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CD4 T Cell–Dependent and –Independent Roles for IFN-γ in Blood-Stage Malaria

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Production of IFN-γ by CD4 T cells is widely theorized to control *Plasmodium* parasite burden during blood-stage malaria. Surprisingly, the specific and crucial mechanisms through which this highly pleiotropic cytokine acts to confer protection against malarial disease remain largely untested in vivo. Here we used a CD4 T cell–restricted Cre-Lox IFN-γ excision mouse model to test whether and how CD4 T cell–derived IFN-γ controls blood-stage malaria. Although complete absence of IFN-γ compromised control of the acute and the chronic, recrudescent blood-stage infections with *P. c. chabaudi*, we identified a specific, albeit modest, role for CD4 T cell–derived IFN-γ in limiting parasite burden only during the chronic stages of *P. c. chabaudi* malaria. CD4 T cell IFN-γ promoted IgG Ab class switching to the IgG2c isotype during *P. c. chabaudi* malaria in C57BL/6 mice. Unexpectedly, our data do not support gross defects in phagocytic activity in IFN-γ-deficient hosts infected with blood-stage malaria. Together, our data confirm CD4 T cell–dependent roles for IFN-γ but suggest CD4 T cell–independent roles for IFN-γ in immune responses to blood-stage malaria. *The Journal of Immunology*, **2023, 210: 1305–1313.**

A highly effective malaria vaccine would greatly reduce the formidable toll malaria currently imposes on global health (1). However, the ability to strategically manipulate key determinants of immunity to the *Plasmodium* infections that cause malaria in the form of an efficacious vaccine remains frustratingly elusive.

The inflammatory cytokine IFN-γ has been widely implicated in control of both the asymptomatic liver stage and the morbidity-driving blood stage of malaria (2). Herein, we focus on the blood stage of malaria, during which *Plasmodium* parasites cause disease by replicating within and then destroying RBCs. Accordingly, control of parasite burden during blood-stage malaria requires identification and removal of *Plasmodium*-infected RBCs and/or extracellular parasites transiting between host RBCs. A crucial role for IFN-γ in suppressing blood-stage parasite replication has been clearly demonstrated in murine models, where elevated parasitemia was seen in IFN-γ-deficient *Ifng*$^{-/-}$ mice (3, 4), in receptor-deficient *Ifngr*$^{-/-}$ mice (5, 6), or upon Ab-mediated IFN-γ neutralization (7).

CD4 T cells are also required for clearing parasitemia during blood-stage malaria in mice (8, 9). Because IFN-γ production is the hallmark of Th1-type CD4 T cells (10), Th1 cells emerged as obvious candidates for producing protective IFN-γ during blood-stage malaria. Early work posited a biphasic model where Th1-type CD4 T cells suppress early parasitemia, and later emerging Th2-type CD4 T cells reduce chronic parasitemia (11). Later work, however, revealed a more complex and plastic CD4 T cell response and that T follicular helper (Tfh) cells rather than Th2 cells likely control parasitemia during chronic blood-stage malaria (11–14).

Despite the clear complexity of the CD4 T cell response to blood-stage malaria, a crucial role for CD4 T cell–derived IFN-γ in controlling blood-stage parasite burden has remained a largely untested assumption. Indirect evidence supporting this model includes studies in humans that identified IFN-γ-producing CD4 T cells as correlating with effective antimalarial immunity (15, 16). In mice, CD4 T cells clearly produce IFN-γ during blood-stage malaria, with plasma IFN-γ content markedly reduced upon depletion of CD4 T cells (17). However, numerous leukocytes are competent IFN-γ producers during blood-stage malaria, including NK cells, γδ T cells, and CD8 T cells (18, 19). To date, the most direct test of the theorized role for CD4 T cell–derived IFN-γ was a singular study where reconstitution of CD4 T cell–depleted mice with an exogenously raised IFN-γ-producing Th1-type CD4 T cell clone facilitated parasite clearance (20). Whether the endogenous CD4 T cell compartment mediates control of parasite burden in an IFN-γ-dependent manner remained untested.

Here we sought to directly test if CD4 T cell–derived IFN-γ is crucial for controlling parasite burden during blood-stage murine malaria. To this end, we generated transgenic CD4CreER^{T2} *Ifng*$^{flox/KO}$ (for short, CD4Cre*Ifng)* mice in which a tamoxifen-responsive CreER^{T2} protein is driven by CD4 promoter elements (21) with a Cre-excisable *Ifng*$^{flox/KO}$ locus. This results in a mouse in which tamoxifen-inducible excision of *Ifng* is restricted to mature CD4 T cells. Unlike prior studies that nonspecifically abolished all IFN-γ signaling, our system minimizes off-target effects by only curtailing IFN-γ production in CD4 T cells during a limited temporal window.

We selected the *Plasmodium chabaudi chabaudi* CB isolate (PccCB) model of murine malaria to interrogate the role of CD4 T cell–derived IFN-γ in controlling blood-stage malaria, because Pcc malaria is the only rodent system that recapitulates both the acute and chronic blood-stage infections that are seen in humans. In the PccCB model, acute malaria comprises an initial wave of high parasitemia that lasts ~1 wk, which is followed by a chronic infection where low-grade recrudescent parasitemia persists for months (22). Additional advantages of the *P. c. chabaudi* model also include a robust circadian synchrony of parasitized RBC lysis and sequestration

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Abbreviations used in this article: d7pi, day 7 postinfection; PccAS, *Plasmodium chabaudi chabaudi* AS isolate; PccCB, *Plasmodium chabaudi chabaudi* CB isolate; Tfh, T follicular helper.

of parasitized RBCs into endothelial vessels—both features of human malaria theorized to be relevant to pathology (22–24).

In this study, we show that ablating CD4 T cell IFN-γ production had no discernible impact on controlling acute PccCB parasitemia and only moderately exacerbated parasite burden during chronic PccCB infection. Excision of the *Ifng*$^{flox/KO}$ locus resulted in a marked reduction of CD4 T cells competent to produce IFN-γ that persisted for weeks and translated into significantly reduced serum IFN-γ levels at the peak of CD4 T cell activation (day 7 postinfection [d7pi]). Compromised control of chronic parasitemia in the CD4 T cell IFN-γ-ablated mice was not associated with reduced numbers or diminished phagocytic capacity of myeloid cell subsets. Instead, loss of CD4 T cell–derived IFN-γ was paired with reduced class switching of parasite-specific Abs to the IgG2c isotype, suggesting that reduced control of chronic parasitemia could be driven by functional impairments in the quality of the Ab response. In concert, our results highlight the importance of IFN-γ-independent CD4 T cell effector mechanisms and alternative sources of IFN-γ in controlling parasite burden during primary episodes of blood-stage malaria in mice.

Materials and Methods

Mice and tamoxifen treatment

Female C57BL6 (B6) mice and *Ifng*$^{-/-}$ mice on the B6 background (25) were sourced from The Jackson Laboratory. *Ifng*$^{-/-}$ mice were maintained in-house at the University of Iowa. *Ifng*$^{flox/wt}$ mice were produced at the University of Iowa Genome Editing Facility using a CRISPR knock-in strategy (26) (guides: TAAACTCAACAAAGCTGACG, CATGCTGGGTAAATGCACTG) to introduce LoxP flanking sites to B6SJLF1/J × B6 (The Jackson Laboratory) embryos. After backcrossing of H-2 $^{b/b}$ founders to B6, *Ifng*$^{flox/wt}$ mice were intercrossed to produce *Ifng*$^{flox/flox}$ progeny. The CD4 T cell–specific inducible IFN-γ ablation mice were generated by crossing *Ifng*$^{flox/flox}$ mice to B6.CD4CreER^{T2} mice (The Jackson Laboratory) (21), producing litters of CD4CreER$^{T2+/-}$ *Ifng*$^{flox/flox}$ mice, which were then crossed with *Ifng*$^{-/-}$ mice to yield the final CD4CreER^{T2} *Ifng*$^{flox/KO}$ mice, hereafter called CD4Cre*Ifng*. The MHC haplotype of the final CD4Cre*Ifn* mice was determined to be H-2$^{b/b}$. The breeding scheme produces litters of CD4CreER^{T2}-positive and -negative mice. In all experiments with CD4Cre*Ifng* mice, Cre^{+} and Cre^{-} littermates were identified and compared with each other, and genotypes were confirmed by PCR. Tamoxifen (Sigma-Aldrich) treatments were performed by i.p. injection of tamoxifen suspended in corn oil and 10% ethanol. Three doses of 4 mg were administered in the week prior to infection (days −7, −5, and −3 relative to infection). Tamoxifen-induced excision was confirmed by intracellular cytokine staining of either peripheral blood or spleen samples after PMA/ionomycin stimulation for all experiments.

Infections

All infections were performed by i.v. injection at the retro-orbital sinus. For PccCB or *P. c. chabaudi* AS (PccAS) experiments, mice were infected with 1×10^5 PccCB-infected RBCs freshly harvested from BALB/c donors that were previously infected with cryopreserved stabilities. PccCB and PccAS were obtained from Patrick Duffy (National Institute of Allergy and Infectious Diseases). For *P. yoelii* 17XNL (Py17XNL) experiments, mice were injected i.v. with 1×10^6 cryopreserved, infected RBCs. Py 17XNL was obtained from BEI and maintained as cryopreserved stabilities produced from a C57BL/6 mouse infected from sporozoites (27). *ActA-Listeria monocytogenes* strain DP-L1942 was a gift from Dan Portnoy (University of California, Berkeley) (28) and was maintained as previously described (25). For i.v. challenge, 1×10^7 CFU were used (25).

Parasitemia and anemia

Parasitemia and anemia were assessed by flow cytometry on glutaraldehyde-fixed peripheral blood as previously described (29), with infected RBCs defined as Hoechst^{+}/CD45^{-}/Ter-119^{+} cells.

Intracellular cytokine staining

Splenocytes were harvested and stimulated with 5 μg/ml PMA, 5 μg/ml ionomycin, and 5 μg/ml brefeldin A, followed by staining using the FoxP3 transcription factor kit (Tonbo) for permeabilization.

Table I. Intracellular cytokine stain and Tfh stain

Marker	Clone	Fluorochrome	Manufacturer
CD4	RM4-5	Allophycocyanin/eFluor 780	eBioscience
CD8	53-6.7	PE/Dazzle-594	BioLegend
CD11a	M17/4	Brilliant Violet 510	BD Biosciences
CD44	IM7	RedFluor 710	Tonbo
PD1	RMP10-30	PE/Cy7	BioLegend
CXCR4	L128D7	Brilliant Violet 785	BioLegend
IFN-γ	XMG1.2	PE	Tonbo
TNF-α	MP6-XT22	PerCP/eFluor 710	eBioscience

IFN-γ ELISA

Serum IFN-γ concentrations were determined in duplicate technical replicates using the IFN-γ Femto High Sensitivity Mouse Uncoated ELISA kit (Thermo Fisher).

Parasite endpoint titer ELISA

PccCB-specific endpoint Ab titers were determined via ELISA by probing sera samples against Nunc MaxiSorp plates coated with 5 μg/mL crude parasite Ag prepared as a lysate from infected peripheral blood as previously described (30). HRP-conjugated IgG and IgG2c Abs (Novus) were used for detection. Samples were processed in technical duplicate, and sigmodal 4PL curve fit was used to calculate endpoint titers relative to naive sera.

Phagocytosis and myeloid population assay

Organs were collected 20 min after i.v. injection of 9×10^9 Fluoresbrite BB carboxylate microspheres (0.5-μm diameter; Polysciences). Splenocytes were prepared by homogenization over 70-μm filters. Livers were homogenized via GentleMACS (Miltenyi Biotec), followed by 30-min 37°C digestion in Liver Digest Buffer (Life Technologies), filtration through 100-μm filters, and removal of debris with 35% Percoll gradient. RBCs were removed with Vitalyse (CMDG).

Flow cytometry

Samples were run on an LSR Fortessa flow cytometer (BD Biosciences). Staining panels are outlined in Tables I, II, and III.

Statistics

All statistical analysis was performed in GraphPad Prism using paired *t* tests to compare two groups. ANOVA was used to compare multiple groups, with normally distributed data analyzed by Welch's ANOVA with the Brown-Forsythe test and non-normally distributed data analyzed by ANOVA with the Kruskal-Wallis posttest. Statistically significant ($p < 0.05$) *p* values are indicated.

Results

Ablation of IFN-γ production by CD4 T cells

To specifically investigate the role of CD4 T cell–derived IFN-γ in controlling blood-stage malaria, we created a transgenic CD4Cre*Ifng* mouse model (21) where IFN-γ production can be inducibly ablated in mature CD4 T cells. In this system, C57BL/6 CD4CreER^{T2} *Ifng*$^{flox/KO}$ mice (hereafter CD4Cre*Ifng*) harbor a single floxed *Ifng* allele that can be efficiently excised via tamoxifen-inducible activity of CreERT2 expressed from the CD4 promoter.

To assess the extent to which CD4 IFN-γ production is reduced in our CD4Cre*Ifng* mice, we measured IFN-γ production in splenocytes harvested at d24pi with PccCB (Table I). Because MHC class II epitopes recognized by substantial populations of CD4 T cells have not been identified for blood-stage murine malaria, we focused our analysis on dually CD11a^{hi}CD44^{hi} Ag-experienced cells (27) as a proxy for CD4 T cells responding to PccCB (Fig. 1A) and an alternative CD11a^{hi}CD8a^{int} expression pattern for the identification of Ag-experienced CD8 T cells (31). We observed a robust 14-fold decrease in the proportion of Ag-experienced CD4 T cells producing IFN-γ in Cre^{+} versus Cre^{-} littermates, with no significant reductions in TNF-α production by CD4 T cells or IFN-γ production by CD8 T cells (Fig. 1B–1D). Although Cre activity in these mice is overwhelmingly restricted to mature CD4 T cells

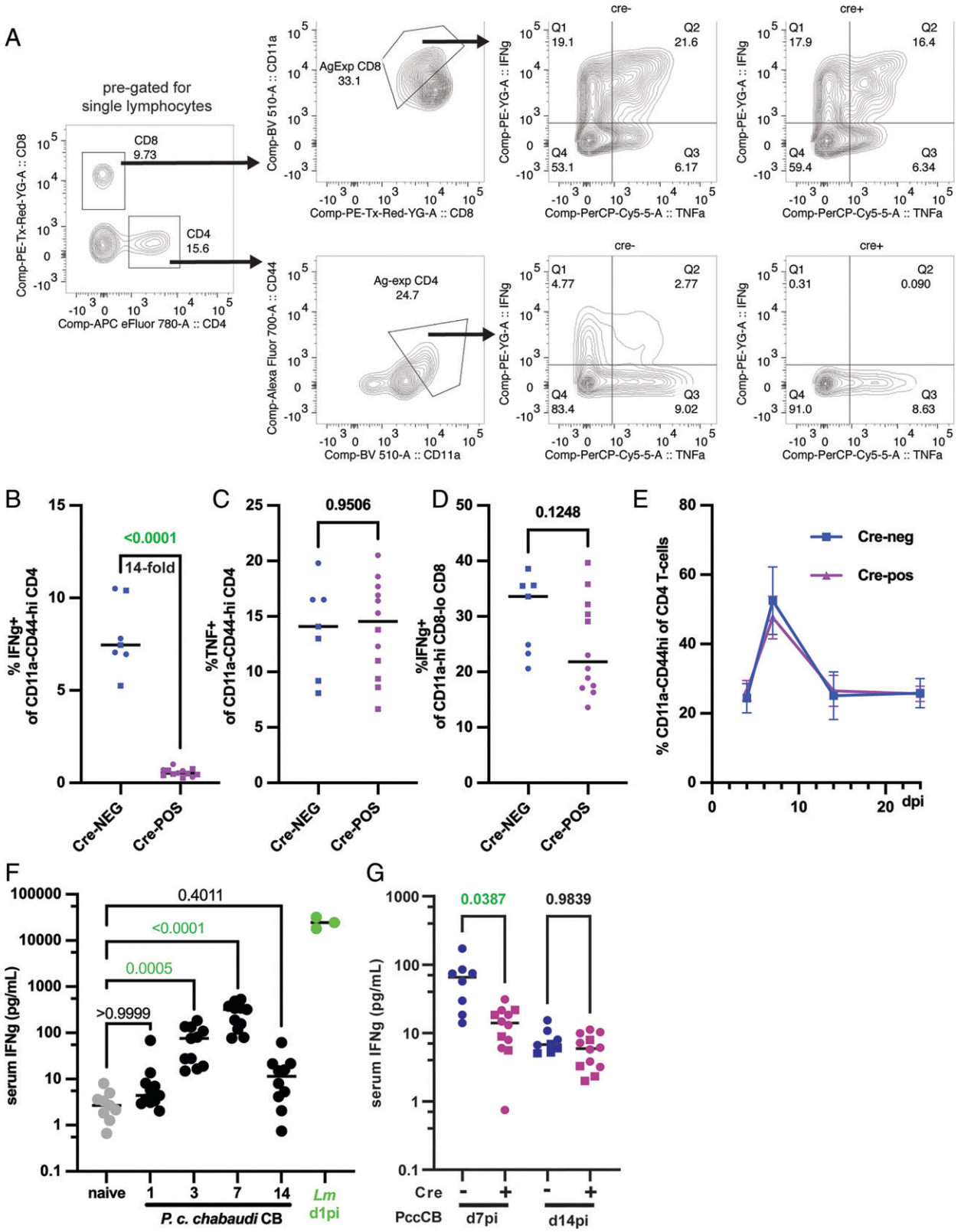

FIGURE 1. Cytokine production in d24pi splenocytes from PccCB-infected mice stimulated with PMA/ionomycin. (**A**) Gating strategy and representative cytokine staining of splenocytes from tamoxifen-treated *ifng*$^{fl/fl}$ CD4ERT2cre^{-} and cre^{+} mice. Proportions of CD11a^{hi}/CD44^{hi} CD4 T cells producing IFN-γ (**B**) and TNF-α (**C**) and CD11a^{hi}/CD8^{lo} CD8 T cells producing IFN-γ (**D**), with *p* values derived from Mann-Whitney tests indicated. (**E** and **F**) Expansion kinetics for peripheral blood Ag-experienced CD4 T cells shown as group mean ± SEM (E). IFN-γ was measured by serum ELISA in wild-type C57BL/6 mice versus a d1pi *Listeria monocytogenes* control (F) and PccCB d7pi and d14pi in CD4Cre*Ifng* mice (**G**), with *p* values from Kruskal-Wallis posttest and one-way ANOVA indicated. Experiments in (A–F) show data from two experiments; (G) shows data from four experiments. Each symbol represents one mouse.

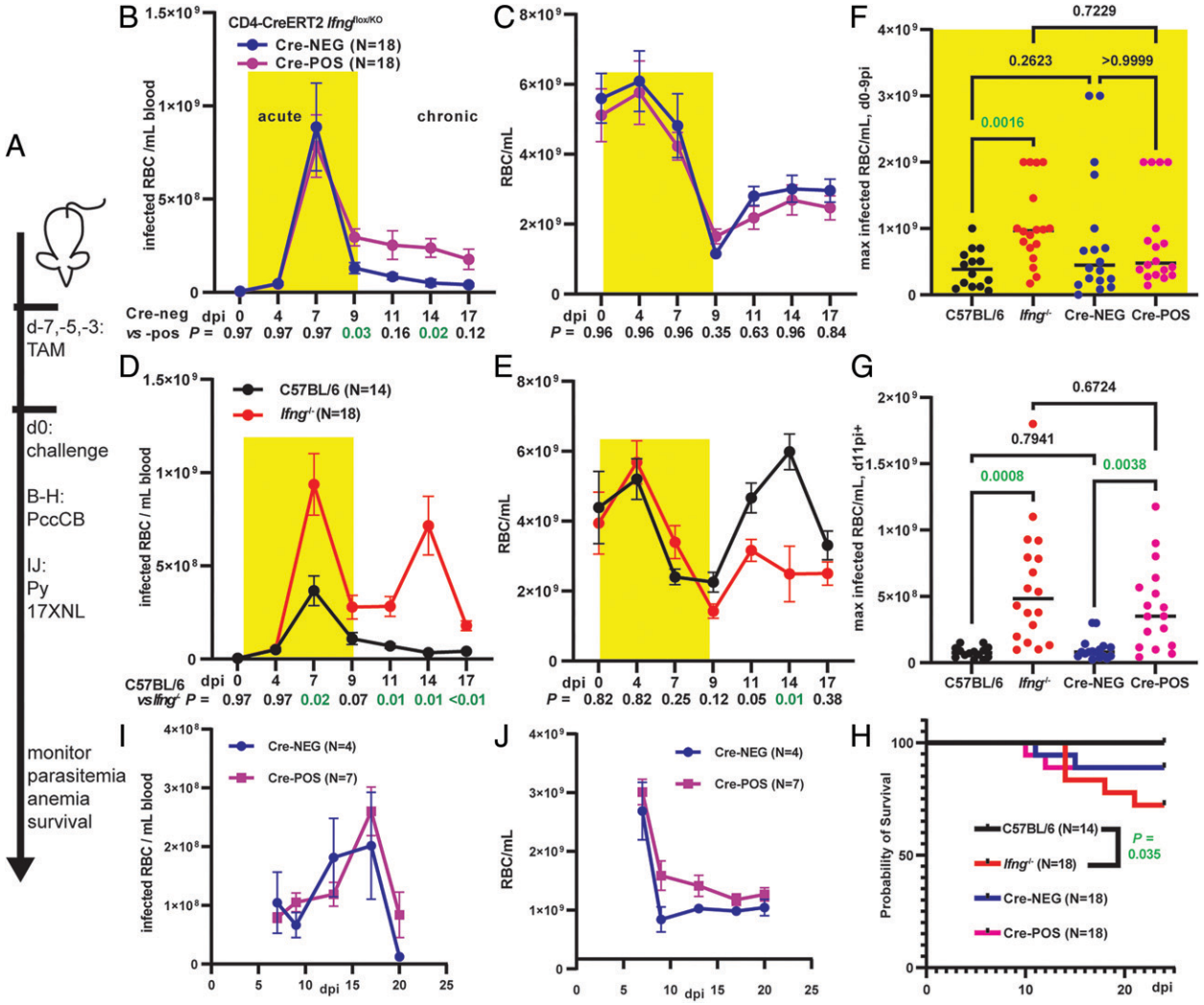

FIGURE 2. (**A**) CD4Cre*Ifng* mice were pretreated with tamoxifen and then challenged with blood-stage malaria (parasitemia [**B**, **F**, and **G**], anemia [**C**], and survival [**H**]) after challenge with 1×10^5 PccCB-infected RBCs of tamoxifen-treated Cre^+ versus Cre^- CD4Cre*Ifng* mice (B and C) or age-matched (non-tamoxifen-treated) C57BL/6J and $Ifng^{-/-}$ females (**D** and **E**). Plot shading indicates acute (yellow) and chronic (no shading) infection stages. Mean parasitemia measurements calculated for groups (error bars, SEM) are shown as kinetic curves (B–E) (*p* values, Holm-Sidak test; error bars, SEM) and maximum parasitemia observed in single mice during acute (d0–d9pi; F) or chronic (d11–17pi; G) stages of infection (*p* values, Brown-Forsythe test with Welch ANOVA). Survival curves (H) (*p* values, Gehan-Breslow-Wilcoxon test). Data from three experiments with C57BL/6 ($n = 18$) and $Ifng^{-/-}$ ($n = 14$) mice and four experiments with CD4Cre*Ifng* mice ($n = 18$) are shown. Parasitemia (**I**) and anemia (**J**) after challenge with 1×10^6 *P. yoelii* 17XNL-infected RBCs of tamoxifen-treated Cre^+ versus Cre^- CD4Cre*Ifng* mice. *P. yoelii* data are from one experiment in which all mice survived and no statistically significant differences between Cre^+ ($n = 7$) and Cre^- ($n = 4$) littermates were observed.

(21), we cannot formally exclude whether deletion of IFN-γ in cells such as CD4-expressing monocytes may also contribute to the observed parasitemia alterations.

To test whether disrupting CD4 IFN-γ production led to a gross alteration of the CD4 response to PccCB, we tracked the kinetics of $CD11a^{hi}CD44^{hi}$ Ag-experienced CD4 T cells in the peripheral blood. No discernible differences were detected comparing Cre^+ and Cre^- mice (Fig. 1E), suggesting that the overall CD4 T cell response remains intact even when IFN-γ production is ablated.

We next asked whether the achieved reduction CD4 T cell IFN-γ production is sufficiently potent to alter systemic IFN-γ levels. In wild-type C57BL/6 mice, we observed a peak in serum IFN-γ content at PccCB d7pi (Fig. 1F), coinciding with maximal expansion of $CD11a^{hi}CD44^{hi}$ CD4 T cells in the peripheral blood (Fig. 1E). Serum IFN-γ content during PccCB malaria was orders of magnitude less than that observed early during acute infection with *Listeria monocytogenes* (Fig. 1F). Nevertheless, we still detected a statistically significant fivefold decrease in serum IFN-γ content at PccCB d7pi in Cre^+ versus Cre^- CD4Cre*Ifng* mice (Fig. 1G). Together, these data demonstrate the fidelity of our model for inducible deletion of IFN-γ from CD4 T cells.

CD4 T cell–derived IFN-γ and control of parasite burden during chronic PccCB malaria

To specifically investigate the role of CD4 T cell–derived IFN-γ in controlling blood-stage malaria, we challenged our CD4Cre*Ifng* mice and Cre-negative littermate controls with blood-stage PccCB parasites to test the widely hypothesized crucial role of CD4 T cell–derived IFN-γ in controlling parasite burden during blood-stage malaria. Mice were pretreated with tamoxifen, and parasitemia, anemia, and survival were monitored during blood-stage PccCB

Table II. Parasitemia and anemia

Marker	Clone	Fluorochrome	Manufacturer
CD45	30-F11	Allophycocyanin	Tonbo
Ter-119	Ter-119	Allophycocyanin/Cy7	BioLegend
Parasite DNA		Hoechst stain	Thermo Fisher

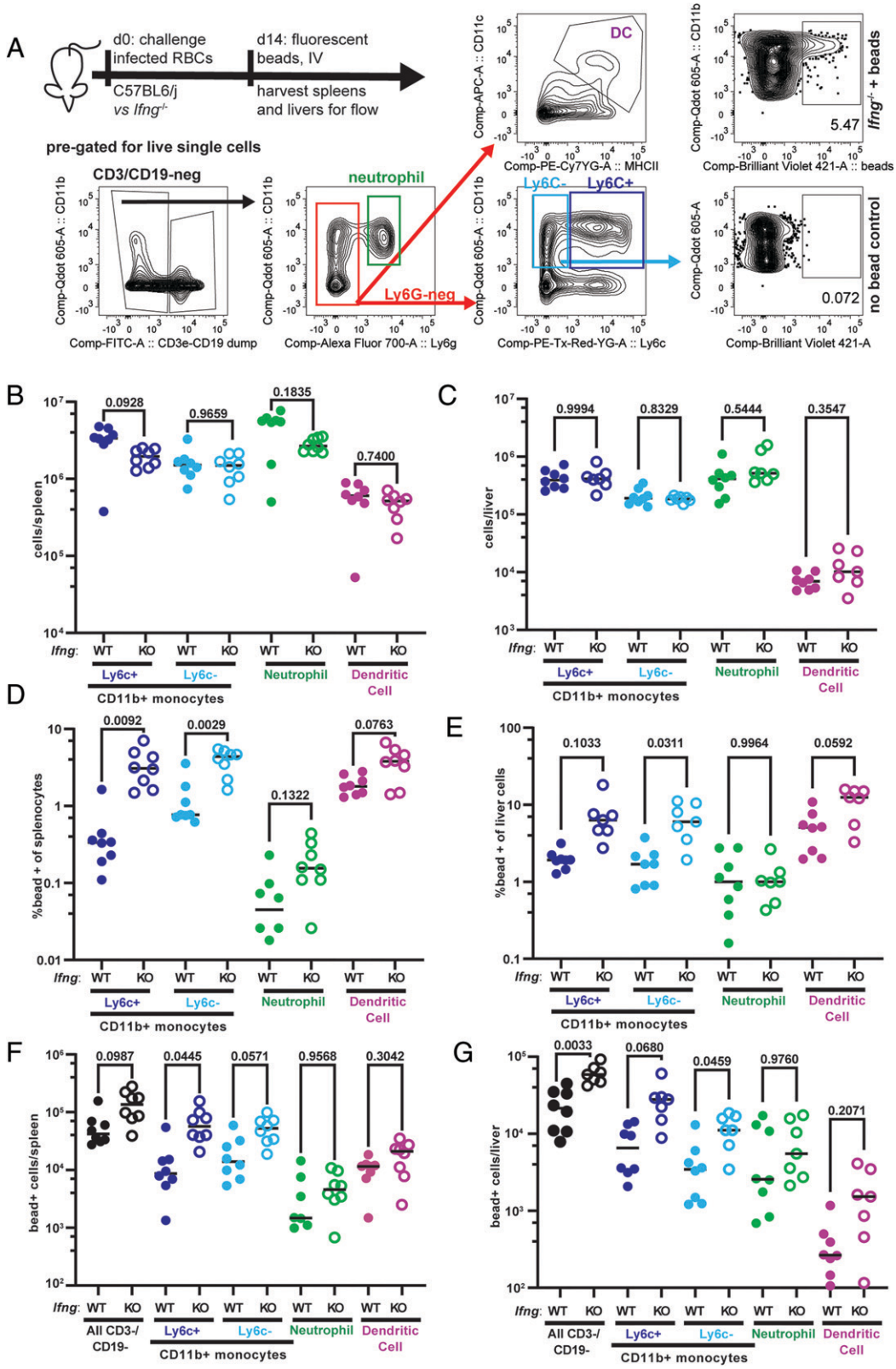

FIGURE 3. (**A**) Experimental schematic and gating strategy for assessing myeloid populations and phagocytosis. (**B** and **C**) Myeloid populations shown as number of cells per organ in the spleens (B) and livers (C) at d14pi. (**D**–**G**) Bead capture shown as percent (D and E) or absolute number of bead positive cells per organ (F and G, spleen; D and F, liver) at d14pi. *p* values from Brown-Forsythe posttest and Welch ANOVA are indicated. Data are from two experiments, each with four mice per group.

malaria (Fig. 2A, Table II). During the first acute parasitemia wave, we observed no discernible impact of Cre-mediated CD4 IFN-γ ablation on parasitemia (Fig. 2B). During the chronic phase of PccCB malaria (d9pi+), we observed a consistent but modest increase in parasite burden in Cre^+ mice compared with Cre^- littermates (Fig. 2B). Anemia was not impacted by Cre-mediated CD4 IFN-γ ablation during either acute or chronic malaria (Fig. 2C).

The relatively minor impact of ablating IFN-γ production in CD4 T cells on parasitemia was unexpected. Thus, we next queried the impact of complete disruption of IFN-γ production on PccCB malaria by comparing parasitemia and anemia in wild-type C57BL6/J and *Ifng*$^{-/-}$ mice. Consistent with prior reports (4), we observed a dramatic increase in acute and chronic parasitemias in the *Ifng*$^{-/-}$ mice (Fig. 2D). Anemia was also exacerbated in the *Ifng*$^{-/-}$ mice during

Table III. Myeloid panel

Marker	Clone	Fluorochrome	Manufacturer
Live-Dead	n/a	Near IR 780	Invitrogen
CD3e	145-2C11	FITC	BD Biosciences
CD19	1D3	FITC	eBioscience
Ly-6g	1A8	Alexa Fluor 700	BioLegend
Ly-6c	HK1.4	PE/Dazzle 594	BioLegend
CD11b	M1/70	Brilliant Violet 785	BioLegend
CD11c	N418	Allophycocyanin	Tonbo
MHCII	114.15.2	PE/Cy7	Thermo Fisher
F4/80	BM8.1	PerCP/Cy5.5	Tonbo

the chronic phase of infection (Fig. 2E), and survival subtly but significantly decreased (Fig. 2H). Non-tamoxifen-treated C57BL/6 mice consistently developed lower acute parasitemia than those observed in experiments with tamoxifen-treated Cre^- mice (Fig. 1B versus Fig. 1D), likely due to the previously reported ability of tamoxifen to exacerbate parasitemia during blood-stage malaria (1).

Because the recrudescent parasitemia episodes of PccCB malaria occur asynchronously, the magnitude of these episodes can be obscured by tracking mean parasitemia in groups of mice. To account for this limitation, we compared the maximum observed parasitemia in individual mice across the entire time course of the infection (Fig. 2F, 2G). During the acute infection, *Ifng*$^{-/-}$ mice again displayed significantly exacerbated maximum parasitemia values relative to C57BL/6, whereas Cre^+ versus Cre^- CD4Cre*Ifng* mice did not significantly differ from one another (Fig. 2F). However, during the chronic phase of infection, Cre^+ CD4Cre*Ifng* mice developed elevated parasite burdens (versus Cre^- littermate controls) of similar severity to the elevated parasitemia of *Ifng*$^{-/-}$ mice (Fig. 2G).

Combined, these results suggest that IFN-γ is indeed a crucial player in suppressing parasite burden during blood-stage PccCB malaria. However, IFN-γ production by CD4 T cells only contributes to managing parasite burden during the chronic phase of PccCB malaria and appears functionally irrelevant to limiting acute parasitemia. The IFN-γ crucial for controlling acute parasitemia that leads to dramatically elevated acute parasitemia in *Ifng*$^{-/-}$ mice must be produced by other cell populations, of which several candidates have emerged in other work (18).

To probe whether the limited ability of CD4 T cell–derived IFN-γ to control acute parasitemia is a quirk of the PccCB model, we compared parasitemia and anemia kinetics in the CD4Cre*Ifng* mice in another acute resolving model of murine blood-stage malaria, *P. yoelii* 17XNL (32). In this system, we could not discern any exacerbation of parasitemia in Cre^+ CD4 IFN-γ-ablated mice, and instead we observed a nonsignificant trend toward improved control of parasitemia and anemia in Cre^+ mice versus Cre^- littermate controls (Fig. 2I, 2J).

Assessment of phagocytosis

Effective control of parasite burden during blood-stage malaria requires identification and removal of parasitized RBCs. The spleen is well recognized as a prime site for the removal of parasite RBCs due to its inherent blood filtration function (4, 32–34), whereas the liver is infrequently assessed as a player during blood-stage malaria but also is well positioned as a site for parasitized RBC clearance because blood and phagocytes are closely juxtaposed in the liver sinusoids (33, 34). IFN-γ is classically considered an activator of macrophage phagocytosis but can also inhibit phagocytosis in some scenarios (35), such as nonopsonic phagocytosis (36, 37). During PccAS malaria, IFN-γ was found to enhance the effector function of splenic macrophages (4). Accordingly, we hypothesized that a failure for IFN-γ to activate phagocytic mechanisms may explain the poor control of parasite burden observed in *Ifng*$^{-/-}$ mice and CD4 IFN-γ-ablated mice.

We tested whether the strikingly poor control of chronic parasitemia observed in *Ifng*$^{-/-}$ mice (Fig. 2D) could be explained by deficient IFN-γ signaling leading to alterations in the presence of phagocytic myeloid cells in the spleen or liver or the phagocytic capacity of such cells. At d14pi with PccCB, fluorescent beads were introduced i.v. into *Ifng*$^{-/-}$ mice and C57BL/6J mice, and splenic and liver myeloid populations were assessed for abundance and bead capture (Fig. 3A, Table III). We observed no significant differences in the abundance of $Ly6c^+CD11b^+$ or $Ly6c^-CD11b^+$ monocytes or macrophages, neutrophils, or dendritic cells in the spleen or liver (Fig. 3B, 3C), although trending decreases in $Ly6c^+CD11b^+$ monocytes and neutrophils were seen in the spleens of *Ifng*$^{-/-}$ mice. Instead, we observed increased proportions of bead-positive $CD11b^+$ monocytes and dendritic cells in both the spleen and liver in *Ifng*$^{-/-}$ mice versus wild-type C57BL/6J controls (Fig. 3D, 3E) and increased absolute quantities of bead-positive myeloid cells per organ (Fig. 3F, 3G). Enhanced phagocytosis in *Ifng*$^{-/-}$ mice does not support a model where compromised phagocytosis leads to the poor control of parasitemia we observed in *Ifng*$^{-/-}$ mice. Of note, phagocytosis of parasitized RBCs may occur in a mechanistically distinct manner that is not reflected by our bead-based experiment, in particular in an Ag-specific process such as opsonization-dependent phagocytosis, and further experiments would be necessary to address this possibility. Accordingly, we focused our remaining efforts on investigating disruptions in humoral immunity in the absence of IFN-γ, given that altered parasite control in Cre^+ CD4Cre*Ifng* mice only manifested after the first week of infection.

Disruption of Ab class switching

Abundant evidence implicates IFN-γ in the tuning of Ab class switching toward IgG2a (35, 38, 39). Hence, we measured PccCB-specific IgG2c (the functional equivalent of IgG2a expressed by C57BL/6 mice [40]) Ab titers at d24pi. As expected, IgG2c-specific titers were reduced in sera from *Ifng*$^{-/-}$ versus C57BL/6 mice (Fig. 4A). IgG2c titers were similarly reduced in sera from Cre+ versus Cre– CD4Cre*Ifng* mice (Fig. 4A). The similarly reduced IgG2c titers in Cre+ Cre– CD4Cre*Ifng* and *Ifng*$^{-/-}$ mice suggest that CD4 T cell–derived IFN-γ is indeed crucial in driving class switching to the IgG2c isotype and further supports the robust excision of *Ifng* in our mice. Importantly, we did not observe significant differences in PccCB-specific titers of total IgG isotypes, comparing C57BL/6 with *Ifng*$^{-/-}$ mice or comparing Cre+ and Cre– CD4Cre*Ifng* mice (Fig. 4B). The lack of titer reduction for all IgG supports a model where class switching is specifically reduced rather than an overall inhibition of parasite-specific humoral responses.

We next asked whether the ablation of CD4 IFN-γ production could disrupt humoral responses by disrupting efficient formation of $CD4^+$ Tfh cells important for development of *Plasmodium*-specific immunity. No differences in splenic Tfh or germinal center Tfh (defined by CXCR5 and PD1 expression; Fig. 4C) abundance, in absolute or proportional terms, were observed at PccCB d24pi (Fig. 4D–4G).

We finally asked if reduced IgG2c switching in Cre^+ CD4Cre*Ifng* mice had functional consequences for the control of parasite burden, using *Ifng*$^{-/-}$ mice infected with a *P. c. chabaudi* strain, AS, that requires humoral immunity for control (20), to highlight CD4-derived IFN-γ-dependent changes in Ab-mediated control. After the peak of acute parasitemia, PccAS-infected *Ifng*$^{-/-}$ mice received sera produced by either naive mice or tamoxifen-treated CD4Cre*Ifng* mice at PccCB d24pi (Fig. 4H). As expected, control of parasite

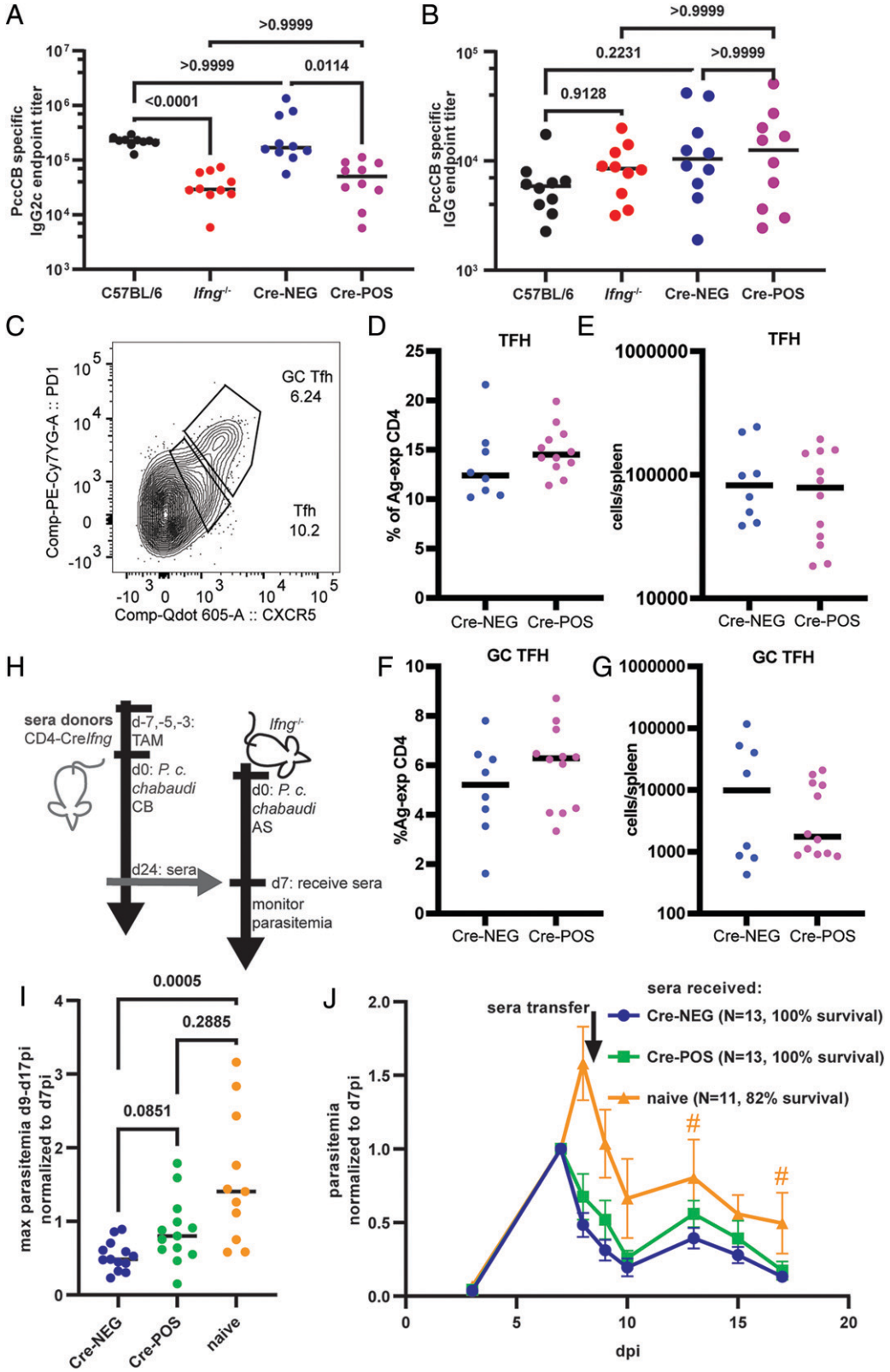

FIGURE 4. (**A** and **B**) PccCB-specific IgG2c (A) and total IgG (B) endpoint titers in d24pi sera. *p* values from one-way ANOVA and Kruskal-Wallis posttest are shown. (**C–G**) Splenic Tfh cells ($CXCR5^{hi}$ PD^{hi}; C, gating for Tfh, following pregating for single lymphocytes by forward scatter/side scatter and $CD11a^{hi}/Cd44^{hi}$ $CD4^{+}$ cells) at PccCB d24pi, with proportion (D and F) or absolute numbers (E and G) indicated (D and E, Tfh; F and G, germinal center Tfh) indicated. Data from two independent experiments are shown. (**H–J**) Sera from PccCB d24pi or naive mice were transferred into $Ifng^{-/-}$ mice at peak acute PccAS parasitemia (H). Parasitemia (normalized to time of sera transfer) is shown as maximum observed values in individual mice (I) or mean values ± SEM for groups (J). Hashtags (#) indicate mortality. *p* values indicate Kruskal-Wallis posttest and one-way ANOVA. Data from three experiments are shown.

burden was least effective in mice receiving sera from naive donors (Fig. 4I, 4J). Compared with naive sera controls, the maximum observed parasitemia following transfer was significantly reduced only for mice receiving sera from Cre^- CD4Cre*Ifng* mice (Fig. 4I, 4J). A nonsignificant trend toward enhanced parasitemia in Cre^+ versus Cre^- CD4Cre*Ifng* mice was also observed (Fig. 4I, 4J). Combined, these results suggest that Ab responses to *P. c. chabaudi* infection that develop in the absence of CD4 T cell–derived IFN-γ are very subtly inferior at conferring control of parasite burden.

Discussion

In total, our results reveal a striking role for IFN-γ in suppressing parasite burden during PccCB malaria. Consistent with previous studies (4), total disruption of the *Ifng* locus dramatically exacerbated parasitemia during acute and chronic PccCB malaria (Fig. 1E, 1F).

To build upon this finding, we used an inducible CD4-specific Cre/Lox excision system to define the contribution of CD4 T cell IFN-γ production to IFN-γ-dependent parasitemia suppression. Although the peak of CD4 T cell activation occurred contemporaneously with the first peak of acute parasitemia at approximately d7pi, we could not discern any role for CD4 T cell–derived IFN-γ in controlling this first parasitemia wave (Fig. 1B). Inefficient ablation of CD4 T cell IFN-γ production is unlikely to explain the lack of an acute parasitemia phenotype, because ablation was sufficiently potent to (1) be robustly detected at d24pi in splenic CD4 T cells, nearly 1 full month after tamoxifen-induced *Ifng* excision (Fig. 1B); (2) significantly reduce serum IFN-γ content at the d7pi peak of CD4 T cell activation and serum IFN-γ accumulation (Fig. 1G); and (3) significantly alter Ab class switching (Fig. 4A).

Accordingly, we conclude that other sources of IFN-γ are crucial to controlling parasite burden during the acute phase of PccCB malaria. Future studies should investigate whether IFN-γ producers identified in other studies, including NK, NKT, γδ T cells, and CD8 T cells (18), are crucial sources of this protective cytokine as either sole or cooperative actors.

Our CD4Cre*Ifng* mice did reveal a moderate yet significant role for CD4 T cell–derived IFN-γ in controlling recrudescent parasitemia during chronic PccCB malaria (Fig. 2B, 2G). More efficient suppression of parasite burden observed in CD4 T cell IFN-γ-intact mice may stem from a superior humoral response via more efficient Ab class switching. Although this defect in isotype class switching manifested as enhanced parasitemia during the chronic phase of PccCB malaria, the crucial window of activity for CD4 T cell–derived IFN-γ likely lies earlier, at approximately d7pi, when serum IFN-γ content and the expansion of activated CD4 T cells both peak (Fig. 1E–1G), and significant reductions in serum IFN-γ were discernible between Cre^+ versus Cre^- mice in the CD4Cre*Ifng* model (Fig. 1G). Notably, during blood-stage malaria, we never observed accumulation of IFN-γ in the serum at levels similar to those observed early in other infections, such as *Listeria monocytogenes* (Fig. 1F). This observation may suggest that IFN-γ signaling acts on primarily a local rather than a system level during blood-stage malaria or simply that serum IFN-γ is rapidly consumed during blood-stage malaria.

An intriguing question for further follow-up is whether IFN-γ production by memory phenotype CD4 T cells would more rapidly or potently control parasite burden during subsequent blood-stage malaria episodes. Several studies in mice show that IFN-γ-producing memory CD4 T cells can confer potent immunity (41, 42). Because humans residing in malaria-endemic regions experience many episodes of malaria throughout their lives and accordingly develop expansive and complex memory lymphocyte compartments, it is likely that memory CD4 T cells are the true analogs of the protective IFN-γ-producing memory cells observed in human studies. Further use of the mouse model to refine the mechanism by which IFN-γ, produced by both CD4 T cells and other cell populations, should help to guide efforts to design efficacious vaccines targeting the blood stage of malaria.

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Disclosures

The authors have no financial conflicts of interest.

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