



Role of IL-10 in inhibiting protective immune responses against infection with heterologous *Plasmodium* parasites

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ARTICLE INFO

Keywords:

Malaria
Protection
Pathogenesis
CD4⁺ T cells
IL-10
Heterologous parasites
Secondary response

ABSTRACT

Malaria is induced by infection with *Plasmodium* parasites, which are genetically diverse, and the immune response to *Plasmodium* infection has both allele-specific and cross-reactive components. To determine the role of the cross-reactive immune response in the protection and disease manifestation in heterologous *Plasmodium* infection, we used infection models of *P. chabaudi chabaudi* (Pcc) and *P. berghei* ANKA (PbA). CD4⁺ T cells primed with Pcc infection exhibited strong cross-reactivity to PbA antigens. We infected C57BL/6 mice with Pcc and subsequently treated them with an anti-*Plasmodium* drug. The Pcc-primed mice exhibited reduced parasitemia and showed no signs of experimental cerebral malaria after infection with PbA. CD4⁺ T cells from the Pcc-primed mice produced high levels of IFN- γ and IL-10 in response to PbA early after PbA infection. The blockade of IL-10 signaling with anti-IL-10 receptor antibody increased the proportion of activated CD4⁺ and $\gamma\delta$ T cells and the IFN- γ production by CD4⁺ T cells in response to PbA antigens, while markedly reducing the levels of parasitemia. In contrast, IL-10 blockade did not have a significant effect on parasitemia levels in unprimed mice after PbA infection. These data suggest a potent regulatory role of IL-10 in the cross-reactive memory response to the infection with heterologous *Plasmodium* parasites leading to the inhibition of the protective immunity and pathogenesis.

1. Introduction

Malaria is a life-threatening disease caused by *Plasmodium* parasites and is one of the most important infectious diseases worldwide: WHO estimated 219 million cases of malaria and 435,000 malaria-related deaths in 2017 [1]. An increasing body of evidence indicates that T and B cell immune responses are pivotal for the protection against blood-stage infection with *Plasmodium* parasites [2–4]. CD4⁺ T cells produce cytokines including IFN- γ , which activate macrophages and stimulate B cells to become plasma cells that produce protective antibodies [5]. Mice with IFN- γ or its receptor knocked out and those treated with anti-IFN- γ antibody have been shown to have defects in controlling *Plasmodium* infection [6], indicating that IFN- γ plays a pivotal role in the

protective immunity against blood-stage *Plasmodium* infection. However, the immune response incurs collateral damage to the host tissues when it is not well controlled; in fact, much of the pathology associated with malaria is considered to be caused by the immune response [4,7,8]. In the mouse model of experimental cerebral malaria (ECM), infection of C57BL/6 (B6) mice with *P. berghei* ANKA (PbA) induces cerebral disease at peripheral blood parasitemia levels that are not high enough to induce pathology in other strains of *Plasmodium* parasites [9,10]. Studies suggest that brain-infiltrating CD8⁺ T cells play a critical role in the pathogenesis of ECM and that IFN- γ -producing CD4⁺ T cells promote the disease [11–13]. Thus, adaptive immune responses against blood-stage infection with *Plasmodium* parasites could be simultaneously protective and pathogenic for the host.

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<https://doi.org/10.1016/j.parint.2019.01.003>

Received 8 December 2018; Received in revised form 4 January 2019; Accepted 7 January 2019

Available online 09 January 2019

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Plasmodium parasites are genetically diverse and multiple species and strains can infect humans in malaria-endemic areas [14]. The immune response induced by natural infection can target various *Plasmodium* antigens; the immune response thus has species- and strain-specific components [15,16]. Experimentally, the immune response to infection with parasites belonging to species or strains different from the ones causing the previous infection is less pronounced, suggesting the species and strain-specific nature of anti-malarial immune response [4,7,17,18]. In addition, vaccine trials of blood-stage candidate antigens suggested that the efficacy of vaccination was reduced with non-vaccine type parasites and thus has allele-specific components [3] [19]. However, other studies suggested that the immune response to *Plasmodium* infection has a cross-reactive component: e.g., immunity induced by subpatent blood-stage infection with *Plasmodium chabaudi chabaudi* AS (Pcc) induced anti-*P. chabaudi* IgG antibody production and T cell response that were protective against subsequent challenge infection with homologous parasites as well as the heterologous *P. chabaudi chabaudi* CB [20]; and mice immunized with attenuated Pcc were protected against challenge infection with heterologous parasites, *P. vinckei* and *P. yoelii* [21]. Taken together, these studies suggest that both allele-specific and cross-reactive components exist in the immune response against *Plasmodium* infection.

People living in the malaria-endemic region obtain resistance to disease manifestations after repeated infections [7]. However, individuals acquire only partial protection, partly because the immune response to *Plasmodium* parasites has an allele-specific component [19]. In addition, the immune response may modify disease manifestations caused by subsequent infection with heterologous *Plasmodium* parasites.

In this study, we investigated the effect of cross-reactive immune response induced by prior infection with *Plasmodium* parasites on the protection against and the manifestation of subsequent infection with heterologous parasites. We used Pcc for priming, which is often used as a model of *P. falciparum* because it invades both normocytes and reticulocytes, and the infected red blood cells (RBCs) adhere to host endothelial cells [22]. In addition, T cells play a major role in the protective immunity against Pcc infection, and memory T cells convey protection against subsequent challenge infection with homologous parasites [23,24].

2. Material and methods

2.1. Animals and *Plasmodium* parasites

B6 mice were purchased from SLC (Shizuoka, Japan). Mice were used at 6–10 weeks of age. The animal experiments were approved by the Institutional Animal Care and Use Committee of Nagasaki University and were conducted according to the guidelines for Animal Experimentation at Nagasaki University.

Pcc was provided by Dr. R. Culleton (Nagasaki University, Nagasaki, Japan). PbA and PbA expressing GFP (PbA-GFP) [25] was provided by Dr. M. Yuda (Mie University, Tsu, Japan). Mice were infected intraperitoneally with parasitized RBCs (5×10^4). The levels of parasitemia were determined by microscopic examination of standard thin blood smears stained with a Diff-Quik staining kit (Sysmex, Kobe, Japan) for Pcc and PbA. When mice were infected with PbA-GFP, the parasitemia levels were determined using FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA).

For the drug treatment, starting at 6 or 14 days after infection with Pcc, mice were intraperitoneally given chloroquine (10 µg/g weight; Sigma-Aldrich) in PBS for 7 consecutive days and sulfadiazine (30 mg/L; Sigma-Aldrich) in drinking water for 14 or 10 days (experiments in Fig. 6). Mice were monitored daily, starting on day 4 of the infection, or every second day after day 5 of infection. After infection with PbA, the clinical scores were defined by the presence of the typical pathological signs, as described previously [26]: ruffled fur, hunching, wobbly gait,

limb paralysis, convulsions, and coma; each sign was given a score of 1.

To block IL-10 signaling, mice received anti-IL-10R mAb (400 µg; 1B1-3A; Bio X cell, West Lebanon, NH, USA) or rat IgG (400 µg; Sigma-Aldrich, St. Louis, MO, USA) in PBS intraperitoneally every 2 days, starting at the time of infection with PbA or PbA-GFP.

2.2. Assessment of the integrity of the blood–brain barrier

Seven days after infection with PbA or Pcc, mice were injected intravenously with 200 µL of Evans Blue (2%; Wako, Osaka, Japan) in PBS. One hour later, the mice were anesthetized and perfused with 15 mL of cold PBS. After sacrifice, brains were isolated, and Evans Blue was extracted by incubating in 2 mL PBS containing 4% paraformaldehyde, at room temperature, for 48 h. The supernatant was collected, and the absorbance was read at 600 nm using a densitometer (Biochrom WPA CO8000 Cell density meter; Biochrom, Cambridge, UK).

2.3. Flow cytometry

Spleen cells were treated with Gey's solution to lyse erythrocytes and were stained for cell surface molecules with the following monoclonal antibodies for 30 min at 4 °C: BV510-anti-CD3 (17A2), APCCy7-anti-CD3, BV711-anti-CD4 (RM4-5), PECy7-anti-CD4 (RM4-5), APCCy7-anti-CD4 (GK1.5), APCCy7-anti-CD8 (53–6.7), FITC-anti-CD11a (M17/4), PE-anti-CD49d (R1–2), FITC-anti-CD44 (IM7), PE-anti-CD62L (MEL-14), PECy7-anti-CD19 (eBio1D3), BV650-anti-B220 (RA3-6B21), APC-anti-B220, Alexa Fluor 488-anti-NK1.1 (PK136), PE-anti-TCRγδ (GL3), and APC-anti-γδTCR mAb. Staining was performed with biotin-anti-CD62L (MEL-14) monoclonal antibodies, followed by incubation with PE-streptavidin. All antibodies were purchased from Biolegend (San Diego, CA, USA), Tonbo Biosciences (San Diego, CA, USA), or eBioscience (San Diego, CA, USA). To exclude dead cells from the analysis, 7-aminoactinomycin D (7-AAD) was added prior to the analysis. For intracellular staining, splenocytes were stained with PECy5-anti-CD3 (145-2C11), BV510-anti-CD3(17A2), APC-anti-CD4 (GK1.5), and FITC-anti-CD25 (PC61.5) monoclonal antibodies for 30 min at 4 °C, fixed and permeabilized using Foxp3 / Transcription Factor Staining Buffer set (eBioscience), and stained with PECy7- or PE-anti-Foxp3 (FJK-16 s) monoclonal antibodies. The stained cells were analyzed using FACSCanto II or LSRFortessa X-20 cell analyzer (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

2.4. Cell culture and ELISA

CD4⁺ T cells were enriched (> 95%) from the spleen using anti-CD4 IMag (BD Biosciences) according to the manufacturer's instructions. Cells were suspended in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, penicillin/streptomycin, 2-mercaptoethanol (5×10^{-5} M), non-essential amino acids (0.1 mM), and sodium pyruvate (1 mM). To examine antigen-specific responses, CD4⁺ T cells (2×10^5) were cultured in 96-well flat-bottom plates in the presence of dendritic cells (1×10^4) with or without Pcc or PbA crude antigen (freeze–thaw lysate of 5×10^6 infected RBCs) for 2 days.

The levels of IFN-γ, IL-2, and IL-10 in the culture supernatant were determined by sandwich ELISA using anti-IFN-γ (R4-6A2; Biolegend) and biotin-anti-IFN-γ (XMG1.2; eBioscience), anti-IL-2 (JES6-1A12; Biolegend) and biotin-anti-IL-2 (JES6-5H4; eBioscience), and anti-IL-10 monoclonal antibodies (Ready SET Go! Kit, eBioscience), respectively, as described previously [27].

The levels of anti-Pcc or -PbA IgG1, IgG2b, and IgG2c antibodies in sera were determined by ELISA. ELISA plates were coated with freeze–thaw lysate of Pcc or PbA (1×10^6 infected RBCs/well) in PBS at room temperature for 2 h. Coated plates were washed with PBS containing 0.2% Tween 20 (washing buffer) and blocked with PBS

containing 10% FCS for 30 min at room temperature. After washing, serum (80× dilution) was added, and plates were incubated overnight at 4 °C. The plates were washed, incubated with biotin-conjugated rabbit anti-mouse IgG1 or IgG2b antibodies (ZyMED, San Francisco, CA, USA) or biotin-conjugated rabbit anti-mouse IgG2c antibodies (BETHYL Laboratories, Montgomery, TX, USA) for 1 h, washed, and incubated with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min. After washing, 1 mg/mL solution of 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) in distilled water was added to each well, and the absorbance was read at 405 nm using an iMark Microplate Absorbance Reader (BIO-RAD, Hercules, CA, USA).

2.5. Statistical analysis

For comparisons between two groups, two-tailed unpaired Student's *t*-tests was used. When three or more groups were compared, an overall difference between the groups was determined using one-way ANOVA or two-way ANOVA for cytokine production analysis. When one-way or two-way ANOVA was significant, differences between individual groups were estimated using Bonferroni's test.

3. Results

3.1. Cross-reactivity of CD4⁺ T cells from Pcc-primed mice to heterologous PbA antigens

To determine the cross-reactive response of CD4⁺ T cells against *Plasmodium* antigens of different species, B6 mice were infected with Pcc, and IFN- γ production of splenic CD4⁺ T cells in response to crude Pcc and PbA antigens was determined 7 days later. CD4⁺ T cells from Pcc-primed mice produced IFN- γ in response to PbA antigens, although the levels were approximately half of those produced in response to Pcc antigens (Fig. 1A). Similarly, PbA-primed CD4⁺ T cells produced IFN- γ in response to Pcc antigens at levels slightly less than those in response to PbA antigens (Fig. S1). These results indicated that CD4⁺ T cells from mice infected with *Plasmodium* parasites exhibit strong cross-reactive responses against heterologous *Plasmodium* antigens.

To determine whether the cross-reactive responses are maintained in memory T cells in the absence of active infection, B6 mice were infected with Pcc and were treated with an anti-malaria drug, chloroquine or sulfadiazine, starting at 14 days after infection (Fig. 1B). > 2 weeks after termination of the treatment, the immune response of splenic CD4⁺ T cells was evaluated (Fig. 1C, D, Fig. S2, S3). The proportions of CD4⁺ and CD8⁺ T cells, B cells, NK cells, and regulatory T (Treg) cells in the spleen of Pcc-primed mice were not significantly different from those in uninfected mice, while the proportion of $\gamma\delta$ T cells was increased (Fig. 1C). The upregulation of integrins CD11a and CD49d is often used as a surrogate marker of antigen-specifically activated CD4⁺ T cells [28,29]. The proportion of specific CD11a^{hi}CD49d^{hi} CD4⁺ T cells in Pcc-primed mice was not significantly different from that in uninfected mice, while the proportion of both effector memory (CD44^{hi}CD62L^{lo}) and central memory (CD44^{hi}CD62L^{hi}) CD4⁺ T cells and CD25⁺Foxp3⁻ CD4⁺ T cells was increased (Fig. 1C). Coordinate upregulation of CD11a and down-regulation of CD8 α is used to define antigen-specific CD8⁺ T cells [29]. The proportion of activated (CD11a^{hi}CD8 α ^{lo}) CD8⁺ T cells and central memory (CD44^{hi}CD62L^{hi}) CD8⁺ T cells in Pcc-primed mice was not significantly different from that in uninfected mice, while the proportion of effector memory (CD44^{hi}CD62L^{lo}) CD8⁺ T cells was increased (Fig. 1C). CD4⁺ T cells from Pcc-primed mice produced IFN- γ , IL-2, and IL-10 in response to not only to Pcc antigens, but also PbA antigens (Fig. 1D), indicating that the cross-reactive memory response of CD4⁺ T cells to PbA antigens was maintained > 1 month after treatment. In the serum of these mice, IgG1, IgG2b, and IgG2c antibodies specific for Pcc antigens were detected, and IgG2b and IgG2c antibodies exhibited

cross-reactivity to PbA antigens (Fig. 1E). These results imply that memory CD4⁺ T and B cells cross-reactive to PbA antigens are induced and maintained after Pcc infection followed by treatment.

3.2. Modulation of the immune pathology induced by PbA infection in Pcc-primed mice

We next evaluated the effect of cross-reactive immune response on disease manifestation in subsequent challenge infections. B6 mice were infected with Pcc, treated with anti-*Plasmodium* drugs for 14 days, and infected with PbA after additional 16–27 days (Fig. 2). Control unprimed mice exhibited increased parasitemia and succumbed to death due to ECM 8–10 days after PbA-infection. However, Pcc-primed mice exhibited low levels of parasitemia up to 9 days after PbA infection, did not develop symptoms of ECM or weight loss, and did not succumb to death; however, they did show increased parasitemia after 10 days of the infection and maintained high levels of parasitemia; therefore, they were sacrificed 20–30 days after the infection (Fig. 2A). To determine the integrity of the blood–brain barrier, mice were injected with Evans Blue 7 days after PbA infection. As positive and negative controls, naïve mice were infected with PbA or Pcc, respectively. Brains of Pcc-primed mice excluded the dye after PbA infection, while those of unprimed mice did not, indicating that the integrity of blood–brain barrier was maintained in the primed mice (Fig. 2B). These data suggest that the immune response is modulated in Pcc-primed mice and hinders the development of ECM after the infection with PbA.

We thus examined the immune response of these mice more in detail. Pcc-primed and unprimed control mice were infected with PbA, and T cell responses were evaluated 3 and 7 days later (Fig. 3A). No significant differences were detected in the proportions of CD11a^{hi}CD49d^{hi} and CD44^{hi}CD62L^{lo} CD4⁺ T cells and CD11a^{hi}CD8 α ^{lo} and CD44^{hi}CD62L^{lo} CD8⁺ T cells 3 and 7 days after infection with PbA (Fig. 3B, C, S4). The proportion of $\gamma\delta$ T cells, which was higher in Pcc-primed mice (Fig. 1C), remained high 7 days after infection with PbA, while that of B cells decreased (Fig. 3C). We compared cytokine production by CD4⁺ T cells from unprimed and Pcc-primed mice in response to Pcc and PbA antigens 3 and 7 days after infection with PbA (Fig. 3D, E). CD4⁺ T cells from Pcc-primed mice produced both IFN- γ and IL-10, but not IL-2, in response to PbA antigens at levels much higher than those from unprimed mice 3 days after PbA infection, suggesting that cross-reactive memory CD4⁺ T cells were activated by PbA-infection (Fig. 3D). However, 7 days after infection, CD4⁺ T cells from the primed mice produced IFN- γ in response to PbA antigens at levels lower than those from unprimed mice, while they produced IL-10 at similar levels (Fig. 3E). These results suggest that CD4⁺ T cells from Pcc-primed mice exhibited an early recall IFN- γ response to PbA antigens, although this response was not sustained after infection, while the IL-10 response was maintained at high levels.

3.3. The effect of IL-10 on challenge infection with heterologous *Plasmodium* parasites

Since the recall IFN- γ response of CD4⁺ T cells was not sustained in Pcc-primed mice after challenge infection with PbA, we hypothesized that IL-10 produced by CD4⁺ T cells modulated the T cell response. To evaluate the role of IL-10 in the recall response of CD4⁺ T cells, IL-10 signaling was blocked in vivo by administration of anti-IL-10R antibodies after challenge infection with PbA (Fig. 4A). The levels of parasitemia did not increase in IL-10-blocked Pcc-primed mice until 11 days after PbA infection, while they increased in control Pcc-primed mice after 8 days of PbA infection (Fig. 4B). The IL-10-blocked mice exhibited severer loss of body weight and clinical symptoms, suggesting the pathology was mediated by the immune system. When we observed these mice for a longer period, 3 out of 5 mice survived both among the IL-10-blocked and the Pcc-primed IgG-treated mice and exhibited very low (0.06 ± 0.01%) and high (48.3 ± 25.6%) parasitemia at 28 days

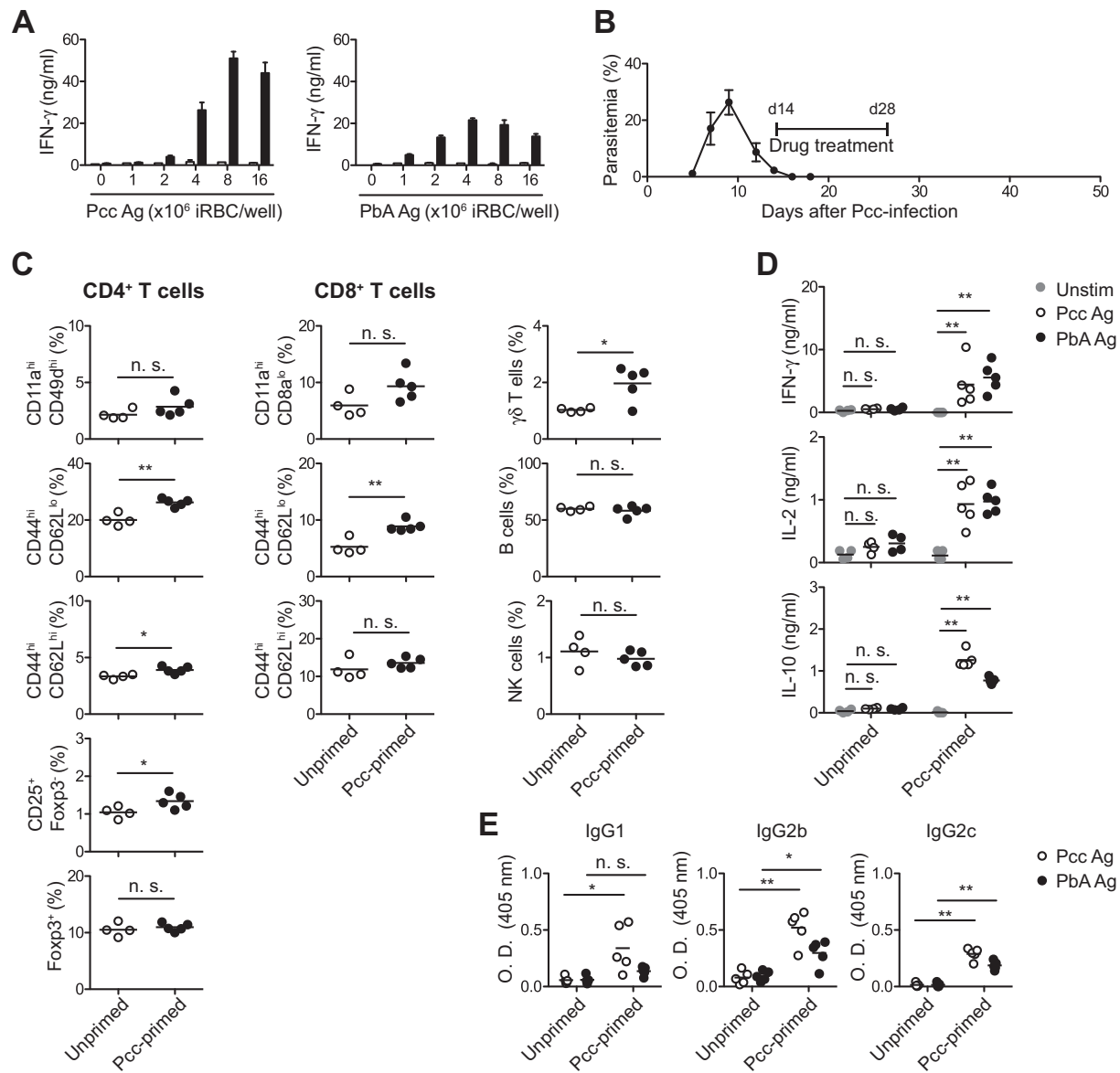


Fig. 1. CD4⁺ T cell response in mice infected with heterologous malaria antigens. (A) Splenic CD4⁺ T cells from uninfected mice (open bar) or mice infected with Pcc for 7 days (closed bar) were stimulated with dendritic cells and different doses of Pcc (left) or PbA (right) crude antigens for 2 days. The levels of IFN- γ in the supernatants were determined by ELISA. Data are presented as mean values \pm SEM. (B–E) Mice were infected with Pcc, treated with chloroquine and sulfadiazine between 14 and 28 days after the infection. (B) The parasitemia levels were determined. The graph shows mean parasitemia values \pm SEM. (C) Spleen cells were prepared 43 days after infection, and their surface phenotypes were evaluated using flow cytometry. Statistical assessment was performed by unpaired Student's *t*-test. * *P* < .05; ** *P* < .01; n.s., not significant. (D) Purified CD4⁺ T cells were stimulated without (gray circle) and with Pcc (open circle) or PbA (closed circle) antigens for 2 days in the presence of dendritic cells, and the cytokine levels in the supernatant were determined by ELISA. Data are presented as mean values \pm SEM. Statistical assessment was performed by two-way ANOVA followed by Bonferroni's test. ** *P* < 0.01; n.s., not significant. (E) Representative data of 4 similar results are shown. Sera were collected from mice (*n* = 5) 43 days after infection, and the levels of Pcc- (open circle) or PbA-specific (closed circle) IgG1, IgG2b, and IgG2c antibodies were determined by ELISA. Data are presented as mean values \pm SEM. Statistical assessment was performed by one-way ANOVA followed by Bonferroni's test. **P* < 0.05; ***P* < 0.01; n.s., not significant.

after infection, respectively, supporting the notion that the parasitemia was better controlled in IL-10-blocked mice. Seven days after the challenge infection with PbA, the immune response of these mice was examined. The proportion of CD4⁺ and CD8⁺ T cells was not significantly different in IL-10-blocked mice than in the controls, while the proportion of $\gamma\delta$ T cells and B cells increased, and that of NK cells decreased (Fig. S5A, 4C). The proportion of CD11a^{hi}CD49d^{hi} cells and CD25⁺Foxp3⁻ CD4⁺ T cells increased in IL-10-blocked mice, suggesting that clonal expansion of specific CD4⁺ T cells was enhanced; the proportion of CD8⁺ T cells was not increased (Fig. 4C). CD4⁺ T cells from IL-10-blocked mice exhibited IFN- γ production in response to

Pcc and PbA antigens at levels higher than those from control mice, although the levels of IL-2 production did not differ significantly (Fig. 4D). The levels of PbA-specific IgG1, IgG2b, and IgG2c were not significantly affected by IL-10 blockade (Fig. 4E). These results suggested that IL-10 inhibited clonal expansion and IFN- γ production by Pcc-primed cross-reactive CD4⁺ T cells during the recall response to PbA challenge infection.

Since the blockade of IL-10 signaling had a significant effect on the outcome of PbA infection in Pcc-primed mice, we next evaluated the effect of IL-10 blockade in PbA infection in naïve mice (Fig. 5A). When naïve mice were infected with PbA, no significant effects of the

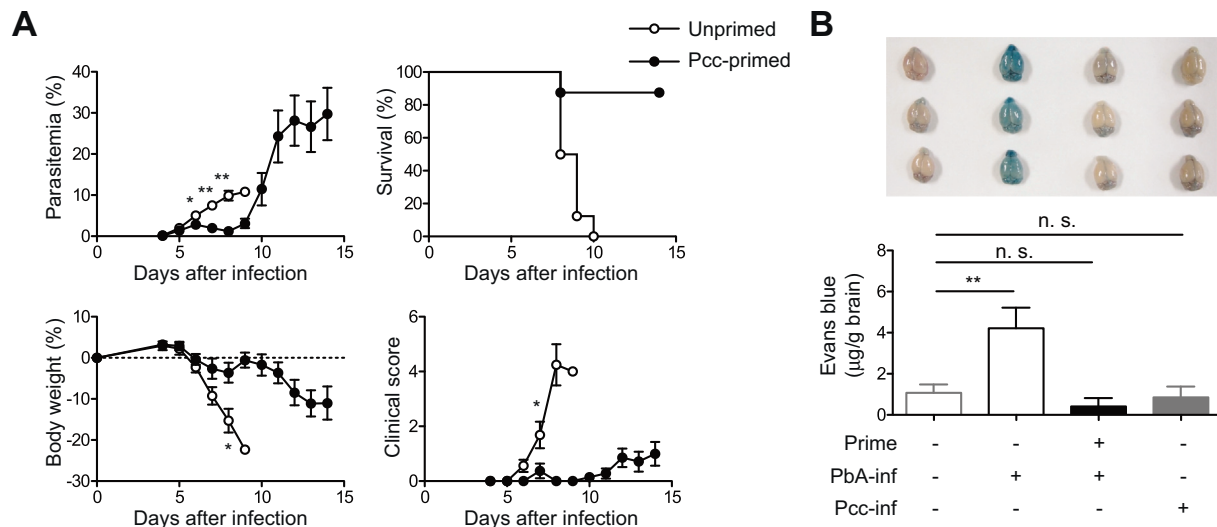


Fig. 2. Modulation of pathogenic immune response in Pcc-primed mice in response to PbA infection. Mice were infected with Pcc and treated with chloroquine and sulfadiazine between 14 and 28 days after the infection. (A) Sixteen to 27 days after the final treatment, the Pcc-primed (closed symbol) and unprimed (open symbol) mice were infected with PbA-GFP, and their parasitemia, survival, body weight, and clinical scores were monitored. Statistical assessment was performed by unpaired Student's *t*-test. * *P* < .05, ** *P* < .01. Data are pooled from 2 experiments (total 8 mice/group). Seven days after infection with PbA (PbA-inf) or Pcc (Pcc-inf), the infected and control uninfected mice were injected with Evans Blue, sacrificed 1 h later, and their brains were isolated. The levels of Evans Blue in the brain tissues were determined. Data presented are means ± SEM of a pool of 3 experiments with 4–8 mice/group. Statistical assessment was performed by one-way ANOVA followed by Bonferroni's test. ** *P* < 0.01; n.s., not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

blockade on the levels of parasitemia, clinical manifestations, or death were observed (Fig. 5B). However, the proportion of CD25⁺CD4⁺ T cells and CD4⁺ T cells producing IFN- γ in response to PbA antigens was higher in the blocked mice than in the control mice, suggesting that IL-10 had an inhibitory effect on the activation of PbA-specific CD4⁺ T cells after infection in naïve mice (Fig. 5C, D). No significant effect was observed in the levels of $\gamma\delta$ T cells, B cells, NK cells, or PbA-specific antibodies. Taken together, while CD4⁺ T cell activation was enhanced, IL-10 blockade did not have a significant impact on the clinical manifestations in mice primarily infected with PbA.

3.4. Priming of cross-reactive immunity occurs in a short period after *Plasmodium* infection

We next examined the length of time required to gain cross-reactive immunity to the subsequent infection with PbA. Mice were infected with Pcc and treated with anti-*Plasmodium* drugs for 10 days starting 6 or 14 days after infection (Fig. 6A). > 2 months after the infection, the immune response of mice treated from day 6 and from day 14 was analyzed. The proportion of CD11a^{hi}CD49d^{hi} and CD44^{hi}CD62L^{lo} CD4⁺ T cells was not significantly different (Fig. 6B). CD4⁺ T cells from both groups produced IFN- γ , IL-2 and IL-10 at similar levels in response to both Pcc and PbA antigens (Fig. 6C). Moreover, the levels of IgG1, IgG2b, and IgG2c antibodies specific for Pcc and PbA antigens were similar in the two groups (Fig. 6D). When the mice were re-infected with Pcc, both groups exhibited limited parasitemia (Fig. 6E). When these mice were infected with PbA, both groups exhibited a delayed onset of parasitemia and did not succumb to death due to ECM, although the mice treated since day 6 showed more severe clinical manifestations than the mice treated since day 14 (Fig. 6F). Taken together, the memory immune response against disease induced by heterologous *Plasmodium* infection was obtained as early as 6 days after Pcc infection, and anti-disease immunity was enhanced by the prolonged infection period.

4. Discussion

CD4⁺ T cells primed by the infection with Pcc exhibited strong cross-reactivity to heterologous PbA antigens and produced IFN- γ , a cytokine critical for the protection against blood-stage *Plasmodium* infection [5,6]. We infected mice with Pcc and treated them with anti-*Plasmodium* drugs for 14–28 days after infection to eliminate the residual parasites that could have affected the subsequent immune response to PbA. The mice were then infected with heterologous PbA parasites. The results showed that these Pcc-primed mice did not develop ECM after infection with PbA. This is likely due to the reduction of parasitemia 7–9 days after challenge infection with PbA and modulation of the immune response. Preexisting antibodies and cross-reactive memory CD4⁺ T cells may have inhibited the parasitemia levels after PbA-infection. In particular, CD4⁺ T cells from Pcc-primed PbA-infected mice produced IFN- γ and IL-10 at levels higher than unprimed mice on day 3 of the infection, suggesting that this response was mediated by memory CD4⁺ T cells. However, 7 days after PbA-infection, IFN- γ production by CD4⁺ T cells from the primed mice did not increase further, while IL-10 production continued to increase from day 3 to day 7. ECM is induced by a combination of parasitized RBCs that adhere to the brain endothelial cells and an inflammatory immune response that leads to the migration of pathogenic CD8⁺ T cells to the brain [30]. Injection of recombinant IL-10 has been shown to protect against ECM [31]. We suspect that both the reduction of parasitemia and the modulation of the immune responses in response to PbA infection, including the increased IL-10 production in Pcc-primed mice, contribute to the lack of ECM development in Pcc-primed mice in the present study.

IL-10 is a potent immunoregulatory cytokine and is crucial for the prevention of inflammatory pathologies. It suppresses antigen presentation and T cell activation mediated by dendritic cells and macrophages and inhibits the production of proinflammatory cytokines [32,33]. It also directly inhibits T cell proliferation and cytokine production [34]. While exhibiting levels of parasitemia similar to those of wild-type mice, IL-10^{-/-} mice infected with Pcc displayed more severe signs of disease, an increased death rate, and higher plasma levels of

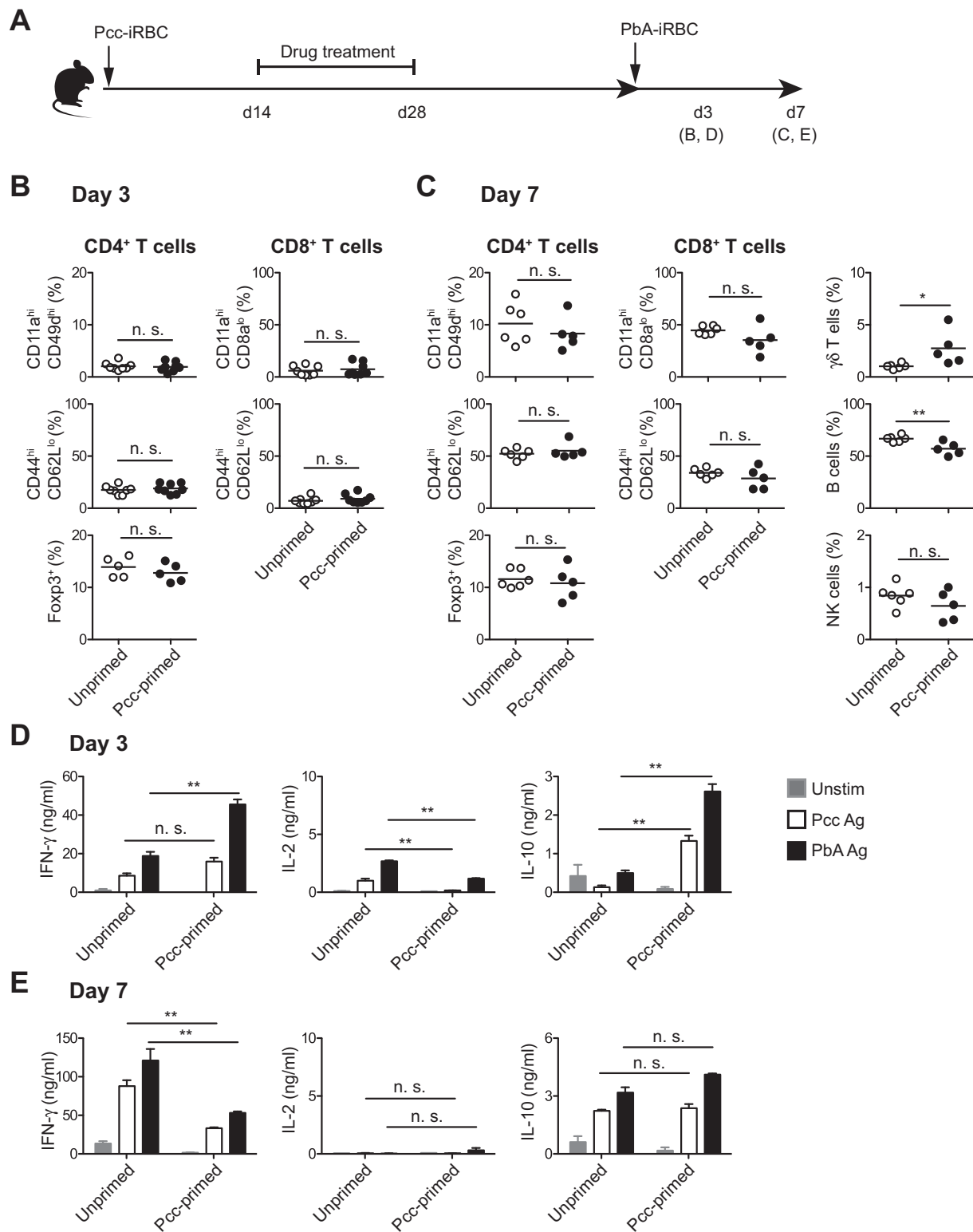


Fig. 3. CD4⁺ T-cell responses of Pcc-primed mice in response to homologous and heterologous malaria antigens. Mice were infected (Pcc-prim) or uninfected (unprim) with Pcc and treated with chloroquine and sulfadiazine between 14 and 28 days after the infection. (A) Experimental scheme. After additional 17–87 days, both groups of mice were infected with PbA, and their splenocytes were prepared 3 (B) or 7 (C) days after the infection, stained with antibodies, and analyzed using flow cytometry. The proportions of CD4⁺ T cell subpopulations from unprimed and Pcc-primed mice are shown. Data are pooled from 4 (B) or 2 (C) experiments. Statistical assessment was performed unpaired Student's *t*-test. **P* < 0.05; ** *P* < 0.01; n.s., not significant. CD4⁺ T cells were prepared from unprimed and Pcc-primed mice 3 (D) or 7 (E) days after infection with PbA and co-cultured with dendritic cells in the absence (gray bar) or presence of Pcc (open bar) or PbA (closed bar) antigens for 2 days. The levels of IFN-γ, IL-2, and IL-10 in the supernatants were determined by ELISA. Data presented are means ± SEM. Statistical assessment was performed by two-way ANOVA followed by Bonferroni's test. ** *P* < .01; n.s., not significant. Results are representative of 3 independent experiments.

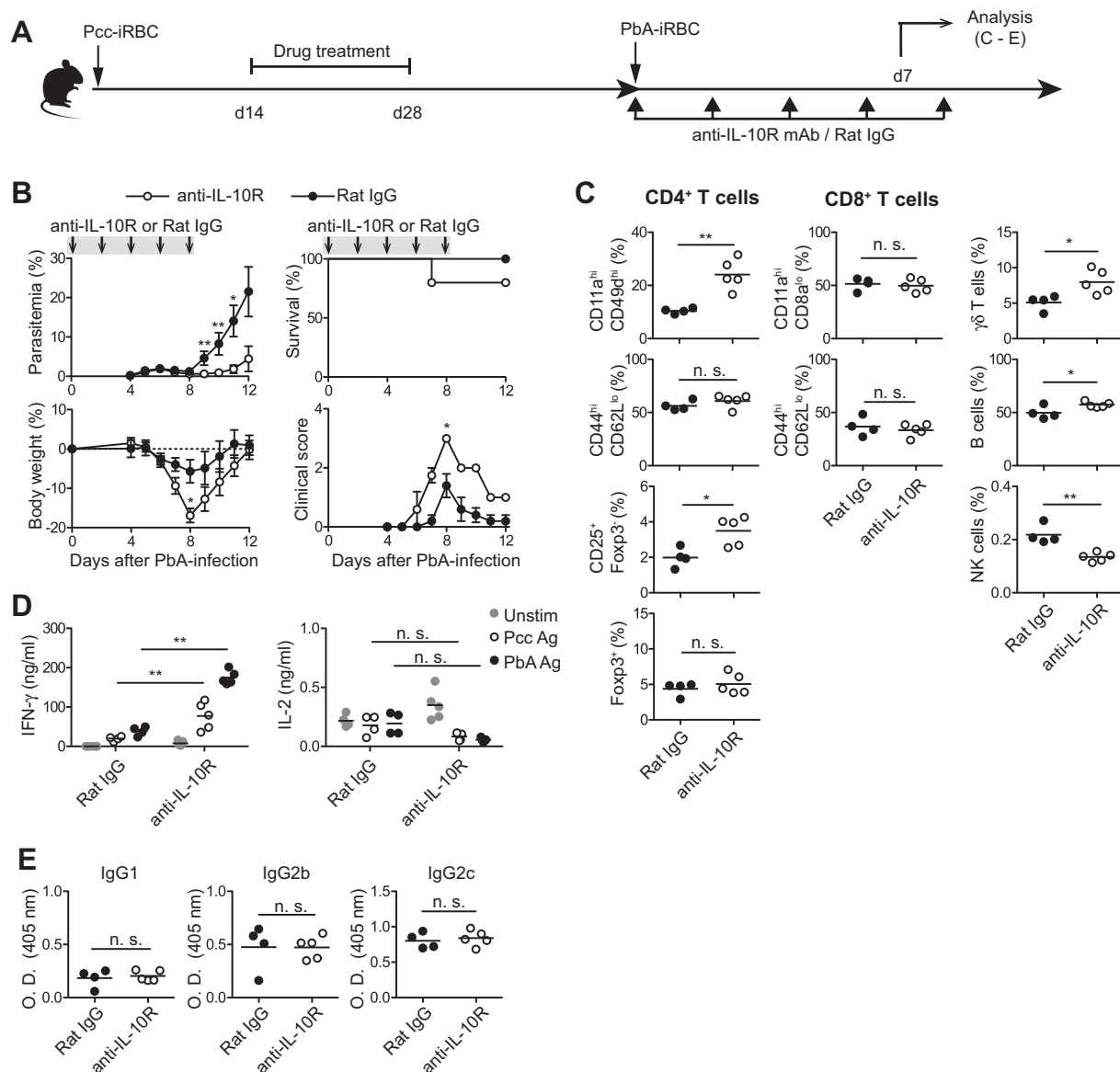


Fig. 4. IL-10R blockade promoted protective and pathological immune responses in Pcc-primed mice after PbA-infection. Mice were infected with Pcc and treated with chloroquine and sulfadiazine between 14 and 28 days after the infection. (A) Experimental scheme. Twenty-four to 44 days after the treatment, the mice were infected with PbA-GFP (B) or PbA (C–E) and treated with anti-IL-10R antibodies (open circle) or control rat IgG (closed circle) every 2 days. (B) The levels of parasitemia, survival, body weight, and clinical scores were monitored. Data are presented as mean values \pm SEM. Statistical assessment between anti-IL-10R-treated and untreated mice was performed by unpaired Student's *t*-test. * $P < 0.05$, ** $P < 0.01$. (C) Seven days after infection with PbA, splenocytes were stained with antibodies and analyzed using flow cytometry. Statistical assessment was performed unpaired Student's *t*-test. * $P < 0.05$; ** $P < 0.01$; n.s., not significant. (D) Seven days after infection with PbA, $CD4^+$ T cells were stimulated without (gray circle) or with Pcc (open circle) or PbA (closed circle) antigens. The levels of cytokines in the supernatants were determined by ELISA. Data presented are means \pm SEM. Statistical assessment was performed by two-way ANOVA followed by Bonferroni's test. ** $P < 0.01$, n.s. not significant. (E) Sera were collected from mice 7 days after infection with PbA. The levels of PbA-specific IgG1, IgG2b, and IgG2c antibodies in the sera were determined by ELISA. Statistical assessment was performed by unpaired Student's *t*-test. n.s., not significant.

IFN- γ and TNF- α [35–37]. Furthermore, infection with Pcc, which did not induce severe brain damage in wild-type mice, induced symptoms and pathology similar to ECM with cerebral edema and hemorrhage in IL-10 $^{-/-}$ B6 mice [38]. Moreover, PbA infection combined with IL-10R blockade induced increased incidence of ECM in BALB/c mice, which normally do not develop ECM [39]. In our model of Pcc-primed PbA-infected mice, parasitemia levels were inhibited in IL-10-blocked mice, indicating a critical role of IL-10 in inhibiting the protective immune responses against PbA. The proportion of activated $CD4^+$ T cells significantly increased, while the proportion of activated $CD8^+$ T cells did not. The proportion of $\gamma\delta$ T cells and B cells also increased, while levels of serum anti-*Plasmodium* antibodies were unchanged, and the

proportion of NK cells was reduced in IL-10R-blocked mice. These results suggest that enhanced clonal expansion and IFN- γ production of specific $CD4^+$ and $\gamma\delta$ T cell subsets is the main driving force for the inhibition of parasitemia in IL-10R-blocked mice. This is consistent with previous studies that suggest a critical role of both $CD4^+$ and $\gamma\delta$ T cells in the protective immunity against blood-stage infection with Pcc [6,40,41]. In addition, $\gamma\delta$ T cells express CD40L and activate dendritic cells via CD40 signaling, resulting in increased activation of $\alpha\beta$ T cells in the *P. berghei* XAT infection model [42]. In contrast to the primed mice, parasitemia levels in unprimed mice was not significantly affected by IL-10 blockade after PbA infection, and the proportion of $\gamma\delta$ T cell was not increased. In these mice, production of IFN- γ by $CD4^+$ T

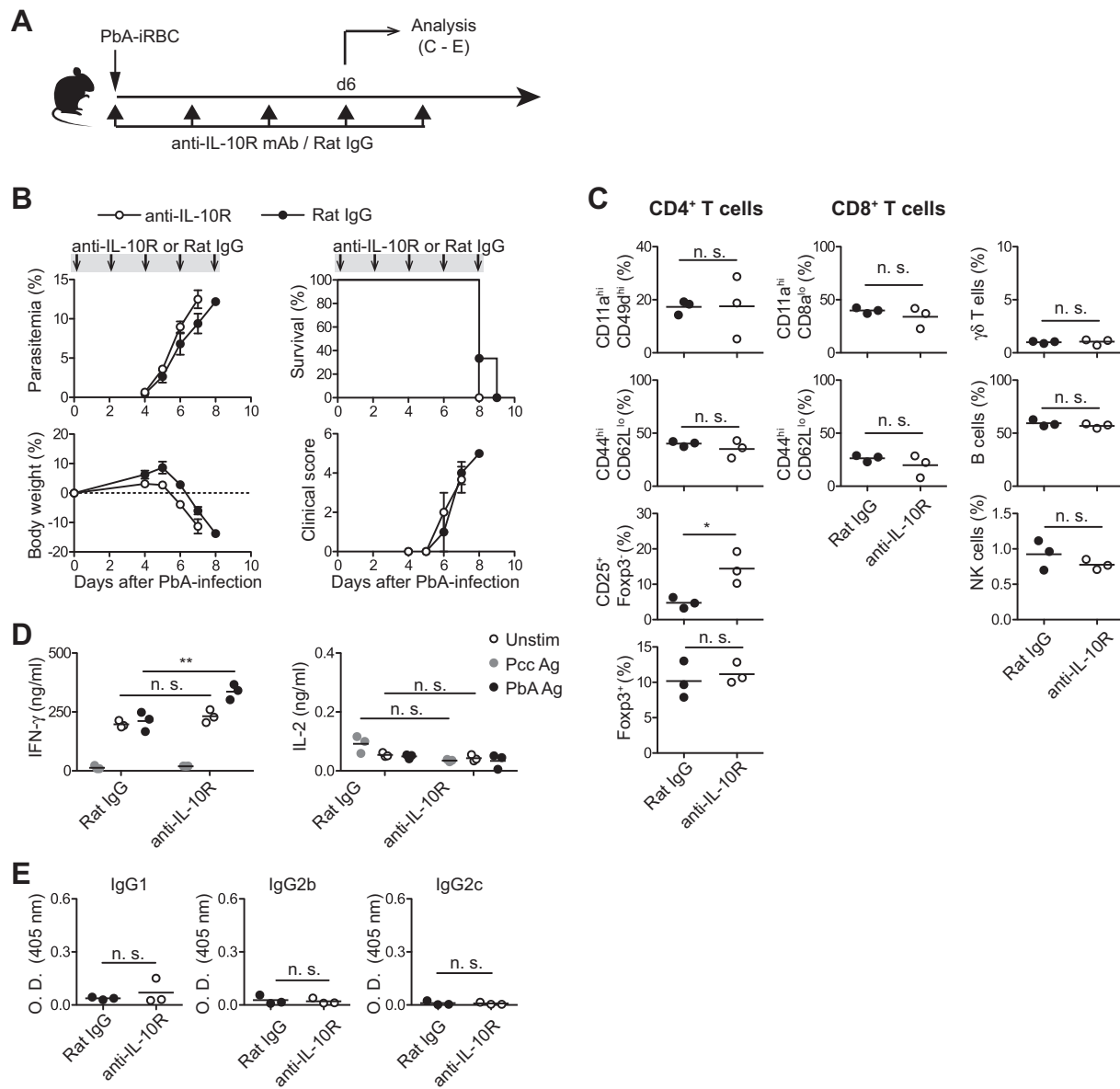
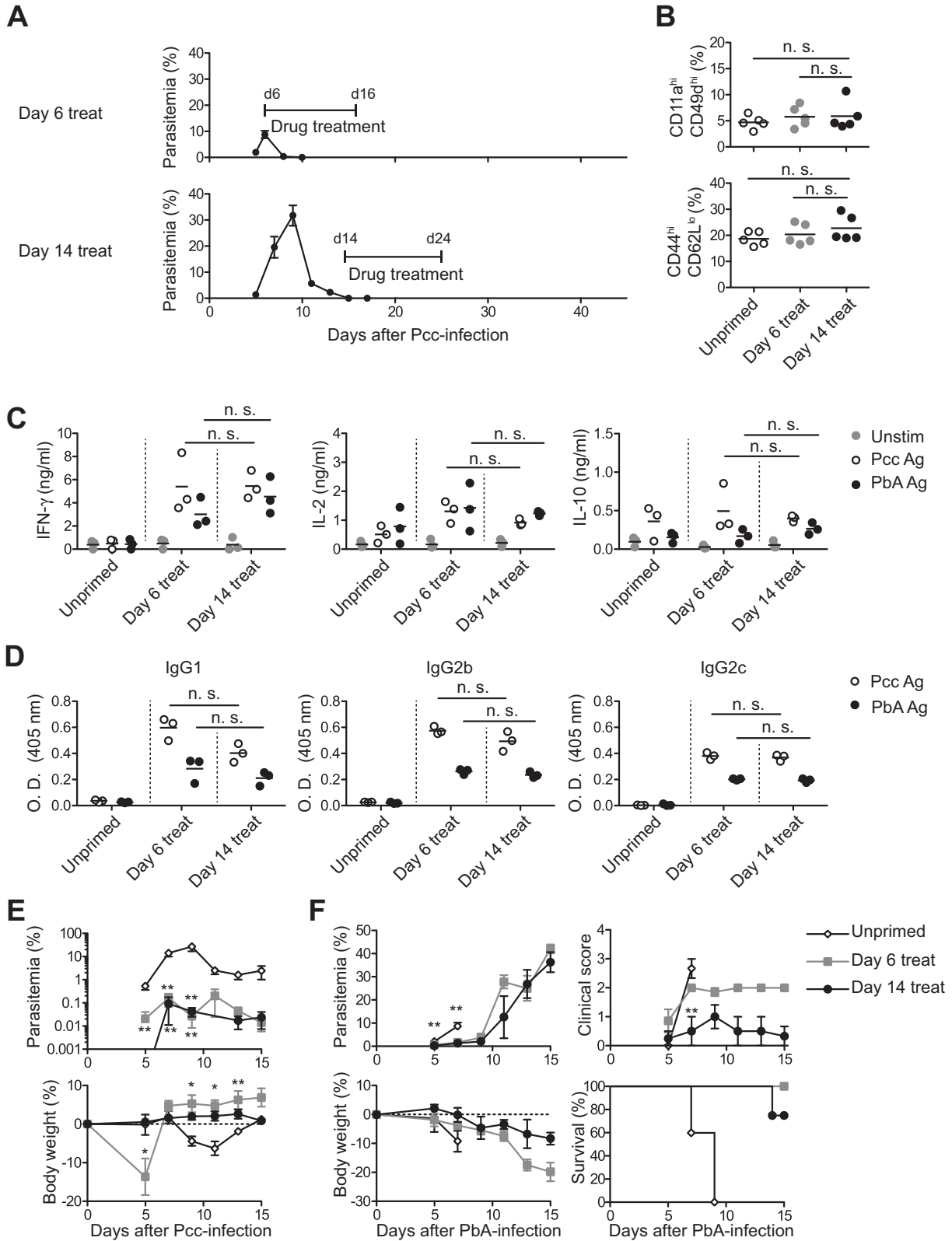


Fig. 5. IL-10R blockade did not affect the pathogenic immune response in unprimed mice after PbA-infection. (A) Mice were treated with anti-IL-10R antibodies (open circle) or control rat IgG (closed circle) every 2 days from the date of infection with PbA (3 mice/group). (B) The levels of parasitemia, survival, body weight, and clinical scores were monitored. Data are presented as mean values \pm SEM. (C) Six days after infection with PbA, splenocytes from anti-IL-10R-treated (open) and rat IgG-treated (closed) mice were stained with antibodies and analyzed using flow cytometry. * $P < 0.05$; n.s., not significant. (D) Six days after infection with PbA, CD4⁺ T cells were stimulated without (gray circle) or with Pcc (open circle) or PbA (closed circle) antigens. The levels of cytokines in the supernatants were determined by ELISA. Statistical assessment between the anti-IL-10R-treated and the control group was performed by two-way ANOVA followed by Bonferroni's test. ** $P < 0.01$, n.s. not significant. (E) Sera were collected from mice 6 days after infection with PbA (3 mice/group). The levels of PbA-specific IgG1, IgG2b, and IgG2c antibodies in the sera were determined by ELISA. Statistical assessment was performed by unpaired Student's *t*-test. n.s., not significant.

cells in response to PbA antigens was only slightly increased, suggesting that the effect of IL-10 is relatively negligible during the primary response to PbA infection. CD4⁺ T cells are the major source of IL-10 during infection with *Plasmodium* parasites [43,44]. Consistent with previous studies, our results suggest that memory CD4⁺ T cells produce high levels of IL-10 in *Plasmodium*-primed mice during the early period of heterologous *Plasmodium* infection [45]. In human studies, it is reported that *P. falciparum*-specific CD4⁺ T cells in children living in endemic regions of malaria produce both IFN- γ and IL-10 [46–48]. In particular, these cells were higher in children with highly frequent recent malaria episodes, while children with less episodes were associated with production of TNF- α without IL-10. These IFN- γ /IL-10-producing CD4⁺ T cells were not directly associated with the protection; rather, they were suggested to be associated with the reduced risk of

pathogenic inflammation or clinical malaria, and thus with asymptomatic infection. In endemic regions, *Plasmodium*-specific memory type CD4⁺ T cells may rapidly produce IL-10 in response to homologous or heterologous *Plasmodium* parasites and inhibit the activation and clonal expansion of CD4⁺ T cells and other immune cells, including $\gamma\delta$ T cells, leading to inhibition of the pathogenesis at the expense of protective immunity. These features highlight a pivotal regulatory effect of IL-10-producing CD4⁺ T cells in the immune response of *Plasmodium*-primed individuals to heterologous *Plasmodium* parasites.

Since Pcc-priming strongly affected the immune response to subsequent heterologous PbA infection, we examined the duration and magnitude of Pcc infection required for the priming. We exposed the immune system of mice to Pcc infection treated for 14 or 6 days, representing long and short exposure, respectively, and treated the mice



(caption on next page)

with anti-*Plasmodium* drugs. However, we found no significant differences in T cell proportions, cytokine production by parasite-specific CD4⁺ T cells, or specific antibody levels between the two groups, suggesting that the priming of the adaptive immune response was achieved mostly within the first 6 days of live Pcc infection. However,

when the mice were re-infected with PbA, the clinical scores were worse in the mice exposed for 6 days than in the mice exposed for 14 days, suggesting that there are immunological parameters that require a longer period of *Plasmodium*-infection to acquire the ability to protect against disease manifestation in subsequent heterologous

Fig. 6. Mice acquire a cross-reactive memory response in a short period after the infection with Pcc. Mice were not infected (Unprimed) or infected with Pcc, and after 6 (Day 6 treat) or 14 (Day 14 treat) days treated with chloroquine and sulfadiazine for 10 days. (A) The parasitemia levels were monitored (8 mice/group). (B) Sixty-four to 76 days after Pcc infection, splenocytes were stained with antibodies and analyzed using flow cytometry. Data are pooled from 4 experiments. (C) CD4⁺ T cells were stimulated without (gray circle) or with Pcc (open circle) or PbA (closed circle) antigens for 2 days, and the levels of cytokines in the supernatants were determined by ELISA. Data pooled from 3 experiments are shown. (D) Sera were collected from the mice 64–76 days after infection (3 experiments). The levels of Pcc- (open circle) or PbA-specific (closed circle) IgG1, IgG2b, and IgG2c antibodies in were determined by ELISA. Statistical assessment was performed by one-way ANOVA followed by Bonferroni's test (B–D). n.s., not significant. (E) Uninfected mice and mice with treatment starting 6 and 14 days after infection (4 mice/group) mice were infected with Pcc, and their parasitemia levels and body weight were monitored. Data are shown as mean values \pm SEM. (F) Uninfected mice (5 mice) and mice with treatment starting 6 (7 mice) and 14 days (4 mice) after infection were infected with PbA, and their parasitemia levels, body weight, clinical scores, and survival are shown as mean values \pm SEM. Data are pooled from 2 experiments. Statistical assessment was performed in comparison to the unprimed mice by one-way ANOVA followed by Bonferroni's test (E, F). ** P < 0.01, * P < 0.05.

infections; however, further research is required to identify these specific parameters.

An increasing body of evidence indicates that T-cell-derived IL-10 is critical for downregulating inflammatory pathology during blood-stage *Plasmodium* infection [43]. Our study showed that IL-10 plays a pivotal role in inhibiting the protective immune response against secondary infection with heterologous parasites. When the protective memory immune response is strong enough, IL-10 may protect the host from pathology induced by an excessive immune response. We suspect, however, that when the host is infected with heterologous parasites, cross-reactive immunity may not be sufficiently powerful, and IL-10 may inhibit the protection. In malaria-endemic areas, multiple strains and species of *Plasmodium* parasites are present, and IL-10 may have a regulatory role in both protection and pathology, depending on the combination of the immune response and types of parasites. These features highlight the importance of IL-10 in balancing the protective and pathogenic immune responses and suggest that IL-10 may influence different outcomes, depending on the present parasite strains.

Acknowledgements

We thank Dr. R. Culleton providing Pcc and Dr. M. Yuda for providing PbA and PbA-GFP. We also thank Dr. K. Kimura for animal care.

Funding sources

This work was supported by grants-in-aid for scientific research from Japan Society for the Promotion of Science (16H05183) to K.Y.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.parint.2019.01.003>.

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