Transcription Factor T-bet in B Cells Modulates Germinal Center Polarization and Antibody Affinity Maturation in Response to Malaria

Graphical Abstract

Highlights

- Antibody responses play a pivotal role in naturally acquired immunity to malaria
- Inflammatory responses upregulate T-bet in germinal center B cells during malaria
- T-bet favors commitment of B cells to the dark zone of the germinal center
- T-bet expression in germinal center B cells improves antibody affinity maturation

Authors

Ann Ly, Yang Liao, Halina Pietrzak, ..., Wei Shi, Axel Kallies, Diana S. Hansen

Correspondence

axel.kallies@unimelb.edu.au (A.K.), hansen@wehi.edu.au (D.S.H.)

In Brief

Antibody responses play a pivotal role in clinical immunity to malaria. Ly et al. report that germinal center (GC) B cells upregulate the transcription factor T-bet during infection. T-bet favors commitment of B cells to the GC dark zone, thereby augmenting immunoglobulin gene mutation rates and antibody affinity maturation.
Transcription Factor T-bet in B Cells Modulates Germinal Center Polarization and Antibody Affinity Maturation in Response to Malaria

Ann Ly,1,2 Yang Liao,1,2 Halina Pietrzak,1,2 Lisa J. Ioannidis,1,2 Tom Sidwell,2 Renee Gloury,6 Marcel Doerflinger,1,2 Tony Triglia,1,2 Raymond Z. Qin,1,2 Joanna R. Groom,1,2 Gabriele T. Belz,1,2 Kim L. Good-Jacobson,4,5 Wei Shi,1,3 Axel Kallies,6,* and Diana S. Hansen1,2,7,*

1The Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria 3052, Australia
2Department of Medical Biology, The University of Melbourne, Parkville, Victoria 3010, Australia
3Department of Computing and Information Systems, The University of Melbourne, Parkville, Victoria 3010, Australia
4Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia
5Infection and Immunity Program, Biomedicine Discovery Institute, Monash University, Clayton, Victoria 3800, Australia
6The University of Melbourne, Department of Microbiology and Immunology, The Peter Doherty Institute for Infection and Immunity, 792 Elizabeth Street, Melbourne, Victoria 3000, Australia
7Lead Contact
*Correspondence: axel.kallies@unimelb.edu.au (A.K.), hansen@wehi.edu.au (D.S.H.)
https://doi.org/10.1016/j.celrep.2019.10.087

SUMMARY

Despite the key role that antibodies play in protection, the cellular processes mediating the acquisition of humoral immunity against malaria are not fully understood. Using an infection model of severe malaria, we find that germinal center (GC) B cells upregulate the transcription factor T-bet during infection. Molecular and cellular analyses reveal that T-bet in B cells is required not only for IgG2c switching but also favors commitment of B cells to the dark zone of the GC. T-bet was found to regulate the expression of Rgs13 and CXCR3, both of which contribute to the impaired GC polarization observed in the absence of T-bet, resulting in reduced IghV gene mutations and lower antibody avidity. These results demonstrate that T-bet modulates GC dynamics, thereby promoting the differentiation of B cells with increased affinity for antigen.

INTRODUCTION

Malaria remains one of the most harmful infectious diseases of humans, causing more than 200 million clinical cases annually (World Health Organization, 2015). Most cases of severe disease are caused by Plasmodium falciparum. Clinical manifestations range from fever to complicated syndromes, including severe anemia, acute respiratory distress, renal failure, seizures, and coma (White and Ho, 1992). Blood-stage malaria parasites, responsible for the induction disease symptoms, express parasitic proteins on the surface of infected erythrocytes, which allows them to bind to vascular endothelial cells. This process, known as parasite sequestration, induces obstructions in the blood flow, resulting in hypoxia and hemorrhages (Miller et al., 2002) associated with organ-specific syndromes. A large body of work indicates that in addition to parasite sequestration, inflammatory responses also contribute to severe disease. High levels of TNF (Molyneux et al., 1993), IFN-γ, IL-1β (Pongponratn et al., 2003), and CXCL10 (Armah et al., 2005) have been associated with disease severity.

Individuals living in malaria-endemic areas develop clinical immunity only after many years of repeated exposure to the parasite. This naturally acquired immunity is not sterilizing but prevents clinical episodes by substantially controlling parasite densities (Marsh and Kinyanjui, 2006). Passive transfer experiments (Cohen et al., 1961) demonstrated that antibodies are a key component of clinical immunity to malaria, with roles that include inhibition of parasite invasion into red blood cells (RBCs) (Blackman et al., 1990) and opsonizing parasites for phagocytosis by effector cells (Hill et al., 2013).

The development of antibody-mediated immunity after exposure to antigen requires the establishment of germinal center (GC) reactions in secondary lymphoid organs. During this process, activated B cells undergo somatic hypermutations of their immunoglobulin (Ig) genes, followed by selective expansion and survival of B cells with high-affinity antigen receptors. These processes take place in two distinct structural and functional compartments within the GC. Whereas proliferation and somatic Ig hypermutation occur in the dark zone (DZ), selection of high-affinity antibody-expressing clones takes place in the light zone (LZ). GCs give rise high-affinity plasma cells and memory B cells. While plasma cells migrate to the bone marrow and continue to produce antibodies for years, memory B cells recirculate and become activated upon re-encounter with their cognate antigen. GC reactions are supported by T follicular helper (Tfh) cells, which express the chemokine receptor CXCR5 that allows them to migrate to B cell areas and provide help to B cells by a number of different mechanisms, including the production of IL-21 (Vinuesa et al., 2005).

The cellular mechanisms underlying the slow acquisition of antibody-mediated immunity to malaria are not fully understood. Using the P. berghei ANKA mouse infection, which recapitulates many features of human P. falciparum malaria (Hansen, 2012; Miller et al., 2002; Schofield and Grau, 2005), we have previously
found that pro-inflammatory cytokines mediating clinical symptoms compromise T<sub>FH</sub> cell differentiation (Ryg-Cornejo et al., 2016). P. berghei ANKA infection was characterized by the accumulation of T<sub>FH</sub> cell precursors, which co-express T<sub>bet</sub> cell-associated molecules such as the transcription factor T-bet and the chemokine receptor CXCR3. IFN-γ and TNF neutralization or genetic ablation of T-bet restored T<sub>FH</sub> cell differentiation and GC formation in the spleen (Ryg-Cornejo et al., 2016). Similar to our findings in mice, acute symptomatic P. falciparum malaria infections were found to activate circulating T<sub>H1</sub>-like T<sub>FH</sub> cells, with limited helper capacity (Obeng-Adjei et al., 2015).

In addition to its well-characterized role in driving T<sub>H1</sub> cell polarization in CD4<sup>+</sup> T cells, T-bet can be found in other lineages, including B cells (Kallies and Good-Jacobson, 2017). The best defined role for T-bet in B cells is its requirement for IgG<sub>2c</sub> isotype class switching (Gerth et al., 2003; Peng et al., 2002; Wang et al., 2012). T-bet was also found to be upregulated in B cells associated with aging (Rubtsova et al., 2015), in memory B cells elicited in response to viral infection (Barnett et al., 2016; Knox et al., 2017; Rubtsova et al., 2013), and in CD11c<sup>+</sup> B cells associated with autoimmune conditions such as systemic lupus erythematosus (SLE) (Rubtsova et al., 2017). In malaria, T-bet has been detected in a subset of auto-reactive anti-body-secreting cells that contribute to autoimmune anemia in mice (Rivera-Correia et al., 2017) and during acute P. falciparum infection, in a population of atypical memory B cells (Obeng-Adjei et al., 2017) reported to have poor effector function (Portugal et al., 2015). Despite studies describing expression of T-bet in GC B cells (Guthmiller et al., 2017; Piovesan et al., 2017; Sheikh et al., 2013), its precise role in modulating GC B cell function during infection remains elusive. To address this question, we examined GC development to Plasmodium infection in mice that lack T-bet specifically in their B cell compartment. Our results revealed that T-bet supports a transcriptional program required for commitment of B cells to the DZ of the GC. T-bet thereby influences GC polarization, resulting in augmented Ig affinity mutation and improved antibody affinity maturation. Overall, our data show that T-bet directs B cell responses during malaria infection in two independent ways. While T cell intrinsic T-bet governs the magnitude of the GC response, T-bet expression in B cells controls the quality of the GC response.

**RESULTS**

**Expression of T-bet in T Cells and B Cells Controls GC B Cell Responses to Malaria**

Using the P. berghei ANKA infection model, we have previously shown that T-bet upregulation associated with acute malaria inhibits T<sub>FH</sub> cell differentiation during infection (Ryg-Cornejo et al., 2016). To test if T-bet was also involved in B cell-intrinsic processes in response to malaria, we infected C57BL/6 mice with P. berghei ANKA and examined T-bet expression in gated CD19<sup>+</sup>CD38<sup>low</sup>GL7<sup>+</sup> GC B cells at different times post-infection (p.i.) using flow cytometry. As P. berghei ANKA infection is lethal in C57BL/6 mice, infected animals were cured with anti-malarial drugs after the onset of disease symptoms on day 5 p.i. to allow assessment of immune responses at later times as described (Ryg-Cornejo et al., 2016). As early as day 6 p.i., more than half of splenic GC B cells expressed T-bet (Figure 1A). Notably, about 60% of all splenic GC B cells expressed the chemokine receptor CXCR3, a well-defined transcriptional target of T-bet, on days 6–9 p.i. (Figure 1B). The absolute number of GC B cells expressing CXCR3 peaked at day 12 p.i. and was strongly correlated with the expression of T-bet in this compartment (Figure 1C). These data suggest that T-bet contributes to the control of GC B cell response to malaria not only via regulating T<sub>FH</sub> cell differentiation but also in a B cell intrinsic manner.

To examine the relative contribution of T-bet in T and B cells to GC formation and function, we infected Tbx21<sup>fl/fl</sup>Cd4<sup>Cre</sup> (lacking T-bet in T cells), Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> mice (lacking T-bet in mature follicular B cells), and control mice with P. berghei ANKA. Consistent with our previous results, the absence of T-bet in CD4<sup>+</sup> T cells significantly increased frequencies and absolute numbers of mature T<sub>FH</sub> cells (CD4<sup>+</sup>CXCR5<sup>high</sup>PD-1<sup>high</sup>) produced in response to infection (Figures S1A and S1B), which resulted in a 3-fold increase in the number of GC B cells in Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> mice compared with controls (Figures 1E and 1F) and improved the establishment of GC structures in the spleen (Figure 1G). In contrast, deletion of T-bet in B cells resulted in only a minor increase of GC B cells in spleens of Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> mice compared with Cd23<sup>Cre</sup> controls (Figures 1H–1J), and frequencies or numbers of T<sub>FH</sub> cells were unaltered (Figures S1C and S1D). As expected, B cells in Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> malaria-infected mice did not express CXCR3 (Figure 1B) and did not class-switch to IgG<sub>2c</sub> (Figure 1D). Instead, higher frequencies of T-bet-deficient GC B cells expressed IgG<sub>1</sub> compared with wild-type (WT) mice (Figure 1D). Together these results indicate that T-bet affects GC development in response to malaria in two different ways. While T-bet expression in T cells limits the magnitude of the GC response, T-bet in B cells controls the quality of the GC response.

**T-bet Modulates the Transcriptional Profile of GC B Cells in Response to Malaria**

To define how the expression of T-bet affects GC B cells in response to infection, the transcriptional profile of sorted GC B cells from P. berghei ANKA-infected Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> and Cd23<sup>Cre</sup> mice was examined using RNA sequencing (RNA-Seq). We found that 407 genes were differentially expressed between both groups. While key genes required for GC development and function, including Bcl-6, Cxcr5, and activation-induced cytidine deaminase (Aicda), required for somatic hypermutation of Ig genes, were not affected, genes encoding molecules required for T cell help, including Cd86, Cd83, and Cd40, were significantly upregulated in the absence of T-bet (Figure 2A). Using a transcriptional signature of GC B cells, which we described previously (Gloury et al., 2016), we identified 17 GC cell-specific genes, expression of which was deregulated in the absence of T-bet. Notably, 15 of these genes, including Mcm3, Mcm4, Mcm7, Pola1, and Tpx2, essential for DNA replication during cell division, were significantly downregulated, while the other two genes, Hs-Q6 and the negative regulator of G
protein signaling $Rgs13$, were upregulated in $Tbx21^{fl/fl}Cd23^{Cre}$ compared with WT GC B cells (Figure 2B).

GC B cells cyclically transit between the DZ and LZ of GCs to undergo rounds of proliferation and affinity selection (Allen et al., 2007). DZ and LZ GC B cells differ substantially in their gene expression profiles, with LZ GC B cells characterized by the expression of immune cell activation-related genes, while DZ GC B cells show increased expression of genes involved in the control of cell cycle (Victora et al., 2012). Interestingly, Gene Ontology analysis showed that pathways involved in regulation of immune responses and control of cell cycle were deregulated in the absence of T-bet (Figure S2; Figures 2C and 2D), suggesting that T-bet may influence DZ/LZ polarization. Indeed, expression of genes involved in regulation of cell motility such as $Cxcr3$ and $Cxcl10$ and genes involved in the control of cell division such as $Trp73$, $Tpx2$, and $Polh$ were significantly downregulated in T-bet-deficient compared with control GC B cells (Figures 2A and 2B).

To further examine if T-bet played a role in the control of GC DZ/LZ polarization, we compared our RNA-seq data with a transcriptional signature of genes previously described (Victora et al., 2012) to define DZ and LZ GC B cells. Our analysis identified 32 DZ/LZ-specific genes differentially expressed between T-bet-deficient and WT GC B cells (Figure 3A). Whereas DZ-defining genes such as $Rrm1$, $Cdca8$, $Anxa2$, and $Relm$, involved in the control of cell proliferation (Figures 3B and 3C), were reduced, LZ-defining genes, including $Cd86$, $Cd40$, and the negative regulators of NF-κB $Nfkbi$ and $Nfkbid$ (Figures 3B and 3C), were significantly enriched in T-bet-deficient GC B
Overall, these results suggest that T-bet promotes DZ over LZ GC B cell programming.

To determine if T-bet also regulated chromatin accessibility in GC B cells, we used the assay for transposase-accessible chromatin using sequencing (ATAC-seq) to detect open chromatin regions in sorted GC B cells from malaria-infected Tbx21\textsuperscript{fl/fl}Cd23\textsuperscript{Cre} mice and compared them with T-bet-expressing (CXCR3+) GC B cells from malaria-infected control mice. This analysis revealed 705 (up) and 171 (down) differentially accessible (DA) peaks between T-bet-deficient and control GC B cells. This included Cxcr3, which was less accessible in T-bet-deficient GC B cells compared with controls (Figures 3D and 3E). Consistent with the RNA-seq analysis, a significant enrichment (p = 0.02147) of DZ/LZ signature genes was found among peaks DA between Tbx21\textsuperscript{fl/fl}Cd23\textsuperscript{Cre} versus control GC B cells.

T-bet Influences GC Polarization in Response to Malaria Infection

To examine T-bet distribution in GC B cells, flow cytometric analysis of DZ and LZ GC B cells of malaria-infected WT mice was performed using a gating strategy based on CD86 and CXCR4 expression published previously (Victora et al., 2010). On day 6 p.i., T-bet expression was confined mainly to the LZ (Figure S3). From day 9 p.i. onward, T-bet could be detected in both DZ and LZ GC B cells, although its expression remained slightly higher in the LZ (Figures S3A and S3B).

To test if T-bet differentially affected LZ and DZ GC B cell development, the expression of CXCR4 and CD86 among GC B cells from malaria-infected Tbx21\textsuperscript{fl/fl}Cd23\textsuperscript{Cre} and Cd23\textsuperscript{Cre} mice was examined. Consistent with our transcriptional profiling that had shown an enrichment of LZ genes in T-bet-deficient GC B cells, the proportion of LZ GC B cells was significantly higher in Tbx21\textsuperscript{fl/fl}Cd23\textsuperscript{Cre} versus control mice (Figure 4), resulting in a significantly reduced DZ/LZ GC B cell ratio in Tbx21\textsuperscript{fl/fl}Cd23\textsuperscript{Cre} compared with control mice (Figure 4F).
Consistent with the unaltered representation of DZ GC B cells in Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> mice, we detected no difference in the number of proliferating GC B cells, identified by BrdU incorporation (Figures S3C and S3D). Moreover, cell cycle analysis performed by sequential labeling with EdU followed by BrdU revealed no differences in proliferation rates of DZ and LZ GC B cells of malaria-infected Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> or control mice (Figures S3E–S3G).

To determine if the effect of T-bet in GC polarization was B cell intrinsic, chimeric mice reconstituted with a 1:1 mixture of congenically marked WT (Ly5.1<sup>+</sup>) and Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> bone marrow were infected with P. berghei ANKA and GC B cells responses assessed in each compartment. Chimeric mice containing a mixture of Ly5.1<sup>+</sup> WT and Ly5.2<sup>+</sup> Cd23<sup>Cre</sup> bone marrow cells were included as controls. Whereas control chimeric mice displayed equal proportions of DZ and LZ cells in each compartment (Figures 4J and 4K), Ly5.2<sup>+</sup> Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> GC B cells showed significantly increased frequencies of LZ cells and decreased frequencies of DZ cells compared with Ly5.1<sup>+</sup> WT controls (Figures 4G–4I).

Thus, intrinsic expression of T-bet in B cells influences GC polarization by limiting LZ differentiation and favoring the transition to a DZ transcriptional program.

**T-bet Modulates Migration of GC B Cells to the DZ**

Our data show that CXCR3 is expressed in GC B cells in a T-bet-dependent manner. The upregulation of CXCR3-binding chemokines such as CXCL10 is a well-documented feature of human and murine malaria (Armah et al., 2007; Nie et al., 2009). Thus, we sought to determine the role CXCR3 in GC B cells. In vitro chemotaxis assays revealed that CXCR3 was functional in WT GC B cells, as cells from malaria-infected mice readily migrated in response to CXCL10, whereas cells from in Tbx21<sup>fl/fl</sup>Cd23<sup>Cre</sup> mice did not (Figure 5A). Next, chimeric mice reconstituted with a 1:1 mix of congenically marked Ly5.1<sup>+</sup> WT and Ly5.2<sup>+</sup> Cxcr3<sup>−/−</sup> bone marrow were infected with P. berghei ANKA and GC B cells responses assessed in each compartment. Frequencies and numbers of GC B cells early during infection were similar between the WT and Cxcr3<sup>−/−</sup> control mice. In contrast, on day 12 p.i., the absence of CXCR3 resulted in a reduction of total GC B cell numbers compared with WT cells (Figures 5B and 5C). Moreover, the proportion of DZ GC B cells as well as the DZ/LZ cell ratio was significantly reduced in the absence of Cxcr3<sup>−/−</sup> mice (Figures 5D and 5E).

Thus, intrinsic expression of T-bet in B cells influences GC polarization by limiting LZ differentiation and favoring the transition to a DZ transcriptional program.
The access of GC B cells into the DZ requires CXCR4-mediated cell migration (Bannard et al., 2013). To examine if this pathway was affected by T-bet, CXCR4 expression and the chemotactic response to its ligand, CXCL12, were determined in Tbx21^{fl/fl}Cd23^{Cre} and Cd23^{Cre} B cells of P. berghei-ANKA-infected mice. The capacity of Tbx21^{fl/fl}Cd23^{Cre} GC B cells to migrate in response to CXCL12 was reduced by 30% compared with control cells (Figure 5F). As CXCR4 expression in DZ and LZ GC B cells was not different between T-bet-deficient and control B cells (Figure 5G), these data suggest that T-bet modulates responsiveness of B cells by regulating the expression of chemotactic mediators other than CXCR4. One of the GC B cell-specific genes significantly upregulated in the absence of T-bet was the regulator of G protein signaling 13 (rgs13) (Figure 2), a negative regulator of signaling in response to chemokines, known to reduce responsiveness to CXCL12 (Johnson and Druey, 2002; Shi et al., 2002). To test if increased expression of rgs13 as observed in T-bet-deficient GC B cells may contribute to impaired migration to the DZ, we infected a human GC B cell lymphoma cell line (HS-Sultan) that constitutively expresses rgs13 (Han et al., 2006) with a GST-tagged T-bet-expressing lentivirus. Lentivirus infection was confirmed using

Figure 4. Lack of T-bet in B Cells Results in an Increase of the GC LZ Compartment during Malaria Infection

(A–F) Tbx21^{fl/fl}Cd23^{Cre} and Cd23^{Cre} mice were infected with P. berghei ANKA and treated with anti-malarial drugs from day 5 p.i. Proportions (A–C) and absolute numbers (D and E) of CD86^{lo}CXCR4^{hi} DZ (A, B, and D) and CD86^{hi}CXCR4^{lo} LZ (A, C, and E) GC B cells were calculated among gated CD19^{+}CD38^{lo}GL7^{+} GC B cells. Ratios of DZ to LZ GC B cell numbers were determined in malaria-infected Tbx21^{fl/fl}Cd23^{Cre} and Cd23^{Cre} mice (F).

(G–K) Lethally irradiated C57BL/6 mice were reconstituted with 1:1 ratios of Ly5.1^{+} WT and Ly5.2^{+} Tbx21^{fl/fl}Cd23^{Cre} /C0 bones (G–I). Ly5.1^{+} WT and Ly5.2^{+} Cd23^{Cre} chimeras were included as controls (J and K). Representative flow cytometry plots showing frequencies of DZ and LZ GC B cells at different times p.i. within the WT (Ly5.1^{+}) and Tbx21^{fl/fl}Cd23^{Cre} (Ly5.2^{+}) compartments (G). Frequencies of DZ (H and J) and LZ (I and K) GC B cells were determined at different times p.i. within the Ly5.1^{+} and Ly5.2^{+} compartments.

Data are representative of two experiments. Graphs depict means of four mice ± SEM. Representative histograms and dot plots are shown. **p < 0.01, ***p < 0.001, and ****p < 0.0001. See also Figure S3.
flow cytometry and RT-PCR (Figures S4A and S4B). Rgs13 levels in infected cells were determined using RT-PCR, and their capacity to migrate in response to CXCL12 was examined. Expression of T-bet in HS-Sultan cells reduced rgs13 mRNA transcripts by >50% compared with cells infected with control lentivirus (Figure 5H), which resulted in a 2.5-fold increase in the capacity of HS-Sultan cells to migrate in response to CXCL12 compared with controls (Figure 5I). Interestingly, T-bet expression significantly improved the forward migration index of HS-Sultan cells in response to chemotactic stimulus but not their velocity (Figures S4C and S4D). Thus, together these results indicate that T-bet influences responsiveness to chemokines required for GC polarization.

**T-bet Regulates the Quality of Memory B Cells and Plasma Cells Induced in Response to Malaria**

We then sought to determine if the T-bet-mediated transition to a DZ transcriptional program subsequently influenced the GC output during *P. berghei* ANKA infection, including memory B cell and plasma cell formation (Figure 6A). Total numbers of isotype-switched plasma cells were significantly higher in *Tbx21<sup>fl/fl</sup>*Cd23<sup>Cre</sup> mice compared with controls on day 9 p.i. (Figure 6B). When IgG<sub>1</sub> and IgG<sub>2c</sub> responses were analyzed, we found that consistent with the well-defined role of T-bet in IgG<sub>2c</sub> class-switch (Gerth et al., 2003; Peng et al., 2002), *Tbx21<sup>fl/fl</sup>*Cd23<sup>Cre</sup> mice almost exclusively generated IgG<sub>1</sub> plasma cells, while control mice generated a response dominated by IgG<sub>2c</sub> plasma cells (Figure 6C). This was also reflected in serum antibody titers specific for *P. berghei* ANKA protein merozoite surface protein-119 (MSP-119) (Figure S5). However, despite the differences in isotype distribution, total parasite-specific IgG levels were not significantly different between *Tbx21<sup>fl/fl</sup>*Cd23<sup>Cre</sup> and controls (Figure S5). As expected, *Tbx21<sup>fl/fl</sup>*Cd23<sup>Cre</sup> mice lacked IgG<sub>2c</sub> memory B cells, while this was the dominant IgG isotype in WT mice (Figure 6E). Notably, *Tbx21<sup>fl/fl</sup>*Cd23<sup>Cre</sup> mice compared with WT mice showed increased numbers of isotype-switched memory B cells early (day 9) in the response (Figure 6F), suggesting that in the absence of T-bet, memory B cells might leave the GC prematurely.

Recent studies have identified various surface molecules as markers of memory B cell precursors. Whereas CCR6 expression was found to define memory B cell precursors in the LZ with low affinity for antigen (Suan et al., 2017), ephrin-B1 was used to identify highly mutated memory B cell precursors (Laidlaw et al., 2017; Wang et al., 2017). Using this gating strategy (Figure 6G), we found that CCR6 was upregulated in LZ WT but not DZ GC B cells in spleens of *Tbx21<sup>fl/fl</sup>*Cd23<sup>Cre</sup> and Cd23<sup>Cre</sup> malaria-infected mice early during infection and declined rapidly thereafter (Figure 6H). Strikingly, on day 9 p.i., the absolute
numbers of CCR6+ LZ GC B cells were 3 times higher in Tbx21^fl/fl^Cd23^Cre^ mice compared with controls (Figure 6H), suggesting that T-bet reduces the number of low-affinity memory B cell precursors early during the response. In contrast, ephrin-B1+ memory precursors, which peaked at 12 days p.i., were substantially reduced in Tbx21^fl/fl^Cd23^Cre^ compared with control mice (Figure 6G). Together, these data indicate that T-bet influences the quality and kinetics of plasma cell and memory B cell generation in response to malaria.

Malaria-Driven Upregulation of T-bet in B Cells Promotes Antibody Affinity Maturation

Our data showed that the absence of T-bet in B cells affects GC polarization and suggested that T-bet may be required to prevent premature cessation of GC cycling and exit from the GC. To test this hypothesis, we examined hypermutation rates in IghV genes of sorted GC B cells from Tbx21^fl/fl^Cd23^Cre^ and Cd23^Cre^ mice. As the locations of mutations induced by P. berghei ANKA infection in IghV genes are unknown, we pursued a bioinformatics approach by which mutations in IghV exons were identified using RNA-seq on days 10 and 15 p.i. Sequences of naive follicular B cells were included as controls. We identified 61 dominant (>3-fold above background) mutations in IghV genes of GC B cells generated in response to infection (Table S1). Consistent with active positive selection, 96% of these mutations increased in frequency from day 10 to day 15 p.i. On day 10 p.i., most of the mutations were at similar frequency (68%) between Tbx21^fl/fl^Cd23^Cre^ and control mice, 22% were significantly increased, and 10% were decreased with T-bet deletion (Figures 7A and 7B). In contrast, on day 15 p.i., the overall frequency of mutations was significantly reduced in GC B cells from Tbx21^fl/fl^Cd23^Cre^ mice compared to Cd23^Cre^ control mice (p = 0.0365). Notably, frequencies of 55% of mutations were significantly different between the two groups (Figures 7C and 7D), and 73% of mutation frequencies were significantly reduced in the absence of T-bet. To examine changes in mutation frequency over time, we focused on mutations that showed clear evidence of selection during the GC response. Two types of mutations were identified in this category: (1)
“highly selected late mutations,” positively selected mutations that showed low (<3%) frequency on day 10 p.i. and displayed at least a 5-fold increase between day 10 and day 15 p.i., reaching at least 5% frequency during this time, and (2) “early common mutations,” high (>5%) frequency on day 10 p.i. Strikingly, mutation frequency changes were significantly lower for highly selected late mutations in Tbx21flox/Cd23Cre compared with control GC B cells (Figures 6E and 6F). In contrast, mutation frequency changes were significantly higher for early common mutations in Tbx21flox/Cd23Cre compared with control GC B cells (Figures 7G and 7H). Overall these data show that T-bet limits somatic hypermutation early but promotes antibody affinity maturation later during the GC response.

To further this analysis, an ELISA-based assay to measure antibody avidity was developed (Figure S6) by incubating serum samples from malaria-infected mice with decreasing concentrations of recombinant P. berghei ANKA MSP-1,19, which makes it possible to obtain specific half maximal effective concentration (EC50) values for antibodies induced in response to infection. Sera avidity from both groups gradually increased from day 9 to day 24 p.i. (Figures 7I and 7J). However, from day 12 p.i. onward, antibody avidity in sera from Tbx21flox/Cd23Cre mice was significantly lower than that of Cd23Cre controls (Figures 7I and 7J). Together these results support a model by which T-bet induces efficient GC polarization and prevents premature exit of antigen-experienced B cells from the GC reaction, thereby promoting antibody affinity maturation.

**DISCUSSION**

Recent studies in humans and infection models revealed that inflammatory cytokines that contribute to the induction of symptomatic malaria such as IFN-γ, upregulate the expression of T-bet in TFr+ cells, which prevents their normal differentiation and capacity to provide help for antibody responses (Obeng-Adjei et al., 2015; Ryg-Cornejo et al., 2016). Here we found that although expression of T-bet in T cells reduces the magnitude of the GC response to infection, its expression in B cells supports a transcriptional program that influences polarization of the GC and thereby promotes antibody-affinity maturation.

The best characterized role of T-bet in B cells is class switch toward IgG2c in response to IFN-γ signaling (Gerth et al., 2003; Peng et al., 2002). T-bet also induces the upregulation of CXCR3 in these cells and their recruitment to sites of inflammation (Moser et al., 2006), which is important for the control of viral infections (Barnett et al., 2016; Knox et al., 2017; Rubtsova et al., 2013). Similarly, migration of CXCR3-expressing lymphocytes to sites of parasite sequestration is a hallmark of P. berghei ANKA infection (Hansen et al., 2007; Ioannidis et al., 2016; Nie et al., 2009). Although CXCR3 deficiency did not alter GC B cell responses after immunization with NP-KLH (Piovesan et al., 2017), in response to malaria, loss of CXCR3 reduced the numbers of GC B cells, suggesting that CXCR3 upregulation in B cells in plays role in amplifying the magnitude of the GC response during acute infection. Further research is required to identify the cellular sources of CXCR3-binding chemokines involved in the control of GC responses under inflammatory conditions.

Our results revealed that in malaria infection, T-bet supports a transcriptional program that results in a preferential enrichment of DZ GC B cells. Recent studies identified other transcription factors such as FOXO1, c-Myc, and AP4 instructing the GC DZ program (Chou et al., 2016; Dominguez-Sola et al., 2012, 2015; Sander et al., 2015). In response to malaria, the absence of T-bet did not affect the expression of these transcription factors (not shown), suggesting that T-bet provides an additional pathway for induction of the GC DZ program. The fact that mice with a B cell-specific deficiency in T-bet showed an enrichment of the LZ compartment, suggested that T-bet expression in GC B cells might promote re-cycling of GC B cells into the DZ. Several lines of evidence lend support to this model. First, the size of the DZ compartment in Tbx21flox/Cd23Cre and CXCR3-deficient mice was proportionally reduced. Second, T-bet-deficient GC B cells displayed reduced capacity to migrate in response to the CXCR4 chemokine CXCL12, required for DZ entry. Third, mice with T-bet-deficient B cells induced significantly increased numbers of low-affinity GC LZ memory B cell precursors. Most important, T-bet-deficient GC B cells showed substantially reduced frequency of highly selected mutations in their IgH genes were, which resulted in significantly lower antibody avidity for parasite-specific antigen compared with controls. Importantly, Aicda expression in GC B cells was unchanged by the absence of T-bet, supporting the notion that impaired Ig somatic mutation rates were due to altered GC B cell LZ-DZ transition. Together these results suggest that the T-bet-induced instruction of DZ program might prevent the exit of low-affinity antigen-experienced B cells from the GC reaction.

Interestingly, other studies found that c-Myc is expressed in a subset of LZ GC B cells, poised for DZ re-entry (Dominguez-Sola et al., 2012). Moreover, c-Myc-expressing B cells in the LZ that receive cognate T cell help upregulate the expression of AP4, which amplifies the DZ transcriptional profile (Chou et al., 2016). Thus, these findings support the notion that in addition to cognate T cell help, additional signals such as inflammatory cytokine milieu may also modulate GC dynamics and influence the quality of the resulting antibody response to infection.

The chemokine receptor CXCR4 is crucial for efficient access of GC B cells into the DZ (Bannard et al., 2013). Similar to our results with T-bet-deficient B cells, low IgHv somatic hypermutation rates have been found in Cxcr4−/− GC B cells. Chemokine receptors such as CXCR4 use GTP-binding protein (G-proteins) as signal transducers (Johnson and Druey, 2002), and their responsiveness to chemotactic stimuli can be negatively modulated by a family of proteins controlling GTPase activity called regulators of G protein signaling (RGS). A GC B cell-specific RGS, named RGS13 (Shi et al., 2002), has been shown to reduce CXCR4 responsiveness to its ligand, CXCL12, in mouse and human GC B cells (Hwang et al., 2013; Shi et al., 2002). Interestingly, Rgs13 expression levels were significantly upregulated in response to malaria in T-bet-deficient GC B cells, which display reduced chemotactic response to CXCL12 and are underrepresented in the DZ compartment, suggesting that CXCR4-mediated migration is modulated by T-bet. In support of this view, in vitro studies using a human GC B cell lymphoma cell line demonstrated that T-bet represses Rgs13 expression in...
B cells, which is accompanied by an improvement in their capacity to migrate in response to CXCL12. Thus, our results support a model by which T-bet might play an important role enhancing the responsiveness of LZ GC B cells to CXCL12 to allow DZ re-cycling, before CXCR4 expression reaches the level required to sustain efficient localization of GC B cells in the DZ. In addition, the transcriptional profiling shown here revealed that the expression of more than 400 genes is affected by T-bet in GC B cells during malaria. Further work to identify other transcriptional targets of T-bet in these cells will be required to understand how this transcription factor modulates antibody affinity maturation in different infection/disease settings.

It is accepted that environmental factors may influence epigenetic marks, leading to diverse biological effects including that T-bet influences chromatin accessibility of GC B cells during malaria infection. In general, both LZ and DZ genes remained accessible in the absence of T-bet, allowing speculation that a non-inflammatory environment might favor a more permissive epigenetic landscape.

Our results support the concept that inflammatory cues drive T-bet expression in B cells, thereby augmenting antibody affinity maturation. The implications of these processes are of interest for vaccine development against chronic viral infections, where T-bet+ memory B cells and plasma cells have been found to play a pivotal role in the control of infection (Barnett et al., 2016; Knox et al., 2017; Rubtsova et al., 2013) and enhanced mutation rates might favor the development of protective, broadly neutralizing antibodies (Kwong et al., 2013). In contrast,
T-bet-driven increase in affinity maturation might have detrimental effects in autoimmune conditions such as SLE (Rubtsova et al., 2017; Wang et al., 2018), in which CD11c+ T-bet+ memory B cells play a pathogenic role. In malaria, although the upregulation of T-bet driven by acute infection appears to reduce the overall magnitude of the GC response by modulating TFH cell differentiation (Obeng-Adjei et al., 2015; Ryg-Comejo et al., 2016), it is conceivable that over time, continual expression of T-bet in B cells in endemic settings might result in antibodies of enhanced affinity, able to maintain parasite densities below the threshold that induces disease symptoms. T-bet+ memory B cells have been detected during both human malaria and mouse infection models, with conflicting results in their effector function and association with disease outcomes (Changrob et al., 2018; Fontana et al., 2016; Obeng-Adjei et al., 2017; Pérez-Mazilah et al., 2018). Further work will be required to establish if antibody responses produced by T-bet+ B cells play a role in the acquisition of clinical immunity to malaria.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at http://doi.org/10.1016/j.cell.2019.10.087.

ACKNOWLEDGMENTS

We thank Prof. Di Yu for scientific advice. This work was supported by the Australian Government National Health and Medical Research Council Independent Research Institute Infrastructure Support Scheme (IRISS) and project grants 1058665 and 1137989, the Australian Academy of Science (D.S.H.), fellowships from the Sylvia and Charles Viertel Foundation, the National Health and Medical Research Council and Commonwealth Serum Laboratory (A.K., K.L.G.-J., and W.S.), and the Victorian State Government Operational Infrastructure Support.

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


