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CD11c+ T-bet+ B cells require IL-21 and IFN-γ from T_{FH1} cells, and intrinsic Bcl-6 expression, but develop normally in the absence of T-bet

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Abstract

CD11c+ T-bet+ B cells generated during ehrlichial infection require CD4+ T cell help and IL-21 signaling for their development, but the exact T cell subset required had not been known. Here we show, in a mouse model of *Ehrlichia muris*, that T_{FH1} cells provide help to CD11c+ T-bet+ B cells via the dual secretion of IL-21 and IFN-γ in a CD40:CD40L-dependent manner. T_{FH1} cell help was delivered in two phases: IFN-γ signals were provided early in infection, whereas CD40:CD40L help was provided late in infection. In contrast to T-bet+ T cells, T-bet+ B cells, did not develop in the absence of B cell-intrinsic Bcl-6, but were generated in the absence of T-bet. T-bet-deficient memory B cells were largely indistinguishable from their wild-type counterparts, although they no longer underwent switching to IgG2c. These data suggest that a primary function of T-bet in B cells during ehrlichial infection is to promote appropriate class switching, not lineage specification. Thus, CD11c+ memory B cells develop normally without T-bet, but require Bcl-6 and specialized help from dual cytokine-producing T_{FH1} cells.

Introduction

T-bet+ B cells are now recognized as a distinct effector/memory B cells subset elicited by infection, aging, and autoimmunity (1–8). T-bet+ B cells have been identified during many infections, including malaria, HIV, and hepatitis C and B infections (9–12). Moreover, several studies have implicated T-bet+ B cells in autoimmune diseases, including SLE (13–15), arthritis (16, 17), and Sjøgren's disease (18). We first identified CD11c+ B cells during murine infection with the obligate intracellular bacterium *Ehrlichia muris* (19) and later showed that all of the CD11c+ B cells also expressed T-bet (20). CD11c+ T-bet+ B cells generated early following infection differentiate into antigen-specific IgM-secreting plasmablasts (21). By day 30 post-infection, however, CD11c+ T-bet+ B cells are found largely as IgM memory cells, although smaller subsets of switched and germinal center (GC)

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B cells are also found within the population (22). The low numbers of switched and GC B cells are likely a consequence of the disruption of splenic architecture observed during *E. muris* infection, which is characterized by a lack of formal GCs and ablation of conventional splenic organization (21, 23). Other work from our laboratory demonstrated that long-term T-bet+ B cells elicited during ehrlichial infection function as memory cells (20); these memory cells also undergo self-renewal, which presumably functions to maintain the population during low-level chronic infection. Ablation of the T-bet+ B cells also abrogated the pathogen-specific switched antibody response following secondary challenge, demonstrating that T-bet+ memory B cells are important for humoral immunity (22).

The development of T-bet+ memory B cells is likely dependent on the inflammatory milieu associated with Type 1 responses encountered during both infection and autoimmunity. Within this inflammatory milieu, IFN-γ, IL-21, and TLR signaling have been shown to be crucial for the development of T-bet+ B cells (24, 25). Moreover, CD11c+ T-bet+ B cell development requires CD4+ T cell help, and it has been proposed that T-bet+ B cells receive this help from T_{FH} cells (26, 27).

T_{FH} cells are a population of CD4+ T cells that provide help to GC B cells via various ligands and cytokines, including CD40L, ICOS, IL-21, and IL-4 (28) and as such, T_{FH} cells are critical for the formation of high affinity ASCs and memory B cells. It is now clear that multiple subsets of T_{FH} cells exist, including populations of T_{FH13}, T_{FH17}, T_{FH2} and T_{FH1} cells (29, 30). Each of these populations express surface markers, transcription factors, and cytokines characteristic of their non-T_{FH} cell counterparts. For example, T_{FH1} cells express the T_{FH} cell surface markers CXCR5, PD-1, and the transcription factor Bcl-6, but also the T_{H1}-associated factors CXCR3 and T-bet (28, 29). These hybrid T_{FH} cells are capable of secreting IFN-γ and IL-21, and similar to T_{H1} cells, arise in response to IL-12, IL-4, and IL-2 (31). T_{FH1} cells have now been shown to be elicited during type I responses to many different pathogens including, HIV, influenza, LCMV and malaria (27, 32–36). T_{FH1} cells generated during HIV contribute to host defense, as they were negatively correlated with viral load and positively correlated with anti-p24 IgG (34). T_{FH1} cells have also been shown to provide help to memory B cells during recall responses following influenza vaccination, where the T cells were positively correlated with anti-influenza antibody responses (32). In contrast, T_{FH1} cells elicited during recurrent malaria infection have been proposed to drive the generation of T-bet+ memory B cells, although these cells exhibited reduced memory function (27). These studies, and others, have demonstrated the requirement for both IL-21 and IFN-γ in the generation of T-bet+ B cells, and have suggested that T_{FH1} cells play an important role in T-bet+ B cell development.

In this study, we demonstrate that T_{FH1} cells are critical for the generation of CD11c+ T-bet + B cells. We also show that while T cell-intrinsic T-bet expression is required for the generation of CD11c+ T-bet+ B cells, T-bet expression in B cells is dispensable for the development of CD11c+ B cells that are identical in phenotype to their wild type counterparts. This research demonstrates a central and perhaps obligatory role for T_{FH1} cells in the generation of CD11c+ T-bet+ B cells in infection and autoimmunity.

Materials and Methods

Mice.

C57BL/6J, CD19^{Cre} (B6.129P2(C)-Cd19$^{tm1(cre)Cgn}$/J), Bcl6^{fl} (B6.129S(FVB)-Bcl6$^{tm1.1Dent}$/J), CD4^{cre} (B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ), Tbx21^{fl} (B6.129-Tbx21^{tm2Srnr}/J), Mb1^{cre} (B6.C(Cg)-Cd79a$^{tm1(cre)Reth}$/EhobJ), IFN-γ reporter (C.129S4(B6)-IFN-γ$^{tm3.1Lky}$/J), IL-21 reporter (B6.Cg-Il21$^{tm1.1Hm}$/DcrJ), CD40L-deficient (B6.129S2-Cd40lg^{tm1Imx}/J), and IFN-γ-deficient (B6.129S7-IFN-γ^{tm1Ts}/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and bred in the SUNY Upstate Medical University Animal Care Facility (Syracuse, NY), in accordance with institutional guidelines for animal welfare. All mice used for experiments were at least 6 weeks old, and both male and female mice were used unless otherwise stated.

Infections and antibody administration.

Mice were infected intraperitoneally (i.p.) with 5×10^4 *E. muris* bacterial copies, as determined by qPCR, and as previously described (37). Anti-CD40L monoclonal antibody (BE0017–1) and isotype control polyclonal Armenian hamster IgG (clone BE0091) were purchased from BioXcell (West Lebanon, NH) and were administered every other day from days 16 to 30 post-infection, or every other day from days 30 to 37 post-infection, as previously described (38). Anti-IFN-γ monoclonal antibody (clone R4–6A2) was purchased from BioXcell (West Lebanon, NH) and 500μg administered once every three days from days 16 to 30 post-infection.

Flow cytometry and antibodies.

Spleens were disaggregated using a 70μm cell strainer (BD Falcon), and erythrocytes removed by incubation with ACK lysis Buffer (Quality Biological Inc). Cells were treated with anti-CD16/32 (2.4G2) prior to incubation with the following antibodies: PerCpCy5.5-conjugated anti-CD19 (6D5), Alexafluor 700-conjugated anti-CD19 (6D5), APC-eFluor 780-conjugated anti-CD11c (N418), V500-conjugated anti-B220 (RA3–6B2), Alexafluor 647-conjugated anti-T-bet (4B10), PerCpCy5.5-conjugated anti-T-bet (4B10), Alexafluor 647-conjugated anti-Bcl-6 (K112–91), FITC-conjugated anti-PD-1 (29F.1A12), PE-conjugated anti-CCR6 (29–2L17), PerCpCy5.5-conjugated anti-CD4 (RM4–4), PECy7-conjugated anti-ICOS (C398.4A), Alexafluor 700-conjugated anti-CD3 (17A2), APCeFluor 780-conjugated anti-CD44 (IM7), Brilliant Violet 421-conjugated anti-CXCR5 (L138D7), BV510-conjugated anti-CXCR3 (CXCR3–173), V500-conjugated anti-CD3 (500A2), PE-conjugated anti-IFN-γ (XMG1.2), Alexafluor 647-conjugated anti-IL-21 (mhalx21).

The cells were stained at 4°C for 30 min, washed, and analyzed. For intracellular staining, surface stained cells were fixed/permeabilized for 40 minutes at 4°C using the Transcription Factor Buffer set Fixation/permeabilization buffer (BD Pharmingen), washed, stained at 4°C for 30 minutes, washed, and analyzed. For cytokine staining, splenocytes were cultured with BioLegend cell activation cocktail containing Phorbol 12-myristate 13-acetate (PMA), ionomycin, and Brefeldin A for 4 hours at 37°C. Surface stained cells were fixed/permeabilized for 20 minutes at 4°C using BD Cytofix/Cytoperm, washed, stained at 4°C for 30 minutes, washed, and analyzed. Unstained cells were used to establish the flow cytometer

voltage settings, and single-color positive controls were used to adjust compensation. Data were acquired on a BD Fortessa flow cytometer with Diva software (BD Bioscience), and were analyzed with FlowJo software (Tree Star, Inc.).

ELISAs.

Serum ELISAs were performed as previously described (22). Recombinant OMP-19 was coated on Flat-Bottom Immuno plates (Thermo Scientific) and antigen-specific IgM, pan IgG, IgG1, IgG2b, IgG2c, and IgG3 serum antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL). Serum ELISAs for IFN-γ were performed using the Mouse IFN-γ ELISA Ready-SET-Go! kit (eBioscience) according to the manufacturer's instructions.

Statistical analysis.

Statistical analysis was performed using Prism 8 (GraphPad). Statistical significance was represented as shown: ns: $p > 0.05$, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, and ****: $p < 0.0001$. Statistical tests performed are indicated in the figure legend. The column in each plot indicates the arithmetic mean of the dataset and upper and lower bounds indicate standard deviation of the dataset.

Results

T-bet+ T cells, but not conventional T_{FH} cells, were required for CD11c+ T-bet+ B cell development

We have previously demonstrated that CD11c-expressing T-bet+ memory B cells are not generated in the absence of CD4+ T cells or IL-21 signaling (22). Similarly, this B cell subset did not develop in the absence of the T cell co-stimulatory molecule CD40L (Figure 1A, Supplementary Figure S1A). These requirements indicated that CD11c+ T-bet+ B cells likely receive CD4+ T cell help from a CD40L+ T cell population capable of secreting IL-21. As *E. muris* infection elicits a large population of CXCR5+ PD-1+ Bcl-6+ T cells (38); (see also Supplementary Figure S1B), we hypothesized that conventional T_{FH} cells were the source of CD4+ T cell help for the CD11c+ T-bet+ B cells. To address this question, we infected CD4-cre x Bcl-$6^{fl/fl}$ mice, which lack conventional T_{FH} cells (39), and monitored the T and B cell populations elicited by infection. CD11c+ T-bet+ memory B cells were detected on day 30 post-infection in mice lacking CXCR5+ PD-1+ T_{FH} cells, indicating that CD11c+ T-bet+ B cells received T cell help from a CD4+ T cell population other than conventional T_{FH} cells (Figure1B, Supplementary Figure S1C). Consistent with this hypothesis, *E. muris* infection also generated a large population of T-bet+ CXCR3+ T cells. These T-bet+ T cells expressed high levels of ICOS and CD44, and transcribed IFN-γ on day 16 post-infection (Figure 1C). Although IFN-γ drives the formation of T-bet+ B cells (25, 26), most B220+ cells, including CD11c+ T-bet+ B cells, were absent in IFN-γ-deficient mice on day 18 post-infection, emphasizing the importance of IFN-γ for the immune response during ehrlichial infection (Figure 1D). To determine if the IFN-γ-producing T-bet+ T cells were a source of CD4+ T cell help for the CD11c+ T-bet+ B cells, we next examined infected CD4-cre x T-bet-$^{fl/fl}$ mice, which lack T-bet+ T cells. CD11c+ T-bet+ B cells were not generated in the absence of T-bet+ T cells, suggesting that the T-bet+

T cells were the source of CD4+ T cell help to the CD11c+ T-bet+ B cells (Figure 1E). Consistent with published studies (40), we also identified a population of T-bet^{high} cells which expressed CD11c, and a population of T-bet^{low} cells which did not express CD11c. These T-bet^{low} cells were still present in the absence of T-bet+ T cells. Although the total number of CD11c+ and T-bet+ B cells was similar in the wild-type and T-bet+ T cell-deficient mice, this was like a consequence of increased splenomegaly in the latter strain, which inflated negligible numbers of CD11c+ and T-bet^{high} B cells (Supplemental Figure S1D). T_{FH} cells were still present in CD4-cre x Tbet$^{fl/fl}$ mice, albeit at significantly reduced levels, and likewise, T-bet+ T cells were present in CD4-cre x Bcl-6$^{fl/fl}$ mice at reduced levels (Supplemental Figure S1E and F).

IFN-γ IL-21 double-producing T_{FH1} cells provide help to CD11c+ T-bet+ B cells

Given that the generation of CD11c+ T-bet+ B cells required IL-21, but not conventional T_{FH} cells, we next sought to determine the cellular source of IL-21. Analysis of IL-21 reporter mice on day 16 post-infection, revealed that both CXCR3+ CXCR5+ and CXCR3+ CXCR5-negative T cells transcribed IL-21 (Figure 1F, Supplemental Figure S2A). Likewise, data from *in vitro* stimulation of T-bet+ T cells indicated that wild-type T-bet+ T cells were capable of producing IL-21 and IFN-γ simultaneously (Figure 1G). In addition to producing IL-21, a large portion of the CXCR3+ T cells expressed the T_{FH} markers CXCR5 and PD-1, as well as Bcl-6 (Supplemental Figure S2B). However, CXCR5-negative CXCR3+ T cells produced more IFN-γ than their CXCR5+ CXCR3+ counterparts (Supplementary Figure S2C). These data are consistent with other studies that have described a similar subset of T_{FH} cells (27, 32–36); the emerging consensus is that these T cells are Type 1 follicular helper (T_{FH1}) cells, consistent with their role in driving type I B cells responses (28).

To further address whether the T-bet+ T cells generated in *E. muris* infection were T_{FH1} cells, we performed RNA sequencing analysis, using CXCR3+ CD4+ T cells obtained from infected mice on day 16 post-infection. CXCR3 was used as a surrogate marker for T-bet, as all of the T-bet+ T cells expressed CXCR3 (Supplementary Figure S3A). The CXCR3+ T cells expressed genes associated with T_{FH} cells including *Cxcr5*, *Il21*, and *Il12rb*, as well as those associated with T_{H1} cells, including *Tbx21*, *Ccr5, Prdm1,* and *Ifng* (Supplementary Figure S3B). These data provide additional support for the interpretation that T-bet+ T cells elicited in *E. muris* infection are T_{FH1} cells. Thus, we conclude that CD11c+ T-bet+ B cells generated during ehrlichial infection receive IL-21, IFN-γ and CD40L signals from IL-21 and IFN-γ dual-producing T_{FH1} cells.

CD11c+ T-bet+ B cells have biphasic requirements for T cell help

Following 4-nitrophenol immunization, mature GCs appear by approximately day 7 post-immunization (41, 42). In contrast, the humoral response to *E. muris* is delayed, such that splenic GL7+ B cells do not appear until approximately 21 days post-infection, and antigen-specific IgG is largely undetectable until 28 days post-infection (21). Because of this delayed response, we next addressed when differentiating CD11c+ T-bet+ B cells received help from T_{FH1} cells during infection. In previous studies, treatment of mice with anti-CD40L from days 2 to 16 post-infection, had only a modest effect on the generation of CD11c+ T-bet+ B cells detected on day 16 post-infection, which suggested that the B cells

acquired CD40-mediated help later following infection (23). To determine if CD11c+ T-bet+ B cells received CD40L help after day 16 post-infection, infected mice were treated with anti-CD40L antibody from days 16 to 30 post-infection, and were analyzed on day 30 post-infection. CD11c+ T-bet+ B cells were substantially reduced on day 30 post-infection following anti-CD40L treatment from days 16 to 30 post-infection (Figure 2A). Moreover, anti-CD40L treatment after day 30 post-infection, when the CD11c+ T-bet+ B cell population is fully matured, only minimally affected CD11c+ T-bet+ B cell frequency (Figure 2B). These data indicate that CD11c+ T-bet+ B cells receive a substantial portion of CD40L help from T cells between days 16 and 30 post-infection, after infection has begun to resolve, much later than is typical for other infections or immunizations. In contrast, CD11c+ T-bet+ B cells required IFN-γ signaling early in infection, as the B cells developed normally following IFN-γ depletion on days 16–30 post-infection, but failed to develop in IFN-γ-deficient mice by day 18 post-infection (Figure 2C). Consistent with this early requirement for IFN-γ, serum IFN-γ was detected on day 8 post-infection but was largely absent after day 16 post-infection (Figure 2D). These data suggest that CD11c+ T-bet+ B cells have a biphasic requirement for help, requiring IFN-γ early during infection, and CD40:CD40L interactions late.

Bcl-6 is required for the development of CD11c+ T-bet+ B cells

The involvement of T_{FH1} cells, and our previous data showing that approximately 50% of CD11c+ B cells express GL7 on day 16 post-infection (23), suggested that CD11c+ T-bet+ B cells develop in GCs. Consistent with this hypothesis, 15–20% of T-bet+ B cells expressed the GC-associated transcription factor Bcl-6 on day 16 post-infection, although expression was largely absent by day 30 post-infection (Figure 3A). However, *E. muris* infection causes a disruption of splenic architecture and a loss of splenic GCs (21, 23), suggesting that CD11c+ T-bet+ memory B cells are generated in a GC-independent fashion. GC-independent memory cells have been described previously, and can be generated in the absence of Bcl-6 in B cells (43, 44). To determine if CD11c+ T-bet+ B cells develop in the absence of Bcl-6, we monitored CD11c+ T-bet+ B cells in infected GC-deficient CD19-cre x Bcl-$6^{fl/fl}$ mice. Unlike GC-independent memory B cells that have been described, CD11c+ T-bet+ memory B cells, elicited by ehrlichial infection, were not present after day 30 post-infection in the absence of Bcl-6 nor were T-bet+ B cells present on day 16 post-infection (Figure 3B). Commensurate with the loss of T-bet+ B cells, fewer switched antibodies, most notably IgG2c, were detected by serum ELISA on day 30 post-infection indicating that the T-bet+ B cells were critical for the switched-antibody response to *E. muris* (Figure 3C) (22). Thus, while developing T-bet+ B cells received T cell help apparently independent of GCs, as these structures are disrupted during ehrlichial infection (21, 23), development of the T-bet+ B cells required at least transient expression of Bcl-6 early in their development. These data indicate that in the absence of formal GC structures, GC-like help from T_{FH1} cells is necessary to drive the development of T-bet+ B cells.

CD11c+ B cells develop in the absence of T-bet

Although we have shown that IL-21 signaling is crucial for the generation of CD11c+ T-bet+ B cells, other studies have demonstrated that IL-21 signaling can drive the formation of CD11c+ B cells *in vitro,* without inducing T-bet expression (25). In addition, studies in other

experimental models have shown that B cells with characteristics of T-bet+ B cells can be generated in the absence of T-bet (14, 45–47). Therefore, we next addressed whether T-bet was required for CD11c+ T-bet+ B cell development following ehrlichial infection using Mb1-cre x T-bet$^{fl/fl}$ mice, where T-bet was eliminated in all B cells. The frequency of CD11c + B cells was increased in *E. muris*-infected, B cell-specific, T-bet-deficient mice, although the absolute number remained the same, and the B cells were largely identical in phenotype to T-bet-sufficient CD11c+ memory B cells (Figure 4A). No differences were observed in the expression of several characteristic T-bet+ B cell surface markers, including CD11c, CD73, CD38, PD-L2, and CD80 (Figure 4B). However, as has been shown in other experimental models, T-bet-deficient B cells produced little antigen-specific IgG2c, and instead generated IgG of other isotypes not normally found during ehrlichial infections (Figure 4C) (48). These results suggest that the primary role of T-bet expression among B cells during ehrlichial infection, is the regulation of antibody class switching, not lineage determination.

Discussion

Here we demonstrate that T_{FH1} cells provide both IFN-γ and IL-21 to developing CD11c+ T-bet+ B cells. These data suggest that CD11c+ T-bet+ B cells require multi-functional helper T cells, as classical T_{FH} cells were insufficient to drive CD11c+ T-bet+ B cell generation. Although other studies have demonstrated T_{FH1} cells could drive expression of T-bet in naïve B cells *in vitro* (26, 27), our work shows that dual cytokine-producing T_{FH1} cells are necessary for the generation of CD11c+ T-bet+ B cells *in vivo*. Given that T_{FH1} cells and T-bet+ B cells arise in many of the same infections and diseases, T_{FH1} cells likely give rise to T-bet+ B cells in other contexts as well, implicating T_{FH1} cells as a key mediator of type I humoral immunity and antibody-mediated autoimmunity (26, 36, 45, 49). Thus, our studies have implications for understanding how protective and pathogenic T-bet+ B cells arise in many different immunological contexts.

We also demonstrate that while CD11c+ T-bet^{high} B cells were not present in the absence of T_{FH1} cells, CD11c-negative T-bet^{low} B cells were still detected. These findings suggest that CD11c+ and CD11c-negative T-bet+ B cells have disparate requirements for T cell help. Alternatively, it is possible that T cells help to drive expression of CD11c and T-bet in B cells, and in the absence of T cell help, T-bet+ B cells remain CD11c-negative and T-bet^{low}.

The dual production of both IFN-γ and IL-21 by T_{FH1} cells suggests that simultaneous secretion of both cytokines from the same helper T cell is crucial for the generation of CD11c+ T-bet+ B cells. This interpretation is supported by previous research which has demonstrated that T helper cytokines are released in a directed fashion from immune synapses, allowing for strict cell-to-cell signaling (28, 50, 51). This dual cytokine requirement likely explain why classical T_{FH} cells were dispensable for the formation of CD11c+ T-bet+ B cells. However, as the temporal requirement for IL-21 was not addressed in this study, further work is required to validate this conclusion.

The T_{FH1} helper signals CD40:CD40L and IFN-γ were required at different times: IFN-γ was needed early following infection, within the first 16 days, while CD40:CD40L signaling

was critical after day 16 post-infection. The biphasic nature of this T cell help suggests that T_{FH1} cells undergo temporal changes, producing IFN-γ early and signaling via CD40L late in infection. Alternatively, it is possible that a second cell population, perhaps NKT cells (52), is responsible for providing early IFN-γ signals to developing T-bet+ B cells, and T_{FH1} cells provide CD40L-mediated help later in infection. However, given the drastic effects on the total B cell population in IFN-γ-deficient mice during *E. muris* infection, further work is needed to confirm these hypotheses. The apparently delayed CD40:CD40L interactions between T_{FH1} cells and CD11c+ T-bet+ B cells may be a result of disruptions in splenic organization that occur during *E. muris* infection, as splenomegaly and increased splenic hematopoiesis begins to resolve after day 16 post-infection (21, 53). These delayed interactions may be relevant in other infections that generate T-bet+ B cells, where similar changes in splenic organization have been observed (54).

A portion of the relevant helper T cells in our studies expressed the T_{H1} marker CXCR3 and the transcription factor T-bet, but not the T_{FH} marker CXCR5 or the transcription factor Bcl-6. The most likely explanation of this observation is that the CXCR3+ T cells form a single T_{FH1} population, although other interpretations are possible. In addition to T_{FH1} cells, *E. muris* infection may also elicit T_{H1} cells. These T_{H1} cells may be sufficient to provide help to developing T-bet+ B cells in the absence of T_{FH1} cells, and may compensate for the loss of T_{FH1} cells in CD4-cre x Bcl-$6^{fl/fl}$ mice. This interpretation is supported by the observation that Blimp-1 mRNA, a transcription factor known to antagonize Bcl-6 and to be highly expressed among non-T_{FH} T cells (28, 55), was increased among CXCR3+ T cells during infection. Alternatively, it is possible that all of the responding T-bet+ T cells function as T_{FH1} cells, even in the absence of Bcl-6, as both CXCR5-positive and negative T cells were capable of producing IL-21 and IFN-γ. Consistent with this interpretation, it has been reported that T_{FH1} cells persist in the absence of Bcl-6 in malaria infection (33). In contrast, CXCR5+ T cells were not detected in the absence of Bcl-6 in our studies, suggesting that even though CXCR3+ T cells were present, they exhibited fewer characteristics of T_{FH} cells. This interpretation suggests that T_{FH1} cells fall within a continuum, between canonical T_{FH}-like cells and T_{H1}-like cells. In this model, some CXCR3+ T cells exhibit characteristics more consistent with a canonical T_{FH} phenotype i.e., they express CXCR5, whereas other CXCR3+ T cells exhibit characteristics more consistent with a T_{H1}, phenotype i.e., they do not express CXCR5, and produce more IFN-γ. These data highlight the plasticity of follicular helper T cells, which has been discussed previously (28). Regardless of their surface marker phenotype, the T-bet-expressing CD4+ T cells formally function as T_{FH1} cells, by providing IL-21, IFN-γ, and CD40L help.

The paucity of GCs in *E. muris*-infected mice is likely a consequence of the highly inflammatory cytokine milieu generated during infection (23). T_{FH1} cells may contribute to this inflammatory response, as they produce and/or transcribe both IFN-γ and TNF-α, and we have shown that TNF-α contributes to splenic disorganization. The absence of formal GCs during ehrlichial infection had suggested that CD11c+ T-bet+ B cells develop independently of GCs. Indeed, similar to GC-independent memory B cells, CD11c+ T-bet+ B cells exhibit low numbers of BCR mutations (22, 56). However, unlike GC-independent memory B cells, T-bet+ B cells expressed Bcl-6 early in their development, and their generation required Bcl-6 expression. This requirement for Bcl-6 in B cells suggests that T-

bet+ B cells are not formal GC-independent memory B cells. Rather, T-bet+ B cells elicited by ehrlichial infection, exhibit characteristics of GC B cells, and require Bcl-6, even in the absence of canonical GCs. Thus, while not found in formal GCs, developing CD11c+ T-bet+ B cells receive GC-like help from T_{FH1} cells. The absence of formal GCs likely explains why most CD11c+ T-bet+ B cells do not undergo extensive SHM and CSR. Furthermore, the lack of a highly selective GC environment may explain the uncharacteristically high frequency of T-bet+ B cells detected in spleens of ehrlichial-infected mice, and may also explain the lack of clonal selection among T-bet+ B cells (20).

Although T-bet+ B cells are now widely considered to play important roles in many disease contexts, we demonstrate that T-bet is not required for lineage determination in our model. CD11c+ B cells, which appear nearly identical to canonical T-bet+ B cells, still develop normally in the absence of T-bet, although T-bet was important for IgG2c switching, as previously reported (24, 45–47). Our data suggest that the primary function of T-bet expression in developing CD11c+ T-bet+ memory B cells during *E. muris* infection is not to determine lineage specification, but rather, to drive class switching to isotypes that provide protective immunity. In this regard, although depletion of T-bet in B cells has been shown to ameliorate symptoms of SLE in a mouse model of lupus, this outcome was likely due to changes in autoantibody isotype, as IgG2c is well-known to contribute to disease pathogenesis in SLE (57). Our findings also contrast with studies of T-bet+ B cells during malaria infection, where T-bet contributes to B cell affinity maturation in GCs. However, T-bet is unlikely to have any effects on the affinity maturation of T-bet+ B cells during *E. muris* infection, as these B cells develop in the absence of GCs. Therefore, while T-bet expression may not contribute to the generation of CD11c+ memory B cells in ehrlichial infection, its expression still has important consequences for type I immunity and autoimmune disease pathogenesis, in particular, via its role in antibody isotype determination. Thus, T-bet may play a more important role in T cells, where it drives the formation of T_{FH1} cells, which are required for the generation of T-bet+ B cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Points

1. Developing CD11c+ T-bet+ B cells require T_{FH1} cells that produce both IFN-γ and IL-21
2. Obligate IFN-γ and CD40L signals are provided at distinct times following infection
3. CD11c+ B cells lacking T-bet undergo normal differentiation but improper switching

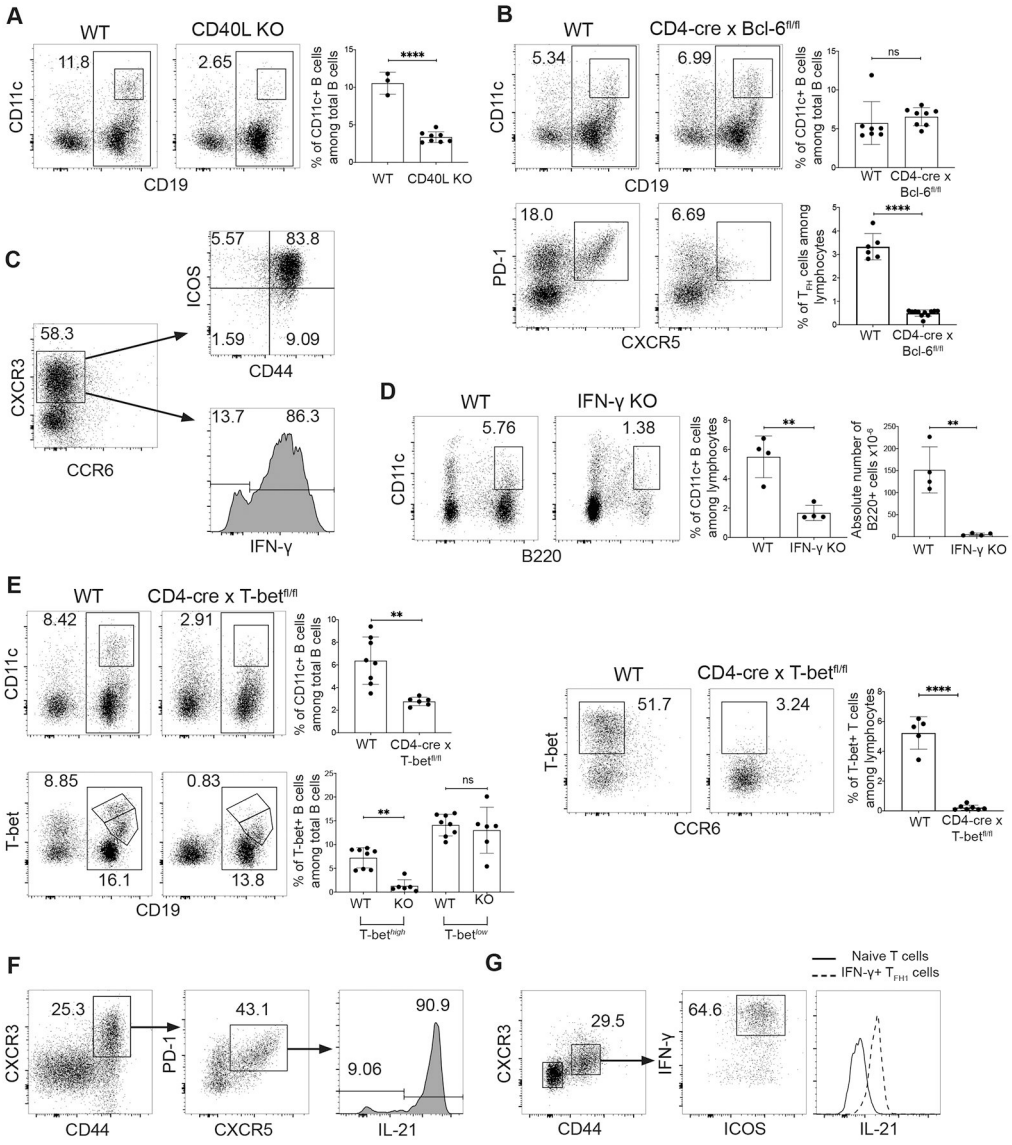

Figure 1: IFN-γ IL-21 double-producing T_{FH1} cells provide help to CD11c+ T-bet+ B cells

(A) Splenocytes from *E. muris*-infected CD40L-deficient or wild-type mice were analyzed on day 30 post-infection. The dot plots show the percentages of CD11c+ CD19+ B cells among total B cells. Graphs represent aggregate data from two independent experiments. Statistical significance was determined using a two-tailed un-paired t test ($p < 0.0001$, df = 9).

(B) Splenocytes from *E. muris*-infected CD4-cre x Bcl-$6^{fl/fl}$ or Bcl-$6^{fl/fl}$ control mice were analyzed on days 30 (top plots) and 16 (bottom plots) post-infection. Representative dot plots show the percentages of CD11c+ CD19+ B cells among total B cells and the percentages of PD-1+ CXCR5+ cells among CD3+ CD4+ T cells. Graphs represent aggregate data from three independent experiments. Statistical significance was determined using two-tailed un-paired t tests (top: $p < 0.4559$, df =13; bottom: $p = 0.0001$, df = 15).

(C) CD3+ CD4+ CXCR3+ CCR6-negative T cells from *E. muris*-infected, female, IFN-γ-reporter mice were analyzed for the expression of ICOS, CD44 and IFN-γ 16 days post-infection. Plots are representative of 4 mice.
(D) Splenocytes from *E. muris*-infected, female, IFN-γ-deficient or wild-type mice were analyzed on day 18 post-infection. Representative dot plots show the percentages of CD11c+ B220+ B cells among total lymphocytes. Graphs represent aggregate data from two independent experiments. Statistical significance was determined using two-tailed un-paired t tests (left: $p = 0.0023$, $df = 6$; right: $p = 0.0014$, $df = 6$).
(E) Splenocytes from *E. muris*-infected CD4-cre x T-bet$^{fl/fl}$ or T-bet$^{fl/fl}$ control mice were analyzed on days 30 (left plot) and 16 (right plot) post-infection. Representative dot plots show the percentages of CD11c+ CD19+ B cells among total B cells (top left), the percentages of T-bet^{high} and T-bet^{low} CD19+ B cells among total B cells (bottom left), and the percentages of T-bet+ CCR6-negative cells among CD3+ CD4+ T cells (right). Graphs represent aggregate data from three independent experiments. Statistical significance was determined using two-tailed un-paired t tests (top left: $p < 0.0030$, $df = 12$; right: $p = 0.0042$, $df = 7$) and an ordinary one-way ANOVA ($p < 0.0001$, $F = 28.36$, $df = 27$) with Sidak's multiple comparisons test (T-bet^{high}: $p = 0.0015$, $df = 24$; T-bet^{low}: $p = 0.7372$, $df = 24$).
(F) CD3+ CD4+ CXCR3+ CCR6-negative PD-1+ CXCR5+ T cells from *E. muris*-infected, female, IL-21-reporter mice were analyzed for the expression of IL-21 and CD44 16 days post-infection. Plots are representative of 4 mice.
(G) Splenocytes from *E. muris*-infected, female, wild-type mice on day 16 post-infection were cultured with a cell activation cocktail containing Brefeldin A for 4 hours at 37°C. CD3+ CD4+ CXCR3+ CD44+ T cells were analyzed for IFN-γ, ICOS, and IL-21 expression. Plots are representative of 5 mice. Not significant (n.s.) > 0.05, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$.

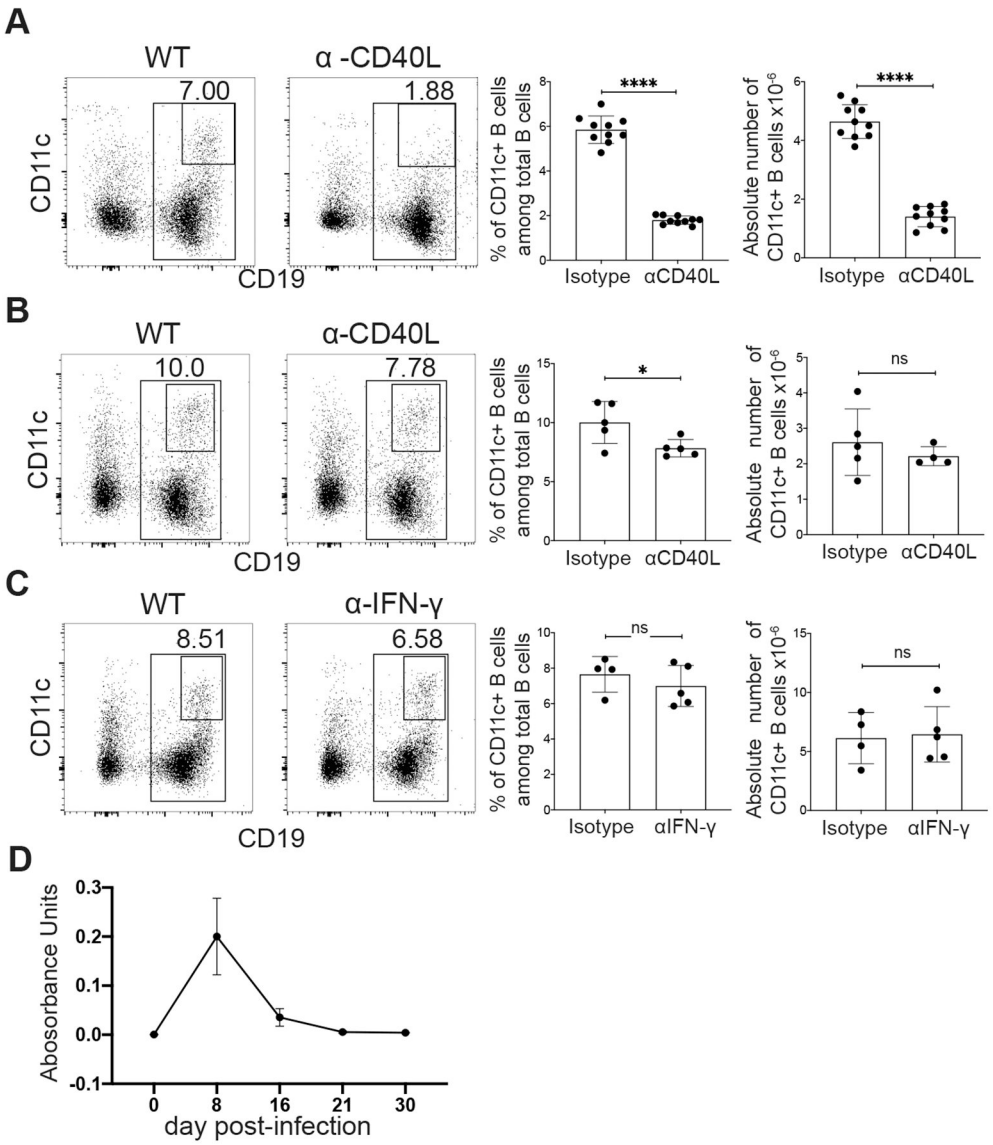

Figure 2: CD11c+ T-bet+ B cells have biphasic requirements for T cell help

(A) *E. muris*-infected, female, wild-type mice were treated with either anti-CD40L antibody or an isotype-matched irrelevant antibody every other day from days 16 to 30 post-infection and splenocytes were analyzed on day 30 post-infection. Representative dot plots show the numbers and percentages of CD11c+ CD19+ B cells among total B cells. Graphs represent aggregate data from two independent experiments. Statistical significance was determined using two-tailed un-paired t tests (left: $p < 0.0001$, df = 18; right: $p < 0.001$, df = df = 18). (B) *E. muris*-infected, female, wild-type mice were treated with either anti-CD40L antibody or an isotype-matched irrelevant antibody every other day from days 30 to 37 post-infection and splenocytes were analyzed on day 37 post-infection. Representative dot plots show the numbers and percentages of CD11c+ CD19+ B cells among total B cells. Aggregate data are shown in the plots on the right. Statistical significance was determined using two-tailed un-paired t tests (left: $p = 0.0346$, df = 8; right: $p = 0.4484$, df = 7).

(C) *E. muris*-infected, female, wild-type mice were treated with either anti-IFN-γ antibody or vehicle control once every three days from days 16 to 30 post-infection and splenocytes were analyzed on day 30 post-infection. Representative dot plots show the numbers and percentages of CD11c+ CD19+ B cells among total B cells. Graphs represent aggregate data from two independent experiments. Statistical significance was determined using two-tailed un-paired t tests (left: $p = 0.4008$, $df = 7$; right: $p = 0.8405$, $df = 7$).

(D) Sera from *E. muris*-infected, female, wild-type mice harvested on days 0, 8, 16, 21, and 30 post-infection were analyzed by ELISA for IFN-γ. Dots represent the arithmetic mean of five mice and the upper and lower bounds represent the standard deviation. n.s.> 0.05, ****$p < 0.0001$.

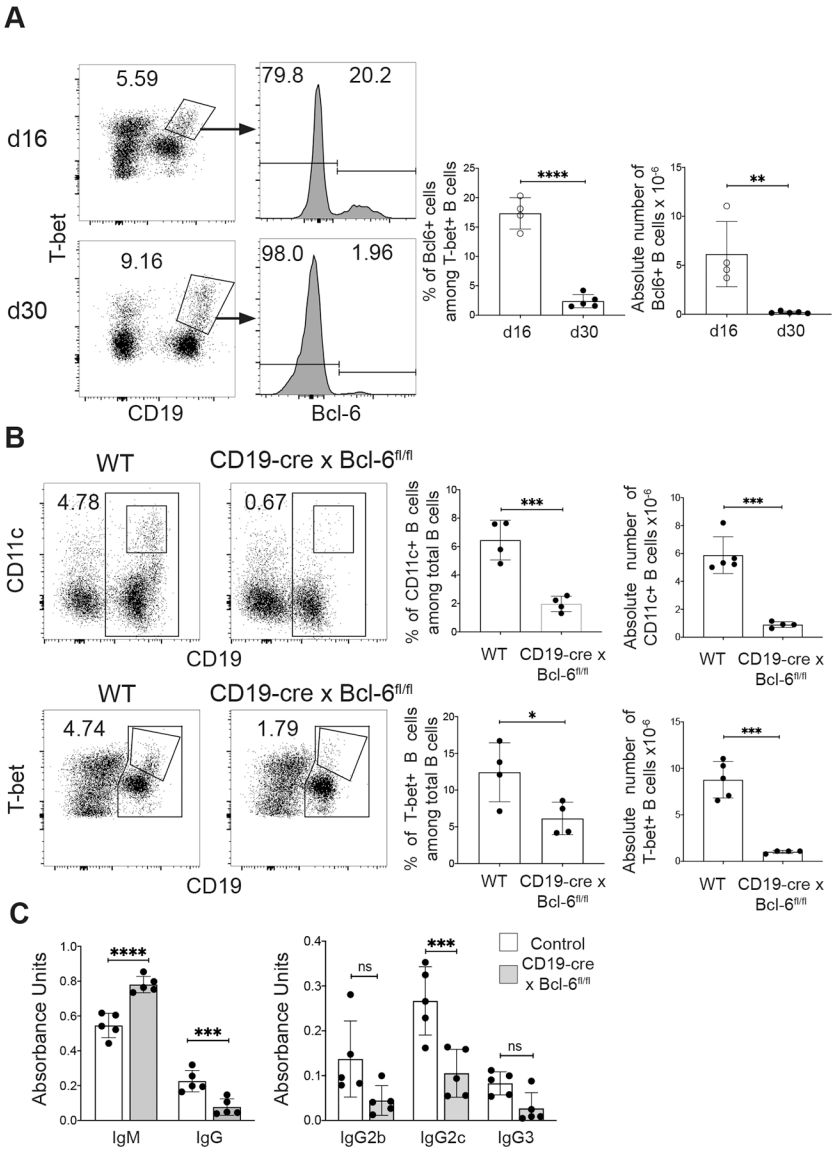

Figure 3: Bcl-6 is required for the development of CD11c+ T-bet+ B cells

(A) T-bet+ CD19+ B cells from *E. muris*-infected, female, wild-type (black circles) or Bcl-6$^{fl/fl}$ control (open circles) mice were analyzed for expression of Bcl-6 on days 16 and 30 post-infection. Aggregate data are shown in the plots on the right. Statistical significance was determined using two-tailed un-paired t tests (left: $p < 0.0001$, df = 7; right: $p < 0.0048$, df = 7).

(B) Splenocytes from *E. muris*-infected CD19-cre x Bcl-6$^{fl/fl}$ or Bcl-6$^{fl/fl}$ control mice were analyzed on days 43 (top plot) and 16 (bottom plot) post-infection. Representative dot plots show the percentages of CD11c+ CD19+ B cells and the percentages of T-bet+ CD19+ B cells among total B cells. Aggregate data are shown in the plots on the right. Statistical significance was determined using two-tailed un-paired t tests (top left: $p = 0.0010$, df = 6; top right: $p < 0.0001$, df = 7; bottom left: $p = 0.0334$, df = 6; bottom right: $p < 0.0001$, df = 7).

(C) Sera from *E. muris*-infected CD19-cre x Bcl6$^{fl/fl}$ or wild-type mice on day 30 post-infection was analyzed by ELISA for IgM, pan IgG, IgG2b, IgG2c, and IgG3. Sera was collected in two independent experiments. Statistical significance was determined using an ordinary one-way ANOVA ($p < 0.0001$, $F = 18.21$, $df = 11$) with Sidak's multiple comparisons test (IgM: $p < 0.0001$, IgG: $p = 0.0008$, IgG2b: $p = 0.0653$, IgG2c: $p < 0.0003$, IgG3: $p = 0.4925$, $df = 40$). n.s.> 0.05, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

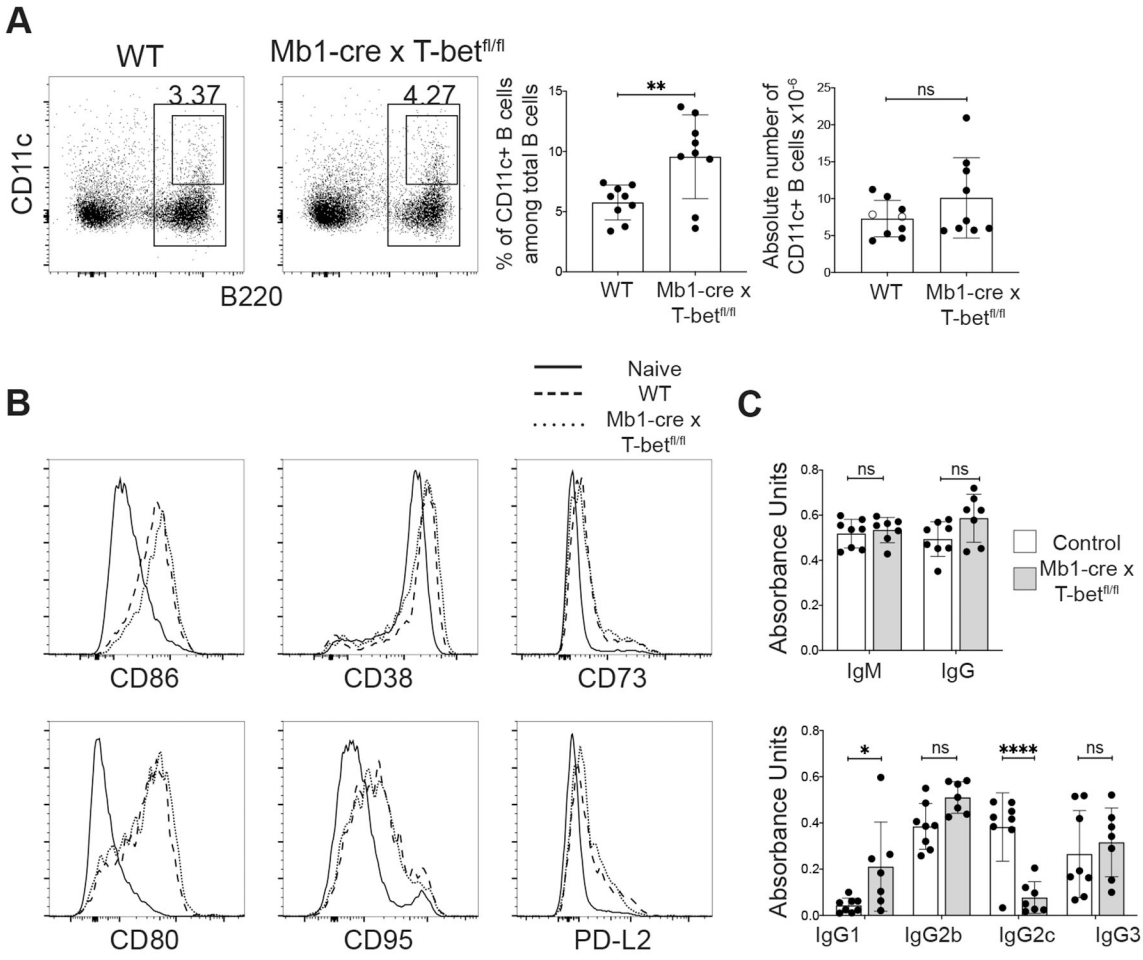

Figure 4: CD11c+ B cells develop in the absence of T-bet

(A) Splenocytes from *E. muris*-infected Mb1-cre x T-bet$^{fl/fl}$ (black circles), Mb1-cre x T-bet$^{fl/+}$ control mice (black circles), or T-bet$^{fl/fl}$ control mice (open circles) were analyzed on day 30 post-infection. Representative dot plots show the percentages of CD11c+ B220+ B cells among total B cells. The graphs represent aggregate data from two independent experiments. Statistical significance was determined using a two-tailed un-paired t test (left: $p = 0.0081$, $df = 16$; right: $p = 0.1754$, $df = 16$).

(B) CD11c+ B220+ B cells from *E. muris*-infected Mb1-cre x T-bet$^{fl/fl}$ or T-bet$^{fl/fl}$ mice were analyzed for expression of CD86, CD38, CD73, CD80, CD95, and PD-L2, thirty days post-infection.

(C) Sera from *E. muris*-infected Mb1-cre x T-bet$^{fl/fl}$ or T-bet$^{fl/fl}$ mice on day 30 post-infection were analyzed by ELISA for IgM, pan IgG, IgG1, IgG2b, IgG2c, and IgG3. Sera were collected in two independent experiments. Statistical significance was determined using an ordinary one-way ANOVA ($p < 0.0001$, $F = 18.21$, $df = 11$) with Sidak's multiple comparisons test (IgM: $p > 0.9999$, IgG: $p = 0.5553$, IgG1: $p = 0.0380$, IgG2b: $p = 0.2164$, IgG2c: $p < 0.0001$, IgG3: $p = 0.9537$, $df = 78$). n.s. > 0.05, *$p < 0.05$, ****$p < 0.0001$.