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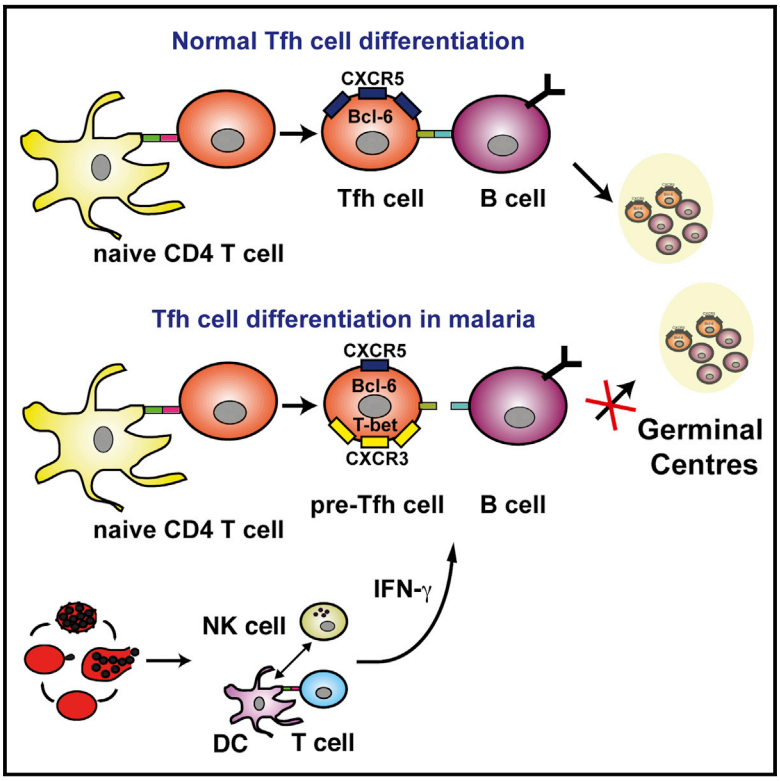
Severe Malaria Infections Impair Germinal Center Responses by Inhibiting T Follicular Helper Cell Differentiation

Graphical Abstract



Highlights

- Immunity to malaria develops after many years of exposure to *Plasmodium* parasites
- Severe malaria infection inhibits the establishment of germinal centers in the spleen
- T follicular helper cell differentiation is impaired during severe infection
- TNF and IFN-γ blockade or T-bet deletion restores Tfh cell differentiation

Authors

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In Brief

Immunity to malaria takes years to develop despite repeated exposure to *Plasmodium* parasites. Ryg-Cornejo et al. report that severe malaria infection impairs germinal center responses by inhibiting T follicular helper cell differentiation. The same pro-inflammatory responses that drive malarial pathogenesis were found to mediate the inhibition of B-cell-mediated immunity.

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Severe Malaria Infections Impair Germinal Center Responses by Inhibiting T Follicular Helper Cell Differentiation

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SUMMARY

Naturally acquired immunity to malaria develops only after years of repeated exposure to *Plasmodium* parasites. Despite the key role antibodies play in protection, the cellular processes underlying the slow acquisition of immunity remain unknown. Using mouse models, we show that severe malaria infection inhibits the establishment of germinal centers (GCs) in the spleen. We demonstrate that infection induces high frequencies of T follicular helper (Tfh) cell precursors but results in impaired Tfh cell differentiation. Despite high expression of Bcl-6 and IL-21, precursor Tfh cells induced during infection displayed low levels of PD-1 and CXCR5 and co-expressed Th1-associated molecules such as T-bet and CXCR3. Blockade of the inflammatory cytokines TNF and IFN-γ or T-bet deletion restored Tfh cell differentiation and GC responses to infection. Thus, this study demonstrates that the same pro-inflammatory mediators that drive severe malaria pathology have detrimental effects on the induction of protective B cell responses.

INTRODUCTION

Malaria is one of the most serious infectious human diseases, and there are ~250 million clinical cases of it annually. Most cases of severe disease are caused by *Plasmodium falciparum*, which remains an important cause of mortality and morbidity worldwide. Humans with no previous experience of malaria almost invariably become ill on their first exposure to the parasite (Miller et al., 2002). They develop a febrile illness that may become severe and, in a proportion of cases, leads to death. Severe malaria cases are associated with various disease syndromes, including respiratory distress, metabolic acidosis, renal failure, pulmonary edema, and cerebral malaria (White and Ho, 1992). Although the precise mechanisms leading to severe disease are not completely understood, it is accepted that the sequestration of blood-stage parasites in the blood vessels of target organs and a strong pro-inflammatory response are important determinants of disease induction. High levels of cytokines such as tumor necrosis factor (TNF) (Molyneux et al., 1993), interferon γ (IFN-γ), and interleukin 1β (IL-1β) (Pongponratn et al., 2003) as well as chemokines, including CCL4, CXCL10, and CXCL8 (Armah et al., 2007; Jain et al., 2008), have been found to be associated with disease severity. Although severe clinical manifestations of malaria ameliorate following a few infections, immune effector mechanisms capable of controlling parasite growth develop only after repeated exposure over a number of years. This form of protection does not result in sterilizing immunity but prevents clinical episodes by substantially reducing the parasite burden. Naturally acquired immunity is known to require antibody responses because passive transfer of sera from clinically immune individuals significantly protects non-immune recipients from high parasitemia and disease symptoms (Cohen et al., 1961).

Despite the key role antibodies play in protection against malaria, little is known about the cellular processes underlying the slow acquisition of humoral immunity. Epidemiological evidence suggests that antibodies to *P. falciparum* antigens are generated inefficiently, and responses are lost in the absence of ongoing exposure (Kinyanjui et al., 2007, 2009). These findings suggest that the development of B cell memory might be compromised during infection. Recent studies have indicated that children and young adults in areas of high seasonal transmission are characterized by a delayed development of memory B cells specific for *P. falciparum* despite repeated exposure to the parasite (Weiss et al., 2010). In contrast, individuals living in areas of low transmission that experience fewer clinical episodes appear to generate malaria-specific memory B cells that are stable even in the absence of frequent boosting (Wipasa et al., 2010). Overall, these observations suggest that the inflammatory responses contributing to clinical episodes of severe malaria have a

detrimental effect on the development of humoral immunity. In support of this view, histological studies have revealed that human fatal malaria infections are accompanied by dramatic changes in splenic architecture (Urban et al., 2005), including dissolution of the marginal zone, loss of B cells, and reduced formation of germinal centers (GCs) required for the development of memory B cells and long-lived plasma cells.

Using murine malaria models, a few studies have investigated alterations occurring in spleens during infection and their consequences for the development of B cell responses (Achtman et al., 2003; Beattie et al., 2006; Carvalho et al., 2007). Most of the available information has been generated using the non-lethal *Plasmodium chabaudi* infection model and demonstrates that, despite transient changes of the splenic architecture, infected mice are able to mount adequate antibody responses and control parasitemia (Achtman et al., 2003; Ndungu et al., 2009). Unlike *P. chabaudi*, infection of mice with *Plasmodium berghei* ANKA results in induction of severe malaria. This infection has many features in common with human disease and is the best available model for certain aspects of clinical malaria (Hansen, 2012). As in humans, sequestration of parasitized red blood cells (pRBCs) in target organs and a strong inflammatory response have been identified as the main determinants of disease (Hansen, 2012). Inflammatory responses mediated by cytokines and chemokines such as TNF (Grau et al., 1987), IFN-γ (Grau et al., 1989), and CXCL10 (Nie et al., 2009) and effector cells such as $CD8^+$T (Belnoue et al., 2002), natural killer T (NKT) cells (Hansen et al., 2003), and natural killer (NK) cells (Hansen et al., 2007) contribute to the induction of severe malaria in this model. Loss of splenic architecture has been found to accompany the onset of severe disease in this model (Carvalho et al., 2007), but the implications of these processes for the induction of humoral responses to the parasite are unclear.

Acquisition of humoral immunity requires a specialized subset of T cells, named T follicular helper (Tfh) cells, that orchestrate the GC reaction and the differentiation of memory B cells and long-lived plasma cells (Crotty, 2011). A number of features define Tfh cells, including expression of the transcriptional regulator Bcl-6, which induces the expression of CXCR5 and migration of Tfh cells into the GC. Tfh cells are also characterized by high PD-1 and inducible T cell co-stimulator (ICOS) expression, strong IL-21 secretion, and low CCR7 expression (Tangye et al., 2013). Tfh cell differentiation requires initial contacts with dendritic cells (Choi et al., 2011) and prolonged interactions with GC B cells to reinforce the Tfh cell phenotype (Crotty, 2014). Similar to other T helper subsets, Tfh cell differentiation is modulated by cytokine signals. IL-6 and IL-21 are positive mediators of Tfh cell differentiation (Karnowski et al., 2012), whereas IL-2 inhibits Tfh fate (Ballesteros-Tato et al., 2012). Furthermore, inflammatory cytokines, including IL-12, type I IFNs, and IFN-γ, have been found to modulate these processes (Lee et al., 2012; Nakayamada et al., 2011; Oestreich et al., 2012; Ray et al., 2014). Interestingly, these cytokines are produced in response to rodent malaria infections (Hansen, 2012), but it is unknown whether they contribute to defective B cell responses. To address this question, humoral responses were investigated using the *P. berghei* ANKA model of severe malaria. Our results reveal that the same pro-inflammatory pathways mediating severe disease syndromes have a detrimental effect in the development of B cell responses by inhibiting Tfh cell differentiation and compromising GC responses required for the efficient induction of antibody-mediated immunity.

RESULTS

Inefficient Induction of Antibody-Secreting Plasma Cells during Severe Malaria Infection

To investigate the role of antibody responses in the control of parasitemia, C57BL/6 wild-type (WT) and $Prdm1^{fl/fl}Cd19^{Cre}$ mice in which B cell-specific deletion of the transcription factor Blimp-1 impairs the development of plasma cells (Shapiro-Shelef et al., 2003) were infected with *P. chabaudi chabaudi* AS or *P. berghei* ANKA. Strikingly, the absence of plasma cells in mice infected with the self-resolving *P. chabaudi chabaudi* AS strain resulted in the development of high parasitemia and death (Figures 1A and 1B), confirming a key role for antibody responses in the control of parasite replication during a mild malaria infection. In contrast, *P. berghei* ANKA-infected $Prdm1^{fl/fl}Cd19^{Cre}$ mice developed cerebral malaria at rates identical to WT control animals (Figure 1C), with highly similar parasitemia levels between the two groups (Figure 1D), suggesting that antibody responses are unable to contribute to the control of parasitemia during a severe malaria infection.

To further investigate the effect of severe malaria on the development of B cell responses to infection, we compared antibody responses of C57BL/6 mice after infection with *P. berghei* ANKA or after immunization with irradiated parasites (irradiated *P. berghei* ANKA [iPbA]). Because *P. berghei* ANKA infection is invariably lethal in C57BL/6 mice, infected animals were cured with anti-malarial drugs after the onset of disease symptoms on day 5 post-infection (p.i.) to allow assessment of responses at late times post-exposure. At this time, infected mice experienced ~5% parasitemia, which is equivalent to $\sim 3 \times 10^8$ pRBCs. To ensure a similar antigenic load in the two groups of experimental mice, three doses of 1×10^8 pRBCs, 2–3 days apart, were administered during the immunization schedule with iPbA. Therefore, this approach allows the comparison of immune responses to *P. berghei* ANKA in the presence or absence of the disease symptoms and inflammatory responses associated with severe malaria (Figure S1). One week after treatment completion, iPbA-immunized C57BL/6 mice had mounted a parasite-specific antibody response five times higher than *P. berghei* ANKA-infected mice (Figure 1E).

The induction of plasma cell responses was investigated using both WT and $Prdm1^{GFP}$ reporter mice, which express GFP in all antibody-secreting cells (Kallies et al., 2004). Plasma cell differentiation in response to *P. berghei* ANKA infection was impaired severely in comparison with mice immunized with iPbA (Figures 1F–1H). Furthermore, the proportion of isotype-switched plasma cells was diminished severely (Figure 1F). Together, these results suggest that severe malaria compromises the development of humoral immune responses to infection.

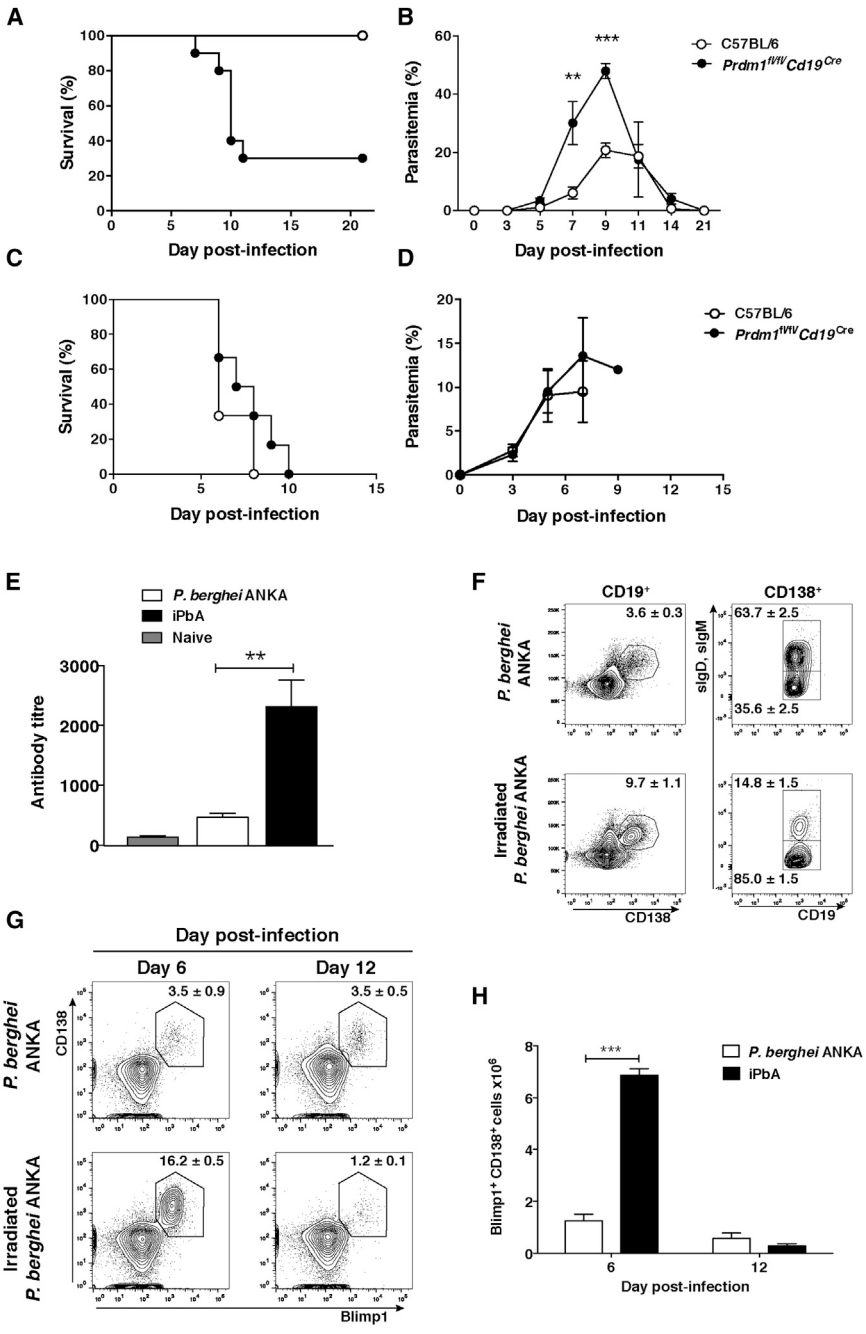


Figure 1. Antibody Responses Are Generated Inefficiently in Response to Severe Malaria Infection

(A–D) *Prdm1*$^{fl/fl}$CD19^{Cre} and C57BL/6 (n = 8–12) mice were infected with *P. chabaudi* AS (A and B) or *P. berghei* ANKA (C and D). Survival (A and C) and parasitemia (B and D) were determined. Each point represents the mean ± SEM. Infections are representative of two experiments. **p < 0.01, ***p < 0.001.

(E) Sera were collected after infection with *P. berghei* ANKA or immunization with iPbA. Parasite-specific IgG responses were measured by ELISA. Columns represents means of six samples ± SEM. **p < 0.01.

(F–H) C57BL/6 (F) or *Prdm1*^{GFP} mice (G and H) were infected with *P. berghei* ANKA or immunized with iPbA. The percentage (F) of CD19$^+$CD138$^+$ cells and the proportion of isotype-switched cells were determined on day 6 p.i. Frequencies (G) and absolute numbers (H) of Blimp-1$^+$CD138$^+$ plasma cells were determined. Data are representative of two experiments. Columns represents means of four mice ± SEM. Representative dot plots are shown. ***p < 0.001.

See also Figure S1.

Severe Malaria Infections Impair Germinal Center Responses

To determine whether *P. berghei* ANKA-mediated severe malaria episodes are accompanied by reduced GC formation in the spleen, as occurs in human malaria, we compared the responses of C57BL/6 mice after infection with *P. berghei* ANKA or after immunization with iPbA. Infected mice were drug-cured as described above, and spleen sections were stained with anti-immunoglobulin D (IgD) to reveal B cell follicles, anti-CD3 to detect T cell zones, and anti-GL7 for detection of GCs. Spleen sections from iPbA-immunized mice maintained their splenic architecture and revealed GC structures in response to immunization as early as day 6 p.i. (Figure 2A). Consistent with previous studies (Achtman et al., 2003; Beattie et al., 2006; Carvalho et al., 2007), sections from malaria-infected mice displayed a disturbed white pulp structure with a striking loss of T cells from T cell zones and a disorganized follicular mantle (Figure 2A). Furthermore, mice experiencing an active infection lacked GCs on days 6 and 9 p.i. (Figure 2A). From day 12 p.i. onward, after infected mice had been treated with anti-malarial drugs and the infection was controlled, the splenic architecture was restored gradually, and GCs started to develop. To confirm these observations, GC B cells were examined further by flow cytometry. The frequency and total numbers of GC B cells (CD19$^+$CD38^{low}GL7$^+$) in *P. berghei* ANKA-infected mice were reduced significantly compared with iPbA-immunized mice until resolution of infection by day 12 p.i. (Figures 2B and 2C).

To investigate the consequences of impaired GC reactions, the frequency and number of memory B cells (CD19$^+$IgD$^-$CD138$^-$CD38^{high}) were analyzed in *P. berghei* ANKA-infected mice or iPbA-immunized counterparts (Figures 2D and 2E). The number of memory B cells induced in response to infection was significantly lower than that obtained after immunization with irradiated parasites (Figure 2E). Moreover, the absolute number of isotype-switched Blimp-1^{high} long-lived antibody-secreting cells was reduced in infected mice compared with iPbA-immunized controls

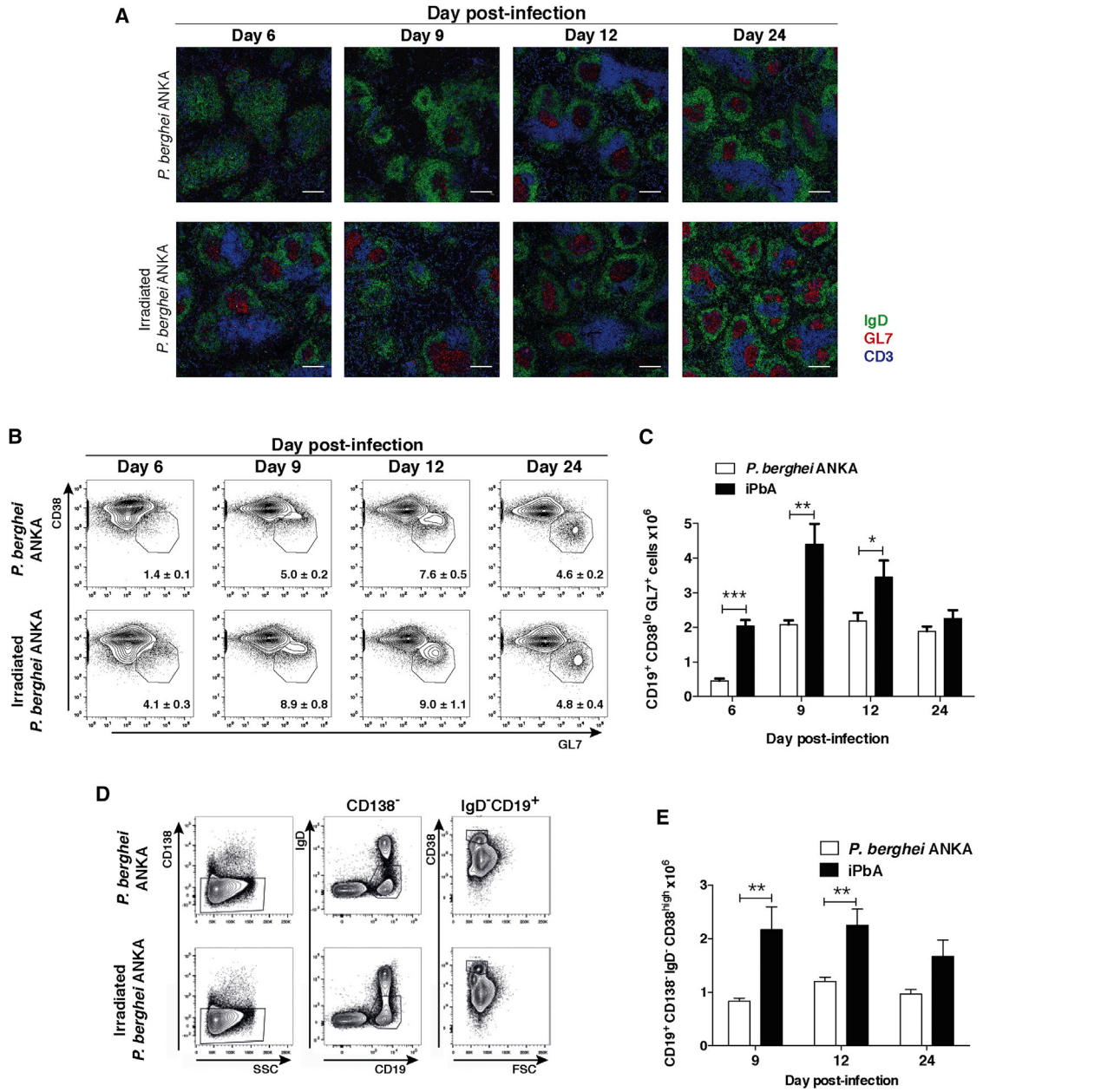


Figure 2. Severe Malaria Compromises Germinal Center Induction

(A) Immunofluorescence analysis of spleen sections from *P. berghei* ANKA-infected or iPbA-immunized mice, identifying GC B cells with anti-GL7, naive B cell follicles with anti-IgD, and T cell zones with anti-CD3. Images are representative of at least 6 animals/group. Scale bars, 200 μm.

(B–E) C57BL/6 mice were infected with *P. berghei* ANKA or immunized with iPbA. The percentage (B and D) and absolute number (C and E) of GC ($CD19^+CD38^{low}GL7^+$) and memory ($CD19^+IgD^-CD138^-CD38^{high}$) B cells were calculated. Data are representative of two experiments. Bars represent the mean of six mice ± SEM. Representative dot plots are shown. *$p < 0.05$, **$p > 0.01$, ***$p < 0.001$.

See also Figure S2.

(Figure S2). Together, these results indicate that severe malaria infection impairs the establishment of GC reactions.

Severe Malaria Infection Inhibits Tfh Cell Differentiation

To investigate whether the impaired GC responses observed in severe malaria-susceptible mice reflected a defect in the Tfh compartment, the frequency and total number of these cells (defined as $CD4^+CXCR5^{high}PD\text{-}1^{high}$) were examined in spleens of *P. berghei* ANKA-infected mice (drug-cured on day 5 p.i.) or mice receiving equivalent doses of iPbA. Figure 3A shows that the percentage of Tfh cells was reduced significantly in mice that experienced a severe malaria infection compared with those

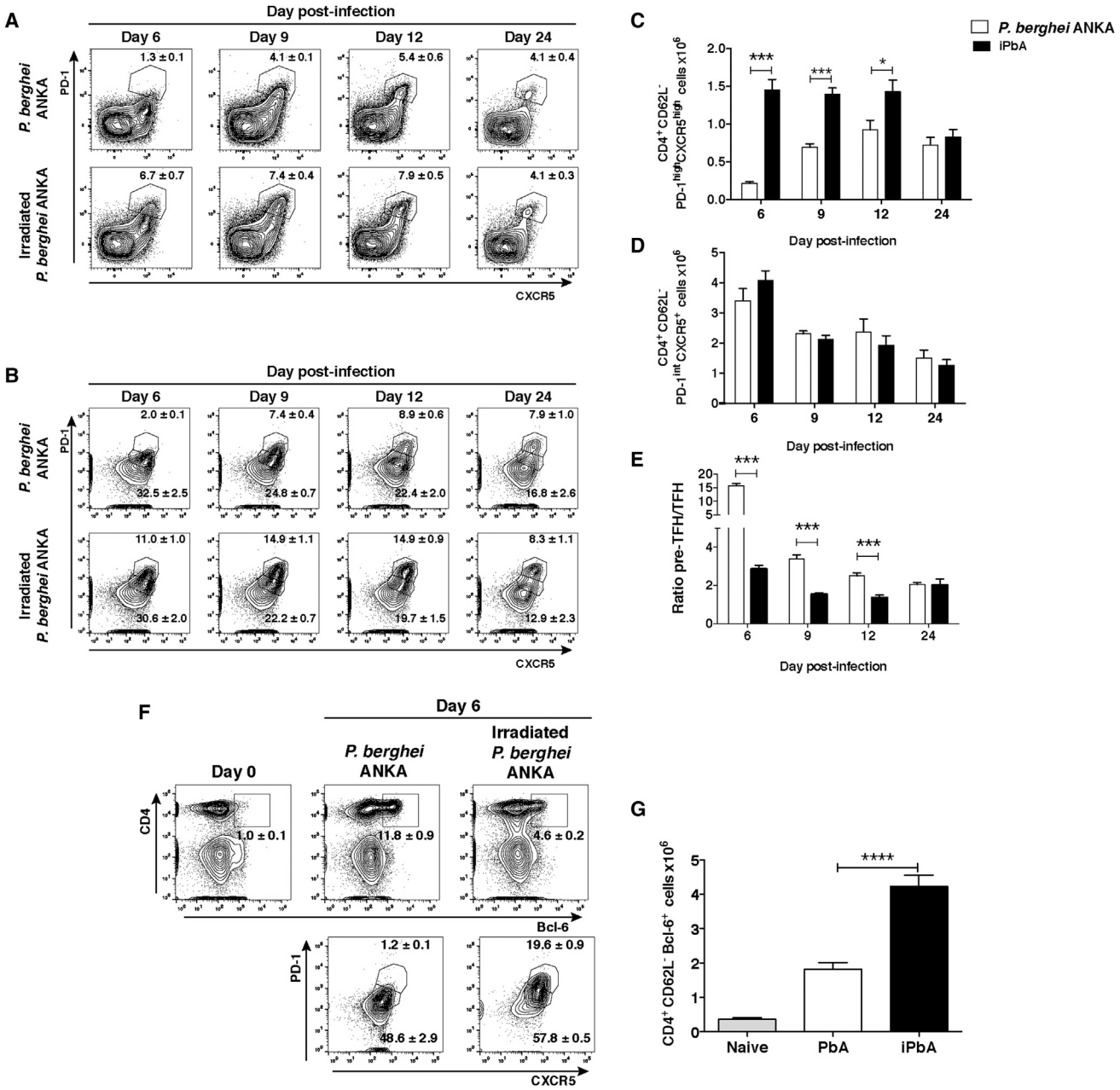


Figure 3. Severe Malaria Inhibits Tfh Cell Differentiation

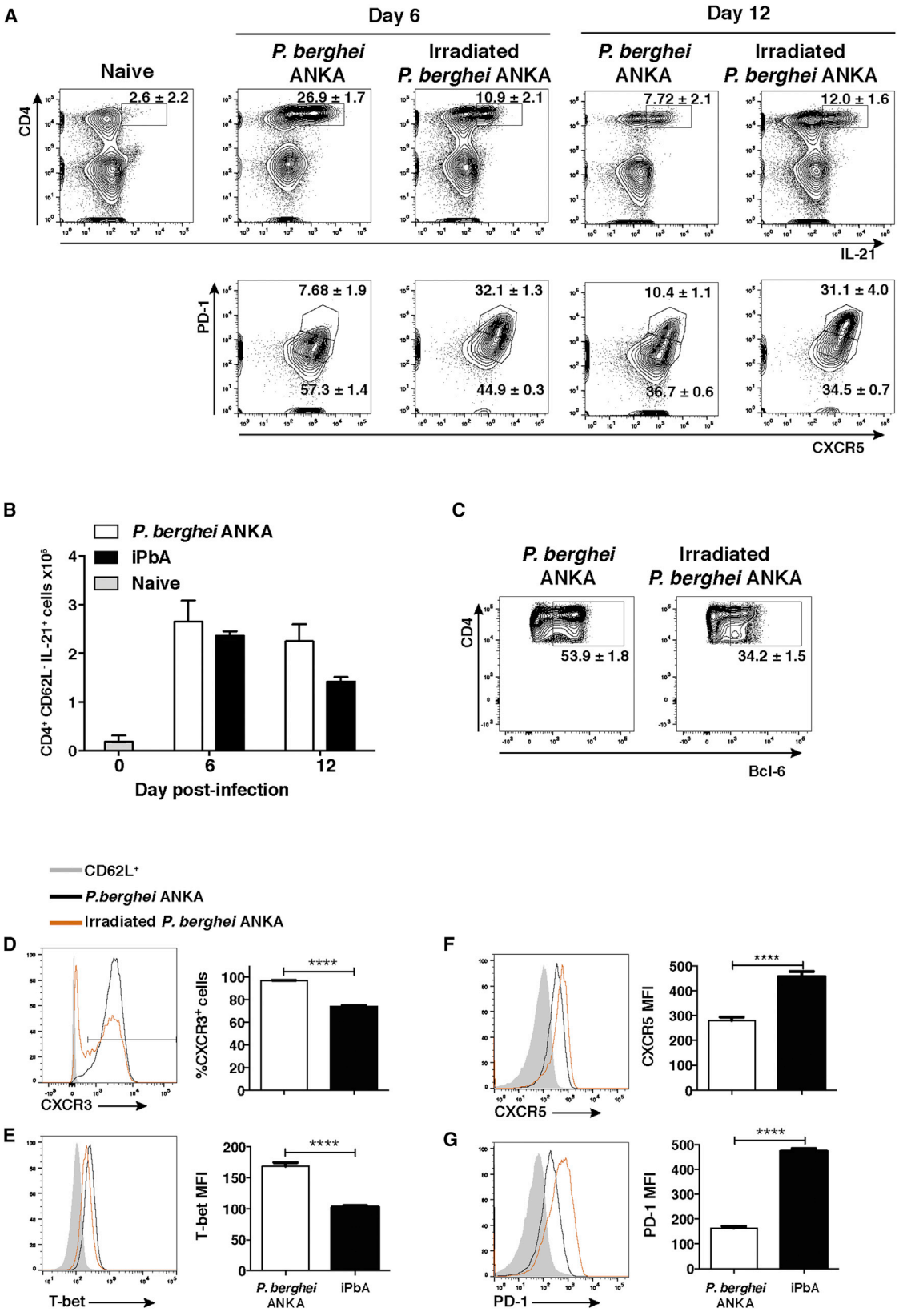
(A–D) C57BL/6 mice were immunized with iPbA or infected with *P. berghei* ANKA. Infected mice were drug-cured on day 5 p.i. The percentage of Tfh cells ($PD\text{-}1^{high}CXCR5^{high}$) (A) was determined at different times p.i. Frequencies and absolute numbers of Tfh cells (B and C) and pre-Tfh cells ($PD\text{-}1^{int}CXCR5^{int}$, B and D) were determined among $CD4^{+}CD62L^{-}$ cells. Data are representative of two experiments. Representative dot plots are shown. Columns show the mean of six to eight mice ± SEM. *$p < 0.05$, ***$p < 0.001$.

(E) Ratio of the number of pre-Tfh cells to Tfh cells determined as in (C) and (D). ***$p > 0.001$.

(F and G). The expression levels of CXCR5 and PD-1 among Bcl-6^{+} CD4^{+} T cells (F) and their absolute numbers (G) were determined on day 6 p.i. Columns show the mean of six mice ± SEM. ****$p < 0.0001$.

See also Figure S3.

immunized with iPbA. Detailed examination of the activated compartment ($CD4^{+}CD62L^{-}$) revealed the presence of a second population of Tfh-like cells expressing intermediate levels of both CXCR5 and PD-1 (Figure 3B). These cells have been described in other infection models and appear to be precursors of fully mature Tfh cells (Ramiscal and Vinuesa, 2013). Consistent with their precursor nature, $CXCR5^{int}PD\text{-}1^{int}$ Tfh cells induced in response to infection did not efficiently localize to GCs or B



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cell follicles (Figure S3). Interestingly, despite a clear defect in the mature Tfh cell compartment (Figures 3A–3C) in malaria-infected mice compared with animals immunized with iPbA, both the percentage and the number of precursor Tfh cells were similar between the two groups (Figures 3B and 3D). Analysis of the ratios between precursor Tfh cells and mature Tfh cells revealed that, although approximately two precursors were found for each mature Tfh cell in animals immunized with iPbA, spleens from *P. berghei* ANKA-infected mice had nearly 15 times as many pre-Tfh cells as mature Tfh cells on day 6 p.i. Together, these results indicate that severe malaria infection compromises the differentiation of precursor Tfh cells into mature Tfh cells.

The transcription factor Bcl-6 is the master regulator of Tfh cell differentiation, and its specific expression allows the identification of Tfh cells. To confirm that the population of CXCR5^{int} PD-1^{int} cells induced in the response to infection belonged to the Tfh cell lineage, the expression of Bcl-6 was analyzed in gated CD4$^+$ T cells 6 days after infection with *P. berghei* ANKA or after immunization with iPbA. Although the frequency of Bcl6$^+$ CD4 T cells was increased in the spleens of *P. berghei* ANKA-infected mice, the absolute number of these cells was significantly higher in spleens of iPbA-immunized mice compared with infected animals (Figures 3F and 3G). Further analysis revealed that both mature Tfh cells and Tfh cell precursors could be found among CD4$^+$Bcl-6$^+$ cells of iPbA-immunized mice. In contrast, the vast majority of CD4$^+$ T cells expressing Bcl-6 in response to infection were CXCR5^{int}PD-1^{int} Tfh cell precursors (Figure 3F), supporting the concept that Tfh cell differentiation is compromised during malaria infection.

Tfh Cells Induced in Response to Malaria Infection Secrete IL-21 and Co-express Th1 Cell Markers

To investigate the capacity of Tfh cells induced in response to malaria to secrete IL-21, *Il21*^{GFP} reporter mice (Lüthje et al., 2012) were infected with *P. berghei* ANKA or immunized with iPbA. Higher frequencies of IL-21$^+$CD4$^+$ T cells were found in infected mice compared with iPbA-immunized controls on day 6 but not on day 12 p.i. (Figure 4A). However, the absolute number of these cells was equivalent between the two groups (Figure 4B). Similar to Bcl-6 expression, IL-21 production after 6 days of infection was found to arise primarily from CXCR5^{int}PD-1^{int} Tfh precursors, whereas a similar percentage of both mature Tfh cells and pre-Tfh cells produced IL-21 in response to immunization with iPbA (Figure 4A). After resolution of infection on day 12 p.i., IL-21 production could be also detected in the CXCR5^{high}PD-1^{high} Tfh cell compartment. Interestingly, 40%–50% of pre-Tfh cells and Tfh cells co-expressed IFN-γ and TNF after infection with *P. berghei* ANKA, whereas, after immunization, only about 20% of pre-Tfh cells and Tfh cells produced these cytokines (Figure S4). Only low frequencies of IL-10-producing Tfh cells could be detected after infection or immunization with irradiated parasites (Figure S4).

Emerging evidence (Weinmann, 2014) suggests that environmental signals mediated by cytokines determine the balance between Th1 and Tfh differentiation by modulating the expression of the transcription factors T-bet and Bcl-6. To determine whether the inflammatory milieu during severe malaria drives helper T cell differentiation toward Th1 development, the expression of T-bet was determined in Tfh lineage cells (Figure 4C) after *P. berghei* ANKA infection or immunization with iPbA. Despite reduced expression of CXCR5 and PD-1 (Figures 4F and 4G), the expression of T-bet was significantly higher in Bcl-6$^+$CD4 T cells in spleens of *P. berghei* ANKA-infected mice compared with iPbA-immunized controls (Figure 4E). The same was true when T-bet expression was examined in pre-Tfh cells or CXCR5^{high}PD1-1^{high} cells (Figure S4). The expression of the chemokine receptor CXCR3, a hallmark of severe malaria infections (Hansen et al., 2007) and a transcriptional target of T-bet, was also examined. Almost 100% of Bcl-6$^+$CD4$^+$ T cells generated in response to infection expressed CXCR3 compared with only 60% of those derived from iPbA-immunized controls. Together, these results demonstrate that severe malaria infection induces the development of a population of CXCR5^{int}PD-1^{int} pre-Tfh cells that share phenotypic features with Th1 lineage cells.

Pro-inflammatory Cytokines Produced during Severe Malaria Infection Inhibit Tfh Cell Differentiation

To determine whether, in the absence of inflammatory signals associated with severe malaria, pre-Tfh cells undergo normal differentiation, an adoptive transfer strategy was pursued. Ly5.2$^+$ *Irf4*$^{fl/fl}$*Cd4*^{Cre} mice, which lack IRF4 expression specifically in the T cell compartment, were used. Because IRF4 is required for Tfh cell differentiation (Bollig et al., 2012), these mice provide an efficient system to assess the helper capacity of adoptively transferred Tfh cells because GC responses are supported exclusively by donor-derived Tfh cells. CD4$^+$CD62L$^-$ cells from Ly5.1$^+$ *P. berghei* ANKA-infected or iPbA-immunized mice were isolated on day 6 p.i. and adoptively transferred into *Irf4*$^{fl/fl}$*Cd4*^{Cre} that were pre-primed with iPbA. Before the adoptive transfer, the majority of Tfh cells from *P. berghei* ANKA-infected mice expressed only intermediate levels of CXCR5 and PD-1 (Figure 5A). In contrast, 6 days after the transfer, nearly 20% of the Ly5.1$^+$ CD4$^+$ T cells were CXCR5^{high}PD-1^{high} cells in animals receiving cells from iPbA-immunized or malaria-infected donors (Figure 5A; Figure S5A). We did not detect Tfh cells among *Irf4*$^{fl/fl}$*Cd4*^{Cre} host cells, validating this model for

Figure 4. Tfh Cells Induced in Response to Malaria Infection Co-express Th1 Cell Markers

(A–G) *Il21*^{GFP} (A and B) or C57BL/6 (C–G) mice were immunized with iPbA or infected with *P. berghei* ANKA. Infected mice were treated with anti-malarial drugs on day 5 p.i.

(A and B) The frequency (A) and absolute number (B) of CD4$^+$IL-21$^+$ cells were determined on days 6 and 12 p.i. The expression levels of CXCR5 and PD-1 in CD4$^+$CD62L$^-$IL-21$^+$ cells (A) were also analyzed. Data are representative of two experiments. Columns represent the mean of three mice ± SEM. Representative dot plots are shown.

(C–G) The phenotype of Bcl-6-expressing CD4 T cells (C) was analyzed. Expression of CXCR3 (D), T-bet (E), CXCR5 (F), and PD-1 (G) is shown in gated CD4$^+$CD62L$^-$Bcl-6$^+$ cells. Data are representative of two experiments. Representative histograms are shown. Columns represent the mean of four to eight mice ± SEM. ****$p < 0.0001$.

See also Figure S4.

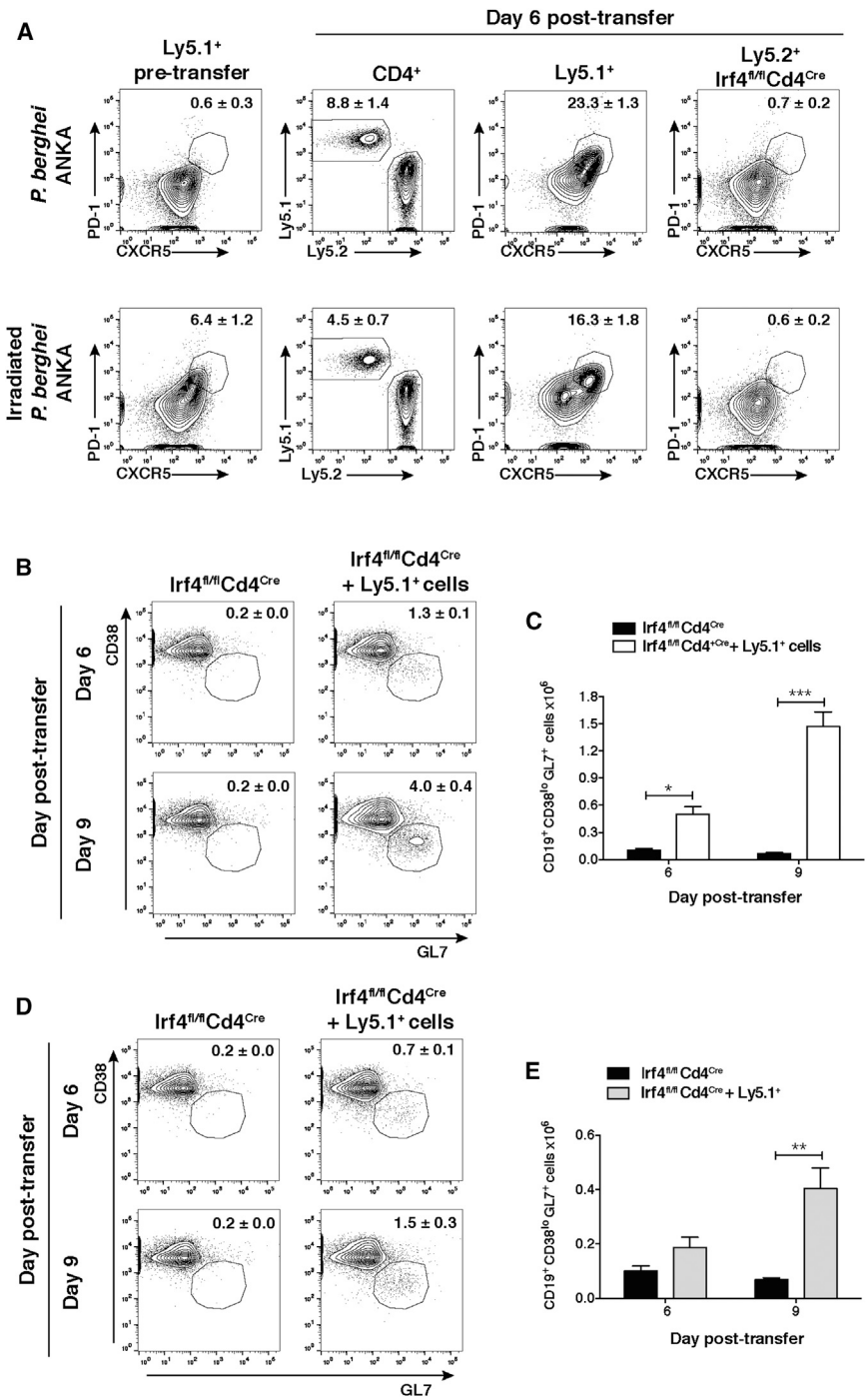


Figure 5. Tfh Cell Precursors Induced in Response to *P. berghei* ANKA Infection Differentiate Normally in the Absence of Inflammatory Signals

Ly5.2$^+$ *Irf4*$^{fl/fl}$*Cd4*^{Cre} mice were immunized with two doses of iPbA. One day after the second dose, mice received CD62L$^-$CD4$^+$ T cells isolated on day 6 p.i. from *P. berghei* ANKA-infected or iPbA-immunized Ly5.1$^+$ donor mice.

(A) The frequency of Ly5.1$^+$PD-1^{high}CXCR5^{high} Tfh cells was determined before and 6 days after adoptive transfer. Analysis of recipient (*Irf4*$^{fl/fl}$*Cd4*^{Cre}, Ly5.2$^+$) CD4$^+$ T cell was included as a control.

(B–E) The percentage (B and D) and absolute number (C and E) of GC B cells (CD19$^+$ CD38^{low}GL7$^+$) were determined at different times after adoptive transfer of CD62L$^-$CD4$^+$ cells from *P. berghei* ANKA-infected (B and C) or iPbA-immunized (D and E) donor mice. Data are representative of two separate experiments. Columns represents the mean of four to eight mice ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S5.

analysis of exogenous Tfh cell function (Figure 5A). Consistent with the precursor nature of pre-Tfh cells, the total number of mature Tfh cells found in recipients receiving cells from malaria-infected infected donors was higher than in recipients that had received cells from iPbA-immunized mice (Figure S5B). Importantly, *Irf4*$^{fl/fl}$*Cd4*^{Cre} mice that had received donor cells from malaria-infected mice developed GC B cells (Figures 5B and 5C) as well as parasite-specific IgM and IgG antibody responses (Figures S5C–S5F) at kinetics comparable with mice that had received cells from immunized donors. Together, these results indicate that, in the absence of inflammatory signals, pre-Tfh cells induced during malaria infection undergo normal differentiation and support GC reactions.

IFN-γ and TNF are inflammatory cytokines known to play a critical role in the induction of severe malaria. To investigate whether these cytokines were involved in the inhibition of GC activity and Tfh cell differentiation observed during infection, mice were infected with *P. berghei* ANKA and treated with anti-IFN-γ- and anti-TNF-neutralizing antibodies or isotype controls. Neutralization of pro-inflammatory cytokines significantly increased the number of mature Tfh cells produced in response to infection compared with isotype control-treated mice (Figures 6A and 6C). Similar results were obtained after infection of *Ifng*$^{-/-}$ mice (Figure S6) or animals treated with anti-IFN-γ alone (data not shown), although the effect was less pronounced than when both IFN-γ and TNF were neutralized. The ratio of pre-Tfh cells to Tfh cells was reduced significantly in anti-cytokine-treated animals compared with isotype controls (Figure 6D). Moreover, neutralization of pro-inflammatory cytokines also resulted in a 3-fold expansion of the splenic GC B cell pool (Figure 6E), a marked improvement of splenic architecture, with increased retention of T cells in T cell zones as well as improved GC structures

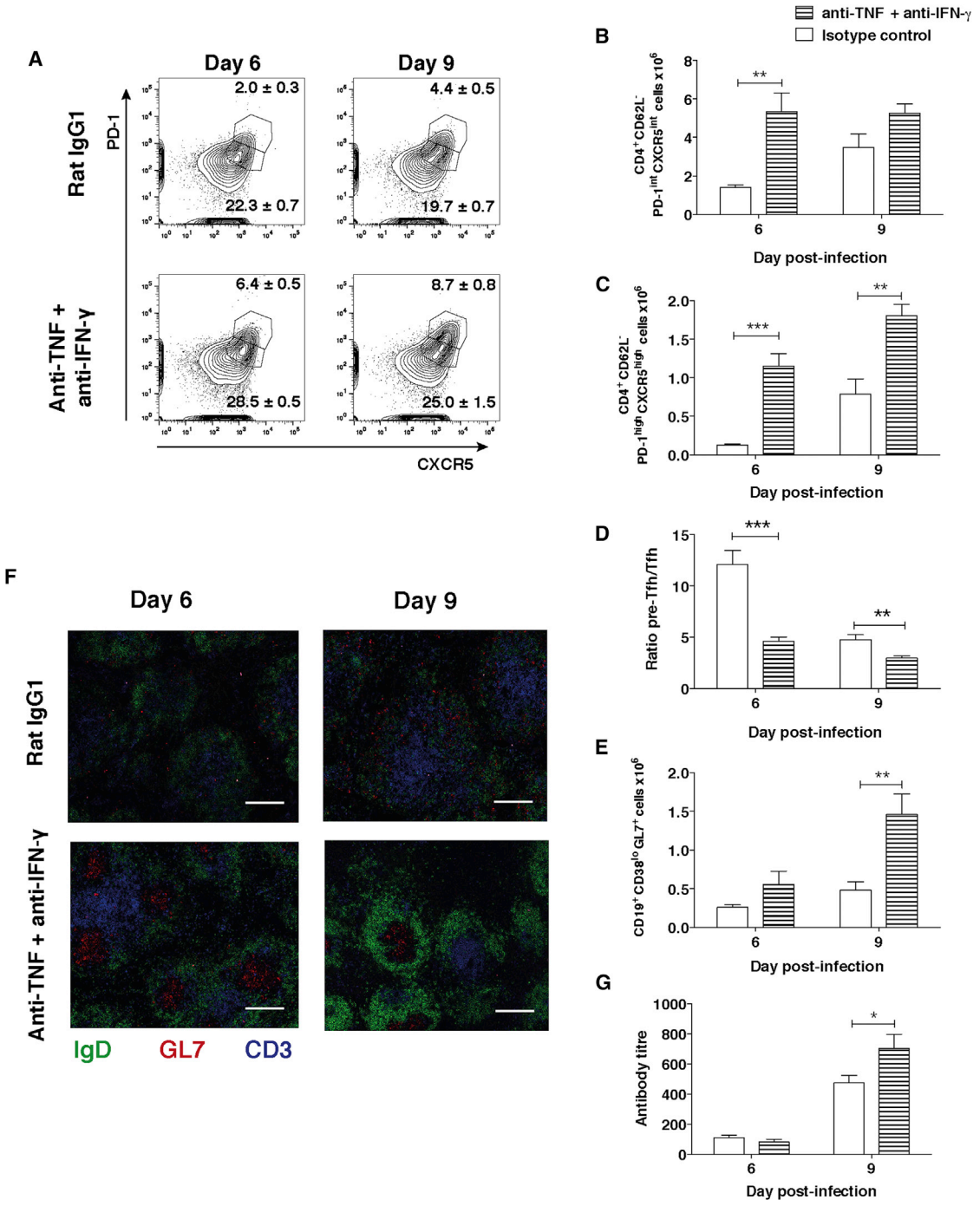
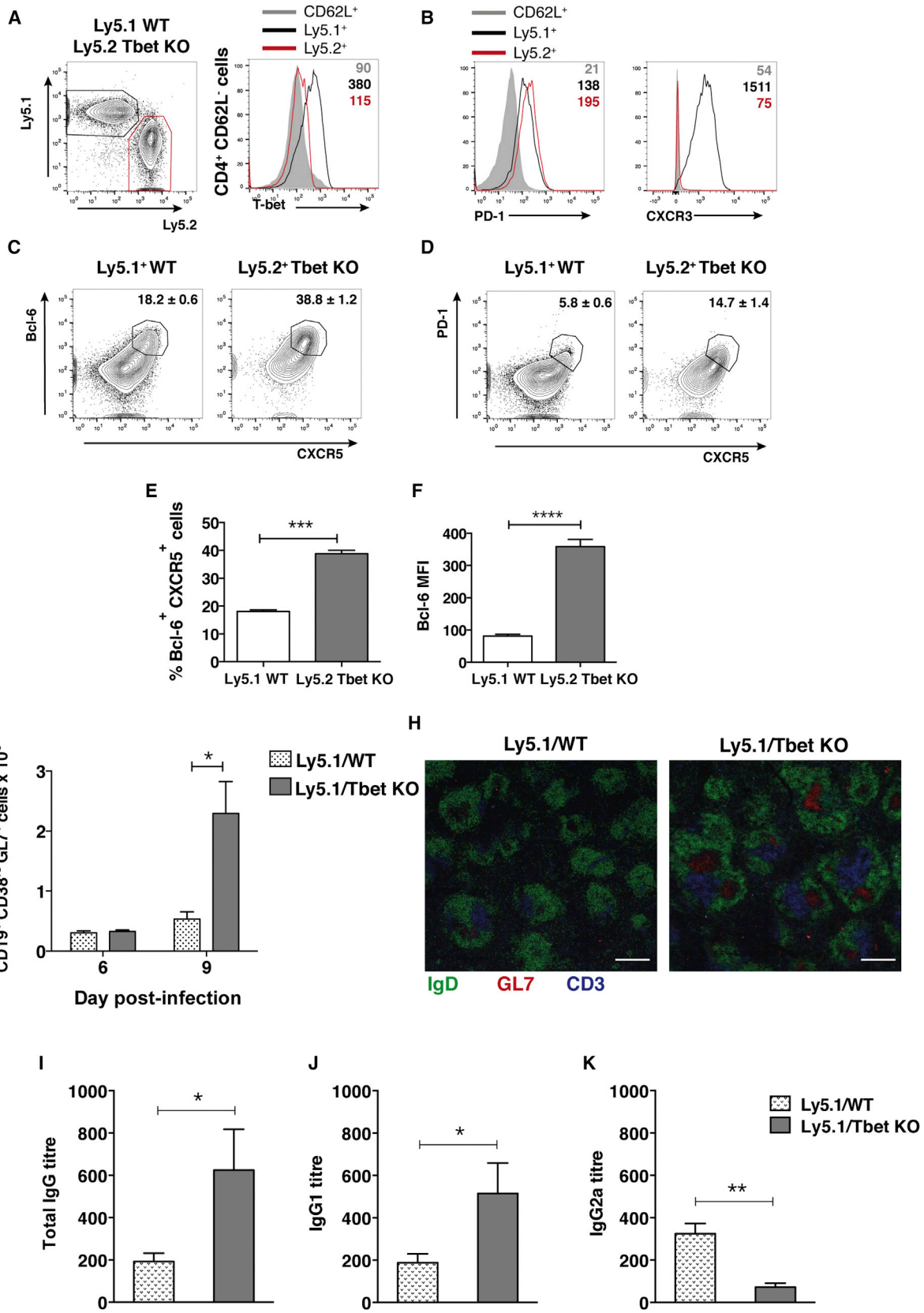


Figure 6. Pro-inflammatory Cytokines Produced during Severe Malaria Infection Inhibit Tfh Cell Differentiation

(A–E and G) *P. berghei* ANKA-infected mice were treated with anti-TNF and anti-IFN-γ neutralizing antibodies or isotype control. The frequency (A), absolute numbers of pre-Tfh ($PD\text{-}1^{int}CXCR5^{int}$) (B) and Tfh ($PD\text{-}1^{high}CXCR5^{high}$) (C) cells, and the ratio of pre-Tfh cells to Tfh cells (D) were determined in $CD4^{+}CD62L^{-}$ T cells. The total numbers of GC B cells ($CD19^{+}CD38^{low}GL7^{+}$) (E) and parasite-specific IgG antibodies were also determined (G). Data are representative of two separate experiments. Columns represent the mean of six mice ± SEM. Representative dot plots are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

(F) Immunofluorescence staining of spleen sections from anti-TNF- and anti-IFN-γ-treated or isotype control-treated mice identifying GC cells with anti-GL7, naive B cell follicles with anti-IgD, and T cell zones with anti-CD3. Scale bars, 200 μm.

See also Figure S6.



(legend on next page)

(Figure 6F) and significantly higher parasite-specific IgG responses to infection (Figure 6G). Together, these results indicate that the pro-inflammatory cytokines IFN-γ and TNF negatively regulate Tfh cell differentiation during severe malaria infection and inhibit GC reactions.

Expression of T-bet Impairs Tfh Cell Differentiation during Severe Malaria

T-bet (encoded by the *Tbx21* gene) is induced by Th1-type inflammation and has been found previously to promote severe malaria pathogenesis (Oakley et al., 2013) and modulate the development of Tfh cell responses (Nakayamada et al., 2011). Therefore, we evaluated the effect of T-bet expression on Tfh cell differentiation throughout infection using a mixed bone marrow chimera approach. Mice were reconstituted with a 1:1 mix of congenically marked Ly5.1$^+$ WT and Ly5.2$^+$ *Tbx21*$^{-/-}$ (T-bet knockout [KO]) bone marrow and infected with *P. berghei* ANKA (Figure 7A). The capacity of each donor compartment to mount Tfh cell responses to infection was assessed by flow cytometry. As expected, T-bet and CXCR3 upregulation occurred by day 6 p.i. in WT but not in *Tbx21*$^{-/-}$ CD62L$^-$CD4$^+$ T cells (Figures 7A and 7B). Importantly, T-bet-deficient CD4$^+$ T cells resulted in significantly higher Bcl-6 expression (Figure 7F) and gave rise to significantly higher frequencies of mature Tfh cells (Figures 7C and 7D) compared with WT cells. No differences were found among compartments of control chimeras reconstituted with Ly5.1$^+$ WT and Ly5.2$^+$ WT bone marrow (data not shown). Chimerism levels in both WT:WT and WT:*Tbx21*$^{-/-}$ remained approximately 1:1 throughout the infection period. Importantly, mixed chimeras containing T-bet-deficient cells allowed the establishment of GC structures in the spleen (Figure 7G), promoted the induction of GC B cells significantly more efficiently (Figure 7H), and gave rise to significantly higher IgG1 but reduced IgG2a parasite-specific antibody titers compared with control chimeras (Figures 7I–7K). Therefore, T-bet negatively regulates Tfh cell development during severe malaria infection.

DISCUSSION

Naturally acquired immunity to malaria develops only after repeated exposure to the parasite over a number of years. The cellular processes underlying the slow and imperfect acquisition of immunity are still not understood. Using a mouse model of severe malaria, this study provides mechanistic insights into cellular processes that are impaired during the development of B cell responses to infection. Our study establishes that compromised Tfh cell responses are responsible for reduced GC activity. Moreover, this study identifies critical factors modulating these processes and demonstrates that the same pro-inflammatory responses mediating severe malaria disease syndromes also have a detrimental effect on the induction of protective immunity by inhibiting Tfh cell differentiation.

Other studies have investigated B cell responses in mouse models of malaria, primarily using the non-lethal *P. chabaudi* infection. Although a concomitant *P. chabaudi* infection has been found to suppress humoral responses to secondary heterologous antigens (Millington et al., 2006; Wilmore et al., 2013), most of the available evidence indicates that, despite transient changes of splenic architecture, *P. chabaudi*-infected mice mount adequate parasite-specific B cell responses required to control parasitemia, with IL-21 production playing a critical role in this process (Achtman et al., 2003; Ndungu et al., 2009; Pérez-Mazliah et al., 2015). Our study is well aligned with those findings because mice lacking plasma cells were unable to control *P. chabaudi* replication, illustrating a key role for antibody responses in the control of infection in a self-resolving model of malaria. In contrast, during *P. berghei* ANKA infection, extrafollicular plasmablast responses as well as GC reactions were generated inefficiently, and Tfh cell differentiation was inhibited, demonstrating that severe malaria affects GC dependent and independent responses.

CD4$^+$ T cells perceive their cytokine environment by signaling through signal transducer and activator of transcription (STAT) proteins that lead to the upregulation of transcriptional regulators that guide differentiation toward distinct T helper subsets. During Tfh cell differentiation, the molecular balance between the opposing helper T cell lineage-specifying transcription factors T-bet and Bcl-6 regulates flexibility in the Th1 and Tfh cell gene programs (Weinmann, 2014). Excessive T-bet expression directly antagonizes Bcl-6 function by masking the DNA binding domain required to regulate the Tfh gene expression program (Oestreich et al., 2012). In line with this concept, the strong inflammatory milieu accompanying severe malaria appeared to block Tfh cell development, giving rise to high frequencies of Tfh cell precursors expressing T-bet and Th1 lineage molecules such as CXCR3. Furthermore, genetic ablation of T-bet during infection allowed efficient differentiation of mature Tfh cells, demonstrating that the expression of this transcription factor directly represses Tfh cell responses during severe malaria

Figure 7. Expression of T-bet Impairs Tfh Cell Differentiation during *P. berghei* ANKA Infection

Irradiated C57BL/6 mice were reconstituted with a 1:1 ratio of Ly5.1$^+$ WT and Ly5.2$^+$ *Tbx21*$^{-/-}$ (T-bet KO) bone marrow and infected with *P. berghei* ANKA.

(A) Representative dot plots showing Ly5.1 and Ly5.2 expression in lymphocytes from chimeric mice and T-bet expression on gated Ly5.1$^+$ or Ly5.2$^+$ CD62L$^-$CD4$^+$ T cells.

(B) PD-1 and CXCR3 expression levels in Bcl-6$^+$CXCR5^{high} CD4$^+$ T cells were also determined within the Ly5.1$^+$ and Ly5.2$^+$ compartments on day 6 p.i.

(C–F) The frequency of Tfh cells, defined as Bcl-6$^+$CXCR5^{high} (C and E) or PD-1^{high}CXCR5^{high} (D) CD4$^+$ T cells, as well as Bcl-6 expression levels (F) were calculated in gated CD4$^+$CD62L$^-$ T cells on day 9 p.i. within the Ly5.1$^+$ and Ly5.2$^+$ compartments.

(G) The percentage of GC B cells (CD19$^+$CD38^{low}GL7$^+$) in WT:*Tbx21*$^{-/-}$ and WT:WT mixed chimeras was determined at different times p.i. with *P. berghei* ANKA.

(H) Immunofluorescence staining of spleen sections from WT:*Tbx21*$^{-/-}$ and WT:WT mixed chimeras identifying GC cells with anti-GL7, B cell follicles with anti-IgD, and T cell zones with anti-CD3. Scale bars, 200 μm.

(I–K) Parasite-specific total IgG (I), IgG$_1$ (J), and IgG$_{2a}$ (K) antibody titers in sera of WT:*Tbx21*$^{-/-}$ and WT:WT mixed chimeras were measured by ELISA on day 9 p.i. Data are representative of two experiments. Representative histograms and dot plots are shown. Columns represent the mean of seven to eight mice ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

infection. IFN-γ, a key mediator of severe disease in the *P. berghei* ANKA model (Grau et al., 1989), also inhibited Tfh cell differentiation, suggesting that signaling through STAT-1 might contribute to the upregulation of T-bet within the Tfh cell compartment.

T-bet expression was also responsible for the upregulation of CXCR3 on Tfh cells of malaria-infected mice. The expression of this receptor is a hallmark of *P. berghei* ANKA infection and allows lymphocytes to migrate to the brains of infected mice, where they contribute to the development of cerebral malaria (Hansen et al., 2007). It is possible that CXCR3 expression among Tfh cells favors their egress outside the spleen, which may contribute to the depletion of a functional Tfh cell compartment required to sustain humoral immunity.

Apart from inducing Th1 features in Tfh cells, inflammatory cytokines also contributed to the loss of splenic architecture characteristic of acute malaria. Blockade of TNF and IFN-γ improved GC formation and also facilitated the retention of T cells within the white pulp. Both cytokines have been found previously to modulate tissue remodeling in other infection models. Whereas IFN-γ responses to lymphocytic choriomeningitis virus (LCMV) have been shown to downregulate the expression of CCL21, required for localization of T cells in T cell zones (Mueller et al., 2007a), TNF produced in response to *Leishmania donovani* mediates the loss of fibroblastic reticular cells, responsible for the production of this homeostatic chemokine (Ato et al., 2002). Because changes in the location of B cells, T cells, and antigen-presenting cells affects access to antigen and cellular interactions, it has been proposed that the loss of secondary lymphoid organ structure impairs immune responses to infection (Ato et al., 2002; Choi et al., 2003; Mueller et al., 2007a, 2007b). Interestingly, Tfh cells are particularly dependent on cell-cell interactions because they rely on B cell-derived signals for their development and maintenance. Surface expression of ICOS (Liu et al., 2015) and CD28 (Salek-Ardakani et al., 2011) as well as signaling lymphocytic-activating molecule (SLAM) family members such as CD84 and Ly108 have emerged as important factors involved in T:B cell engagement required for efficient Tfh cell differentiation (Ramiscal and Vinuesa, 2013). It is conceivable that the disorganized splenic structure characteristic of acute malaria does not facilitate the interactions between B cells and T cells required to sustain Tfh cell differentiation. Therefore, our results support a model in which inflammatory responses to malaria impair GC reactions by two non-exclusive mechanisms: direct upregulation of T-bet in Tfh precursors, which interferes with correct Tfh cell differentiation, and disruption of the splenic architecture, which impairs T:B cell interactions required to sustain this process.

Similar to our findings using a severe malaria infection model, it has been shown recently that acute *P. falciparum* malaria induces the activation of circulating PD-1$^+$CXCR5$^+$CXCR3$^+$ Tfh cells, which appear to have a limited helper capacity (Obeng-Adjei et al., 2015). Although these findings revealed the negative effect a single malaria episode has on the development of humoral immunity, the results shown here also indicate that control of parasitemia and severe disease by treatment with anti-malarial drugs allows the establishment of GC responses. In line with those observations, field studies have shown that individuals living in low transmission settings (Wipasa et al., 2010) are able to efficiently mount parasite-specific B cell memory. Further mechanistic work is required to establish whether frequent clinical episodes are responsible for the delayed acquisition of immunological of B cell memory, as observed in individuals residing in high transmission areas (Weiss et al., 2010). A better understanding of the factors that impair humoral immunity is of vital importance because it may lead to the development of therapeutic approaches to improve the efficacy of immune responses to the malaria parasite.

EXPERIMENTAL PROCEDURES

Mice and Infections

All mice used throughout this study, including *Il21*^{GFP}, *Prdm1*^{GFP}, *Prdm1*$^{fl/fl}$, *Ifng*$^{-/-}$, *Irf4*$^{fl/fl}$, and *Tbx21*$^{-/-}$ mice, were on a C567BL/6J background. Eight- to twelve-week-old mice were infected intravenously (i.v.) with 5×10^4–1×10^5 *P. berghei* ANKA or *P. chabaudi chabaudi* AS pRBCs. Parasitemia was assessed from Giemsa-stained smears of tail blood, and mortality was checked daily. In some experiments, mice were treated with a dose of chloroquine (10 mg/kg) and pyrimethamine (10 mg/kg) intraperitoneally (i.p.) at the onset of severe malaria symptoms on day 5 p.i., followed by treatment with drinking water containing chloroquine and pyrimethamine (10 mg/kg) for 7 days. In immunization experiments, mice received three i.v. injections of 1×10^8 irradiated (20,000 rad) *P. berghei* ANKA pRBCs 2–3 days apart. For cytokine blockade experiments, mice were injected i.p. every other day from day 1 p.i. with anti-IFN-γ (HB170, 0.5 mg) and anti-TNF (XT-22, 0.2 mg) or 0.7 mg of anti-rat IgG1 isotype control antibody. All experiments were performed in compliance with the Walter and Eliza Hall Institute Animal Ethics Committee requirements.

Generation of Bone Marrow Chimeras

Lethally irradiated (two doses of 550 Cy 3 hr apart) Ly5.1 mice were reconstituted with equal numbers of bone marrow cells from Ly5.1 mice and *Tbx21*$^{-/-}$ (Ly5.2) or WT (Ly5.2) controls. Chimeric mice were allowed to reconstitute for 8 weeks before use in malaria infections.

ELISA for Detection of Malaria-Specific Antibodies

Microtiter plates were coated with *P. berghei* ANKA lysate (5 μg/ml) in carbonate buffer (pH 9.6) by overnight incubation at 4°C. Empty sites were blocked with 5% skim milk for 1 hr at 37°C. After washing with 0.05% Tween 20 in PBS, plates were incubated with antisera in serial dilutions for 1 hr at 37°C. The plates were washed and incubated with a peroxidase-conjugated rabbit anti-mouse antibody (Pierce). Isotype titers were determined after incubation with anti-IgM, -IgG1, or -IgG2a antibodies (Zymed), followed by incubation with a peroxidase-conjugated goat anti-rabbit antibody (Pierce). Bound complexes were detected by reaction with tetramethyl-benzidine (KBL) and H_2O_2. Absorbance was read at 450 nm.

Immunofluorescence Staining and Confocal Microscopy

Spleens were snap-frozen in optimum cutting temperature compound, and 5-μm sections were mounted on Polysine slides prior to fixation in 100% ice-cold acetone. Endogenous biotin was blocked using an avidin/biotin blocking kit prior to staining with Alexa 488 anti-IgD (11-26c.2a), biotin-conjugated GL7, and Alexa 647-conjugated CD3 (RM4-5) for 2 hr at room temperature. Secondary streptavidin-conjugated-Alexa 568 was used to detect anti-GL7 staining. In some experiments, allophycocyanin (APC) anti-CD45.1 (A20) was used. Slides were mounted in ProLong Gold antifade medium. Images were acquired with a 20× objective on a Zeiss LSM 780 laser-scanning confocal system.

Flow Cytometry

Splenocyte suspensions were stained with the fixable viability dye efluor-506 (eBioscience) for dead cell exclusion, followed by incubation with anti-CD16/

CD32 antibody in fluorescence-activated cell sorting (FACS) staining buffer. Cells were washed twice prior to incubation with fluorescent antibodies for 45 min at 4°C. For analysis of GC B cells, cells were stained with fluorescein isothiocyanate (FITC) anti-CD38 (90/CD38), phycoerythrin (PE) anti-GL7 and PerCPCy5.5 anti-CD19 (1D3), or PE Cy7anti-CD4 (RM4-5, BioLegend), brilliant violet 650 anti-CD19 (1D3, BioLegend), APCCy7 anti-CD62L (MEL-14, BioLegend), PE anti-PD-1 (J43), PerCPCy5.5 anti-CXCR5 (2G8), and APC anti-CXCR3 (173, BioLegend) for the analysis of Tfh cells. Memory B cells were detected with PECy7 anti-CD38 (90, BioLegend), PerCPCy5.5 anti-CD19 (1D3), FITC anti-CD138 (281-2), and FITC anti-IgD (11-26c.2a). When required, secondary streptavidin conjugates were added for 30 min at 4°C following sample washes (all antibodies were from BD Pharmingen unless indicated otherwise). Anti-CD45.1 (A20, BioLegend) and CD45.2 (104, BioLegend) antibodies were used to identify congenically marked cells. For intracellular staining, cells were stained with surface markers and then fixed/permeabilized using the Foxp3/transcription factor staining buffer set (eBioscience). Antibodies to Bcl-6 (K112-91) and T-bet (4B10) were then added for 45 min at room temperature. The cells were then washed twice, resuspended in PBS, and analyzed with a BD LSR II W or Fortessa X20. Analysis was performed with FlowJo X 10.0.7 (Tree Star).

Adoptive Transfer

Ly5.1 C57BL/6 mice were infected with 5×10^4 *P. berghei* ANKA pRBCs. On day 6 p.i., $CD4^+CD62L^-$ spleen cells were isolated by sorting on a BD Aria W system. In some experiments, pre-Tfh and mature Tfh cells were isolated. Sorted cells were adoptively transferred (1×10^6 cells/mouse) into Ly5.2 $Irf4^{fl/fl}CD4^{Cre}$ or WT recipients immunized previously with two doses of 1×10^8 irradiated pRBCs (2 days apart). Spleen cells and sera from recipient animals were collected on days 2, 6, or 9 post-transfer for assessment of immune responses.

Statistical Analysis

Student's t test was used for data evaluation of datasets with confirmed normal distribution. Other datasets were evaluated using Mann-Whitney nonparametric test. Multiple comparisons were evaluated by t tests corrected with the Holm-Sidak method. Differences in mortality rates of malaria-infected mice were assessed by Cox-Mantel log rank analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.12.006.

AUTHOR CONTRIBUTIONS

V.R.C., D.Y., A.K., and D.S.H. planned the study and interpreted the data. V.R.C., L.J.I., A.L., C.Y.C., J.T., and D.L.H. performed the experiments. S.P.P., M.P., and S.L.N. contributed materials. V.R.C., A.K., and D.S.H. wrote the paper.

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