Parasitology International 74 (2020) 101994

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Activation and IL-10 production of specific CD4$^+$ T cells are regulated by IL-27 during chronic infection with *Plasmodium chabaudi*

Odsuren Sukhbaatar[a,b], Daisuke Kimura[a,1], Mana Miyakoda[a,2], Sayuri Nakamae[a,3], Kazumi Kimura[a], Hiromitsu Hara[c], Hiroki Yoshida[d], Shin-Ichi Inoue[a,b], Katsuyuki Yui[a,b,e,f,*]

[a] Division of Immunology, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
[b] Program for Nurturing Global Leaders in Tropical and Emerging Infectious Diseases, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
[c] Department of Immunology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8544, Japan
[d] Division of Molecular and Cellular Immunoscience, Department of Biomolecular Sciences, Faculty of Medicine, Saga University, 5-1-1 Nabeshima, Saga 849-8501, Japan
[e] School of Tropical Medicine and Global Health, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan
[f] Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan

ARTICLE INFO

Keywords:
Malaria
CD4$^+$ T cells
Cytokine
IL-27
IL-10
Protective immunity
Immunological memory

ABSTRACT

IL-27, a regulatory cytokine, plays critical roles in the prevention of immunopathology during *Plasmodium* infection. We examined these roles in the immune responses against *Plasmodium chabaudi* infection using the *Il-27ra*$^{-/-}$ mice. While IL-27 was expressed at high levels during the early phase of the infection, enhanced CD4$^+$ T cell function and reduction in parasitemia were observed mainly during the chronic phase in the mutant mice. In mice infected with *P. chabaudi* and cured with drug, CD4$^+$ T cells in the *Il-27ra*$^{-/-}$ mice exhibited enhanced CD4$^+$ T-cell responses, indicating the inhibitory role of IL-27 on the protective immune responses. To determine the role of IL-27 in detail, we performed CD4$^+$ T-cell transfer experiments. The *Il-27ra*$^{-/-}$ and *Il27p28*$^{-/-}$ mice were first infected with *P. chabaudi* and then cured using drug treatment. *Plasmodium*-antigen primed CD4$^+$ T cells were prepared from these mice and transferred into the recipient mice, followed by infection with the heterologous parasite *P. berghei* ANKA. *Il-27ra*$^{-/-}$ CD4$^+$ T cells in the infected recipient mice did not produce IL-10, indicating that IL-10 production by primed CD4$^+$ T cells is IL-27 dependent. *Il27p28*$^{-/-}$ CD4$^+$ T cells that were primed in the absence of IL-27 exhibited enhanced recall responses during the challenge infection with *P. berghei* ANKA, implying that IL-27 receptor signaling during the primary infection affects recall responses in the long-term via the regulation of the memory CD4$^+$ T cell generation. These features highlighted direct and time-transcending roles of IL-27 in the regulation of immune responses against chronic infection with *Plasmodium* parasites.

1. Introduction

Malaria is a life-threatening mosquito-borne infectious disease caused by parasites of *Plasmodium* species and is one of the crucial public health problems in the world. World Health Organization estimated that 219 million cases of malaria occurred worldwide, and 435,000 people mainly children less than five years old died due to malaria in 2017 [1,2]. Both cellular and humoral immune responses are essential to limit the replication of the parasites and control severity of the disease. In particular, CD4$^+$ T cells orchestrate protective immunity and produce IFN-γ, which activates macrophages and supports B cells to make antibodies against *Plasmodium* parasites [3–5]. CD4$^+$ T cells also

* Corresponding author at: Division of Immunology, Department of Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
E-mail address: katsu@nagasaki-u.ac.jp (K. Yui).
[1] Daisuke Kimura: Department of Health, Sports, and Nutrition, Faculty of Health and Welfare, Kobe Women's University, 4–7-2 Minatojima-nakamachi, Chuo-ku, Kobe, 650–0046, Japan.
[2] Mana Miyakoda: Research and Education Center for Drug Fostering and Evolution, School of Pharmaceutical Sciences, Nagasaki University, 1–14 Bunkyomachi, Nagasaki 852–8521, Japan.
[3] Sayuri Nakamae: Department of Immune Regulation, Shionogi Global Infectious Diseases Division, Institute of Tropical medicine, Nagasaki University, 1–12-4 Sakamoto, Nagasaki 852–8523, Japan.

https://doi.org/10.1016/j.parint.2019.101994
Received 3 July 2019; Received in revised form 5 August 2019; Accepted 1 October 2019
Available online 18 October 2019

produce macrophage colony-stimulating factor (M-CSF), which induce CD169$^+$ macrophages to control parasites [6].

While the immune response protects against infection with *Plasmodium* parasites, the excessive immunological reaction can contribute pathology and disease manifestation of the host [7]. The balance between pro- and anti-inflammatory responses is critical for the maintenance of the homeostasis while attacking the invading parasites. IL-10 is one of the major inhibitory cytokines that limit inflammatory responses during *Plasmodium* infection [8]. In mice deficient of IL-10, infection with *Plasmodium chabaudi chabaudi* (Pcc) leads to exacerbated pathological conditions accompanied by increased production of cytokines, including IFN-γ produced by CD4$^+$ T cells [9]. IL-10 also inhibits protective immune responses against secondary infection with heterologous *Plasmodium* parasites [10]. CD4$^+$ T cells are the major source of IL-10 that regulates immune responses to prevent pathology during *Plasmodium* infection, although many different cell types can also produce IL-10 [8,11]. These regulatory CD4$^+$ T cells (Tr1 cells) are Foxp3$^-$ and produce IL-10 sometimes in addition to IFN-γ in response to *Plasmodium* antigens to regulate antigen-specific immune responses [11,12]. In human, high production of IL-10 by *P. falciparum*-specific CD4$^+$ T cells correlated asymptomatic infection after malaria season in children living in Mali, suggesting that T-cell derived IL-10 may play a role in reducing the onset of clinical malaria [13]. Consistent with this study, the frequency of CD4$^+$ T cells producing IL-10 was associated with a decreased risk of clinical malaria once infected in Uganda, while the frequency of specific TNF-α-producing CD4$^+$ T cells was associated with increased risk of symptomatic malaria [14].

IL-27 is a heterodimeric cytokine of the IL-12 family, composed of Epstein-Barr virus-induced gene 3 (EBI3) and IL-27p28 subunits produced by macrophages and dendritic cells as well as CD4$^+$ T cells, and signals through the receptor composed of IL-27 receptor α-chain (WSX-1) and gp130 [15–17]. Accumulating evidence suggests that IL-27 is a regulatory cytokine acting on varieties of immune cells [16]. One of the major targets of IL-27 is CD4$^+$ T cells, in which IL-27 inhibits the production of IL-2 [18,19], promotes the production of IL-10 by effector CD4$^+$ T cells [20–22] and suppresses the development to Th17 cells [23]. The effects of IL-27 on *Plasmodium* infection have been studied using IL-27 receptor knock-out (*Il27ra*$^{-/-}$) mice. The *Il27ra*$^{-/-}$ mice were highly susceptible to infection with *P. berghei* NK65 with the development of exacerbated Th1-mediated immune responses leading to a severe liver pathology, although parasites were cleared efficiently [24]. IL-27 receptor signaling regulated CD4$^+$ T cell-intrinsic responsiveness to IL-12 to inhibit terminal differentiation of Th1 cells in a manner independent of IL-10 and Foxp3 [25]. In the *P. berghei* NK65-infected mice after treatment with an anti-*Plasmodium* drug, the composition of memory CD4$^+$ T-cell pool was only slightly different in *Il27ra*$^{-/-}$ mice from those in wild-type mice. However, the *Il27ra*$^{-/-}$ mice displayed enhanced parasite control against challenge infection with the homologous parasites [26]. In Pcc model, generation of IL-10$^+$IFN-γ$^+$ Th1 cells during the acute phase of the parasite infection was shown to be dependent on IL-27 receptor signaling [27]. These studies showed important roles of IL-27 receptor signaling in limiting pathogenic Th1-type immune responses and inducing the IL-10 production. However, its role in the protection and pathogenesis in the chronic model of *Plasmodium* infection has not been investigated.

Pcc is a rodent *Plasmodium* parasite that invades both normocytes and reticulocytes, and thus, considered as a model of human infection with *P. falciparum* [28]. The acute infection of C57BL/6 (B6) mice with Pcc results in rapid increase of the parasitemia reaching approximately 30% in 7–10 days. This acute phase is rapidly resolved by 20 days followed by chronic phase with a low to subpatent levels of parasitemia and occasional recrudescence for several weeks [29]. We previously showed that C57BL/6 (B6) mice infected with Pcc, followed by treatment with the anti-*Plasmodium* drug, exhibit resistance to the challenge infection with heterologous parasites, *P. berghei* ANKA (PbA) [10]. After the challenge infection with PbA, T cells produced high levels of IL-10 in response to *Plasmodium* antigen, which played a critical inhibitory role in the immune response. In this study, we investigated the role of IL-27 receptor signaling in a mouse model of chronic infection with Pcc. CD4$^+$ T cells from *Il27ra*$^{-/-}$ mice exhibited strong protective immune responses during the chronic phase of Pcc infection. We also investigated the immune responses after the drug therapy of Pcc-infected mice, and after the challenge infection with heterologous parasite PbA. This study suggested dual roles of IL-27 receptor signaling; the inhibitory role in the priming of *Plasmodium*-specific CD4$^+$ T cells resulting in the reduction in the recall responses and the promotion of IL-10 production by memory CD4$^+$ T cells during challenge infection with PbA.

2. Materials and methods

2.1. Mice and malaria infection

The *Il27ra*$^{-/-}$ and *Il27p28*$^{-/-}$ mice were described previously [17,30]. B6.SJL-*Ptpc* congenic mice were originally provided by Dr. Y. Takahama (Tokushima University, Japan). B6 and BALB/c mice were purchased from SLC (Shizuoka, Japan). The mice were maintained under specific pathogen-free conditions in the Laboratory Animal Center for Animal Research at Nagasaki University. All mice used in this study were at 6–13 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) were provided by Dr. M. Yuda (Mie University, Tsu, Japan) [31]. Pcc and PbA were inoculated to B6 and BALB/c mice, respectively, from the frozen stock before being used to infect experimental animals. For the infection with the parasites, mice were inoculated intraperitoneally (i.p) with parasitized red blood cells (5×10^4). The levels of parasitemia were evaluated by microscopic examination of standard thin blood smears stained with Diff-Quick staining kit (Sysmex, Kobe, Japan). For PbA-GFP infection, parasitemia was determined as the percentage of GFP$^+$ RBC with uninfected RBCs as a negative control using FACS Canto II (BD Biosciences, Franklin Lakes, NJ, USA). For elimination of Pcc after 12–13 days of the infection, mice were administered Chloroquine (10 μg/g body weight; Sigma-Aldrich, St. Louis, Mo, USA) i.p for 1 wk. and sulfadiazine (30 mg/L; Sigma-Aldrich) in drinking water for 2 wk., as previously described [32]. Mice were monitored every day after day 4 of the challenge infection, and clinical scores were defined by the presence of the pathological signs: ruffled fur, hunching, wobbly gait, limb paralysis, convulsions, and coma as previously described [33]. Each sign was given a score of 1. The body weight (g) was measured and the percent changes were calculated as (the weight on the day / the weight on day 0) × 100%.

2.2. ELISA for tissue cytokines

Spleen was weighed and suspended in ice-cold phosphate buffered saline (PBS) containing 0.1% Igepal CA-630 nonionic detergent (0.1%) (Sigma-Aldrich) and protease inhibitor cocktail (50 μL/10 mg tissue) (Sigma-Aldrich), as described previously [34,35]. After incubation on ice for 10 min, the tissue was homogenized and centrifuged at 1500 rpm at 4 °C for 5 min. The supernatant was aliquoted and stored at −20 °C for ELISA analysis. The levels of IFN-γ in the supernatant were determined by sandwich ELISA using a set of anti-IFN-γ (R4-6A2) and biotin-anti-IFN-γ (XMG1.2) mAbs, as previously described [32]. The levels of IL-10 (Ready SET Go! Kit, eBioscience, San Diego, CA, USA) and IL-27 (Mouse IL-27a Matched Antibody Pair kit, Abcam, Cambridge, UK) were determined using manufacturer's cytokine ELISA kits.

2.3. Flow cytometry

Spleen cells were prepared and erythrocytes were lysed with Gey's solution. Cells were stained with APCCy7- or APC-anti-CD4 (GK1.5), FITC- or PECy7-anti-CD3Ɛ (145-2C11), APCCy7-anti-CD45.1 (A20), BV605-anti-CD45.2 (104), FITC-anti-CD11a (M17/4), FITC-anti-CD44 (IM7), PE-anti-CD49d (R1–2), PE-anti-CD62L (MEL-14), PeCy7-anti-CXCR5 (L138D7), PE-anti-PD-1 (J43), PeCy7-anti-CD19 (eBio1D3), FITC-anti-B220 (RA3-6B2), APC-anti-CD95 (SA367H8), PE-anti-GL7 (GL7) mAbs or PE-streptavidin plus biotin-anti-CD62L (MEL-14) mAb for 30 min at 4 °C. All antibodies were purchased from Biolegend (San Diego, CA, USA), Tonbo Biosciences (San Diego, CA, USA.) or eBioscience. To exclude dead cells from the analysis, 7-aminoactinomycin D (7-AAD) was added prior to the analysis. Cells were analyzed using FACSCanto II or LSRFortessa X-20 cell analyzer (BD Biosciences) and with FlowJo software (Tree Star, Ashland, OR, USA). Intracellular cytokine staining was performed following the manufacturer's instructions (BD Biosciences) as described with slight modification [17]. Cells (3×10^6) were stimulated for 5 h with PMA (50 ng/mL) and ionomycin (1 μg/mL) in RPMI-1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal calf serum (FCS, 10%), L-glutamine (2 mM), penicillin/streptomycin, non-essential amino acids (0.1 mM), sodium pyruvate (1 mM) and 2-mercaptoethanol (5×10^{-5} M) with brefeldin added for the last 2 h. After adding the Fc receptor block (2.4G2), cells were stained with APC-anti-CD4 (GK1.5), APCCy7-anti-CD4 (GK1.5), PECy7- or PECy5-anti-CD3Ɛ (145-2C11), PECy7- or APCCy7-anti-CD45.1 (A20) or APC-anti-CD45.2 (104) mAbs, fixed, permeabilized, and stained with PE-anti-IL-10 (JES5–16E3) and AlexaFlour 488-anti-IFN-γ (XMG1.2) (BioLegend) mAb or their isotype controls and analyzed using FACSCanto II (BD Biosciences) and FlowJo software (Tree Star).

2.4. Cell culture and ELISA

$CD4^+$ T cells (> 95%) were prepared from the spleen using anti-CD4 IMag (BD Biosciences) according to manufacturer's instructions and were suspended in RPMI-1640 medium supplemented with 10% FCS, 2 mM glutamine, penicillin/streptomycin, 2-mercaptoethanol (5×10^{-5} M), non-essential amino acids (0.1 mM) and sodium pyruvate (1 mM). For antigen-specific responses, $CD4^+$ T cells (2×10^5) were cultured in a 96-well flat-bottomed plate 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 RBC) for 48 h, as previously described [31,32]. Dendritic cells were prepared from B6 spleen cells using anti-CD11c magnetic-activated cell sorting (MACS) microbeads and autoMACS (Miltenyi, Gladbach, Germany) following the manufacturer's instruction.

2.5. Measurement of the parasite-specific antibody levels

The levels of parasite-specific IgG1, IgG2b, IgG2c, and IgM antibodies in the serum were determined by ELISA, as previously described [10]. ELISA plates (Nunc MaxiSorp ®, Thermo Fisher Scientific) were coated with freeze-thaw lysate of PbA or Pcc (1×10^6 iRBC equivalent/well) in PBS for 2 h at room temperature, washed 3 times with PBS containing 0.2% Tween 20, and blocked with PBS containing 10% FCS and 0.2% Tween-20 for 30 min at room temperature. After washing, a serial dilution of serum (1/20–1/1280) was added. After overnight incubation at 4 °C, plates were washed 3 times and incubated with biotin-conjugated rabbit anti-mouse IgG1, IgG2b, IgM (ZyMED, San Francisco, CA, USA) or IgG2c (Bethyl Laboratories, Montgomery, TX, USA) antibodies for 1 h. The plates were then washed, and incubated with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) for 30 min. After washing, 4-nitrophenyl phosphate disodium salt hexahydrate (1 mg/mL) (Sigma-Aldrich) was added in each well, and the optical density (OD) was determined at 405 nm using an iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA).

2.6. Adoptive transfers

B6.Ly5.1, *Il27ra*$^{-/-}$ *or Il27p28*$^{-/-}$ mice were infected with Pcc, treated with chloroquine and sulfadiazine between 13 and 27 days after infection. Splenocytes were prepared after 31–49 days of infection, and erythrocytes were lysed using Gey's solution. For the $CD4^+$ T-cell transfer experiments, spleen cells from B6.Ly5.1 and *Il27ra*$^{-/-}$ mice were passed through LS column attached to MidiMACS separator (Miltenyi) to deplete hemozoin. $CD4^+$ T cells were prepared using anti-CD4 IMag (BD Biosciences). $CD4^+$ T cells ($4–7 \times 10^6$) from Pcc-primed B6.Ly5.1 mice were mixed with those from Pcc-primed *Il27ra*$^{-/-}$ or Pcc-primed *Il27p28*$^{-/-}$ mice at 1:1: ratio and transferred intravenously into (B6 × B6.Ly5.1) F_1 mice via the tail vein. The recipient mice were infected with PbA the next day, and their spleen cells were analyzed 7 days after the infection.

2.7. Statistical analysis

For the comparisons of two groups, two-tailed unpaired *t*-tests were used at the significance level of 0.05 ($P < .05$). The paired *t*-test was used to compare a pair of T cells co-transferred into the same mice. Differences in the survival was assessed by the log-rank test. When three or more groups were compared, an overall difference among the groups was determined using one-way ANOVA and two-way ANOVA for cytokine and specific antibody measurements, respectively. If the one-way or two-way ANOVA revealed significant differences, differences between individual groups were estimated using Bonferroni's test.

3. Results

3.1. Reduction of the parasitemia in IL-27ra$^{-/-}$ mice during the chronic phase of Pcc infection

Following infection of B6 mice with Pcc, the levels of IFN-γ, IL-10, and IL-27 in the spleen were determined by ELISA weekly after the infection. The concentration of IFN-γ and IL-27 increased in the spleen, which returned to the baseline level by day 15 and maintained after that (Fig. 1A). IL-10 concentration did not significantly increase during the acute phase and increased on day 29 of the infection, when the splenomegaly ceased. To determine the effects of IL-27 receptor signaling on *Plasmodium* infection, B6 and *Il27ra*$^{-/-}$ mice were infected with Pcc. The levels of parasitemia in *Il27ra*$^{-/-}$ mice were lower than B6 mice on day 12 and during the chronic phase of Pcc infection. The body weight of *Il27ra*$^{-/-}$ mice was lower than that of B6 mice on day 10 of the infection suggesting tissue damage (Fig. 1B), consistent with the previous study demonstrating the immune-mediated pathology and excessive-inflammatory immune response in *Il27ra*$^{-/-}$ mice [24]. We next analyzed $CD4^+$ T-cell immune responses in uninfected mice (day 0) and during acute (day 6) and chronic (day 35) phase of Pcc infection. While the proportions of $CD44^{hi}CD62L^{lo}$ and $CD11a^{hi}CD49d^{hi}$ $CD4^+$ T cells were slightly lower in the spleen of uninfected *Il27ra*$^{-/-}$ mice when compared with B6 mice, the number of $CD4^+$ T cells and their subpopulations were not significantly different during both acute and chronic infection (Fig. 1C, Fig. S1). In uninfected mice, basal level of IL-10 production by $CD4^+$ T cells from *Il27ra*$^{-/-}$ mice was slightly lower than those in B6 mice (Fig. 1D). On day 6 of the infection, production of IFN-γ, IL-10, and IL-27 by $CD4^+$ T cells from *Il27ra*$^{-/-}$ mice in response to Pcc antigens was not significantly different from those of B6 mice. However, 35 days after infection, $CD4^+$ T cells from *Il27ra*$^{-/-}$ mice produced higher levels of IFN-γ in response to Pcc antigens than those from B6 mice, although the levels of IL-10 and IL-27 production were not significantly different.

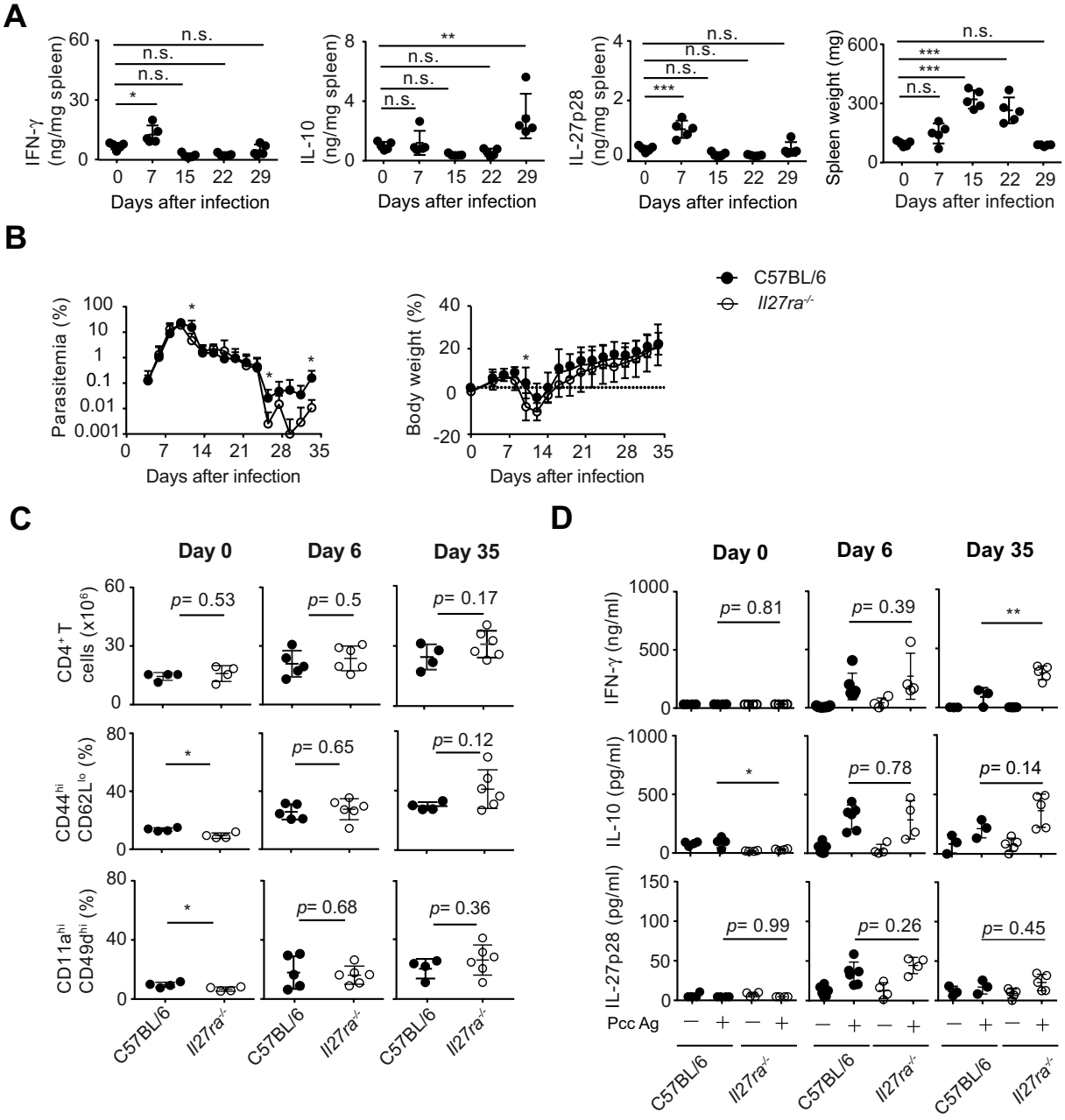

Fig. 1. Reduced parasite burden and enhanced CD4$^+$ T cell responses during the chronic phase of Pcc infection in the absence of IL-27 signaling. (A) B6 mice were infected with Pcc, and their spleens were collected at day 0, day 7 (parasitemia 5.48 ± 2.11%), day 15 (parasitemia 0.68 ± 0.69%), day 22 (parasitemia 0.17 ± 0.17%) and day 29 (parasitemia 0.016 ± 0.02%) (n = 5 mice/group) and weighed. The concentration of IFN-γ, IL-10, and IL-27p28 in the spleen lysate was determined by ELISA. Statistical analysis was performed by one-way ANOVA, followed by Bonferroni's test. *p < .05, **p < .01, ***p < .001; n.s., not significant. (B) B6 (●, n = 7) and *Il27ra*$^{-/-}$ (○, n = 8) mice were infected with Pcc, and the levels of parasitemia and body weight were monitored. The results are a pool of 3 experiments. Statistical analysis was performed by unpaired Student's t-test. *p < .05. (C) Spleen cells were stained for CD3/CD4, CD44/CD62L or CD11a/CD49d on day 0, 6 and 35 after infection. The CD4$^+$ T cell numbers and their proportions are shown. The data on day 0 are from one experiment, and on day 6 and 35 are a pool of four experiments with 1–2 mice/group. Statistical analysis was performed by unpaired Student's t-test. (D) Splenic CD4$^+$ T cells were stimulated with Pcc antigens in the presence of dendritic cells for 48 h, on day 0, 6 and 35 of the infection. The levels of IFN-γ, IL-10, and IL-27p28 in the supernatant were determined by ELISA. The results of day 6 and 35 are a pool of 3 experiments with 1–2 mice/group at each time point. Statistical analysis was performed by unpaired Student's t-test. *p < .05, **p < .01. Means ± SDs are shown by bars.

3.2. Efficient generation of the memory CD4$^+$ T cells in the absence of IL-27R signaling

The difference in the immune response between B6 and *Il27ra*$^{-/-}$ mice could be due to the difference in the parasite load in vivo. To exclude the effect of differential parasitemia levels, mice were treated with the anti-*Plasmodium* drug between 12 and 26 days after Pcc infection, and the immune response was determined 1–3 wk. later (Fig. 2A). The number of CD4$^+$ T cells in Pcc-primed *Il27ra*$^{-/-}$ mice was higher than those in B6 mice, although their subpopulations were not significantly different (Fig. 2B, Fig. S3A). In intracellular cytokine staining, the proportion IFN-γ$^+$ CD4$^+$ T cells from Pcc-primed *Il27ra*$^{-/-}$ mice exhibited a tendency to be higher than those from B6 mice, although it was statistically not significant, and the mean fluorescence

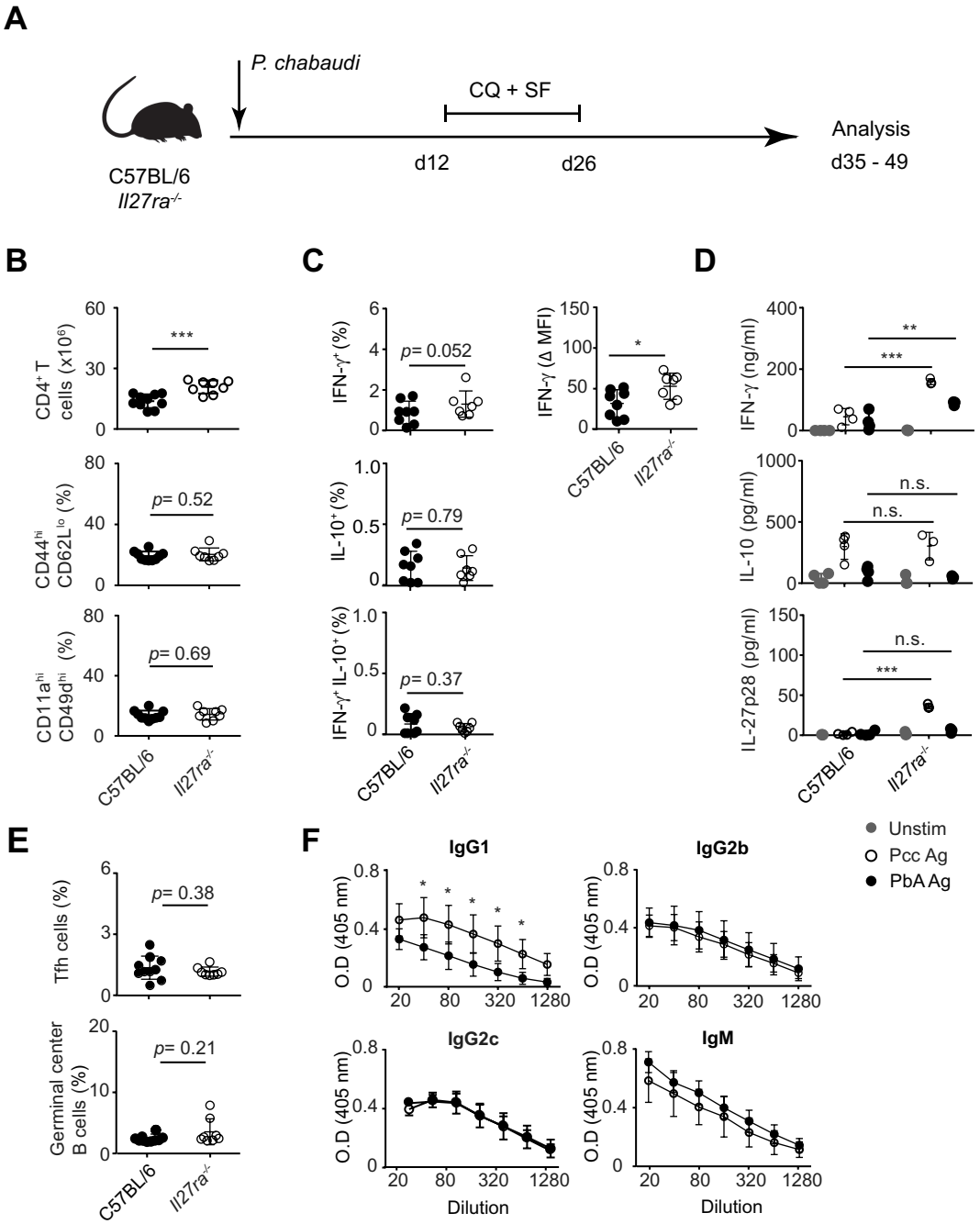

Fig. 2. Increased memory CD4$^+$ T cell responses in the absence of IL-27 signaling. B6 (●) and *Il27ra*$^{-/-}$ (○) mice were infected with Pcc, treated with chloroquine and sulfadiazine between 12 and 26 days after the infection. After additional 9–23 days, spleen cells were stained for CD3/CD4 and CD44/CD62L or CD11a/CD49d. (A) Experimental scheme. (B) Total numbers of CD4$^+$ T cells and the proportions of their subpopulations. Results are a pool of 4 experiments with 1–4 mice/group. Statistical analysis was performed by unpaired Student's *t*-test. ***$p < .001$. (C) Spleen cells from Pcc-primed B6 or *Il27ra*$^{-/-}$ mice were stimulated with PMA and ionomycin, stained for CD3/CD4, fixed/permeabilized, stained for IFN-γ/IL-10, and analyzed using flow cytometry. Proportions of cells expressing IFN-γ$^+$, IL-10, and both IFN-γ and IL-10 are shown. Fluorescence intensity (ΔMFI) of IFN-γ staining in IFN-γ$^+$CD4$^+$ T cells are shown. The results are a pool of 3 experiments with 2–3 mice/group. Statistical analysis was performed by unpaired Student's *t*-test. *$p < .05$. (D) IFN-γ, IL-10 and IL-27 levels in the supernatant of CD4$^+$ T cells from Pcc-primed B6 and *Il27ra*$^{-/-}$ mice were determined by ELISA after culture in the presence of dendritic cells without (●) or with Pcc (○) or PbA (●) antigens. Data are a pool of 2 experiments with 1–2 mice/group. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni's test. **$p < .01$, ***$p < .001$; n.s., not significant. (E) Proportions of Tfh (CXCR5$^+$PD-1$^+$CD3$^+$CD4$^+$) in CD4$^+$CD3$^+$ T cells and germinal center B cells (CD95$^+$GL7$^+$CD19$^+$B220$^+$) in B220$^+$CD19$^+$ B cells in the spleen of Pcc-primed B6 and *Il27ra*$^{-/-}$ mice. Results are a pool of 4 experiments with 1–4 mice/group. Significance was tested by unpaired Student's *t*-test. (F) Serum was collected from the Pcc-infected mice 18–23 days after anti-malarial drug treatment. The levels of Pcc-specific IgG1, IgG2b, IgG2c and IgM in the serum were determined by ELISA. Results are a pool of 3 experiments with 2–3 mice/group. Statistical significance was performed by a two-way ANOVA, followed by Bonferroni's test. *$p < .05$. Means ± SDs are shown by bars.

intensity (ΔMFI) of IFN-γ staining was significantly higher, suggesting the enhancement of IFN-γ-producing CD4$^+$ T cells in the absence of IL-27 receptor signaling (Fig. 2C, Figs. S2, S3C, S4A). The proportion of IL-10$^+$CD4$^+$ T cells were, however, not significantly different. We also determined cytokine productions by CD4$^+$ T cells in response to Pcc as well as heterologous PbA antigens. CD4$^+$ T cells from Pcc-primed

Il27ra$^{-/-}$ mice produced IFN-γ and IL-27 in response to Pcc at levels higher than those from B6 mice, while the levels of IL-10 production were not significantly different (Fig. 2D). In response to the heterologous PbA antigens, CD4$^+$ T cells from Pcc-primed *Il27ra*$^{-/-}$ mice produced IFN-γ, but not IL-10 or IL-27, at levels higher than those in B6 mice (Fig. 2D). We also evaluated humoral immune responses. The proportions of Tfh and germinal center B cells were not significantly different (Fig. 2E, Figs. S3A, S4B). However, serum levels of anti-Pcc IgG1 antibodies in Pcc-primed *Il27ra*$^{-/-}$ mice were higher than those in B6, whereas those of IgG2b, IgG2c (IgG2c in C57BL/6 mice corresponds to IgG2a in BALB/c mice [36]), and IgM antibodies were not significantly different (Fig. 2F, Fig. S4C). This finding was unexpected since CD4$^+$ T cells from *Il27ra*$^{-/-}$ mice exhibited enhanced production of IFN-γ implying the Th1 type immune response. However, IL-27 receptor is expressed on B cells and its direct signaling induces T-bet expression and IgG2a class switching in BALB/c B cells [37]. Therefore, it is plausible that IgG1 class switching was promoted in the absence of IL-27 receptor signaling in B cells.

3.3. Enhanced immune response of Pcc-primed *Il27ra*$^{-/-}$ mice against heterologous PbA parasite

We used an infection model of heterologous PbA to evaluate the recall response of Pcc-primed B6 and *Il27ra*$^{-/-}$ mice, as described previously (Fig. 3A) [10]. Naïve *Il27ra*$^{-/-}$ mice develop experimental cerebral malaria similar to B6 mice and succumb to death within 10 days of the infection with PbA (Fig. S5). When Pcc-primed mice were challenged with PbA, the levels of parasitemia in *Il27ra*$^{-/-}$ mice were less than those in B6 mice. However, Pcc-primed *Il27ra*$^{-/-}$ mice showed a more severe reduction in the body weight, higher clinical scores and earlier death when compared with B6 mice (Fig. 3B). Immune responses were examined 7 days after the challenge infection. Pcc-primed *Il27ra*$^{-/-}$ mice exhibited higher proportions of CD11a^{hi}CD49d^{hi}CD4$^+$ T cells expressing surrogate markers of activated CD4$^+$ T cells in the spleen when compared to the Pcc-primed B6 mice, suggesting the increase in the proportion of *Plasmodium*-specific CD4$^+$ T cells. However, the total number of splenic CD4$^+$ T cells and the proportion of effector memory CD44^{hi}CD62L^{lo}CD4$^+$ T cells were not significantly different (Fig. 3C, Fig. S3B). Staining of intracellular cytokine revealed that the number of IFN-γ$^+$CD4$^+$ T cells was higher in Pcc-primed *Il27ra*$^{-/-}$ mice than Pcc-primed B6 mice, whereas the number of IL-10$^+$ or IFN-γ$^+$IL-10$^+$CD4$^+$ T cells was reduced (Fig. 3D, Fig. S3D). Consistently, CD4$^+$ T cells from Pcc-primed *Il27ra*$^{-/-}$ mice produced higher levels of IFN-γ and IL-27 in response to PbA as well as Pcc antigens when compared with that from B6 mice. In contrast, CD4$^+$ T cells produced lower levels of IL-10 in Pcc-primed *Il27ra*$^{-/-}$ mice than in B6 mice in response to both Pcc and PbA antigens (Fig. 3E). The proportions of Tfh and germinal center B cells were not significantly different (Fig. 3F, Fig. S3B). The levels of PbA-specific IgG1 antibodies in *Il27ra*$^{-/-}$ mice were higher than those in B6, although those of IgG2b, IgG2c, and IgM antibodies were not significantly different (Fig. 3G). Taken together, these studies showed that recall response of memory CD4$^+$ T cells to the heterologous PbA infection was upregulated in *Il27ra*$^{-/-}$ mice with increased IFN-γ and reduced IL-10 production, culminating in the reduction in the levels of parasitemia accompanied by the enhancement of the immune-mediated tissue damage leading to earlier death in *Il27ra*$^{-/-}$ mice when compared with B6 mice.

3.4. Critical role of IL-27 signaling in CD4$^+$ T cells during challenge infection

To determine whether the enhanced immune response in *Il27ra*$^{-/-}$ mice after the challenge infection with PbA was a direct effect of IL-27 on CD4$^+$ T cells or mediated indirectly by the reduced production of IL-10 in other cells, we performed adoptive transfer experiments. CD4$^+$ T cells prepared from Pcc-primed B6 and *Il27ra*$^{-/-}$ mice showed similar phenotype (Fig. S6A, B) and were adoptively transferred at 1:1 ratio into the same mice. These recipient mice were infected with PbA, and their CD4$^+$ T cells were analyzed 7 days after infection (Fig. 4A). In mice infected with PbA, the number of *Il27ra*$^{-/-}$CD4$^+$ T cells, and the proportions of CD44^{hi}CD62L^{lo} and CD11a^{hi}CD49d^{hi}*Il27ra*$^{-/-}$ CD4$^+$ T cells were not significantly different from the co-transferred B6 CD4$^+$ T cells (Fig. 4B, C). The frequencies of IFN-γ producing CD4$^+$ T cells were also not significantly different, but the proportions of *Il27ra*$^{-/-}$ CD4$^+$ T cells, which produced IL-10 or both IL-10 and IFN-γ, were lower than those of B6 CD4$^+$ T cells in the infected mice (Fig. 4D, E). These results suggest that the increase in the proportions of activated CD4$^+$ T cells and IFN-γ-producing CD4$^+$ T cells are not directly linked to the IL-27 receptor signaling in the transferred host, implying the involvement of other cytokines such as IL-10 in the inhibition of T cell responses during the challenge infection. However, IL-27 signaling in antigen-specific memory CD4$^+$ T cells is required to promote their IL-10 production.

IL-27 receptor signaling may modify the immune response during the priming of Pcc infection or the challenge infection with PbA. To determine IL-27 receptor signaling that is critical for the modulation of CD4$^+$ T cell responses, in particular, the increase in IL-10 production, we performed another cell transfer experiment. B6 and *Il27p28*$^{-/-}$ mice were infected with Pcc and cured by the drug treatment. Both mice exhibited similar levels of parasitemia, and CD4$^+$ T cells from these mice showed similar phenotype (Fig. S6C, D) CD4$^+$ T cells were prepared from Pcc-primed *Il27p28*$^{-/-}$ mice, in which they were primed in IL-27-defective environment, and were co-transferred at 1:1 ratio with CD4$^+$ T cells from Pcc-primed B6.Ly5.1 mice into the recipient mice, which were infected with PbA and CD4$^+$ T cells examined after 7 days (Fig. 5A). The proportions of *Il27p28*$^{-/-}$ CD4$^+$ T cells and the frequencies of CD44^{hi}CD62L^{lo}CD4$^+$ cells were higher than those in B6 CD4$^+$ T cells in the infected mice, although those of CD11a^{hi}CD49d^{hi} populations were not significantly different (Fig. 5B, C). The frequencies of *Il27p28*$^{-/-}$ CD4$^+$ T cells which produced IFN-γ was higher than those in B6.Ly5.1 CD4$^+$ T cells, while those producing IL-10 or both IFN-γ and IL-10 were not significantly different (Fig. 5D, E). These findings suggest that CD4$^+$ T cell-priming under the IL-27-defective environment leads an effective generation of memory CD4$^+$ T cells during the Pcc infection. However, those producing IL-10 were not significantly different between *Il27p28*$^{-/-}$ and B6 CD4$^+$ T cells, implying that the IL-27-deficit during priming did not affect the production of IL-10 during the challenge infection with PbA.

4. Discussion

In this study, the role of IL-27 receptor signaling on the immune responses was evaluated during a chronic infection model of Pcc in the *IL-27* receptor gene knock-out mice. High levels of IL-27 production was detected in the spleen during the early phase of Pcc infection when the parasite burden was high. Although in the early phase, the parasite burden was similar, T cells showed similar phenotype and cytokine production between Pcc-infected *Il27ra*$^{-/-}$ and control mice, the significant differences were observed during the chronic phase of the infection in both parasite burden and IFN-γ production by CD4$^+$ T cells. Furthermore, Pcc-primed *Il27ra*$^{-/-}$ mice exhibited enhanced protection against *Plasmodium* parasites and their CD4$^+$ T cells produced excessive IFN-γ suggesting the inhibitory role of IL-27 on the protective immune responses; these studies are in line with the previous studies reported on mice infected with *P. berghei* NK65 [24–26]. However, antigen-specific production of IL-10 by CD4$^+$ T cells was reduced in Pcc-infected *Il27ra*$^{-/-}$ mice after the challenge infection with PbA, which was also consistent with the previous study showing that IL-10 production in CD4$^+$ T cells is dependent on IL-27 during the primary responses to Pcc infection [27].

To determine the role of IL-27 receptor signaling in the recall response of the primed mice, we set up an adoptive transfer of CD4$^+$ T

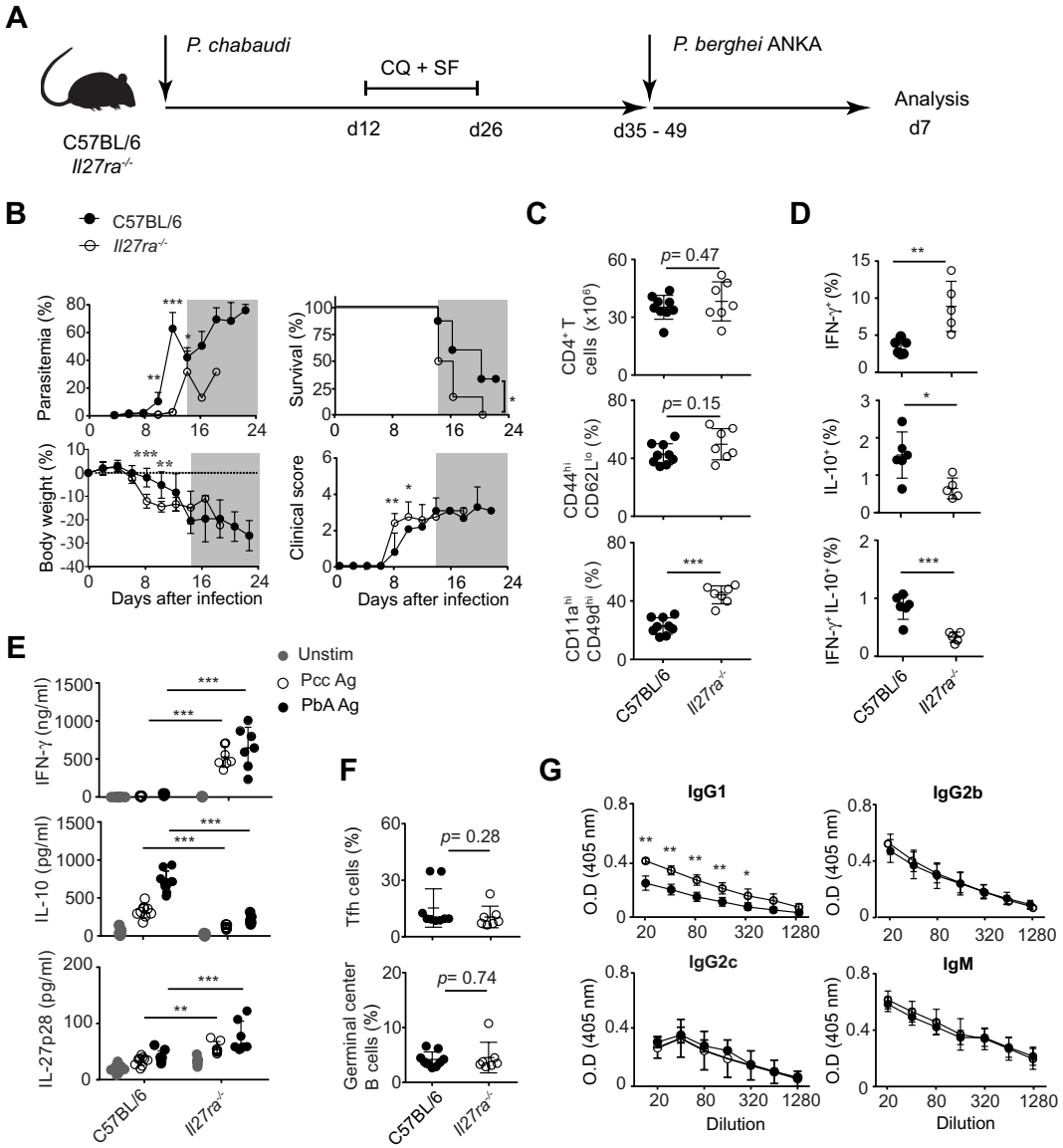

Fig. 3. Pcc-primed *Il27ra*$^{-/-}$ mice displayed enhanced parasite control and immune response against challenge infection with PbA. B6 (●) and *Il27ra*$^{-/-}$ (○) mice were infected with Pcc, treated with chloroquine and sulfadiazine between 12 and 26 days after the infection, and were challenged with PbA-GFP 9–23 days after the treatment. (A) Experimental scheme. (B) Parasitemia, survival rate, body weight changes, and clinical scores were monitored on every 2 days after PbA infection. Gray shadow indicates the data with reduced sample number due to the death of the mice. Results are a pool of 3 experiments with 2–3 mice/group. Statistical analysis of parasitemia, body weight and clinical score was performed by unpaired Student's *t*-test. $*p < .05$, $**p < .01$, $***p < .001$. Differences in the survival was assessed by the log-rank test. $*p < .05$. (C) Seven days after PbA infection, spleen cells were stained for CD3/CD4 and CD44/CD62L or CD11a/CD49d. Total numbers of CD4$^+$ T cells and the proportions of their subpopulations are shown. Results are a pool of 4 experiments with 1–3 mice/group. Statistical analysis was performed by unpaired Student's *t*-test. $***p < .001$. (D) Spleen cells from Pcc-primed B6 or *Il27ra*$^{-/-}$ mice were stimulated with PMA/ionomycin, surface stained for CD3/CD4, fixed/permeabilized and stained for IFN-γ/IL-10, and analyzed using flow cytometry. Summary of the proportions of IFN-γ$^+$, IL-10$^+$ and IFN-γ$^+$IL-10$^+$CD4$^+$ T cells is shown. Results are a pool of 4 experiments with 1–3 mice/group. Statistical analysis was performed by unpaired Student's *t*-test. $*p < .05$, $**p < .01$, $***p < .001$. (E) IFN-γ, IL-10, and IL-27 levels in the supernatant of splenic CD4$^+$ T cells from Pcc-primed B6 and *Il27ra*$^{-/-}$ mice were determined by ELISA after culture in the presence of dendritic cells without (●) or with Pcc (○) or PbA (●) antigens. Results are a pool of 3 experiments with 1–2 mice. Statistical analysis was performed by two-way ANOVA, followed by Bonferroni's test. $**p < .01$, $***p < .001$. (F) Proportions of Tfh (CXCR5$^+$PD-1$^+$CD3$^+$CD4$^+$) in CD4$^+$ T cells and germinal center B cells (CD95$^+$GL7$^+$CD19$^+$B220$^+$) in B cells (CD19$^+$B220$^+$) in the spleen of Pcc-primed B6 and *Il27ra*$^{-/-}$ mice. Results are a pool of 4 experiments with 1–3 mice/group. Significance was tested by unpaired Student's *t*-test. (G) Serum was collected from the mice on day 7 after PbA infection. The levels of PbA-specific IgG1, IgG2b, IgG2c, and IgM in the serum were determined by ELISA. Results are a pool of 3 experiments with 1–3 mice/group. Significance was tested by a two-way ANOVA followed by Bonferroni's test. $*p < .05$, $**p < .01$. Means ± SDs are shown by bars.

cells from mice lacking the IL27 receptor. *Il27ra*$^{-/-}$ mice were infected with Pcc and cured with drug treatment. CD4$^+$ T cells from the mutant and control mice were mixed and transferred to the recipient mice, followed by the challenge infection with PbA. We found no significant differences in cell number, activation in phenotype and IFN-γ production between co-transferred Pcc-primed *Il27ra*$^{-/-}$ and control CD4$^+$ T cells in the recipient mice after challenge infection with PbA, although *Il27ra*$^{-/-}$ CD4$^+$ T cells were expected to show enhanced activation as reported in the previous study [26]. One possibility is that the lack of IL-27 receptor signaling in *Il27ra*$^{-/-}$ mice did not have a significant effect on the clonal expansion of PbA-reactive CD4$^+$ T cells in the primary response to Pcc. Alternatively, the increase in the activated

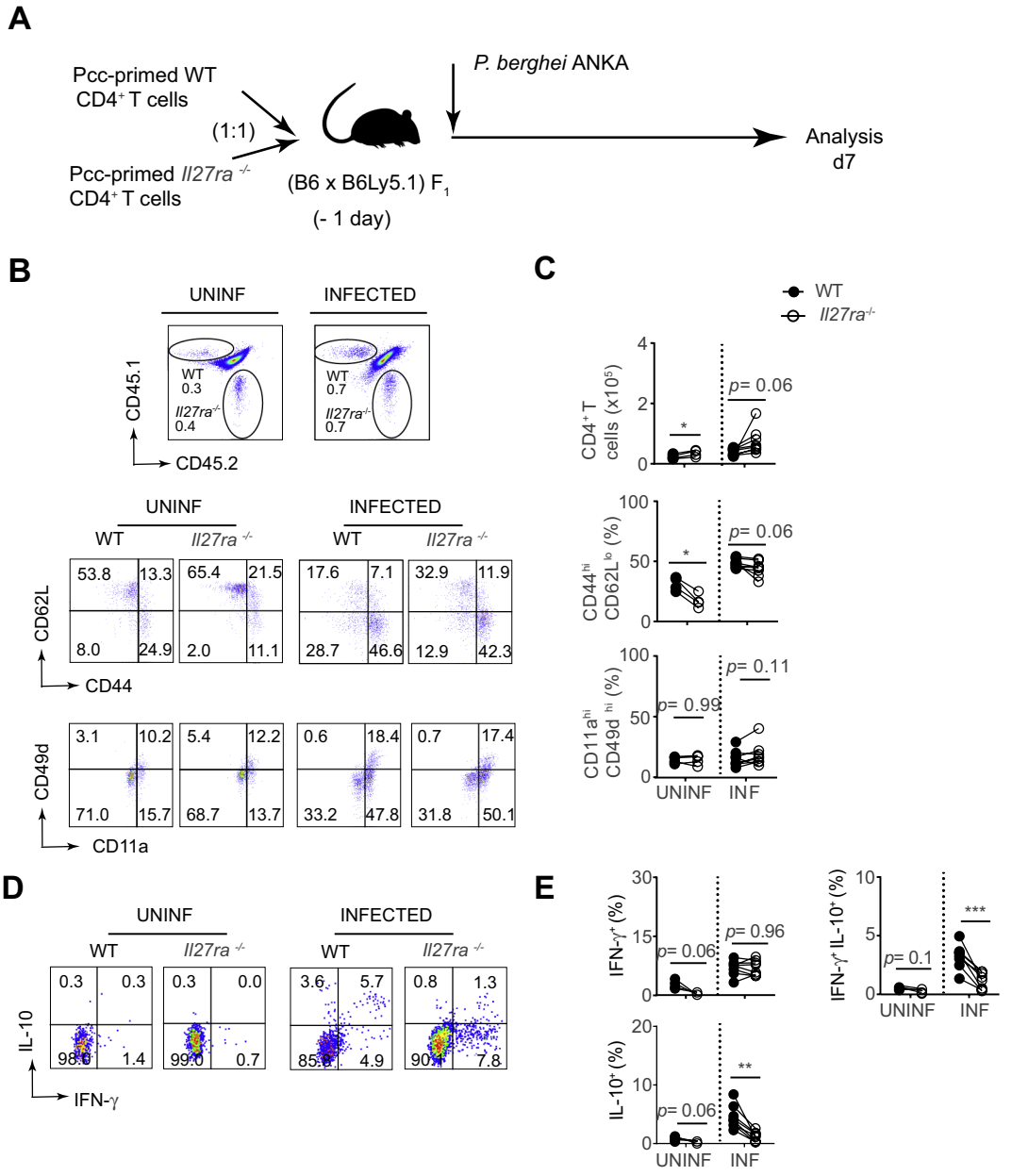

Fig. 4. IL-10 production by *Plasmodium*-specific CD4$^+$ T cells in the secondary response is dependent on IL-27 receptor signaling. B6.Ly5.1 (CD45.1$^+$/2$^-$) and *Il27ra*$^{-/-}$ (CD45.1$^-$/2$^+$) mice were infected with Pcc and treated with chloroquine and sulfadiazine between 12 and 26 days after the infection. Fourteen to twenty-one days later, CD4$^+$ T cells (4–5 × 10^6) were prepared from B6.Ly5.1 (●) and *Il27ra*$^{-/-}$ (○) mice, mixed at a 1:1 ratio, and transferred into (B6 × B6.Ly5.1) F$_1$ (CD45.1$^+$/2$^+$) mice. These mice were infected with PbA on the following day and were analyzed on day 7 of the infection (parasitemia 15–18%). (A) Experimental scheme. (B) CD45.1 and CD45.2 profiles of CD4$^+$ T cells in mice infected and uninfected with PbA for 7 days (upper panel). The profiles of CD44/CD62L and CD11a/CD49d on the gated B6.Ly5.1 (CD45.1$^+$CD45.2$^-$) and *Il27ra*$^{-/-}$ (CD45.1$^-$CD45.2$^+$) CD4$^+$ cells in the recipient's spleen from uninfected and infected mice. Numbers in the quadrants represent proportions (%) of cells. (C) Summary of the total number of CD4$^+$ T cell and the proportions of their subpopulations from B6.Ly5.1 and *Il27ra*$^{-/-}$ mice. The values for Pcc-primed B6.Ly5.1 and Pcc-primed *Il27ra*$^{-/-}$ CD4$^+$ T cells in the same mice are connected with lines. (D) Spleen cells from the recipient (B6 × B6.Ly5.1) F$_1$ mice were stimulated with PMA/ionomycin, surface stained for CD3, CD4, CD45.1, and CD45.2 and fixed/permeabilized, stained for IFN-γ/IL-10, and analyzed by flow cytometry. Numbers in the quadrants represent proportions (%) of cells. (E) Summary of IFN-γ$^+$ (including both IL-10$^+$ and IL-10$^-$), IL-10$^+$ (including both IFN-γ$^+$ and IFN-γ$^-$), and IFN-γ$^+$IL-10$^+$ CD4$^+$ T cells. Results are a pool of 2 experiments with 3–5 mice. Significance was tested by paired *t*-test. **p* < .05, ***p* < .01, ****p* < .001.

Il27ra$^{-/-}$ CD4$^+$ T cells during the primary Pcc infection did not affect their recall response of CD4$^+$ T cells, perhaps because the maintenance of the memory or the secondary response of the PbA-reactive CD4$^+$ T cells requires IL-27 receptor signaling. Despite these unexpected observations, it was clear that IL-10 production by CD4$^+$ T cells in the secondary immune response was dependent on IL-27 receptor signaling. The study showed a critical role of IL-27 signaling in the production of IL-10 by primed CD4$^+$ T cells during the secondary infection with PbA.

CD4$^+$ T cells receive activation signals during the primary infection with Pcc and the challenge infection with PbA. To evaluate the effects of IL-27 receptor signaling on these two activation events, we set up a second model of CD4$^+$ T cell transfer using mice lacking the IL-27. The *Il27p28*$^{-/-}$ mice were primed with Pcc infection and then cured, and CD4$^+$ T cells were transferred to the recipient mice followed by infection with PbA. Under the condition employed, *Il27p28*$^{-/-}$ CD4$^+$ T cells did not receive IL-27 receptor signaling during the priming but

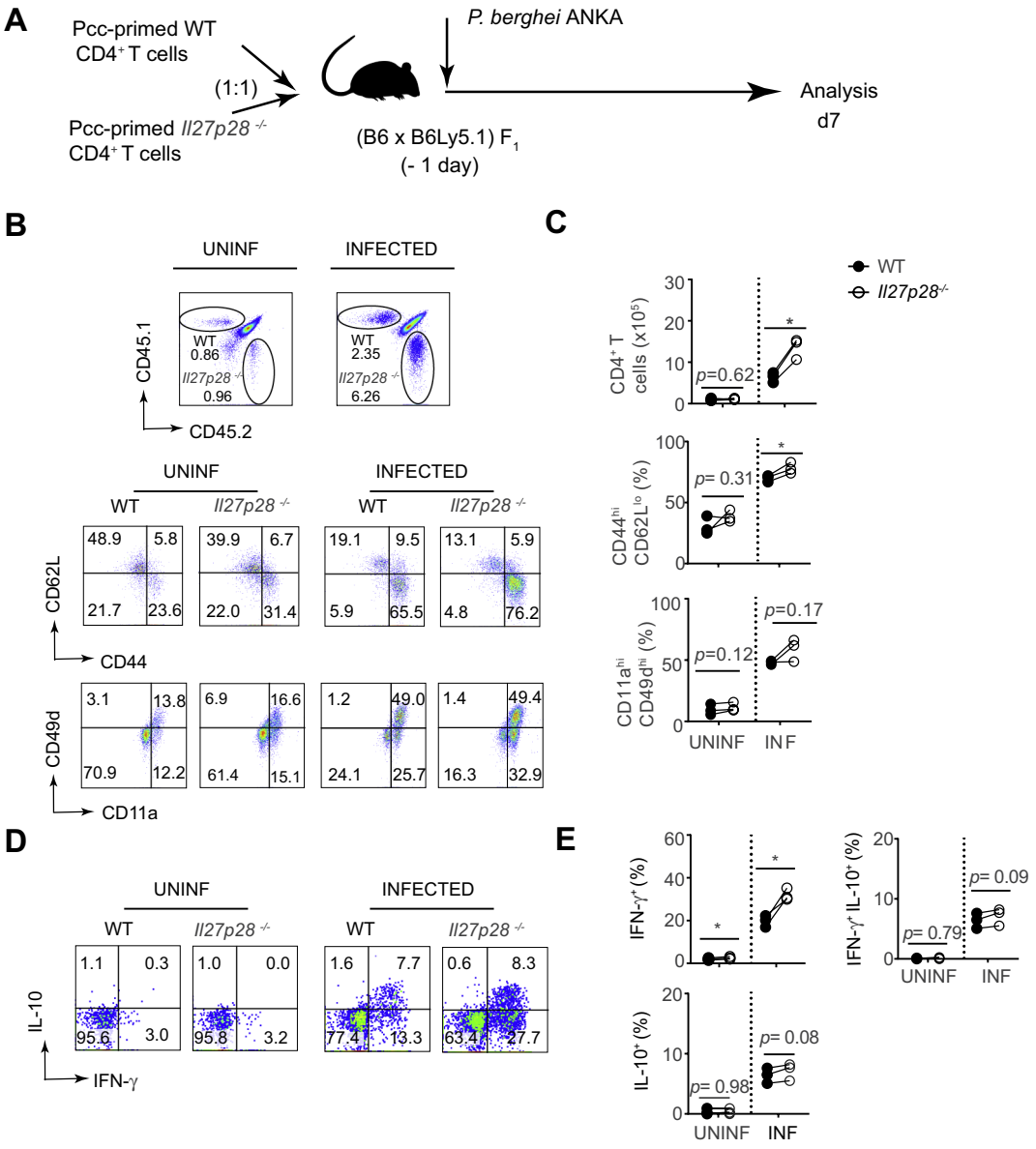

Fig. 5. IL-27 acts directly on *Plasmodium*-specific $CD4^+$ T cells. B6.Ly5.1 ($CD45.1^+/2^-$) and *Il27p28*$^{-/-}$ ($CD45.1^-/2^+$) mice were infected with Pcc and treated with chloroquine and sulfadiazine between 13 and 27 days after the infection. Ten to fifteen days later, $CD4^+$ T cells (7×10^6) were prepared from B6.Ly5.1 (●) and *Il27p28*$^{-/-}$ (○) mice, mixed at a 1:1 ratio, and transferred into (B6 × B6.Ly5.1) F_1 mice. Seven days after infection with PbA (parasitemia 5.6–10.5%), the experiments were performed. (A) Experimental scheme. (B) CD45.1 and CD45.2 profiles of $CD4^+$ T cells in mice infected and uninfected with PbA for 7 days (upper panel). The proportions of CD44/CD62L and CD11a/CD49d in $CD4^+$-gated spleen cells from uninfected and infected mice (lower panel). Numbers in the quadrants represent proportions (%) of cells. (C) Summary of the total number of $CD4^+$ T cell and the proportions of their subpopulations from B6.Ly5.1 and *Il27p28*$^{-/-}$ mice. The values for Pcc-primed B6.Ly5.1 and Pcc-primed *Il27p28*$^{-/-}$ $CD4^+$ T cells in the same mice are connected with lines (3 mice/group). Significance was tested by paired *t*-test. *$p < .05$ (D) Spleen cells from recipient were stimulated with PMA/ionomycin, surface stained for CD3, CD4, CD45.1 and CD45.2, fixed/permeabilized, stained for IFN-γ/IL-10, and analyzed by flow cytometry. Numbers in the quadrants represent proportions (%) of cells. (E) Summary of IFN-γ^+ (including both IL-10^+ and IL-10^-), IL-10^+ (including both IFN-γ^+ and IFN-γ^-), and IFN-γ^+IL-$10^+$$CD4^+$ T cells. The values for $CD4^+$ T cells in the same mice are connected with lines. Results are representative of 2 experiments with similar results. Significance was tested by paired *t*-test. *$p < .05$.

received it during the challenge infection. The *Il27p28*$^-$ $CD4^+$ T cells exhibited an increase in cell number, enhanced activation of phenotypes, and an increase in the proportion of IFN-γ-producing cells when compared with control $CD4^+$ T cells. The enhanced activation of *Il27p28*$^{-/-}$ $CD4^+$ T cells is perhaps due to the increase in the generation of memory $CD4^+$ T cells in the primary response. The difference in the recall response between the primed *Il27ra*$^{-/-}$ and *Il27p28*$^{-/-}$ $CD4^+$ T cells in the transferred host mice in response to PbA-infection suggests a decisive role of IL-27 in the activation and expansion of the primed $CD4^+$ T cells during the challenge infection. Alternatively, the difference in the cell type that expresses the IL-27 receptor and IL-27 might underlie the differential effects of these molecules on the immune responses. One caveat to these interpretations is that the IL-27 might not be a single ligand of the IL-27 receptor and vice versa. IL-27p28 is a component of both IL-27, a dimer formed by p28 and EBI3 subunits, and IL-30, formed by p28 alone [38–40]. It is possible that IL-30, lacking in *Il27p28*$^{-/-}$ mice, signals through the receptor distinct from IL-27 receptor [41], and is responsible for the differential responses of *Il27ra*$^{-/-}$ and *Il27p28*$^{-/-}$ $CD4^+$ T cells in the transferred host mice. Further, detailed experiments are required to determine these possibilities.

In contrast to IFN-γ, IL-10 production by *Il27p28*$^{-/-}$ $CD4^+$ T cells

in the recipient mice was not significantly different from the control wild-type $CD4^+$ T cells. The direct stimulation through the IL-27 receptor is likely to be required for the production of IL-10 by primed $CD4^+$ T cells during the secondary infection with PbA. These results are consistent with studies of *Plasmodium* infection using the IL-10 and IFN-γ reporter mice, which showed that $CD4^+$ T cells producing IL-10 are short-lived after the clearance of the parasites and that the ability to produce IL-10 is less stably maintained [42]. In addition, a cohort study of *P. falciparum* exposure to asymptomatic children in Mali suggested that IL-10 production by $CD4^+$ T cells is dependent on the exposure to *P. falciparum* [13].

These studies revealed the short-term and long-term effects of IL-27 on the host immune responses. In short-term, IL-27 directly regulates immune responses through the induction of IL-10 producing $CD4^+$ T cells, whereas, in long-term, it regulates the immune responses through inhibition of the generation of memory-type $CD4^+$ T cells, which is consistent with a previous study [26]. The long-term effect may explain why the lack of IL-27-receptor signaling became apparent in the chronic phase of Pcc infection, although IL-27 production was most abundant during the early period of the infection in the spleen. An alternative possibility is the source of IL-27 from different cell types, including macrophages, dendritic cells, and T cells during *Plasmodium* infection at a different time and location [16,17]. These cells may differentially regulate the activation and function of antigen-specific $CD4^+$ T cells. Nonetheless, these possibilities should be addressed in more detail in future studies.

Regulatory cytokines, including IL-10 and IL-27, play critical roles in the balance between pro- and anti-inflammatory responses during infection with *Plasmodium* parasites. Our study highlights the direct effect of IL-27 on memory $CD4^+$ T cells to produce IL-10 during recall responses against *Plasmodium* infection and the long-term impacts of IL-27 on $CD4^+$ T cell immune responses. Further studies will define details of mechanisms underlying these time transcending effects of the regulatory cytokines on the immune responses, which is critical for the development of effective vaccine and therapy of malaria.

Funding sources

This work was supported by grants-in-aid for scientific research from Japan Society for the Promotion of Science (16H05183), The Uehara Memorial Foundation and The Naito Foundation to K.Y. and Takeda Science Foundation to D.K.

Acknowledgments

We thank Dr. R. Culleton for providing Pcc and Dr. M. Yuda for providing PbA and PbA-GFP. We also thank M. Yoshida and N. Kawamoto for technical help. We would like to thank Editage for English language editing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.parint.2019.101994.

References

[1] World Health Organization, World Malaria Report 2018, (2018).
[2] E.A. Ashley, A. Pyae Phyo, C.J. Woodrow, Malaria, Lancet 391 (10130) (2018) 1608–1621.
[3] J. Langhorne, F.M. Ndungu, A.M. Sponaas, K. Marsh, Immunity to malaria: more questions than answers, Nat. Immunol. 9 (7) (2008) 725–732.
[4] J.C. Hafalla, O. Silvie, K. Matuschewski, Cell biology and immunology of malaria, Immunol. Rev. 240 (1) (2011) 297–316.
[5] S.P. Kurup, N.S. Butler, J.T. Harty, T cell-mediated immunity to malaria, Nat. Rev. Immunol. 19 (7) (2019) 457–471.
[6] M.F. Fontana, G.L. de Melo, C. Anidi, R. Hamburger, C.Y. Kim, S.Y. Lee, J. Pham, C.C. Kim, Macrophage colony stimulating factor derived from $CD4^+$ T cells contributes to control of a blood-borne infection, PLoS Pathog. 12 (12) (2016) e1006046.
[7] L. Schofield, G.E. Grau, Immunological processes in malaria pathogenesis, Nat. Rev. Immunol. 5 (9) (2005) 722–735.
[8] A.P. Freitas do Rosario, J. Langhorne, T cell-derived IL-10 and its impact on the regulation of host responses during malaria, Int. J. Parasitol. 42 (6) (2012) 549–555.
[9] C. Li, I. Corraliza, J. Langhorne, A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi chabaudi* infection in mice, Infect. Immun. 67 (9) (1999) 4435–4442.
[10] S. Nakamae, D. Kimura, M. Miyakoda, O. Sukhbaatar, S.I. Inoue, K. Yui, Role of IL-10 in inhibiting protective immune responses against infection with heterologous *Plasmodium* parasites, Parasitol. Int. 70 (2019) 5–15.
[11] K.N. Couper, D.G. Blount, M.S. Wilson, J.C. Hafalla, Y. Belkaid, M. Kamanaka, R.A. Flavell, J.B. de Souza, E.M. Riley, IL-10 from $CD4^+CD25^-Foxp3^-CD127^-$ adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection, PLoS Pathog. 4 (2) (2008) e1000004.
[12] M.G. Roncarolo, S. Gregori, R. Bacchetta, M. Battaglia, N. Gagliani, The biology of T regulatory type 1 cells and their therapeutic application in immune-mediated diseases, Immunity 49 (6) (2018) 1004–1019.
[13] S. Portugal, J. Moebius, J. Skinner, S. Doumbo, D. Doumtabe, Y. Kone, S. Dia, K. Kanakabandi, D.E. Sturdevant, K. Virtaneva, S.F. Porcella, S. Li, O.K. Doumbo, K. Kayentao, A. Ongoiba, B. Traore, P.D. Crompton, Exposure-dependent control of malaria-induced inflammation in children, PLoS Pathog. 10 (4) (2014) e1004079.
[14] M.J. Boyle, P. Jagannathan, K. Bowen, T.I. McIntyre, H.M. Vance, L.A. Farrington, A. Schwartz, F. Nankya, K. Naluwu, S. Wamala, E. Sikyomu, J. Rek, B. Greenhouse, E. Arinaitwe, G. Dorsey, M.R. Kamya, M.E. Feeney, The development of *Plasmodium falciparum*-specific IL10 CD4 T cells and protection from malaria in children in an area of high malaria transmission, Front. Immunol. 8 (2017) 1329.
[15] S. Pflanz, J.C. Timans, J. Cheung, R. Rosales, H. Kanzler, J. Gilbert, L. Hibbert, T. Churakova, M. Travis, E. Vaisberg, W.M. Blumenschein, J.D. Mattson, J.L. Wagner, W. To, S. Zurawski, T.K. McClanahan, D.M. Gorman, J.F. Bazan, R. de Waal Malefyt, D. Rennick, R.A. Kastelein, IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive $CD4^+$ T cells, Immunity 16 (6) (2002) 779–790.
[16] H. Yoshida, C.A. Hunter, The immunobiology of interleukin-27, Annu. Rev. Immunol. 33 (2015) 417–443.
[17] D. Kimura, M. Miyakoda, K. Kimura, K. Honma, H. Hara, H. Yoshida, K. Yui, Interleukin-27-producing $CD4^+$ T cells regulate protective immunity during malaria parasite infection, Immunity 44 (3) (2016) 672–682.
[18] T. Owaki, M. Asakawa, S. Kamiya, K. Takeda, F. Fukai, J. Mizuguchi, T. Yoshimoto, IL-27 suppresses CD28-mediated IL-2 production through suppressor of cytokine signaling 3, J. Immunol. 176 (5) (2006) 2773–2780.
[19] A.V. Villarino, J.S. Stumhofer, C.J. Saris, R.A. Kastelein, F.J. de Sauvage, C.A. Hunter, IL-27 limits IL-2 production during Th1 differentiation, J. Immunol. 176 (1) (2006) 237–247.
[20] A. Awasthi, Y. Carrier, J.P. Peron, E. Bettelli, M. Kamanaka, R.A. Flavell, V.K. Kuchroo, M. Oukka, H.L. Weiner, A dominant function for interleukin 27 in generating interleukin 10-producing anti-inflammatory T cells, Nat. Immunol. 8 (12) (2007) 1380–1389.
[21] J.S. Stumhofer, J.S. Silver, A. Laurence, P.M. Porrett, T.H. Harris, L.A. Turka, M. Ernst, C.J. Saris, J.J. O'Shea, C.A. Hunter, Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10, Nat. Immunol. 8 (12) (2007) 1363–1371.
[22] D.C. Fitzgerald, G.X. Zhang, M. El-Behi, Z. Fonseca-Kelly, H. Li, S. Yu, C.J. Saris, B. Gran, B. Ciric, A. Rostami, Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells, Nat. Immunol. 8 (12) (2007) 1372–1379.
[23] J.S. Stumhofer, A. Laurence, E.H. Wilson, E. Huang, C.M. Tato, L.M. Johnson, A.V. Villarino, Q. Huang, A. Yoshimura, D. Sehy, C.J. Saris, J.J. O'Shea, L. Hennighausen, M. Ernst, C.A. Hunter, Interleukin 27 negatively regulates the development of interleukin 17-producing T helper cells during chronic inflammation of the central nervous system, Nat. Immunol. 7 (9) (2006) 937–945.
[24] E.G. Findlay, R. Greig, J.S. Stumhofer, J.C. Hafalla, J.B. de Souza, C.J. Saris, C.A. Hunter, E.M. Riley, K.N. Couper, Essential role for IL-27 receptor signaling in prevention of Th1-mediated immunopathology during malaria infection, J. Immunol. 185 (4) (2010) 2482–2492.
[25] A. Villegas-Mendez, J.B. de Souza, S.W. Lavelle, E. Gwyer Findlay, T.N. Shaw, N. van Rooijen, C.J. Saris, C.A. Hunter, E.M. Riley, K.N. Couper, IL-27 receptor signalling restricts the formation of pathogenic, terminally differentiated Th1 cells during malaria infection by repressing IL-12 dependent signals, PLoS Pathog. 9 (4) (2013) e1003293.
[26] E. Gwyer Findlay, A. Villegas-Mendez, N. O'Regan, J.B. de Souza, L.M. Grady, C.J. Saris, E.M. Riley, K.N. Couper, IL-27 receptor signaling regulates memory $CD4^+$ T cell populations and suppresses rapid inflammatory responses during secondary malaria infection, Infect. Immun. 82 (1) (2014) 10–20.
[27] A.P. Freitas do Rosario, T. Lamb, P. Spence, R. Stephens, A. Lang, A. Roers, W. Muller, A. O'Garra, J. Langhorne, IL-27 promotes IL-10 production by effector Th1 $CD4^+$ T cells: a critical mechanism for protection from severe immunopathology during malaria infection, J. Immunol. 188 (3) (2012) 1178–1190.
[28] R. Stephens, R.L. Culleton, T.J. Lamb, The contribution of *Plasmodium chabaudi* to our understanding of malaria, Trends Parasitol. 28 (2) (2012) 73–82.
[29] A.H. Achtman, R. Stephens, E.T. Cadman, V. Harrison, J. Langhorne, Malaria-specific antibody responses and parasite persistence after infection of mice with *Plasmodium chabaudi chabaudi*, Parasite Immunol. 29 (9) (2007) 435–444.
[30] H. Yoshida, S. Hamano, G. Senaldi, T. Covey, R. Faggioni, S. Mu, M. Xia,

A.C. Wakeham, H. Nishina, J. Potter, C.J. Saris, T.W. Mak, WSX-1 is required for the initiation of Th1 responses and resistance to *L. major* infection, Immunity 15 (4) (2001) 569–578.

[31] T. Tamura, K. Kimura, M. Yuda, K. Yui, Prevention of experimental cerebral malaria by Flt3 ligand during infection with *Plasmodium berghei* ANKA, Infect. Immun. 79 (10) (2011) 3947–3956.

[32] M. Miyakoda, D. Kimura, K. Honma, K. Kimura, M. Yuda, K. Yui, Development of memory $CD8^+$ T cells and their recall responses during blood-stage infection with *Plasmodium berghei* ANKA, J. Immunol. 189 (9) (2012) 4396–4404.

[33] A. Haque, S.E. Best, F.H. Amante, A. Ammerdorffer, F. de Labastida, T. Pereira, G.A. Ramm, C.R. Engwerda, High parasite burdens cause liver damage in mice following *Plasmodium berghei* ANKA infection independently of $CD8^+$ T cell-mediated immune pathology, Infect. Immun. 79 (5) (2011) 1882–1888.

[34] S. Rosengren, G.S. Firestein, D.L. Boyle, Measurement of inflammatory biomarkers in synovial tissue extracts by enzyme-linked immunosorbent assay, Clin. Diagn. Lab. Immunol. 10 (6) (2003) 1002–1010.

[35] K.Z. Matalka, M.F. Tutunji, M. Abu-Baker, Y. Abu Baker, Measurement of protein cytokines in tissue extracts by enzyme-linked immunosorbent assays: application to lipopolysaccharide-induced differential milieu of cytokines, Neuro Endocrinol. Lett. 26 (3) (2005) 231–236.

[36] Z. Zhang, T. Goldschmidt, H. Salter, Possible allelic structure of IgG2a and IgG2c in mice, Mol. Immunol. 50 (3) (2012) 169–171.

[37] T. Yoshimoto, K. Okada, N. Morishima, S. Kamiya, T. Owaki, M. Asakawa, Y. Iwakura, F. Fukai, J. Mizuguchi, Induction of IgG2a class switching in B cells by IL-27, J. Immunol. 173 (4) (2004) 2479–2485.

[38] S. Crabe, A. Guay-Giroux, A.J. Tormo, D. Duluc, R. Lissilaa, F. Guilhot, U. Mavoungou-Bigouagou, F. Lefouili, I. Cognet, W. Ferlin, G. Elson, P. Jeannin, J.F. Gauchat, The IL-27 p28 subunit binds cytokine-like factor 1 to form a cytokine regulating NK and T cell activities requiring IL-6R for signaling, J. Immunol. 183 (12) (2009) 7692–7702.

[39] O. Shimozato, A. Sato, K. Kawamura, M. Chiyo, G. Ma, Q. Li, M. Tagawa, The secreted form of p28 subunit of interleukin (IL)-27 inhibits biological functions of IL-27 and suppresses anti-allogeneic immune responses, Immunology 128 (1 Suppl) (2009) e816–e825.

[40] J.S. Stumhofer, E.D. Tait, W.J. Quinn, N. Hosken 3rd, B. Spudy, R. Goenka, C.A. Fielding, A.C. O'Hara, Y. Chen, M.L. Jones, C.J. Saris, S. Rose-John, D.J. Cua, S.A. Jones, M.M. Elloso, J. Grotzinger, M.P. Cancro, S.D. Levin, C.A. Hunter, A role for IL-27p28 as an antagonist of gp130-mediated signaling, Nat. Immunol. 11 (12) (2010) 1119–1126.

[41] C. Garbers, B. Spudy, S. Aparicio-Siegmund, G.H. Waetzig, J. Sommer, C. Holscher, S. Rose-John, J. Grotzinger, I. Lorenzen, J. Scheller, An interleukin-6 receptor-dependent molecular switch mediates signal transduction of the IL-27 cytokine subunit p28 (IL-30) via a gp130 protein receptor homodimer, J. Biol. Chem. 288 (6) (2013) 4346–4354.

[42] A. Villegas-Mendez, C.A. Inkson, T.N. Shaw, P. Strangward, K.N. Couper, Long-lived $CD4^+IFN\text{-}\gamma^+$ T cells rather than short-lived $CD4^+IFN\text{-}\gamma^+IL\text{-}10^+$ T cells initiate rapid IL-10 production to suppress anamnestic T cell responses during secondary malaria infection, J. Immunol. 197 (8) (2016) 3152–3164.