first 5 days of infection corrected the IgG subclass distribution and restored resistance to blood-stage malaria in KO mice during primary and challenge infections. The ability of IL-12 to modulate the Ab isotype/subclass response is likely to occur via IFN-γ, although a direct effect on B cells cannot be ruled out (28). Interestingly, we previously observed a similar change in Ab response in IFN-γ-deficient mice (13).

Modulation of IgG2a and IgG3 production by IL-12 has been observed in a variety of inbred strains of mice after immunization with various protein or hapten Ags (29, 46, 47) and in several infectious disease models (48, 49). For murine malaria, a protective effect of IL-12 has also been shown in *P. berghei* XAT infection in CBA mice (17). Furthermore, studies in *P. yoelii*-infected BALB/c (30) and *P. berghei* XAT-infected CBA mice (31) also demonstrate that Th1-associated IgG2a and IgG3 are the major protective Ab subclasses in Ab-mediated immunity against blood-stage malaria. Taken together, these results and those of the present study suggest that IL-12-dependent production of protective IgG2a/IgG3 Abs may represent a general protective mechanism against blood-stage infection with murine malaria parasites.

In conclusion, the results presented in this report demonstrate that IL-12, produced during the early phase of blood-stage *P. chabaudi* AS infection, is not only important for induction of IFN-γ-dependent early innate and cell-mediated immune mechanisms critical for control of the acute stage of infection and survival of
the host but also strongly influences Ab-mediated immunity required for suppressing and eventually resolving the chronic phase of infection. We provide conclusive evidence that IL-12 selectively promotes IgG2a, and possibly IgG3, Ab responses, which play a major protective role against blood-stage P. chabaudi AS infection in mice. This finding may have important implications in the design of a vaccination strategy for human malaria.

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References