CD11C+ T-BET+ B CELLS IN INFECTION AND AUTOIMMUNITY

by

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Abstract

Title: 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

and

Pharmacological targeting of T-bet+ B cells via the adenosine 2a receptor ameliorates autoimmune disease in mice

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CD11c+ T-bet+ B cells serve crucial roles in both protective immunity and autoimmunity. However, the ontogeny of these cells remains unclear, and strategies to target them in vivo have yet to be identified. Here, we demonstrate that developing CD11c+ T-bet+ B cells received help in the form of IL-21, IFN-γ, and CD40L from a population of T follicular helper 1 (T_{FH1}) cells outside of formal germinal centers (GC). These T_{FH1} cells provided help to developing CD11c+ T-bet+ B cells in two distinct phases: IFN-γ was provided early following infection, and CD40L was provided later. Unlike the T_{FH1} cells, CD11c+ T-bet+ B cells required the GC-associated transcription factor Bcl-6 for their development, but not T-bet. While the CD11c+ B cells that arose in the absence of T-bet appeared nearly identical to their T-bet-competent counterparts, they did not switch to IgG2c. These data support a model where, in the absence of formal GCs, T_{FH1} cells provide GC-like help to developing CD11c+ T-bet+ B cells and while T-bet is not required for the development of these T-bet+ B cells, it is required for appropriate class-switch recombination (CSR).

Our work also demonstrates that mature CD11c+ T-bet+ B cells, which arise in both immunity and autoimmunity, were eliminated following treatment with the adenosine 2a receptor (A2aR) agonist CGS-21680. Depletion of these CD11c+ T-bet+ B cells occurred in a B cell-intrinsic manner and was corelated with improved disease outcome in a mouse model of lupus. Preliminary data indicated that human CD11c+ B cells expressed the A2aR, and these cells were depleted following CGS-21680 treatment in vitro, suggesting that A2aR-agonist administration may also be effective in the treatment of human autoimmune diseases where CD11c+ B cell play a role. Overall, this work provides novel insight into the development of T-bet+ B cells and identifies the first pharmacological approach to target these cells in vivo.

CHAPTER I: Introduction

Introduction

B cells contribute to humoral immunity via the production of antibodies and subsequent neutralization of invading pathogens. Following exposure to antigen, naïve B cells migrate to the B cell T cell border where they interact with cognate T cells and undergo proliferation (1). Activated B cells can then differentiate into short-lived plasmablasts and migrate to the medullary chords where they secrete low affinity unswitched antibody (2). Alternatively, activated B cells can enter GCs and undergo affinity maturation. During this process GC B cells proliferate rapidly and accumulate mutations in their B cell receptor (BCR). As T cell help is limited in the GC, only B cells with the highest affinity BCRs will bind antigen and receive this help, whereas B cells with low affinity BCRs will die from neglect. These GC B cells will eventually give rise to high affinity class-switched memory cells or bone marrow homing long-lived plasma cells (2). Following secondary pathogen exposure, memory B cells will help sequester the pathogen by differentiating into antibody-secreting cells or by reentering GCs and giving rise to additional high affinity plasma cells.

In addition to the classical naïve, GC, plasma cell, and memory B cell populations, there are many additional B cell subsets. The specific function and ontogeny of these various B cell populations are the subject of continuing research. CD11c-expressing B cells, later shown to express T-bet, represent a relatively novel subset of B cells. One of the first studies to describe CD11c+ B cells, identified them during bacterial infection in mice and showed that the cells function as short-lived antibody-secreting cells and later as memory cells (3, 4). CD11c+ T-bet+ B cells were later found in aged mice, where they contribute to age-related autoimmunity, and also in influenza-infected

mice, where they contribute to the anti-influenza response (5, 6). CD11c+ T-bet+ B cells are now known to be generated in a myriad of infectious and autoimmune diseases, but how these cells develop and what role they play during immunity and autoimmunity is not clear. This chapter will summarize our current understanding of the ontogeny and function of CD11c+ T-bet+ B cells in immunity and autoimmunity and will describe a novel signaling pathway in these B cells that may lead to the development of new treatments for autoimmunity.

T-bet+ B cells in autoimmunity

B cells play a prominent role in systemic lupus erythematosus (SLE) pathogenesis via the generation of anti-DNA antibodies and subsequent formation and deposition of immune complexes (7). $CD19^{hi}$, CD27 and IgD Double Negative (DN) B cells specifically, are thought to contribute to disease pathogenesis, as these B cells correlate with autoantibody titers, Systemic Lupus Activity Measure, and kidney nephritis in SLE patients (8–10). This DN population is now known to be composed of at least two distinct daughter populations, DN1 cells and DN2 cells (11). Like their parent population, DN1 and DN2 cells are negative for CD27 and IgD, but differ in that DN1 cells are positive for CXCR5 and CD21 and negative for CD11c and T-bet, whereas DN2 cells are negative for CXCR5 and CD21, but positive for CD11c and T-bet (11). These T-bet+ DN2 cells have been proposed to be extrafollicularly-derived ASC precursors, due to their similar epigenome, clonal relatedness, and ability to differentiate into ASCs following stimulation (11, 12). It is thought that the lack of proinflammatory negative regulators (e.g. NFKBIA, TNFAIP3, TRAF5, and TRAF4), and their hyper-responsiveness to Toll-

like receptor 7 (TLR7) stimulation, contribute to the expansion of DN2 cells during SLE (11). DN2 cells, rather than DN1 cells, appear to contribute to disease pathology in SLE, as they compose the majority of DN B cells in patients with active SLE and correlate with disease severity (11). Mouse models of lupus have yielded more direct evidence for the involvement of DN2 cells in lupus pathology as depletion of T-bet+ B cells significantly decreases anti-Smith antibody titers in TLR7 immunized mice, and elimination of T-bet expression among B cells ameliorates disease in lupus-prone mice (6, 13). Given the role of B cells, and particularly T-bet+ B cells, in the pathogenesis of SLE, these cells are an attractive therapeutic target.

Similar to SLE patients, Common Variable Immunodeficiency (CVID) patients generate a large number of $CD19^{hi}$ $CD21^{lo}$ B cells. These $CD21^{lo}$ B cells transcribe high levels of T-bet and express many of the same markers as T-bet+ B cells found in other diseases (14, 15). $CD21^{lo}$ B cells have increased expression of CXCR3 and CXCR6 and decreased expression of CXCR4, CXCR5 and CCR7, which presumably allows the cells to migrate away from lymphoid tissues and towards sites of inflammation (15). Consistent with this hypothesis, $CD21^{lo}$ B cells are the dominant B cell subset in the bronchoalveolar space of CVID patients (15). Similar to T-bet+ B cells generated during SLE, $CD21^{lo}$ B cells generated during CVID correlate with autoimmune manifestations among CVID patients and are mostly composed of autoreactive clones (16). Indeed, $CD21^{lo}$ B cells in excess of 20% among total B cells is a diagnostic marker for the autoimmune-prone Ia CVID subgroup (14, 17, 18). It is unknown what factors drive the generation of $CD21^{lo}$ B cells during CVID; however, CVID patients have elevated

quantities of serum BAFF and IFN-γ, factors known to drive the formation of T-bet-expressing B cells in other contexts (14, 19).

T-bet-expressing B cells have also been implicated in the pathogenesis of obesity-related autoimmunity (20). Obesity is associated with autoimmune disease pathogenesis, as human subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) are known to be a generative site for autoantibodies. (20, 21). These autoreactive antibodies are thought to be generated in part by T-bet+ DN B cells, as these cells can compose up to 50% of B cells in the stromal vascular fraction of SAT in obese humans (21, 22). It is thought that expression of CXCL10, CCL2, and CCL5 by adipocytes recruits B cells to adipose tissues, wherein, localized hypoxia causes cell death leading to the presentation of self-antigens to these B cells and the generation of autoreactive DN B cells (23). Indeed, adipocytes have been shown to transcribe the T-bet-inducing factors IL-21 and IFN-γ and coculture of splenic B cells with adipocytes leads to the generation of T-bet+ B cells (23). Therefore, it is thought that adipose tissue functions as a generative site for T-bet+ B cells, which then contribute to obesity-related autoimmunity.

The disease pathogenesis of multiple sclerosis (MS) is complex and not entirely understood, but recent work indicates that disease etiology may be in part mediated by T-bet+ B cells (24). It is thought that T-bet+ B cells contribute to disease pathogenesis in MS via the presentation of self-antigen to T cells in the central nervous system (CNS) and by the production of inflammatory cytokines and granzyme B (25, 26). Indeed, work in other models has demonstrated that T-bet+ B cells can function as potent antigen-presenting cells (APCs), leading to the aberrant activation of CD4+ T cells (27). Consistent with a role for T-bet+ B cells in MS etiology, anti-B cell therapy improves

disease outcome in MS patients, and this effect is thought to be mediated by the blockade of B cell help to T cells, as serum antibodies are unaffected by B cell depletion (26, 28–30). Moreover, CXCR3+ (T-bet+) B cells from MS patients display an increased ability to cross the blood brain barrier, and patients treated with the anti-α4-integrin antibody natalizumab, have reduced accumulation of these CXCR3+ B cells in the CNS and improved disease outcome (26, 31).

T-bet+ B cells have been identified in a number of other autoimmune conditions. $CD21^{lo}$ T-bet+ B cells have been correlated with joint damage in RA patients and can be found in the synovium of joints (15, 32). T-bet+ B cells have also been found in aged individuals as well, where they can account for 5% of circulating B cells in healthy female adults (6). Termed age-related B cells (ABCs), these cells are associated with naturally arising autoantibodies in older individuals, and as such are thought to contribute to age-related autoimmunity (6). T-bet+ B cells have also been found during Sjøgren's disease, Scleraderma, Crohn's disease, and Celiac's disease (33–36). Given the prevalent role of T-bet+ B cells in the pathogenesis of many different autoimmune diseases, research into the ontogeny and function of these cells is critical for the development of novel treatments for autoimmunity.

T-bet+ B cells in infectious disease

Human immunedeficiency virus (HIV) infection is known to cause various B cell-specific abnormalities including, IgG1 and IgG3 hypergammaglobulinemia, a poor response to vaccination, and the development of B cells with a heighted propensity towards neoplastic transformation (37–39). HIV infection also results in the development of a population of "atypical" $CD20^{hi}$, CD27-negative, FcRL4+, CD11c+, T-bet+, $CD21^{lo}$ B cells (40). These T-bet+ B cells are generally termed atypical memory B cells (aMBCs), as they resemble memory B cells, but differ functionally and phenotypically, or tissue-like memory (TLM) B cells, as they resemble tissue-based memory B cells originally found in the tonsils of healthy humans (40, 41). The role of TLM B cells in the HIV immune response is not clear. Some believe that TLM B cells contribute poorly to the HIV response, as they are poor producers of antibodies and cytokines, undergo limited somatic hypermutation (SHM) and CSR, and appear to be "exhausted" (i.e., they exhibit reduced proliferation and activation in response to B cell receptor (BCR) stimulation) (40). This B cell "exhaustion" is likely due to the inhibitory receptors expressed on the surface of the cells, as knockdown of FcRL4 and Siglec-6 enhances BCR signaling among TLM B cells (42). The lack of SHM and CSR among these TLM B cells is likely the result of their exclusion from GCs, which may hinder the formation of a high-affinity antibody response against HIV (43). However, despite appearing to be functionally impaired, TLM B cells account for 60% of the CD4bs broadly neutralizing antibody response to HIV and represent the majority of gp-140-specific B cells (44). Therefore, while T-bet+ TLM B cells may differ from "canonical" memory B cells, they still appear to be critical for HIV immunity.

T-bet+ aMBCs, similar to those derived in HIV, also arise during malaria infection, and these cells can account for 50% of circulating B cells in individuals from malaria endemic areas (45). Malaria-derived aMBCs share a similar gene-expression profile to those generated in HIV-infection, and like HIV-derived aMBCs, malarial aMBCs are CD11c+, T-bet+, CD20+, CD21-negative, and CD27-negative, but differ in that malaria aMBCs express FcRL5 and FcRL3 rather than FcRL4 (46). It is thought that the accumulation of aMBCs during malaria is driven by chronic exposure to the malaria parasite, as individuals from areas of high malaria transmission have more aMBCs than those from areas of low transmission and because aMBCs decline in the absence of malaria exposure (47). Similar to HIV, the role of aMBCs in malarial immunity is also contested. Some postulate that aMBCs are detrimental to malaria immunity and are generated aberrantly, the result of immune evasion, as the parasite is known to deliberately alter the B cell response (48). Consistent with this hypothesis, aMBCs respond weakly to BCR crosslinking and are poor producers of antibody (40). Moreover, B cell-specific deletion of T-bet has been shown to significantly enhance the GC and anti-malaria antibody response in a mouse model of malaria infection (49). However, contrary to their proposed dysfunction, aMBCs are associated with low parasite-density, and immunoglobulin sequencing has demonstrated that aMBC BCRs can be malaria-specific (50, 51). Indeed, aMBC BCR sequences can even be found among circulating antibodies (51). As with aMBCs found in HIV, malaria-derived aMBCs may serve a different, but not necessarily inferior role, than “canonical” memory B cells.

T-bet+ B cells have been identified in a myriad of other viral and bacterial contexts. T-bet+ B cells are elicited in response to hepatitis C virus infection, and they

are correlated with viremia (52, 53). Influenza infection in mice has been shown to elicit T-bet+ B cells, and these cells can make up 20-30% of the antigen-specific B cell pool (5). T-bet+ B cells have been shown to be generated in response to LCMV, MCMV, TB, and vaccination (54–58). The fact that T-bet+ B cells are elicited in such a wide range of infectious diseases may indicate that T-bet+ B cells are not "atypical" at all, but rather a normal subset of B cells elicited during viral and bacterial infections.

Most of the T-bet-expressing B cell subsets identified in these various infectious and autoimmune contexts have been given different names and have been identified based on different markers. Moreover, these various subsets can differ functionally, as autoimmune-derived T-bet+ B cells generate inflammatory cytokines and readily produce antibody, whereas aMBCs derived during infectious disease do not (15, 25, 36). Differences between T-bet+ B cells can even be observed within the same individual. Activated naïve B cells, switched-memory B cells, and DN2 cells, found in the blood of the same SLE patient, all express T-bet, but are each functionally distinct (12). Given this heterogeneity, it has been proposed that expression of T-bet, CD11c, and CD21 are insufficient to identify a discrete population of B cells (12). However, despite these differences, there are still many commonalities between these various subsets. For example, all these subsets express T-bet and the integrin CD11c, and most lack CD27 and CD21 and express FcRL5. Additionally, most T-bet+ B cells arise under conditions of chronic inflammation, and many of them display similar, if not identical, developmental requirements (59). Given these similarities we propose that while there may exist many smaller subpopulations within the T-bet+ B cell population, T-bet still represents a useful

and informative marker to delineate a distinct population of B cells found in many different immune contexts.

The function of T-bet+ B cells

T-bet is member of the T-box family of transcription factors; it is considered the master transcription factor for T_{H1} cells, regulating expression of a myriad of genes important for type I immunity, including IFN-γ and the IL-12R (60). In B cells, T-bet plays a key role in the regulation of hundreds of different genes including Bcl-6, Cxcr5, Aicda, CD86, CD80 and CD40 (61). B cell-intrinsic T-bet expression is critical for B cell-mediated type I immunity, as it is required for class-switching to IgG2a/c, the primary isotype generated in response to viral and bacterial infections (62). T-bet expression in B cells is also required for proper development of GCs and has been implicated in GC polarization (62). Indeed, during malaria infection, T-bet is thought to promote the localization of GC B cells to the dark zone and ultimately contribute to B cell affinity maturation (49). Despite the importance of T-bet in the identification of CD11c+ T-bet+ B cells, a population of nearly identical cells still arises in the absence of T-bet (4, 54, 63). However, these T-bet-negative B cells vary in several important ways. T-bet-negative B cells show transcriptional differences in genes related to migration, glycosylation, differentiation, and proliferation (61). Moreover, these T-bet-negative B cells can exhibit alterations in affinity maturation, and importantly, reduced IgG2a/c class-switching (62). These differences severely impact the function of T-bet-deficient B cells, as mice deficient in B cell-intrinsic T-bet have impaired viral clearance following gammaherpesvirus 68 infection, and lupus-prone mice lacking T-bet expression in B cells

have improved disease outcome compared to T-bet-sufficient control mice (57). These differences highlight the integral role of T-bet expression in B cells during type I immunity.

T-bet+ B cells appear to have a variety of functions during immunity and autoimmunity. The long-lived nature of T-bet+ B cells, their ability to differentiate into ASCs, their surface marker expression profile, and their telomere length suggest that T-bet+ B cells can function as memory cells (64, 65). Work from our laboratory has demonstrated that T-bet+ B cells generated during *E. muris*-infection persist indefinitely, self-renew, and give rise to all effector B cell populations following antigen challenge, formally functioning as memory cells (4, 66). However, T-bet+ B cells generated in many different contexts, express low levels of SHM and CSR, indicating that these cells may serve a different role from "canonical" memory cells (67). Indeed, a lack of affinity maturation may allow T-bet+ B cells to respond to a variety of structurally similar, yet distinct, antigens rather than a single epitope, as we have previously demonstrated (66). In this way, T-bet+ memory B cells may provide a more broadly reactive antibody response such as in HIV, where T-bet+ B cells account for majority of broadly neutralizing antibodies (68). A more broadly reactive antibody response could allow for the binding to more conserved, less immunogenetic epitopes, which would protect against heterologous infections and mutant strains, as in HIV.

T-bet+ B cells have been shown to function in other capacities besides memory. ABCs can function as potent APCs, driving the CD4+ T cell response during type I immunity, but also contributing to aberrant CD4+ T cell activation during autoimmunity (27). T-bet+ B cells are also able to directly contribute to the inflammatory response via

the secretion of inflammatory cytokines such as TNF-α (69). DN2 cells generated in SLE can rapidly differentiate into ASCs following stimulation, indicating that these T-bet+ B cells may function as pre-ASCs, although this ability is also indicative of a memory B cell phenotype (11). It has been proposed that T-bet+ B cells may serve a role as innate B cells given their ability to respond to innate TLR signals and their poor response to BCR crosslinking (15). T-bet+ B cells may also serve a role in mucosal tissue surveillance as the cells have been identified in Peyer's patches (54). Thus there is likely no single function of T-bet+ B cells. Depending on the context, T-bet+ B cells may serve as quiescent memory cells, APCs, pre-ASCs, and/or innate B cells; as such, the function of T-bet+ B cells should be considered within the immunological context in which they are found. However, research into the function of T-bet+ B cells in these various contexts will improve our ability to predict the function of T-bet+ B cells in different diseases and determine if and how they contribute to immunity or disease pathology.

The development of T-bet+ B cells

While T-bet+ B cells appear to serve many different functions, the conditions in which these cells arise seem to be fairly conserved. TLR signals, predominantly TLR7 and 9, along with IFN-γ and IL-21, are known to drive T-bet expression in naïve B cells, and while IL-21, CD40, and BCR signals are insufficient to induce T-bet expression alone, these signals can induce and amplify T-bet expression among B cells treated with TLR7 or 9 (5, 70). However, simultaneous TLR and BCR signals can inhibit the expression of T-bet, suggesting a critical timing component in the generation of T-bet+ B cells (5). Because of this sequential signaling requirement, it has been proposed that TLR

signals poise developing T-bet+ B cells, possibly via epigenetic modification, for differentiation into mature T-bet+ B cells following cytokine and other signaling cues (71).

The requirement for IL-21 and IFN-γ in the generation of T-bet+ B cells implicates Jak/STAT signals as a critical component of T-bet+ B cell development (5). Indeed, STAT1-deficient, although not STAT4-deficient, B cells fail to induce expression of T-bet following IFN-γ treatment (72). Moreover, T-bet itself seems to regulate STAT1, possibly via IFN-γ secretion, as STAT1 is reduced in the absence of T-bet (73).

While T-bet expression in B cells can be induced *in vitro* using only TLR signals and cytokines, *in vivo* generated T-bet+ B cells require CD40L and MHCII, implicating a role for CD4+ T cells in the generation of T-bet+ B cells (4, 64). Other in vivo studies have demonstrated that T-bet+ B cells require IFN-γ, and IL-21, suggesting that T-bet+ B cells may be receiving help from a population of IFN-γ, IL-21 dual-producing T follicular helper (T_{FH}) cells, also known as T_{FH1} cells (4, 74). T_{FH1} cells represent a relatively novel subset of CD4+ T cells characterized by the expression of both T_{FH} and T_{H1} markers (e.g., PD-1, CXCR5, CXCR3, and T-bet) (74). These T cells are capable of secreting both T_{H1} and T_{FH} associated cytokines and are generally elicited under type I polarizing conditions. As such, T_{FH1} cells are generated in many of the same diseases that T-bet+ B cells are found, such as malaria and HIV, further implicating a role for T_{FH1} cells in the generation of T-bet+ B cells (75–80).

In general, T-bet+ B cells appear to arise in type 1 immune responses, as the cells are found in a wide range of viral and bacterial infections, but are not thought to arise in response to type 2 infections (71). Moreover, while ABCs naturally arise in T_{H1}-biased

C57BL6/J mice, these cells are absent in T_{H2}-prone BALB/c mice (5). This observation is consistent with the fact that type I immune responses are characterized by high concentrations of IFN-γ and IL-21, the same cytokines known to drive T-bet-expression in B cells (5). In addition to these cytokine signals, T-bet+ B cells appear to be elicited as a result of antigen activation, as many of the cells have pathogen-specific BCRs (51). Indeed, many of the diseases in which T-bet+ B cells arise are characterized by prolonged antigen exposure and chronic inflammation (59). It is possible that the prolonged presence of antigen and inflammation is responsible for maintaining the T-bet+ B cell population over time (59). Consistent with this hypothesis, the number T-bet+ B cells declines in mice that successfully resolve malaria infection, in contrast to mice that remain chronically infected (81). T-bet+ B cells elicited during autoimmunity are thought be generated by a similar mechanism, as many autoimmune diseases are similarly characterized by type I polarizing conditions, chronic inflammation, and self-antigen exposure (59). These common developmental requirements suggest that while T-bet+ B cells may be heterogeneous, strategies to prevent the development of these B cells may be effective in multiple disease contexts.

Adenosine signaling in immunity and autoimmunity

Given the role of T-bet+ B cells in the pathogenesis of many autoimmune diseases, the development of novel pharmacological techniques to target these cells has become a prominent area of study. The therapeutic potential of adenosine receptor-modulating drugs has been recognized since the 1920s, however, it is only more recently that this class of drugs has been studied for its effects on various lymphocyte populations

(82). Many hematopoietic cells, including T cells, express the extracellular ATPases CD73 and CD39, as well as adenosine receptors, in particularly the A2aR (83, 84). Adenosine signaling in these different cells has varied effects. T cells stimulated in the presence of the A2aR agonist CGS-21680 produce less IFN-γ and IL-2 and have impaired effector function (85). Additionally, A2aR stimulation during T cell activation can promote T cell anergy, increase TGFβ production, and inhibit IL6 production (86). A2aR stimulation can also drive the formation of CD39 and CD73-expressing T_{reg} cells, which inhibit the generation of effector T_{H1} and T_{H17} cells (Mandapathil et al., 2010; Mascanfroni et al., 2015; Zarfek et al., 2008). Adenosine signaling is also critical for the function of GC T_{FH} cells, as CGS-21680 treatment inhibited the generation of T_{FH} cells and impaired the GC response in PE-SRB immunized mice (89). Similar to T_{reg} cells, $CD56^{bright}$ CD16-negative NK cells can also produce adenosine, and likewise impair T cell proliferation (90).

Less is known about the function of adenosine signaling in B cells. CD73 and CD39 are expressed on B cells, particularly B1 cells, and adenosine production by these cells can help ameliorate colitis (91). CD73 can also be found on certain memory B cells, plasmablasts, bone marrow plasma cells, and GC B cells (92). Recent work has also identified a novel population of CD73+ $CD39^{hi}$ B_{reg} cells capable of inhibiting the function of T_{eff} cells via the production of extracellular adenosine (93).

Given its widespread role among immune cells, adenosine signaling, in particular A2aR signaling, is linked to various diseases. The A2aR has been extensively studied in the field of cancer immunology, as some cancerous cells produce extracellular adenosine as a method of immune evasion (94). Indeed, extracellular adenosine in a tumor

environment can be increased 10-20 times over that of nearby tissues, the result of increased hypoxia and cellular stress, but also the result of increased quantities of CD39 and CD73 expressed by the tumor (95, 96). Acting via the A2aR, this extracellular adenosine can induce anergy among T_{eff} cells and increase the generation of T_{reg} cells and the production of immuno-suppressive cytokines such as TGFβ and IL-10, thereby blunting the anti-tumor response (86). Consistent with the role of A2aR in cancer immune evasion, A2aR-deficient mice show improved tumor rejection compared to A2aR-competent controls (84). Similarly, pharmacological blockade of the A2aR significantly improved the anticancer response in multiple murine cancer models and enhanced the efficacy of checkpoint inhibitors (94, 97).

While A2aR-deficient mice reject transplanted tumors, these mice are prone to develop autoimmunity, highlighting the important immunosuppressive role of A2aR (84). Indeed, MOG-treated mice deficient for A2aR generate more activated macrophages and lymphocytes capable of secreting IFN-γ, and ultimately, these mice develop more severe experimental autoimmune encephalitis compared to A2aR-sufficient controls (98). Treatment of lupus-prone mice with an A2aR agonist has been shown to improve disease outcome in these mice (99). A2aR has also been implicated in human autoimmunity as well. Lymphocytes taken from RA patients express more A2aR compared to healthy controls, and treatment of these lymphocytes with the A2aR agonist CGS-21680 blocked production of TNFα, IL-1β and IL-6 (100). Similarly, T_{reg} cells taken from SLE patients display an impaired ability to respond to adenosine signals, as they failed to suppress B cell differentiation following incubation with adenosine (101). Given the widespread expression of the A2aR among lymphocytes and its important role in the regulation of

autoimmunity, A2aR-specific drugs may represent a novel approach to eliminate pathogenic autoimmune-derived lymphocytes, which express A2aR, such as T-bet+ B cells.

Summary

T-bet+ B cells are found in a multitude of different diseases. When elicited in response to infection, T-bet+ B cells can be protective, by functioning as memory cells and by giving rise to neutralizing antibody. When generated during autoimmunity, however, T-bet+ B cells can contribute to disease pathology via the aberrant activation of CD4+ T cells and the production of autoreactive antibodies. The development of T-bet+ B cells in either of these disease contexts appears to be dependent upon chronic inflammation, TLR signaling, and various T helper cell signals. Currently there are no pharmacological approaches to specifically eliminate these T-bet+ B cells.

CHAPTER II: 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

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 (59, 71, 102–107). T-bet+ B cells have been identified during many infections, including malaria, HIV, and hepatitis C and B infections (108–111). Moreover, several studies have implicated T-bet+ B cells in autoimmune diseases, including SLE (65, 112, 113), arthritis (114, 115), and Sjøgren's disease (116). We first identified CD11c+ B cells during murine infection with the obligate intracellular bacterium *Ehrlichia muris* (117) and later showed that all of the CD11c+ B cells also expressed T-bet (118). CD11c+ T-bet+ B cells generated early following infection differentiate into antigen-specific IgM-secreting plasmablasts (119). By day 30 post-infection, however, CD11c+ T-bet+ B cells are found largely as IgM memory cells, although smaller subsets of switched and GC B cells are also found within the population (120). 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 (119, 121). Other work from our laboratory demonstrated that long-term T-bet+ B cells elicited during ehrlichial infection function as memory cells (118); 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 (120).

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 (5, 122). 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 (80, 123).

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 (124) 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 (125, 126). 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 (124, 125). 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 (127). T_{FH1} cells have now been shown to be elicited during type I responses to many different pathogens including, HIV, influenza, LCMV and malaria (75–80). 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 (77). 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 (75). 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 (80). 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 5x10^4 *E. muris* bacterial copies, as determined by qPCR, and as previously described (128). 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 (129). 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 (120). 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 (120). 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 (129); (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 (130), 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 (5, 123), 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**).

Figure 1

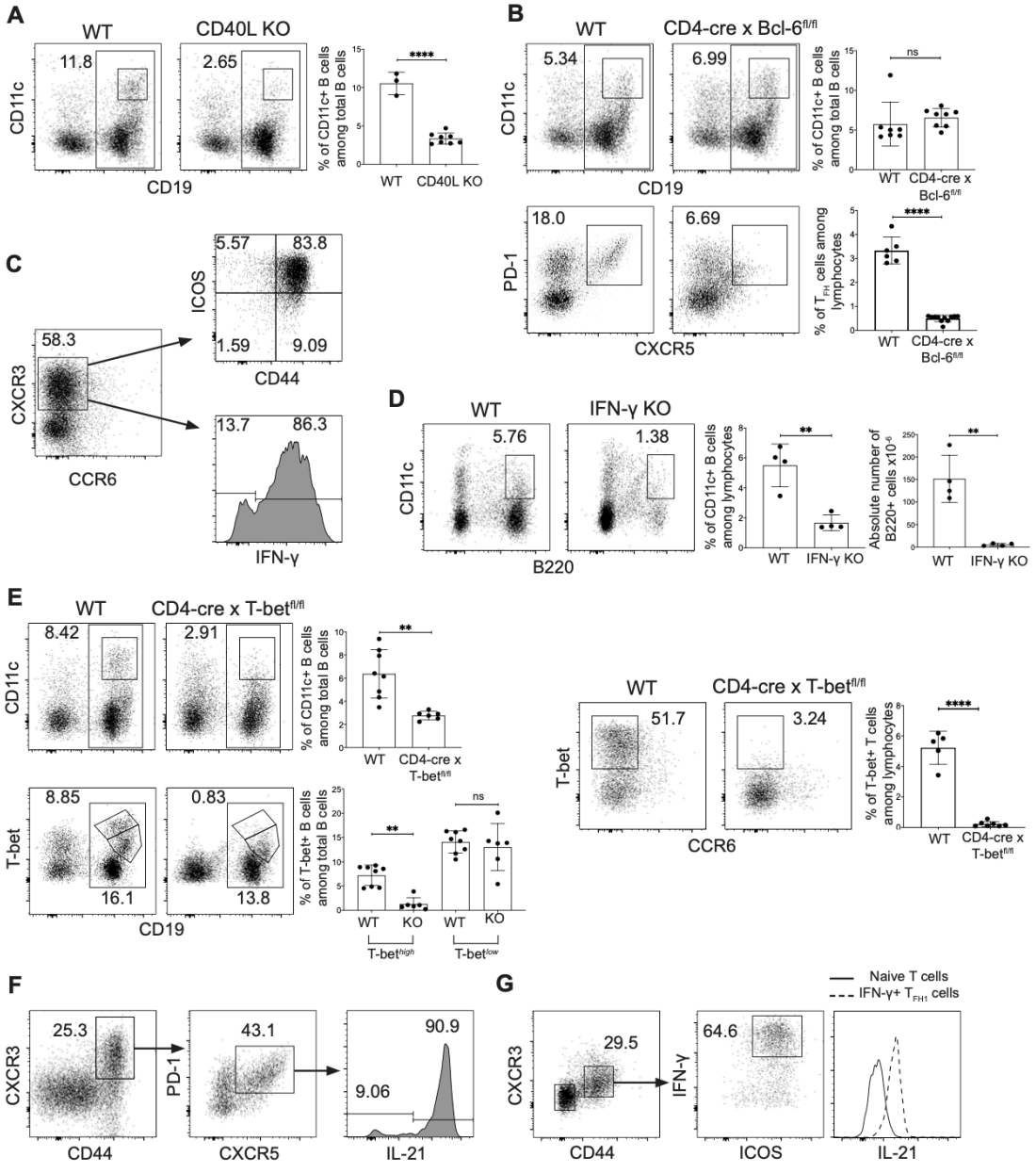

A
WT
CD40L KO
11.8
2.65
CD11c
CD19
% of CD11c+ B cells among total B cells
****
WT
CD40L KO
B
WT
CD4-cre x Bcl-6fl/fl
5.34
6.99
CD11c
CD19
% of CD11c+ B cells among total B cells
ns
WT
CD4-cre x Bcl-6fl/fl
18.0
6.69
PD-1
CXCR5
% of TFH cells among lymphocytes
****
WT
CD4-cre x Bcl-6fl/fl
C
5.57
83.8
1.59
9.09
ICOS
CD44
58.3
CXCR3
CCR6
13.7
86.3
IFN-γ
D
WT
IFN-γ KO
5.76
1.38
CD11c
B220
% of CD11c+ B cells among lymphocytes
**
WT
IFN-γ KO
Absolute number of B220+ cells x10-6
**
WT
IFN-γ KO
E
WT
CD4-cre x T-betfl/fl
8.42
2.91
CD11c
% of CD11c+ B cells among total B cells
**
WT
CD4-cre x T-betfl/fl
8.85
0.83
16.1
13.8
T-bet
CD19
% of T-bet+ B cells among total B cells
**
ns
WT
KO
WT
KO
T-bethigh
T-betlow
WT
CD4-cre x T-betfl/fl
51.7
3.24
T-bet
CCR6
% of T-bet+ T cells among lymphocytes
****
WT
CD4-cre x T-betfl/fl
F
25.3
43.1
90.9
9.06
CXCR3
CD44
PD-1
CXCR5
IL-21
G
Naive T cells
IFN-γ+ TFH1 cells
29.5
64.6
CXCR3
CD44
IFN-γ
ICOS
IL-21

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.

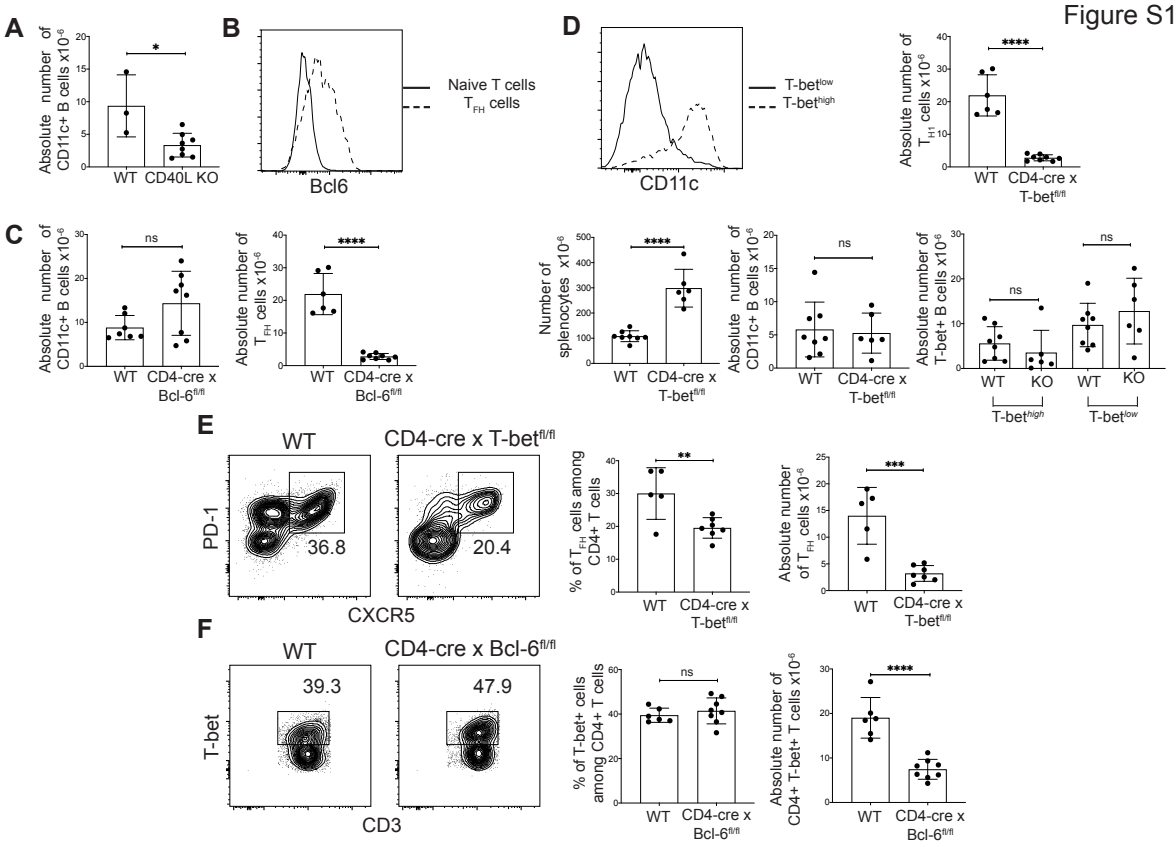
(D) Splenocytes from *E. muris*-infected, female, IFN-γ-deficient or wild-type mice were analyzed on day 18 post-infection. Representative dot plots show the percentages of CD11c+ B220+ B cells among total lymphocytes. Graphs represent aggregate data from two independent experiments. Statistical significance 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 S1


A
B
C
D
E
F
Absolute number of CD11c+ B cells x10^-6
WT
CD40L KO
Naive T cells
T_FH cells
Bcl6
T-bet^low
T-bet^high
CD11c
Absolute number of T_H1 cells x10^-6
CD4-cre x T-bet^fl/fl
Absolute number of T_FH cells x10^-6
CD4-cre x Bcl-6^fl/fl
Number of splenocytes x10^-6
Absolute number of T-bet+ B cells x10^-6
KO
T-bet^high
T-bet^low
ns
WT
PD-1
CXCR5
36.8
20.4
% of T_FH cells among CD4+ T cells
T-bet
CD3
39.3
47.9
% of T-bet+ cells among CD4+ T cells
Absolute number of CD4+ T-bet+ T cells x10^-6

Supplementary Figure S1: T-bet+ T cells, but not conventional T_{FH} cells, were required for CD11c+ T-bet+ B cell development

(A) Splenocytes from *E. muris*-infected CD40L-deficient or wild-type mice were analyzed on day 30 post-infection. The graph represents aggregate data from two independent experiments. Statistical significance was determined using a two-tailed un-paired t test ($p < 0.0104$, $df = 9$).

(B) Splenocytes from *E. muris*-infected female Bcl-$6^{fl/fl}$ mice were analyzed on day 16 post-infection. The representative histogram shows Bcl-6 expression among CD3+ CD4+ CD44-negative T cells and among CD3+ CD4+ CXCR5+ PD-1+ T cells.

(C) Splenocytes from *E. muris*-infected CD4-cre x Bcl-$6^{fl/fl}$ or Bcl-$6^{fl/fl}$ control mice were analyzed on day 30 post-infection. Graphs represent aggregate data from three independent experiments. Statistical significance was determined using two-tailed un-paired t tests (left :$p = 0.0818$, $df = 13$; right: $p < 0.0001$, $df = 12$).

(D) Splenocytes from *E. muris*-infected T-$bet^{fl/fl}$ control mice were analyzed on day 30 post-infection. The representative histogram shows CD11c expression among T-bet^{high} and T-bet^{low} B cells. Graphs represent aggregate data from three independent experiments. Statistical significance was determined using two-tailed un-paired t tests (top right: $p < 0.0001$, $df = 10$; bottom left: $p < 0.0001$, $df = 12$; bottom middle: $p = 0.7911$, $df = 12$) and an ordinary one-way ANOVA ($p = 0.0193$, $F = 3.994$, $df = 27$) with Sidak's multiple comparisons test (bottom right: T-bet^{high}: $p = 0.7244$, $df = 24$; T-bet^{low}: $p = 0.4862$, $df = 24$).

(E) Splenocytes from *E. muris*-infected wild-type T-$bet^{fl/fl}$ or CD4-cre x T-$bet^{fl/fl}$ mice were analyzed on day 16 post-infection. Representative dot plots show the percentage of PD1+ CXCR5+ cells among CD3+ CD4+ T cells. Graphs represent aggregate data from two independent experiments. Statistical significance was determined using two-tailed un-paired t tests (left plot: $p = 0.009$, $df = 10$; right plot: $p = 0.0004$, $df = 10$).

(F) Splenocytes from *E. muris*-infected CD4-cre x Bcl-$6^{fl/fl}$ or Bcl-$6^{fl/fl}$ control mice were analyzed on day 16 post-infection. Representative dot plots show the percentage of CD3+ T-bet+ cells among CD3+ CD4+ T cells. Graphs represent aggregate data from two independent experiments. Statistical significance was determined using two-tailed un-paired t tests (left: $p = 0.4739$, $df = 12$; right: $p < 0.0001$, $df = 12$).

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 (131), 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**).

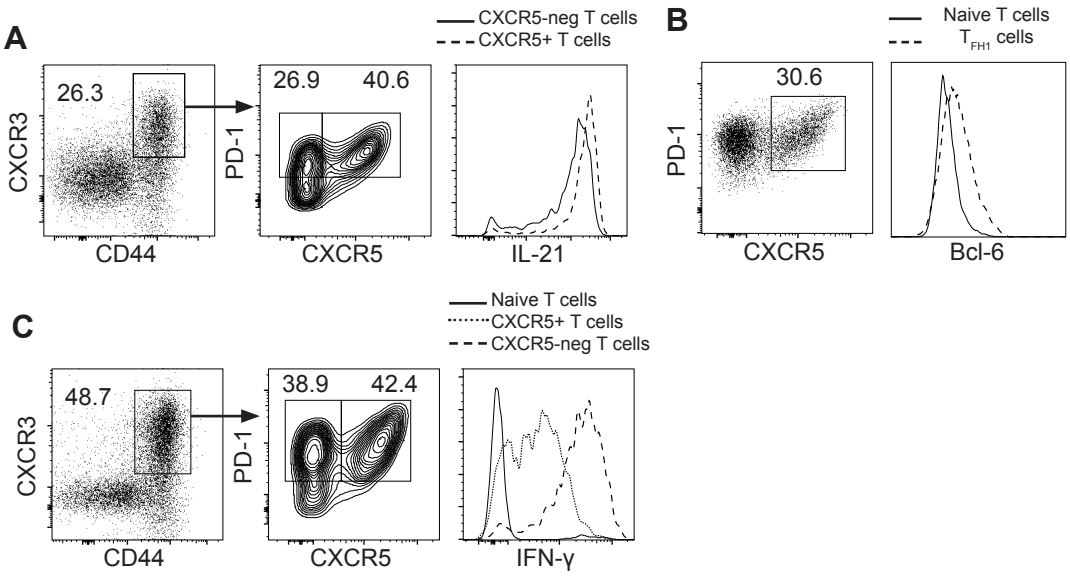
IFN-γ IL-21 double-producing T_{FH1} cells provide help to CD11c+ T-bet+ B cells

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 (75–80); 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 (124).

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.

Figure S2


A
CXCR5-neg T cells
CXCR5+ T cells
26.3
CXCR3
CD44
26.9
40.6
PD-1
CXCR5
IL-21
B
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T FH1 cells
30.6
PD-1
CXCR5
Bcl-6
C
Naive T cells
CXCR5+ T cells
CXCR5-neg T cells
48.7
CXCR3
CD44
38.9
42.4
PD-1
CXCR5
IFN-γ

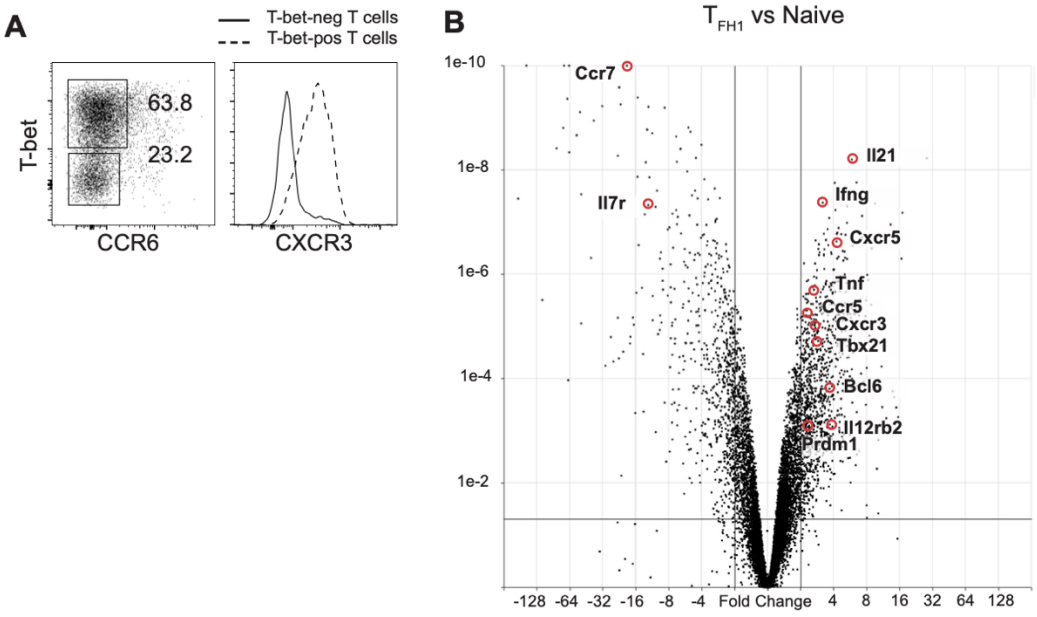
Supplementary Figure S2: IFN-γ IL-21 double-producing T_{FH1} cells provide help to CD11c+ T-bet+ B cells

(A) CD3+ CD4+ CXCR3+ CD44+ CXCR5+ and CXCR5-negative T cells from *E. muris*-infected IL-21-reporter mice were analyzed for the expression of IL-21 16 days post-infection.

(B) Splenocytes from *E. muris*-infected wild-type Bcl-$6^{fl/fl}$ mice were analyzed on day 16 post-infection. Representative dot plots show Bcl-6 expression among CD3+ CD4+ CD44-negative T cells and CD3+ CD4+ CXCR3+ CXCR5+ PD-1+ T cells.

(C) CD3+ CD4+ CXCR3-negative CD44-negative naïve T cells and CD3+ CD4+ CXCR3+ CD44+ CXCR5+ and CXCR5-negative T cells from *E. muris*-infected female IFN-γ-reporter mice were analyzed for the expression of IFN-γ 16 days post-infection. n.s. > 0.05, **p < 0.01, ***p < 0.001 ****p < 0.0001.

Figure S3


A
T-bet-neg T cells
T-bet-pos T cells
T-bet
63.8
23.2
CCR6
CXCR3
B
T_FH1 vs Naive
1e-10
1e-8
1e-6
1e-4
1e-2
Ccr7
Il7r
Il21
Ifng
Cxcr5
Tnf
Ccr5
Cxcr3
Tbx21
Bcl6
Il12rb2
Prdm1
-128 -64 -32 -16 -8 -4 Fold Change 4 8 16 32 64 128

Supplementary Figure S3: *E. muris*-induced T_{FH1} cells display characteristics of both T_{FH} and T_{H1} cells.

(A) Splenocytes from *E. muris*-infected wild-type T-bet$^{fl/fl}$ mice were analyzed on day 16 post-infection. Representative plots show CXCR3 expression from CD3+ CD4+ T-bet+ CCR6+ T cells and CD3+ CD4+ T-bet-negative CCR6+ T cells.

(B) Volcano plot of differentially expressed genes between flow cytometrically purified CD3+ CD4+ CD44+ CXCR3+ T_{FH1} cells and CD3+ CD4+ CD44- negative naïve T cells from *E. muris*-infected wild-type female mice 16 days post-infection. Differentially expressed genes are indicated on the plot.

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 (2, 132). 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 (119). 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 (121). 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.

Figure 2

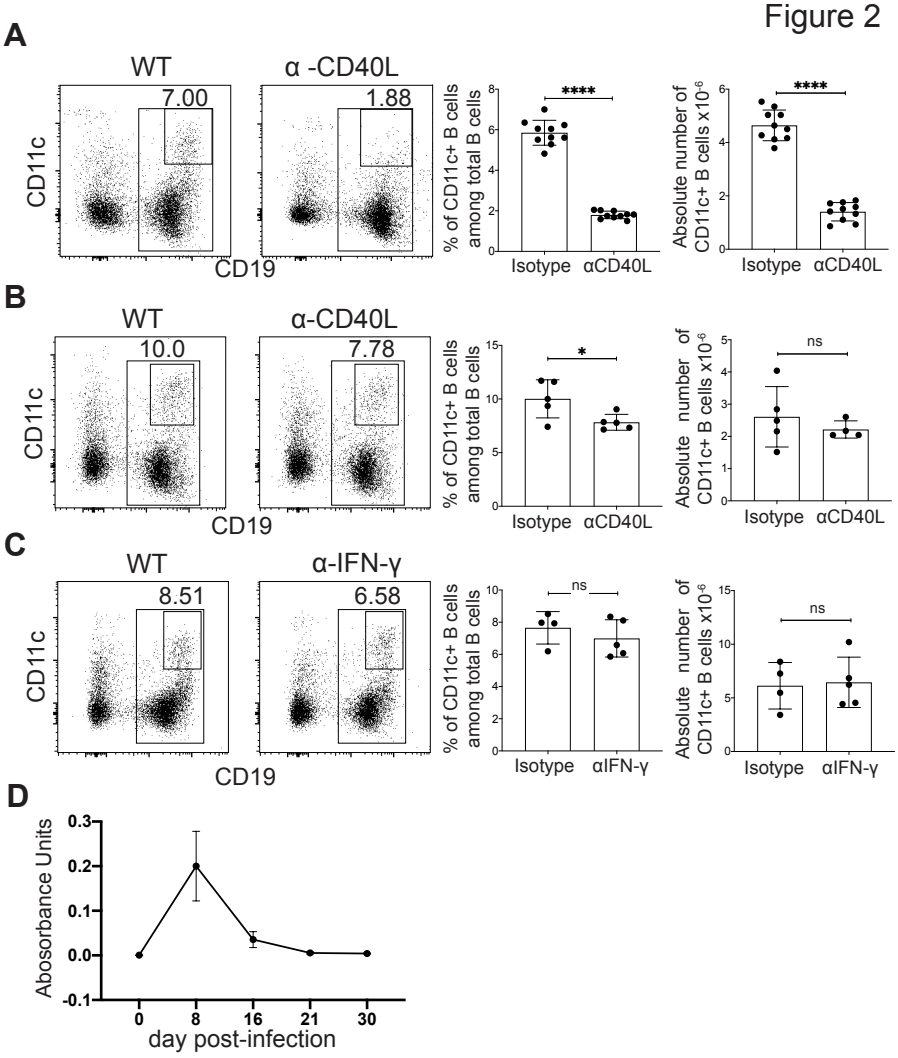

A
WT
α -CD40L
7.00
1.88
CD11c
CD19
% of CD11c+ B cells among total B cells
****
Isotype
αCD40L
Absolute number of CD11c+ B cells x10-6
****
Isotype
αCD40L
B
WT
α-CD40L
10.0
7.78
CD11c
CD19
% of CD11c+ B cells among total B cells
*
Isotype
αCD40L
Absolute number of CD11c+ B cells x10-6
ns
Isotype
αCD40L
C
WT
α-IFN-γ
8.51
6.58
CD11c
CD19
% of CD11c+ B cells among total B cells
ns
Isotype
αIFN-γ
Absolute number of CD11c+ B cells x10-6
ns
Isotype
αIFN-γ
D
Absorbance Units
0.3
0.2
0.1
0.0
-0.1
0
8
16
21
30
day post-infection

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). This data was provided by Maria Popescu.

(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. This data was provided by Berenice Cabrera-Martinez.

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 (121), 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 (119, 121), 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 (133, 134). 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**) (120). Thus, while developing T-bet+ B cells received T cell help apparently independent of GCs, as these structures are disrupted during ehrlichial infection (119, 121), 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.

Figure 3

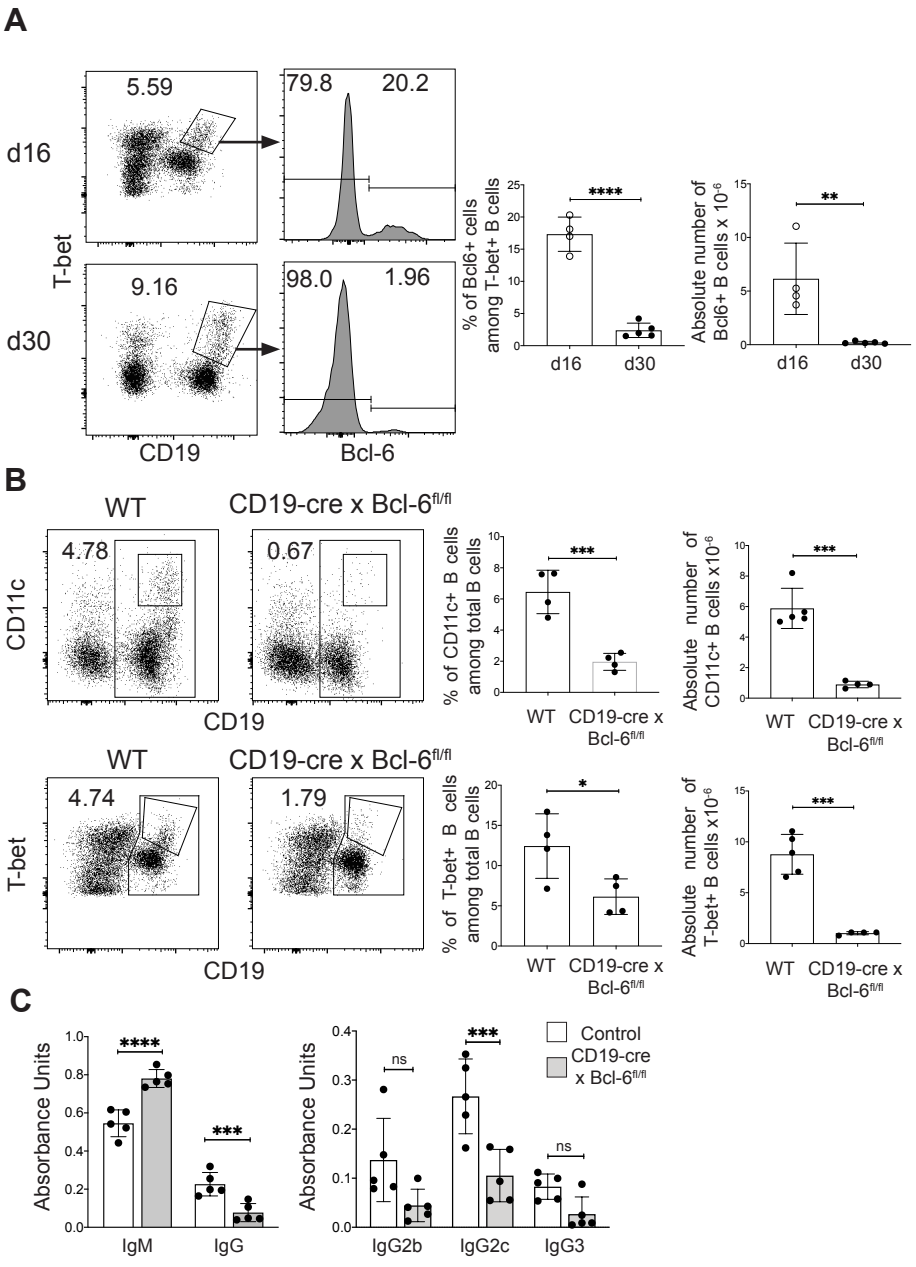

A
d16
d30
T-bet
5.59
79.8
20.2
9.16
98.0
1.96
CD19
Bcl-6
% of Bcl6+ cells among T-bet+ B cells
****
Absolute number of Bcl6+ B cells x 10-6
**
d16
d30
B
WT
CD19-cre x Bcl-6fl/fl
CD11c
4.78
0.67
CD19
% of CD11c+ B cells among total B cells
***
Absolute number of CD11c+ B cells x10-6
T-bet
4.74
1.79
% of T-bet+ B cells among total B cells
*
Absolute number of T-bet+ B cells x10-6
C
Absorbance Units
IgM
IgG
IgG2b
IgG2c
IgG3
ns
Control
CD19-cre x Bcl-6fl/fl

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 Bcl$6^{fl/fl}$ 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, *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$. Krista Newell contributed to this data.

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 (5). 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 (62, 113, 135, 136). 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**) (137). 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.

Figure 4

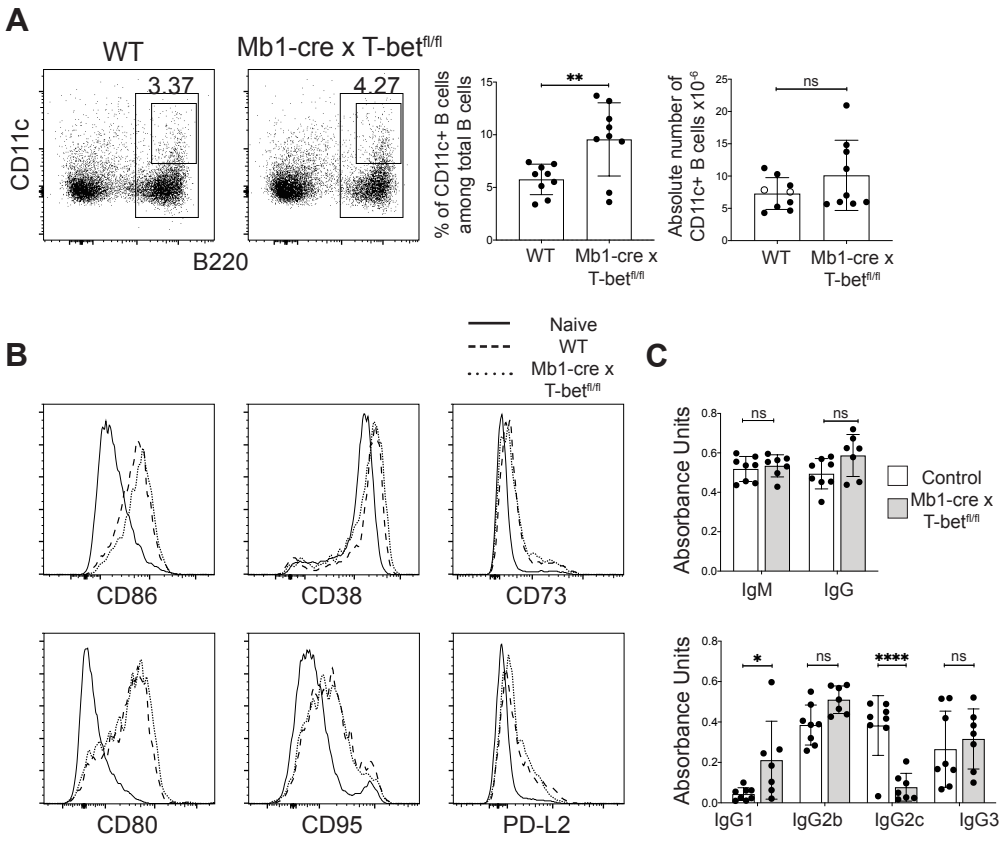

A
WT
Mb1-cre x T-bet^fl/fl
3.37
4.27
CD11c
B220
% of CD11c+ B cells among total B cells
**
WT
Mb1-cre x T-bet^fl/fl
Absolute number of CD11c+ B cells x10^-6
ns
WT
Mb1-cre x T-bet^fl/fl
B
Naive
WT
Mb1-cre x T-bet^fl/fl
CD86
CD38
CD73
CD80
CD95
PD-L2
C
Absorbance Units
ns
ns
IgM
IgG
Control
Mb1-cre x T-bet^fl/fl
Absorbance Units
*
ns
****
ns
IgG1
IgG2b
IgG2c
IgG3

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). This data was provided by Krista Newell.

(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. This data was provided by Krista Newell.

(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, *$p < 0.05$, ****$p < 0.0001$. This data was provided by Krista Newell.

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* (80, 123), 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 (79, 123, 135, 138). 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 (124, 139, 140). 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 (141), 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 (119, 142). These delayed interactions may be relevant in other infections that generate T-bet+ B cells, where similar changes in splenic organization have been observed (143).

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 (124, 144), 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 (76). 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 (124). 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 (121). 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 (120, 145). 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 (118).

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 (62, 122, 135, 136). 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 (146). 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.

Future Directions

There are multiple questions raised by this manuscript that we intend to pursue. First, we will determine when CD11c+ T-bet+ B cells receive IL-21 signals over the course of *E. muris*-infection. Second, we will determine if IFN-γ signaling is required for the development of CD11c+ T-bet+ B cells. Knowing what signals contribute to the development of CD11c+ T-bet+ B cells and when these signals are required will improve our understanding of the ontogeny of these cells and may help identify new techniques to prevent or enhance their development during infection and autoimmunity. We will next determine if CD11c+ T-bet^{hi} and CD11c-negative T-bet^{lo} B cells differ transcriptionally. This information will better our understanding of the heterogeneity among T-bet+ B cells and may help us to identify novel functions of these cells. Finally, we will determine if T_{FH1} cells contribute to splenic disorganization during *E. muris*-infection. These data will provide novel insight into the function of T_{FH1} cells and will aid in our understanding of other diseases characterized by these T cells and by GC disruption.

When do CD11c+ T-bet+ B cells receive IL21?

In the current manuscript, we demonstrate that T_{FH1} cells can produce IL-21 and IFN-γ simultaneously, and while it is likely that developing CD11c+ T-bet+ B cells require IL-21 early in infection, we do not formally address this. To answer this question, we will first determine when IL-21 is secreted during *E. muris* infection by performing ELISAs for IL-21 on the serum and spleens of *E. muris*-infected mice at days 0, 8, 16, 21, and 30 post-infection. We will then determine if CD11c+ T-bet+ B cells require IL-21 signals early or late during infection by treating *E. muris*-infected mice with anti-IL-21

antibody from days 0 to 16 and from days 16 to 30 post-infection and flow-cytometrically analyzing splenocytes for the presence of CD11c+ T-bet+ B cells on day 30 post-infection. The absence of CD11c+ T-bet+ B cells in one or more of the treatment regimens will tell us when CD11c+ T-bet+ B cells require IL-21 signals. Alternatively, if CD11c+ T-bet+ B cells are not depleted in either regimen it would indicate that CD11c+ T-bet+ B cells receive IL-21 signals long-term over the course of the infection. As an alternate method, we could utilize IL-21 reporter mice and analyze T_{FH1} cells for the generation of IL-21 at various time points post-infection.

Is IFN-γ signaling required for CD11c+ T-bet+ B cell development?

To determine if IFN-γ signals were required for the development of *E. muris*-derived CD11c+ T-bet+ B cells, we flow-cytometrically analyzed *E. muris*-infected IFN-γ-deficient mice on day 18 post-infection. However, these mice had significant defects in their B cell compartment, complicating interpretation of the data. To more accurately assess the requirement for IFN-γ in the development of CD11c+ T-bet+ B cells, we plan to analyze *E. muris*-infected, B cell-specific IFN-γ receptor 1-deficient (Mb1-cre x $Ifgr1^{flox}$) mice for the presence of CD11c+ T-bet+ B cells. If CD11c+ T-bet+ B cells are not generated in the Mb1-cre x $Ifgr1^{flox}$ mice, it would indicate that B cell-specific IFN-γ signaling is required for the development of these cells. Alternatively, if CD11c+ T-bet+ B cells are still generated in the absence of B cell-intrinsic IFN-γ signaling, it could indicate that IFN-γ does not directly signal through B cells, but through an intermediate cell population such as T_{FH1} cells. As an alternate method, we could administer IFN-γ blocking antibody to *E. muris*-infected wild-type mice from days 0 to 16 post-infection,

during peak IFN-γ secretion, and analyze mice for the presence of CD11c+ T-bet+ B cells.

Do CD11c+ T-bet^{hi} and CD11c-negative T-bet^{lo} B cells differ transcriptionally?

In the current manuscript we demonstrate that the T-bet+ B cell population is composed of both CD11c+ T-bet^{hi} and CD11c-negative T-bet^{lo} B cells. While these subsets differ in their requirement for T cell help, we have yet to identify additional differences in surface marker or transcription factor expression between these two B cell populations. To determine if these subsets differ transcriptionally, we plan to use T-bet-cre^{ERT2} x Rosa26^{eYFP} mice, in which all T-bet-expressing cells are permanently labelled following administration of tamoxifen. We will perform single-cell RNA-sequencing on flow-cytometrically sorted CD11c+ T-bet+ and CD11c-negative T-bet+ B cells from *E. muris*-infected T-bet-cre^{ERT2} x Rosa26^{eYFP} mice. Previous work from our laboratory has demonstrated that CD11c+ T-bet+ and CD11c-negative T-bet+ B cells interconvert in-vivo (118). Single-cell RNA-sequencing will also allow us to perform pseudotime analysis on both B cell subsets and determine if these two populations undergo sequential transcriptomic changes as they interconvert. If CD11c+ T-bet+ and CD11c-negative T-bet+ B cells do not differ transcriptionally, it would indicate that the observed differences between CD11c and T-bet expression are the result of post-transcriptional modifications, in which case we could perform high dimensional mass-spectroscopy and look for differences in phosphorylated proteins. As an alternative method, we could perform bulk RNA-sequencing on flow cytometrically sorted CD11c+ T-bet+ and CD11c-negative T-

bet+ B cells, although this technique would not provide single-cell resolution or allow for pseudotime analysis.

Do T_{FH1} cells contribute to splenic disorganization via the secretion of TNF-α?

Previous work from our laboratory has demonstrated that TNF-α contributes to GC disruption during *E. muris* infection (121). Here we show that T_{FH1} cells transcribe TNF-α, and therefore, may contribute GC disruption during *E. muris*-infection. To test this hypothesis, we plan to flow-cytometrically analyze *E. muris*-elicited T_{FH1} cells for TNF-α production. We also plan to utilize CD4-cre x $Tace^{flox}$ mice, in which CD4+ T cells do not generate TNF-α. We then plan to infect these mice with *E. muris* and analyze them for serum and tissue TNF-α and for GC formation. If the concentration of TNF-α is reduced in the CD4+ T cell-specific TNF-α-deficient mice, and likewise, if GCs are not disrupted in these mice, it would suggest that T_{FH1} cells contribute to GC disruption via the secretion of TNF-α. If T_{FH1} cells do not secrete TNF-α and if the CD4+ T cell-specific TNF-α mice do not display reduced quantities of TNF-α and improved GC formation, it would indicate that TNF-α is likely produced by another population of cells, possibly splenic macrophages. As an alternative, we could generate mixed bone marrow chimeras from TNF-α-deficient mice and CD4+ T cell deficient mice, in which all CD4+ T cells are TNF-α deficient.

CHAPTER III: Pharmacological targeting of T-bet+ B cells via the adenosine 2a receptor ameliorates autoimmune disease in mice

Abstract

CD11c+ T-bet+ B cells are now recognized as an integral component of humoral immunity as well as autoimmunity, in both humans and mice. Pharmacological targeting of pathogenic CD11c+ T-bet+ B cells in autoimmunity may aid in the treatment of diseases such as SLE, arthritis, and Sjøngren's Syndrome, although no such treatments are currently available. Our studies of CD11c+ T-bet+ B cells generated during intracellular bacterial infection identified a number of genes that were differentially expressed relative to naive B cells, including the A2aR. Given the well-studied role of the A2aR in immunity and the fact that multiple A2aR-specific drugs are available, we tested whether A2aR function was critical for the maintenance of CD11c+ T-bet+ B cells. In-vivo administration of the A2aR agonist CGS-21680 depleted established CD11c+ T-bet+ B cells generated in *E. muris*-infected mice, although CD11c-negative T-bet^{lo} B cells were unaffected. This depletion was B cell-intrinsic, as CD11c+ T-bet+ B cells were still present in B cell-specific A2aR-deficient mice following CGS-21680 treatment. CGS-21680 administration also depleted CD11c+ and T-bet+ B cells in mouse models of lupus and this treatment was associated with decreased disease severity. Human CD11c+ B cells also expressed the A2aR, and *in vitro* treatment of human PBMCs reduced the number of these CD11c + B cells. This study identifies the first means of pharmacologically targeting pathogenic CD11c+ T-bet+ B cells, and suggests that A2aR agonists can be used in the treatment of autoimmune diseases associated with CD11c+ T-bet+ B cells.

Introduction

CD11c+ T-bet+ B cells have become accepted as an important subset of B cells involved in both protective immunity and pathogenic immune responses. T-bet+ B develop in diseases characterized by type I immunity, such as malaria, HIV, Influenza, and Hepatitis C infections (40, 45, 52, 147). We have previously demonstrated that developing CD11c+ T-bet+ B cells express the GC-associated marker Bcl-6, and require GC-like help from T_{FH1} cells in the form of IL-21 and IFN-γ (148). Developing CD11c+ T-bet+ B cells eventually give rise to memory cells capable of self-renewing and differentiating into effector cells following secondary challenge (66). Data from our laboratory indicate that CD11c+ T-bet+ memory B cells directly contribute to humoral immunity, as elimination of this population after *E. muris*-infection abrogated the switched antibody response following secondary challenge (4). In addition to their role in type I immunity, CD11c+ T-bet+ B cells are also involved in pathogenic immune responses such as occurs in arthritis, Søgren's disease, and SLE (35, 149).

SLE is a relapsing autoimmune disease affecting 1.5 million people in the US, and 5 million worldwide. It commonly manifests as facial rash, fatigue, and joint pain, and in certain cases can result in renal failure (150). It has been proposed that CD11c+ T-bet+ B cells generated in SLE are poised towards differentiation into ASCs, and thereby contribute to disease pathogenesis via the formation of autoreactive antibodies (6, 13, 151). Moreover, T-bet expression in B cells drives class-switching to IgG2c, an isotype known to contribute to disease severity in autoimmune diseases (6, 54, 63, 152). Indeed, elimination of T-bet expression in murine B cells significantly improved disease outcome in a mouse model of lupus (13). Strategies to target these pathogenic cells in

autoimmunity may aid in the treatment of diseases where these cells have been implicated; however, no such pharmacological treatments currently exist.

Our studies of CD11c+ T-bet+ B cells generated during *E.* muris infection led us to identify a number of genes that were highly expressed, relative to naive B cells, including the G-protein-coupled receptor A2aR (153). A2aR is one of several receptors for extracellular adenosine, and is ubiquitously expressed on most leukocytes, as well as on neurons and endothelial cells (83, 154). Extracellular adenosine is primarily produced via the catabolism of ATP by the ectoenzymes CD73 and CD39, both of which are highly expressed on CD11c+ T-bet+ B cells (155). Extracellular adenosine production by certain subsets of NK cells and T_{reg} cells can be immunosuppressive, and may act by signaling through the A2aR to reduce T_{eff} proliferation and to increase the generation of T_{reg} cells (156, 157). Adenosine signaling can also significantly affect the humoral immune response by suppressing T_{FH} cell proliferation and inhibiting the GC response (89). Adenosine signaling has also been implicated in various autoimmune diseases. Lymphocytes from patients with rheumatoid arthritis express high levels of A2aR, and stimulation of this receptor inhibits production of the proinflammatory cytokines TNFα, IL-1β, and IL-6 by lymphocytes (158–160). Similarly, administration of the A2aR receptor agonist CGS-21680 significantly improved disease outcome in a mouse model of lupus (99). Adenosine signaling has also been implicated in human SLE, as T_{reg} cells taken from SLE patients and incubated with adenosine failed to prevent B cell differentiation, in contrast to their non-treated counterparts (161).

In the present study we demonstrate that A2aR stimulation can deplete CD11c+ T-bet+ B cells from *E. muris*-infected and lupus-prone mice, as well as CD11c+ B cells

from healthy humans. Moreover, A2aR stimulation significantly reduces disease severity in CGS-21680 treated MRL/lpr mice. We also demonstrate that A2aR-mediated depletion is correlated with T-bet and CD11c, as CD11c-negative T-bet^{lo} B cells are not depleted following CGS-21680 administration. This research describes the first targeted pharmacological approach to deplete pathogenic CD11c+ T-bet+ B cells and highlights the therapeutic potential of A2aR stimulation in the treatment of autoimmune diseases where CD11c+ T-bet+ B cells have been implicated in pathogenesis.

Materials and Methods

Mice. C57BL/6J, CD4^{cre} (B6.Cg-Tg(Cd4-cre)1Cwi/BfluJ), Mb1^{cre} (B6.C(Cg)-Cd79a$^{tm1(cre)Reth}$/EhobJ), MRL/lpr (MRL/MpJ-*Fas*^{lpr}/J), SLE^{123} (B6;NZM-*Sle1*$^{NZM2410/Aeg}$ *Sle2*$^{NZM2410/Aeg}$ *Sle3*$^{NZM2410/Aeg}$/LmoJ), and Rosa26^{eYFP} (B6.Cg-*Gt(ROSA)26Sor*$^{tm3(CAG-EYFP)Hze}$/J) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). T-bet-cre^{ERT2}. Adora2a^{flox} (B6;129-*Adora2a*^{tm1Dyj}/J) mice were provided by Dr. Joel Linden, La Jolla Institute for Immunology, La Jolla, CA. AID-cre^{ERT2} mice were provided by Dr. Jean-Claude Weill, ISERM, Paris, France. The mice were kept 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.

Infections and drug administration. Mice were infected intraperitoneally (i.p.) with 5-10x10^4 *E. muris* bacterial copies, as determined by qPCR, and as previously described (128). CGS-21680 hydrochloride (17126) was purchased from Cayman Chemical (Ann Arbor, MI), and mice were injected intraperitoneally with 50µg in 200µl PBS 5% DMSO.

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), FITC-conjugated anti-PD-1 (29F.1A12), PerCpCy5.5-conjugated anti-CD4 (RM4-4), Alexafluor 700-conjugated anti-CD3 (17A2), Brilliant Violet 421-conjugated anti-CXCR5 (L138D7), V500-conjugated anti-CD3 (500A2), Alexafluor 647-conjugated anti-A2aR (7F6-G5-A2).

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. 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 for ANAs were performed by coating Flat-Bottom Immuno plates (Thermo Scientific) with with 100µl of 50µg/ml of ANA (100ng of cardiolipin, 100pg of ApoH) per well in 0.01 M $NaHCO_3$ (pH 9.55) and chromatin-specific antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL).

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.

In vitro culture of human PBMCs. Human PBMCs were purified using BD Vacutainer Specialty Venous Blood Collection Tubes according to the manufacturer's instructions. PBMCs were cultured in complete tumor medium with 1μM of CGS-21680 or vehicle for 48 hours at 37°C at 5% CO2.

Proteinuria. Urine albumin was determined using Fisherbrand 10-SG Urine Reagent Strips by dipping the strips in collected mouse urine and colorimetrically determining protein concentration.

Western Blot. 20ug of protein from sorted B cells was loaded per lane using Bio-Rad TGX gels, and analyzed using a Bio-Rad ChemiDoc. C3 (9662) and β-actin (13E5) antibodies were purchased from Cell Signaling.

Results

A2aR stimulation depleted CD11c+ T-bet+ B cells

We have previously demonstrated that the gene encoding the A2aR is expressed over ten-fold higher in *E. muris*-elicited CD11c+ T-bet+ B cells compared to naïve B cells (153). To determine if A2aR stimulation would deplete established CD11c+ T-bet+ memory B cells, we treated *E. muris*-infected mice with CGS-21680 once daily from days 30 to 37 post-infection, after the CD11c+ T-bet+ B cell population is fully developed. No CD11c+ T-bet+ B cells were detected following CGS-21680 treatment, indicating that A2aR stimulation eliminated the mature CD11c+ T-bet+ B cells, although CD11c-negative T-bet^{lo} B cells were still present (**Figure 1A**). Preliminary data indicated that a single dose of CGS-21680 was sufficient to reduce the number of CD11c+ T-bet+ B cells, and this reduction took place within 48 hours (**Supplemental Figure S1A**). Preliminary data also indicated that T_{FH} cells elicited early during infection were depleted following CGS-21680 treatment every other day from days 0 to 16, consistent with previously published work (89). In contrast, T_{FH} cells present on day 37 post-infection, likely memory T_{FH} cells, were not depleted following CGS-21680 treatment (**Supplemental Figure S1B**). Following CGS-21680 administration, it is possible that CD11c+ T-bet+ B cells appear depleted because they downregulated CD11c and T-bet or because they migrated to other tissues. To determine if CD11c+ T-bet+ B cells changed phenotype or migrated to other tissues following CGS-21680 administration, we utilized T-bet-cre ^{ERT2} x Rosa26^{eYFP} mice, in which T-bet-expressing cells were permanently labelled with eYFP following tamoxifen administration. Following CGS-21680 treatment, there was a significant reduction of eYFP+ B cells in the spleens of T-bet-cre

^{ERT2} x Rosa26^{eYFP} mice, but no change in the frequency of eYFP+ B cells in the lymph nodes, liver, and bone marrow. These data indicate that CD11c+ T-bet+ B cells did not migrate to other tissues or change their phenotype following CGS-21680 treatment, but rather underwent cell death (**Figure 1B**). In agreement with this interpretation, preliminary data from CD11c+ B cells flow-cytometrically sorted from spleens of *E. muris*-infected mice 31 days post-infection, indicated that 24 hours after CGS-administration, CD11c+ B cells had less uncleaved caspase-3. These data suggest that CD11c+ T-bet+ B cells undergo apoptosis following A2aR stimulation (**Supplemental Figure S1C**).

CGS-21680 administration has been shown to affect GC B cells by acting on T_{FH} cells (89). To determine if A2aR-mediated depletion of CD11c+ T-bet+ B cells was B cell-intrinsic, we utilized Mb1-cre x Adora2a^{flox} mice, in which, the A2aR is not expressed on B cells. CD11c+ T-bet+ B cells were still present in Mb1-cre x Adora2a^{flox} mice following CGS-21680 administration on days 30-37 post-infection, indicating that A2aR-mediated depletion of CD11c+ T-bet+ B cells was B cell intrinsic (**Figure 1C and Supplemental Figure 1D**). Moreover, CD11c+ T-bet+ B cells were still depleted in CD4-cre x Adora2a^{flox} mice treated with CGS-21680 from days 30-37 post-infection, indicating that this depletion was not T cell dependent (**Figure 1D and Supplemental Figure 1E**). In contrast to CD11c+ T-bet+ B cells elicited during *E.* muris-infection, ABCs generated in one-year-old mice were not depleted following CGS-21680 administration (**Figure 1E)**. Yet, preliminary data indicated that CD11c+ ABCs taken from healthy humans were reduced following CGS-21680 treatment *in vitro*, consistent with increased expression of A2aR on human CD11c+ B cells (**Figure 1F**). These data

suggest that *in vivo* depletion of human ABCs may require longer A2aR stimulation to induce cell death, compared to in vitro treated human ABCs or CD11c+ T-bet+ B cells generated during *E. muris*-infection. Cumulatively, these data indicate that A2aR-stimulation depleted CD11c+ T-bet+ B cells in a B cell-intrinsic manner, and that human CD11c+ T-bet+ B cells may similarly be susceptible to depletion, as they also express the A2aR.

Figure 1

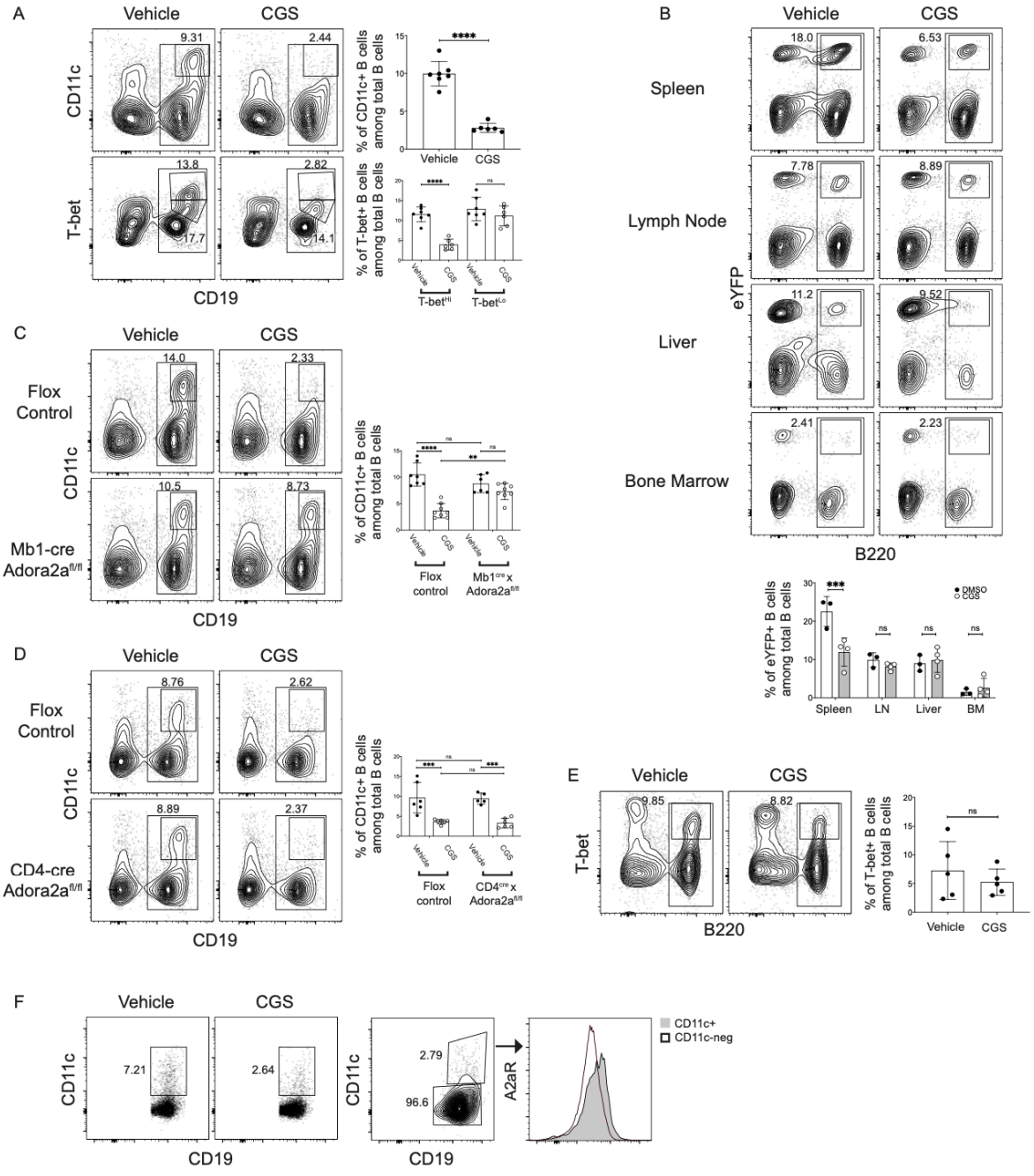

A
Vehicle
CGS
9.31
2.44
CD11c
13.8
2.82
T-bet
17.7
14.1
CD19
% of CD11c+ B cells among total B cells
****
Vehicle
CGS
% of T-bet+ B cells among total B cells
T-bet^Hi
T-bet^Lo
B
Spleen
Lymph Node
Liver
Bone Marrow
18.0
6.53
7.78
8.89
11.2
9.52
2.41
2.23
eYFP
B220
% of eYFP+ B cells among total B cells
DMSO
CGS
***
ns
Spleen
LN
Liver
BM
C
Flox Control
Mb1-cre Adora2a^fl/fl
14.0
2.33
10.5
8.73
Flox control
Mb1^cre x Adora2a^fl/fl
D
Flox Control
CD4-cre Adora2a^fl/fl
8.76
2.62
8.89
2.37
CD4^cre x Adora2a^fl/fl
E
9.85
8.82
% of T-bet+ B cells among total B cells
F
7.21
2.64
2.79
96.6
A2aR
CD11c+
CD11c-neg

Figure 1: A2aR stimulation depleted CD11c+ T-bet+ B cells

(A) *E. muris*-infected wild-type mice were treated with CGS-21680 every other day for seven days starting on day 30 post-infection and splenocytes analyzed on day 37 post-infection. Contour plots show the percentage of CD11c+ CD19+, T-bet^{Hi} CD19+, and T-bet^{lo} CD19+ 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 (top: $p < 0.0001$, $df = 11$) and a one-way ordinary ANOVA (bottom: $p < 0.0001$, $F = 19.60$, $df = 25$) with Sidak's multiple comparisons test (T-bet^{hi}: $p < 0.0001$; T-bet^{lo}: $p = 0.3399$, $df = 22$).

(B) *E. muris*-infected T-bet$^{cre\ ERT2}$ x Rosa26^{eYFP} mice were fed tamoxifen chow from day 15 to day 25 post-infection, treated with CGS-21680 every other day for seven days starting on day 30 post-infection, and splenocytes analyzed on day 37 post-infection. Contour plots show the percentage of CD11c+ B220+ among total B cells. Statistical significance was determined using an ordinary one-way ANOVA ($p < 0.0001$, $F = 18.67$, $df = 27$) with Sidak's multiple comparisons test (spleen: $p = 0.0002$, lymph node = 0.8614, liver = 0.9881, bone marrow = 0.9711, $df = 20$).

(C) *E. muris*-infected Mb1^{cre} x Adora2a^{flox} mice were treated with CGS-21680 every other day for seven days starting on day 30 post-infection and splenocytes analyzed on day 37 post-infection. Contour plots show the percentage of CD11c+ CD19+ cells among total B cells. Graphs represent aggregate data from three independent experiments. Statistical significance was determined using a two-way ANOVA (interaction: $p = 0.0003$, genotype: $p = 0.1532$, treatment: $p < 0.0001$)

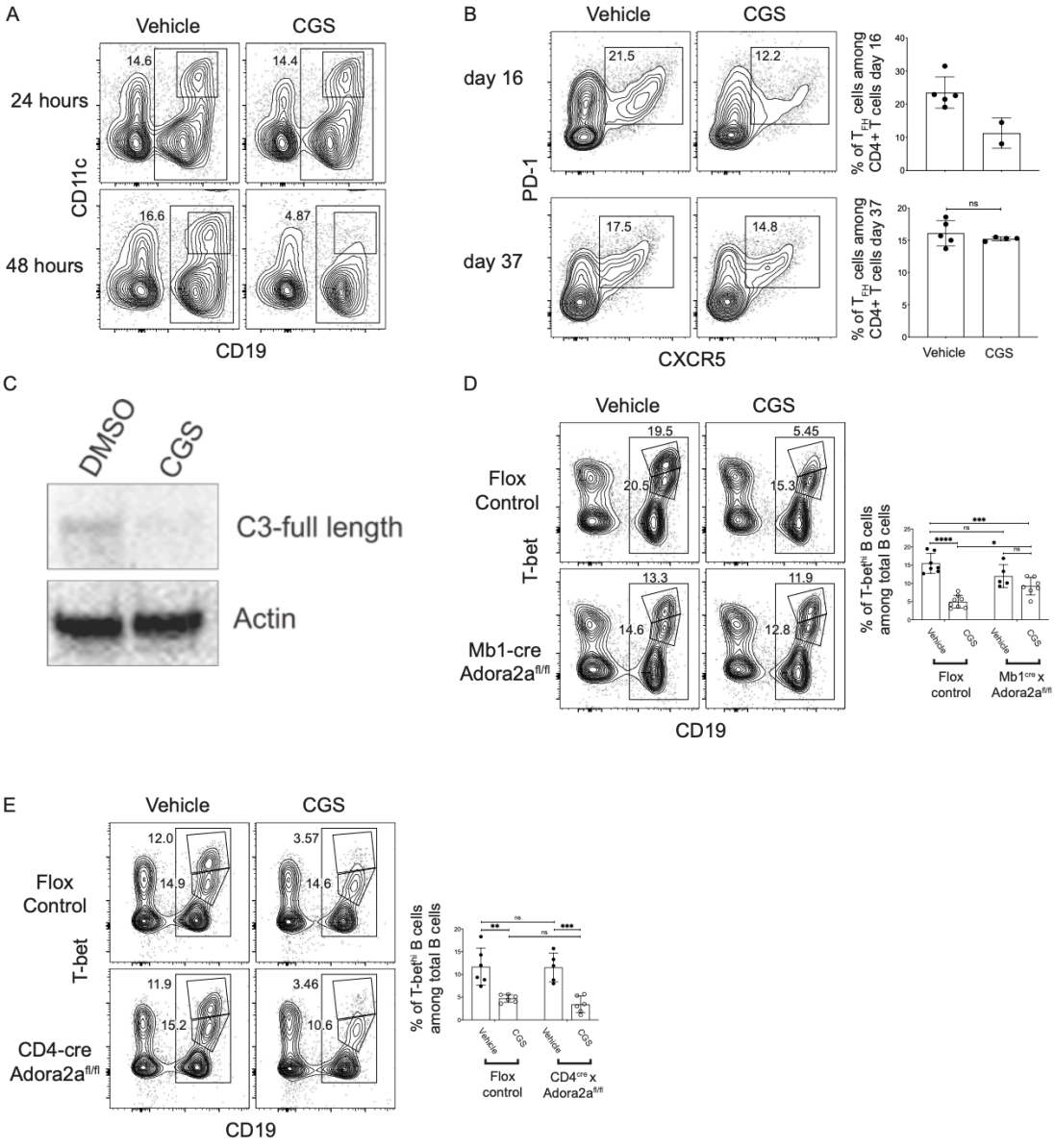
with Sidak's multiple comparisons test (vehicle flox vs vehicle KO: $p = 0.3982$, vehicle flox vs CGS flox: $p < 0.0001$, CGS flox vs CGS KO: $p = 0.0017$, vehicle KO vs CGS KO: $p= 0.5239$).

(D) *E. muris*-infected $CD4^{cre}$ x $Adora2a^{flox}$ mice were treated with CGS-21680 every other day for seven days starting on day 30 post-infection and splenocytes analyzed on day 37 post-infection. Contour plots show the percentage of CD11c+ CD19+ cells among total B cells. Graphs represent aggregate data from three independent experiments. Statistical significance was determined using a two-way ANOVA (interaction: $p = 0.9582$, genotype: $p = 0.7562$, treatment: $p < 0.0001$) with Sidak's multiple comparisons test (vehicle flox vs vehicle KO: $p = 0.9978$, vehicle flox vs CGS flox: $p = 0.0007$, CGS flox vs CGS KO: $p = 0.9931$, vehicle KO vs CGS KO: $p= 0.0009$).

(E) one-year-old mice were treated with CGS-21680 every other day for seven days and splenocytes analyzed. Contour plots show the percentage of CD11c+ CD19+ cells among total B cells. Statistical significance was determined using a two-tailed un-paired t test ($p = 0.4325$, $df = 8$)

(F) PBMCs taken from healthy human donors were cultured in vitro with 1uM of CGS-21680 for 48 hours and then analyzed. Dot plots show the percentage of CD11c+ CD19+ cells among total B cells. The histogram shows A2aR expression among Live, CD3-negative, CD14-negative, CD19+, CD11c+ and CD11c-negative B cells. This data was provided by Berenice Cabrera-Martinez and Krista Newell.

Supplementary Figure 1



Supplementary Figure S1: A2aR stimulation depleted CD11c+ B cells after 24 hours

(A) *E. muris*-infected wild-type mice 30 days post-infection were treated with either CGS-21680 or vehicle control and splenocytes analyzed 24 and 48 hours later. Contour plots show the percentage of CD11c+ B cells among total B cells. This data was provided by Berenice Cabrera-Martinez.

(B) *E. muris*-infected wild-type mice were treated with CGS-21680 every other day from day 0 to 16 post-infection and splenocytes analyzed on day 16 post-infection (top) or every day from day 30 to 37 post-infection and splenocytes analyzed on day 37 post-infection. Contour plots show the percentage of PD-1+ CXCR5+ cells among CD3+ CD4+ T cells. Statistical significance was determined using a two-tailed un-paired t test (left: $p = 0.4113$, $df = 7$).

(C) CD11c+ CD19+ B cells were flow-cytometrically sorted from *E. muris*-infected wild-type mice 24 hours after treatment with CGS-21680 on day 30 post-infection. Sorted cells were analyzed by western blot for full-length Caspase-3. This data was provided by Iwona Buskiewicz

(D) *E. muris*-infected $Mb1^{cre}$ x $Adora2a^{flox}$ mice were treated with CGS-21680 every other day for seven days starting on day 30 post-infection and splenocytes analyzed on day 37 post-infection. Contour plots show the percentage of CD11c+ CD19+ cells among total B cells. Graphs represent aggregate data from three independent experiments. Statistical significance was determined using a two-way ANOVA (interaction: $p = 0.0008$, genotype: $p = 0.6899$, treatment: $p < 0.0001$) with Sidak's multiple comparisons test (vehicle flox vs vehicle KO: $p = 0.1116$,

vehicle flox vs CGS flox: $p < 0.0001$, CGS flox vs CGS KO: $p = 0.0213$, vehicle KO vs CGS KO: $p= 0.3103$).

(E) *E. muris*-infected $CD4^{cre}$ x $Adora2a^{flox}$ mice were treated with CGS-21680 every other day for seven days starting on day 30 post-infection and splenocytes analyzed on day 37 post-infection. Contour plots show the percentage of CD11c+ CD19+ cells among total B cells. Graphs represent aggregate data from three independent experiments. Statistical significance was determined using a two-way ANOVA (interaction: $p = 0.6248$, treatment: $p = 0.0001$, genotype: $p < 0.5317$) with Sidak's multiple comparisons test (vehicle flox vs vehicle KO: $p > 0.9999$, vehicle flox vs CGS flox: $p = 0.0019$, CGS flox vs CGS KO: $p = 0.9625$, vehicle KO vs CGS KO: $p = 0.0006$).

A2aR stimulation ameliorated disease in a mouse model of lupus

The CD11c+ T-bet+ population is expanded in SLE patients, and previous research has demonstrated that CD11c+ T-bet+ B cells contribute to disease pathogenesis in SLE (13, 65). To determine if CGS-21680 treatment would deplete CD11c+ T-bet+ B cells generated during SLE, we treated nine-month-old SLE^{123} mice with CGS-21680 every other day for five days and flow-cytometrically analyzed their splenocytes. CGS-21680 treatment significantly reduced the number of T-bet+ B cells in the spleens of SLE123 mice (**Figure 2A**). Similarly, CD11c+ B cells were reduced in the spleens of 15-week-old MRL/lpr mice treated with CGS-21680 once weekly for six weeks (**Figure 2B**). To determine if CGS-21680 treatment could ameliorate disease symptoms in SLE, MRL/lpr mice were treated with CGS-21680 twice weekly for six weeks, staring at nine weeks of age, and their sera analyzed were for the presence of anti-chromatin antibodies. Fifteen-week-old CGS-21680-treated mice had significantly lower ANAs compared to vehicle controls (**Figures 2C**). Likewise, proteinuria was significantly reduced in MRL/lpr mice treated with CGS-21680 twice weekly starting at eight weeks of age, and preliminary data indicate that CGS-21680 treatment reduced morbidity in treated MRL/lpr mice (**Figure 2D and E**). These data indicated that A2aR stimulation depleted autoimmune-derived CD11c+ T-bet+ B cells and improved disease outcome in a mouse model of lupus.

Figure 2

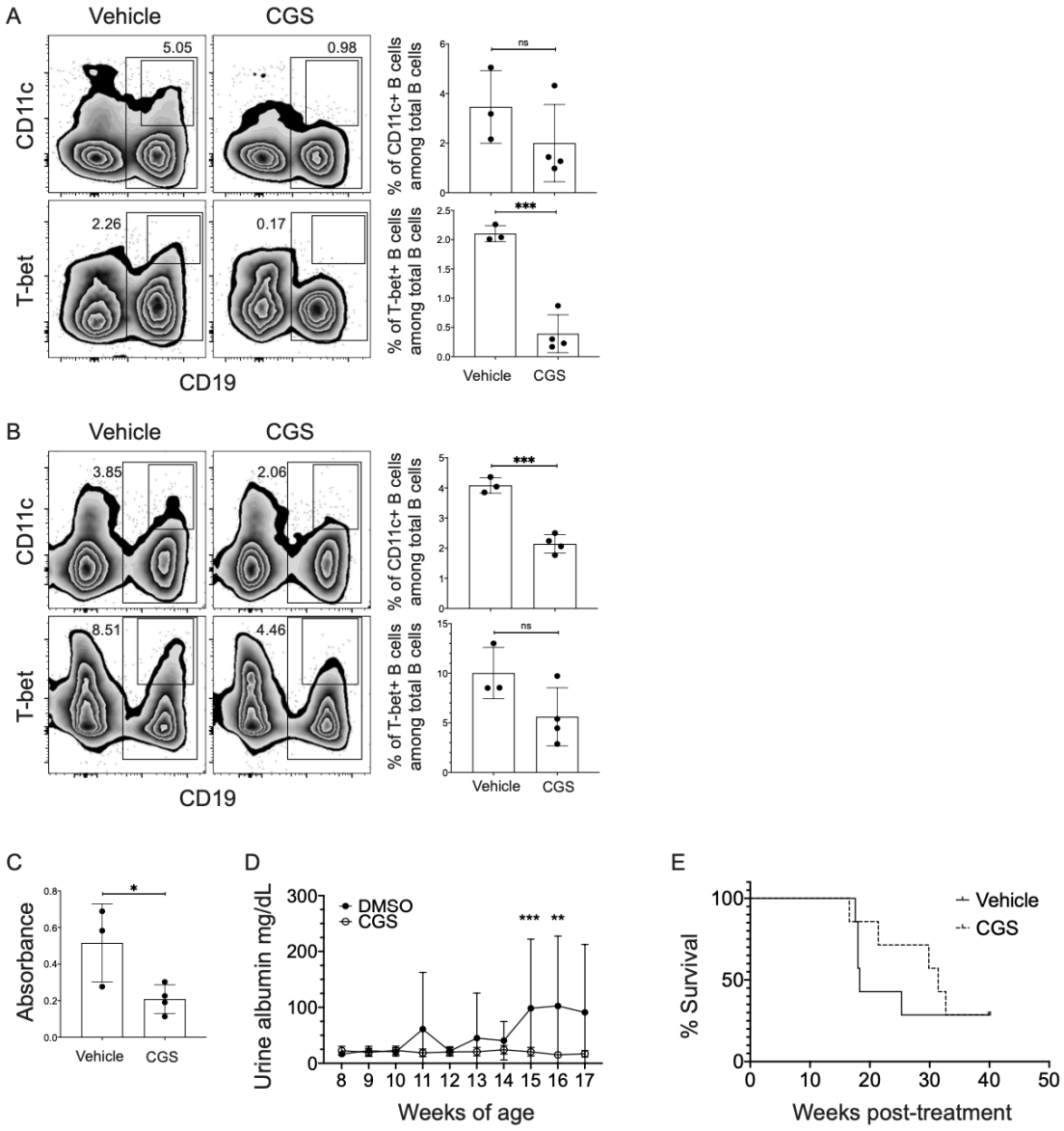

A
Vehicle
CGS
5.05
0.98
2.26
0.17
CD11c
T-bet
CD19
% of CD11c+ B cells among total B cells
% of T-bet+ B cells among total B cells
ns
***
Vehicle
CGS
B
Vehicle
CGS
3.85
2.06
8.51
4.46
CD11c
T-bet
CD19
% of CD11c+ B cells among total B cells
% of T-bet+ B cells among total B cells
***
ns
Vehicle
CGS
C
Absorbance
*
Vehicle
CGS
D
Urine albumin mg/dL
DMSO
CGS
*** **
Weeks of age
E
% Survival
Vehicle
CGS
Weeks post-treatment

Figure 2: A2aR stimulation ameliorated disease in a mouse model of lupus

(A) nine-month-old SLE^{123} mice were treated with CGS-21680 every other day for 5 days and splenocytes analyzed. Zebra plots show the percentage of CD11c+ CD19+, and T-bet+ CD19+ B cells among total B cells. Statistical significance was determined using two-tailed un-paired t tests (top: $p = 0.2641$, $df = 5$) (bottom: $p = 0.0004$, $df = 5$).

(B) Nine-week-old MRL/lpr mice were treated with CGS-21680 once weekly for six weeks and splenocytes analyzed. Zebra plots show the percentage of CD11c+ CD19+, and T-bet+ CD19+ B cells among total B cells. Statistical significance was determined using two-tailed un-paired t tests (top: $p = 0.0003$, $df = 5$) (bottom: $p = 0.0937$, $df = 5$).

(C) Sera from 15-week-old MRL/lpr mice treated with CGS-21680 once weekly for six weeks was analyzed by ELISA for anti-chromatin antibodies. Statistical significance was determined using a two-tailed un-paired t test ($p = 0.0420$, $df = 5$).

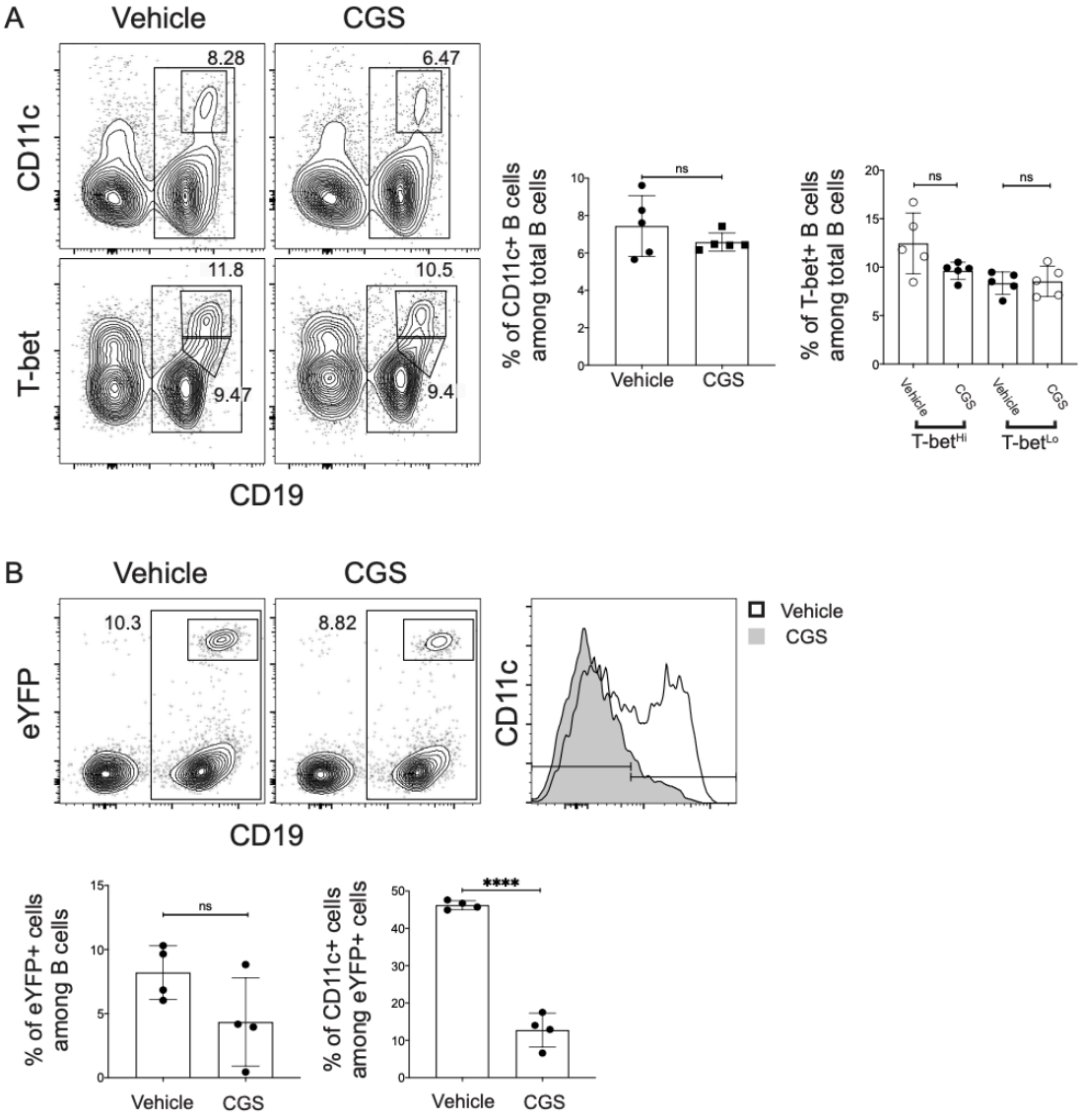
(D) Urine from CGS-21680 or vehicle treated mice was analyzed for albumin. Statistical significance was determined using an ordinary two-way ANOVA (interaction: $p = 0.0351$, age: $p = 0.0993$, treatment: $p < 0.0001$) with Sidak's multiple comparisons test (15: $p = 0.0240$; 16: $p = 0.0183$).

(E) Eight-week-old MRL/lpr mice were treated with CGS-21680 every other day and sacrificed when moribund.

A2aR stimulation depleted CD11c+, but not CD11c-negative T-bet+ B cells

While CD11c+ B cells were completely depleted following CGS-21680 treatment, these B cells reappeared one month following the cessation of treatment, suggesting that a population of B cells resistant to A2aR-mediated depletion was giving rise to the CD11c+ T-bet+ B cells (**Figure 3A**). We previously demonstrated that the T-bet+ B cell population is composed of both CD11c+ and CD11c-negative cells, and that these two subsets interconvert *in vivo* (66). We have also shown that CD11c expression among B cells is correlated with T-bet expression, with CD11c+ B cells expressing high quantities of T-bet and CD11c-negative B cells expressing low quantities. CD11c-negative T-bet^{lo} B cells were still present following CGS-21680 treatment suggesting that this population gave rise to the CD11c+ T-bet^{hi} B cells after the cessation of treatment (see Figure 1A). To confirm this hypothesis, we utilized AID-cre ^{ERT2} x Rosa26^{eYFP} mice, in which both CD11c+ and CD11c-negative T-bet+ B cells are permanently labelled with eYFP following tamoxifen administration (66). Analysis of splenocytes from wild-type and AID-cre ^{ERT2} x Rosa26^{eYFP} mice treated with CGS-21680 from days 30 to 37 post-infection revealed that CD11c-negative T-bet+ B cells were still present following CGS-21680 treatment, whereas all CD11c+ T-bet+ B cells were depleted (**Figure 3B**).These data indicate that CD11c-negative T-bet^{lo} B cells are resistant to A2aR-mediated cell death and suggests that these cells gave rise to CD11c+ T-bet^{hi} B cells following the cessation of CGS-21680 treatment.

Figure 3


A
Vehicle
CGS
8.28
6.47
CD11c
11.8
10.5
T-bet
9.47
9.4
CD19
% of CD11c+ B cells among total B cells
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Vehicle
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Figure 3: A2aR stimulation depleted CD11c+, but not CD11c-negative T-bet+ B cells

(A) *E. muris*-infected wild-type mice were treated with CGS-21680 every other day for seven days starting on day 30 post-infection and splenocytes analyzed one month later. Contour plots show the percentage of CD11c+ CD19+, T-bet^{Hi} CD19+, and T-bet^{lo} CD19+ cells among total B cells. Statistical significance was determined using a two-tailed un-paired t test (left: $p = 0.2890$, $df = 8$) and a one-way ordinary ANOVA ($p = 0.0124$, $F = 2.057$, $df = 19$) with Sidak's multiple comparisons test (T-bet^{hi}: $p = 0.0615$, T-bet^{lo}: $p = 0.9862$).

(B) *E. muris*-infected AID$^{cre\ ERT2}$ x Rosa26^{eYFP} mice were treated with CGS-21680 every other day for seven days after day 30 post-infection and splenocytes analyzed. Dot plots show the percentage of eYFP+ CD19+ cells among total B cells and the histograms shows CD11c expression among eYFP+ CD19+ B cells. Statistical significance was determined using two-tailed un-paired t tests (left: $p = 0.1034$, $df = 6$) (right: $p < 0.0001$).

Discussion

Our work demonstrates that administration of the A2aR agonist CGS-21680 can eliminate CD11c+ T-bet+ B cells generated in *E. muris*-infected and autoimmune prone mice, as well as CD11c+ T-bet+ B cells generated in healthy humans. We also show that A2aR agonist treatment ameliorates disease in a mouse model of lupus. While A2aR stimulation has been shown reduce disease burden in a mouse model of lupus, our work identifies targeted depletion of CD11c+ T-bet+ B cells as a primary mechanism by which A2aR stimulation can limit or perhaps cure disease (99). Our findings suggest that A2aR agonist-mediated depletion of CD11c+ T-bet+ B cells may also alleviate disease burden in other illnesses where CD11c+ T-bet+ B cells have been implicated in disease pathogenesis, such as RA, MS, and Sjøgren's Syndrome (25, 35, 149). The use of the pan B cell-depleting antibody rituximab (anti-CD20) has been effective at ameliorating disease in RA and MS (28, 162). However, this treatment lacks specificity, eliminating most B cells in the patient (90-100% of peripheral B cells) (163). Moreover, these treatments cost on average $36,000 yearly and can take up to six hours to administer (164). Pan depletion of B cells also eliminates suppressive B cells, and it is thought that loss of these B cells contributes to the spike in symptoms observed among some MS patients receiving anti-B cell therapy (24). In contrast to these anti-B cell therapies, A2aR agonists cost less, are administered as a single bolus injection, and are more specific, making them an attractive alternative.

Data from B cell-specific and T cell-specific A2aR-deficient mice indicate that T-bet+ B cells are depleted following CGS-21680 administration in a B cell-intrinsic manner. This finding indicates that CGS-21680 likely acts directly on T-bet+ B cells to

induce cell death, rather than acting on an intermediate cell population, such as T_{FH1} cells. Consistent with this interpretation, while CGS-21680 treatment appeared to reduce the frequency of T_{FH1} cells generated early in infection, T_{FH1} cells present at d30-37 post-infection (presumably memory T_{FH} cells), were not depleted following CGS-21680 administration. These data could indicate that memory T_{FH} cells downregulate A2aR, or that early activated T_{FH1} cells are metabolically poised for A2aR-mediated depletion, whereas their quiescent memory T_{FH1} cell counterparts are not. Alternatively, A2aR stimulation may not induce cell death among T_{FH1} cells at all, but rather may block their differentiation, and as such has limited effects on the mature cells.

While the A2aR signaling cascade has not been well studied in B cells, it has been characterized in other leukocytes, in particular, T cells. In T_{eff} cells, A2aR stimulation results in the activation of PKA, which then inhibits ZAP70, various MAP kinases, and PKC, as well as Notch1 signaling, ultimately blocking T cell activation and the generation of IFN-γ and Granzyme B (165). Other studies have also identified pAKT and NFκB as important components of A2aR signaling in T cells (166, 167). It is likely that the A2aR signals in B cells through similar intermediates, namely, pAKT, PKA, and NFκB, but the precise mechanism by which these signaling components induce cell death is unclear. The timing of cell death following CGS-21680 treatment and the lack of uncleaved caspase-3 among treated CD11c+ T-bet+ B cells strongly suggest that A2aR-stimulation induces apoptosis in these cells, implicating downstream apoptosis signaling proteins such as caspase-9, Bax/Bak, Bcl-2, and others. However, additional research is required to formally address this question.

The depletion of CD11c+ T-bet+ B cells following CGS-21680 administration may ameliorate disease in MRL/lpr mice via several mechanisms. First, as it has been proposed that CD11c+ T-bet+ B cells generated during SLE may be pre-ASCs. If true, the loss of this population would in turn reduce the number of ASCs and autoreactive antibodies (151). This would in turn reduce immune complex deposition in the kidneys of MRL/lpr mice and subsequently reduce the number of infiltrating proinflammatory T cells. Second, as CD11c+ T-bet+ B cells are known to function as potent APCs, CGS-21680 treatment may also help inhibit the aberrant activation of CD4+ T cells, thus reducing T cell-mediated disease pathogenesis (27). Finally, depletion of CD11c+ T-bet+ B cells may lead to a direct reduction of inflammation as SLE-derived T-bet+ B cells are known to secrete proinflammatory cytokines (168).

As A2aR is known to be widely expressed among many different leukocyte populations, CGS-21680 treatment may ameliorate disease in MRL/lpr mice by acting on other subsets of cells in addition to B cells. A2aR stimulation may reduce inflammation by inhibiting the differentiation of T_{eff} cells, by abrogating the secretion of inflammatory cytokines by NK cells and T cells, or by promoting the generation of T_{reg} cells (97, 167, 169). However, given their established role in the pathogenesis of SLE we propose that depletion of CD11c+ T-bet+ B cells is the primary mechanism by which A2aR agonist treatment ameliorates disease in autoimmunity.

Our studies, CGS-21680 was administered to MRL/lpr mice starting at eight weeks of age, prior to disease onset. However, human RA and SLE patients generally receive treatment after they have begun to present with symptoms. Preliminary data suggested that short-term CGS-21680 treatment did not deplete bone marrow ASCs in *E.*

muris-infected mice (data not shown), which may indicate that A2aR stimulation will not deplete established pathogenic ASCs generated during autoimmunity. RA-elicited ASCs are similarly resistant to current B cell depletion therapies, such as rituximab (170). Despite this, long-term rituximab treatment has been shown to be effective at reducing overall autoreactive antibody titers in RA patients (171). It has been proposed that this improvement is due either to the loss of rituximab-susceptible autoreactive B cells, which would normally replenish the ASC population, or it is due to the loss of inflammatory factors that promote the survival of the ASCs (171). We hypothesize that long-term A2aR stimulation will similarly reduce autoantibody titers by one or both mechanisms, as CD11c+ T-bet+ B cells are known to give rise to pathogenic ASCs, and to secrete proinflammatory cytokines (25, 65). It is also possible that A2aR agonists directly act on autoimmune-derived ASCs and induce cell death, although the presence of A2aR on ASCs has not yet been determined.

The difference in susceptibility to A2aR-mediated cell death among CD11c+ and CD11c-negative T-bet+ B cells is likely due to differential expression of A2aR. This differential expression could indicate that T-bet regulates A2aR expression or that T-bet and A2aR are co-regulated. Alternatively, it is possible that CD11c+ T-bet^{hi} B cells and CD11c-negative T-bet^{lo} B cells differ metabolically, such that CD11c+ T-bet+ B cells are poised for A2aR-mediated depletion whereas CD11c-negative B cells are not.

The reemergence of the CD11c+ T-bet^{hi} B cell population following the cessation of CGS-21680 treatment was most likely the result of CD11c-negative T-bet^{lo} B cells giving rise to the CD11c+ T-bet^{hi} B cells over time. This finding suggests that A2aR agonists, given for the treatment of autoimmune disease, would need to be administered

continually to deplete newly arising CD11c+ T-bet^{hi} B cells. However, as CD11c+ and CD11c-negative T-bet+ B cells interconvert, long-term treatment would likely deplete all T-bet-expressing B cells.

Future Directions

We plan to advance the research summarized in this dissertation in several ways. First, we will determine if A2aR-agonist treatment depletes pathogenic ASCs in autoimmune-prone mice. As pathogenic ASCs contribute to the generation of autoantibodies in SLE, understanding the effects of A2aR stimulation on this population will allow us to determine the clinical efficacy of A2aR agonists in the treatment of SLE. Second, we will determine if A2aR stimulation depletes canonical memory B cells. This data will demonstrate whether A2aR agonist treatment affects other related populations of B cells besides CD11c+ T-bet+ B cells, and therefore, if it will negatively impact immunological memory. Next, we will determine if A2aR-stimulation induces apoptosis in CD11c+ T-bet+ B cells. Understanding the mechanism by which A2aR agonists deplete CD11c+ T-bet+ B cells may enable us to enhance the efficacy of the treatment and allow us to develop novel therapeutic approaches to target these cells. Finally, we will determine if A2aR stimulation depletes human SLE-derived CD11c+ T-bet+ B cells. While our data strongly suggest that A2aR agonist treatment will deplete human SLE-derived CD11c+ T-bet+ B cells, this work will directly address this question.

Does long-term A2aR agonist treatment deplete ASCs?

ASCs directly contribute to disease pathogenesis in various autoimmune diseases via the secretion of autoreactive antibodies. However, techniques to target and deplete these cells have generally been ineffective. It is thought that the inability to deplete these pathogenic ASCs is one of the reasons why anti-B cell therapy has been seemingly

ineffective in the treatment of SLE (172). Recent work has demonstrated that long-term anti-B cell therapy can reduce the number of ASCs in the kidneys of autoimmune prone mice, suggesting either that these ASCs can be directly eliminated after repeated treatments, or that depletion of their susceptible progenitors eventually depletes the ASC population (173). In this way, depletion of CD11c+ T-bet+ B cells, which have been shown to give rise to pathogenic ASCs, may prove an effective approach to eliminate this resistant ASC population. To address this hypothesis, we plan to treat 16-week-old MRL/lpr mice with CGS-21680 twice weekly for one month and then analyze spleens, kidneys, and bone marrow for the presence of autoreactive ASCs. If ASCs are not present following long-term CGS-21680 treatment it could indicate that A2aR stimulation directly induces cell death in ASCs, that the elimination of the ASC precursors (i.e. CD11c+ T-bet+ B cell) results in the eventual loss of the ASC population, or that the reduction of proinflammatory cytokines caused by the loss of CD11c+ T-bet+ B cells eventually leads to the depletion of ASCs. If ASCs are not depleted following long-term CGS-21680 treatment it would indicate that A2aR stimulation ameliorates disease in lupus-prone mice by an alternate method, possible by acting on other cell subsets. It could also indicate that A2aR agonist treatment will have limited efficacy in treating established disease and will need to be administered prophylactically. As an alternate method, we could perform similar studies using *E. muris*-infected wild-type mice as CD11c+ T-bet+ B cells are known to give rise to ASCs in this model as well.

Does A2aR-stimulation deplete canonical memory B cells?

While A2aR agonist treatment induces cell death among CD11c+ T-bet+ B cells, its effects on other B cell subsets are unknown. To determine if A2aR stimulation induces cell death in canonical memory B cells (i.e., CD11c and T-bet-negative memory cells), we plan to infect wild-type mice with influenza virus and treat the mice with CGS-21680 every other day for seven days starting on day 30 post-infection. We will use an influenza nucleoprotein tetramer to analyze spleens and lymph nodes from infected mice for the presence of CD11c-negative T-bet-negative antigen-specific memory B cells. If canonical memory B cells are depleted following CGS-21680 treatment, it would indicate that other B cell populations may be susceptible to A2aR-mediated cell death and that further research is required to determine the long-term effects of A2aR stimulation on various B cell populations. As an alternative, we could immunize mice with influenza nucleoprotein and analyze spleens and lymph nodes for antigen-specific memory B cells.

Does A2aR stimulation induce apoptosis in CD11c+ T-bet+ B cells?

Studies using T-bet$^{cre\ ERT2}$ x Rosa26^{eYFP} mice, as well as preliminary western blot data suggest that A2aR stimulation induces apoptosis in CD11c+ T-bet+ B cells. To test this hypothesis, we plan to analyze flow-cytometrically-sorted CD11c+ T-bet+ B cells taken from MRL/lpr mice 24 hours after CGS-21680 administration for the presence phosphorylated AKT and PKA, cleaved Caspases 3 and 8/9, NFκB, and Mcl1, as these signaling molecules are associated either with A2aR signaling and/or with apoptosis (166, 167, 174). If the CD11c+ T-bet+ B cells do not express markers consistent with apoptosis, it would suggest that they undergo cell death via an alternate mechanism, such

as necrosis. As an alternative method, we could perform similar analyses using a B cell line, like RAMOS cells, transfected with A2aR and treated with CGS-21680 in vitro.

Does A2aR stimulation deplete human CD11c+ T-bet+ B cells generated during SLE?

Data from mice and healthy humans strongly suggest that CD11c+ T-bet+ B cells generated during SLE are susceptible to A2aR-mediated depletion. To confirm this, we plan to analyze PBMCs from healthy humans and SLE patients for the presence of CD11c+ T-bet+ B cells before and after the administration of the A2aR agonist Regadenoson, as this drug is given routinely for cardiac stress tests. If CD11c+ T-bet+ B cells are not depleted following Regadenoson treatment it could indicate that SLE-derived CD11c+ T-bet+ B cell are not susceptible to A2aR-medited depletion. However, A more likely explanation is that a single dose of Regadenoson does not induce sufficient A2aR signaling to cause depletion of CD11c+ T-bet+ B cells, as this drug is not as potent an A2aR agonist as CGS-21680. As an alternate approach, we could treat B cells taken from the blood of SLE patients with CGS-21680 in vitro and analyze the samples for the presence of CD11c+ T-bet+ B cells.

CHAPTER IV: Discussion

Chapter 2: 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

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

The work summarized in chapter 2 contributes to our understanding of the ontogeny of CD11c+ T-bet+ B cells in several ways. First, we demonstrate that CD11c+ T-bet+ B cells received help from a specialized T_{FH} subset, T_{FH1} cells, in the form of IL-21, IFN-γ, and CD40. While T_{FH1} cells have been suspected of contributing to the generation of T-bet+ B cells, this work formally confirms this hypothesis. This observation has implications for other disease contexts, such as SLE, where circulating T_{FH} cells and T-bet+ B cells have been associated with disease pathogenesis (113, 175). Our data suggest that SLE-derived T_{FH} cells contribute to disease etiology via the generation of T-bet+ B cells. The identification of this specific helper T cell population reveals a novel cellular target for the treatment of autoimmune diseases associated with T-bet+ B cells.

Our work also suggests that the T_{FH} cells, which have been suspected of providing help to T-bet+ B cells in these other models, are T_{FH1} cells. Indeed, malaria and HIV infections are known to induce the generation of T_{FH1} cells, and given that both of these diseases are associated with the generation of T-bet+ B cells, it is likely that T_{FH1} cells serve as helper cells to T-bet+ B cells in these contexts as well, as has been proposed (80, 176, 177). Alternatively, it is possible that T-bet+ B cells receive CD40, IL-21, and IFN-γ from different populations of helper cells. For example, IFN-γ signals could be provided by NKT cells early in infection and CD40 and IL-21 signals could be provided by canonical T_{FH} cells later (178). However, given the prevalence of T_{FH1} cells in the same

diseases that elicit T-bet+ B cells, and the dual requirement for IFN-γ and IL-21 by developing T-bet+ B cells, it seems likely that both signals are provided by T_{FH1} cells.

Second, our studies demonstrate that CD40L and IFN-γ signaling were required at different times during T-bet+ B cell development. It is known that CD40L and IFN-γ are required for the generation of T-bet+ B cells, however, when these signals are provided had not been addressed. The sequential requirement for these signals indicates that it is not just be the presence of T-bet-inducing signals that drive the accumulation of T-bet+ B cells during immunity and autoimmunity, but rather, when these signals are provided. Indeed, the timing of other signals is known to be important in the development of T-bet+ B cells. For example, while sequential TLR and BCR signals induce T-bet expression in B cells, concurrent TLR and BCR signals block this expression (5, 71). Similarly, IFN-γ signaling may epigenetically poise developing T-bet+ B cells for CD40-induced differentiation into mature T-bet+ B cells, as has been proposed for TLR and BCR signaling (71). Alternatively, it is possible that IFN-γ signals are required early to elicit other populations of helper cells, which then provide help to developing T-bet+ B cells. Similarly, early IFN-γ signals may induce epigenetic changes among T_{FH1} cells themselves, which then allow the T cells to provide the necessary help signals to developing T-bet+ B cells.

Next, we demonstrated that while CD11c+ T-bet+ B cells developed in the absence of formal GCs, they still had similar development requirements to GC-derived B cells (i.e., a requirement for Bcl-6). Given their lack of SHM and GC markers, T-bet+ B cells are generally thought to be generated independently of GCs. In contrast, our findings indicate that T-bet+ B cells generated in E. muris may be GC B cells, and in the

absence of formal GCs the cells lose expression of GC markers, but still receive similar T cell help in a non-GC environment. Indeed, the disruption of formal GC architecture and subsequent reduced clonal selection, is likely what drives the expansion of T-bet+ B cells in, E. muris and possibly in other diseases as well (118). Alternatively, it is possible that the requirement for Bcl-6 does not indicate that T-bet+ B cells are GC B cells at all, but simply that Bcl-6 is required for the differentiation of B cells into T-bet+ memory B cells. While generally considered to be competitive transcription factors, dual expression of T-bet and Bcl-6 is known to occur in developing T_{FH} cells (179). In these T cells, T-bet and Bcl-6 function cooperatively to modify the transcriptional landscape (180).

Finally, our work demonstrates that while Bcl-6 was required for the generation of CD11c+ T-bet+ B cells, T-bet was not. Despite its importance in the identification of CD11c+ T-bet+ B cells, we show that the primary role of T-bet in B cells during E. muris-infection was CSR, not CD11c+ B cells differentiation. While others have demonstrated that T-bet controls CSR, GC polarization, and affinity maturation, we show that in the absence of formal GCs, T-bet still serves an important role in B cell immunity, via isotype determination (62, 122, 136). Appropriate CSR is critical for the humoral immune response as different isotypes are associated with different types of infection, such as bacterial vs helminth infection. Moreover, certain isotypes, namely IgG2a/c, are associated with disease pathogenesis during autoimmunity via completement fixation (146). Indeed, depletion of B cell specific T-bet among lupus-prone mice resulted in reduced serum IgG2c and improved disease outcome (113). As such, our work highlights the importance of T-bet in immunity and autoimmunity even outside of its role in GC formation and affinity maturation.

Together our findings reveal that in the absence of formal GCs, developing CD11c+ T-bet+ B cells receive GC-like help in the form of IFN-γ, IL-21, and CD40 from T_{FH1} cells. These T_{FH1} cells provide this help in two distinct phases: IFN-γ early and CD40L late. Finally, rather than mediating the differentiation of these developing CD11c+ T-bet+ B cells, we show that T-bet instead controls CSR among CD11c+ T-bet+ cells. This research informs our understanding of the function and ontogeny of T-bet+ B cells, and as such may aid in the development of novel techniques to target these cells during autoimmunity.

Chapter 3: Pharmacological targeting of T-bet+ B cells via the adenosine 2a receptor, ameliorates autoimmune disease in mice

In chapter 3, we identified a novel role for A2aR signaling in the maintenance of CD11c+ T-bet+ B cells, and described the first pharmacological technique to target these cells. We first demonstrated that A2aR stimulation induced cell death, likely via apoptosis, in CD11c+ T-bet+ B cells. While A2aR signaling has been shown to be important in the development and function of various leukocyte subsets, including B cells, our research is the first to describe this signaling in T-bet+ B cells; although the role of A2aR signaling in the development and function of these cells is still not clear. A2aR signaling has been shown to increase the generation of T_{Reg} cells and inhibit the differentiation of T_{FH} cells, thus blocking GC reactions and blunting humoral immunity (181–183). Because of this, it has been proposed that the A2aR acts as a negative regulator of the immune response, preventing excessive inflammation and likely preventing the development of autoreactive lymphocytes (184). Given the potent ability

of A2aR agonists to induce cell death in T-bet+ B cells, it is likely that A2aR signaling acts as a negative regulator of these cells as well. For example, extracellular adenosine, likely produced by T_{Reg} cells, may induce anergy or cell death in T-bet+ B cells, as occurs following CGS-21680 administration. In addition to T_{Reg} cells, T-bet+ B cells themselves, may contribute to the generation of extracellular adenosine, as T-bet+ B cells express large quantities of the ectoenzymes CD39 and CD73. As such, T-bet+ B cells may signal through the A2aR in an autocrine fashion, thereby preventing the population from over expanding, such as occurs during autoimmunity.

Genetic elimination of A2aR on B cells did not affect the development or maintenance of *E. muris*-elicited T-bet+ B cells, suggesting that there may be minimal A2aR signaling under physiological conditions. Therefore, A2aR signaling may only occur during specific instances characterized by high levels of extracellular adenosine, such as during hypoxia or inflammation (185). A more likely explanation is that A2aR signaling in T-bet+ B cells is a redundant regulatory mechanism and is only necessary in the absence of other regulatory signals.

We next demonstrated that A2aR stimulation ameliorated autoimmune disease severity in lupus-prone mice. Unlike previous studies, our work identified targeted depletion of CD11c+ T-bet+ B cells as the likely cellular mechanism by which A2aR agonist treatment improved disease outcome in these mice. Our work also demonstrated A2aR stimulation could lower disease burden with a much reduced treatment regimen compared to previous studies (99). T-bet+ B cells are known to contribute to disease etiology in many autoimmune diseases (e.g. SLE, RA, and Sjøgren's disease) and as

such, the ability to pharmacological deplete these cells represents a significant advancement for the treatment of not just SLE, but any autoimmune disease where T-bet+ B cells contribute to disease pathology (116, 149).

The use of A2aR agonists to treat these autoimmune diseases has significant advantages over current B cell targeted therapies. A2aR agonists are cheap, can be administered as a single bolus injection, cause only mild transient side effects, and are already approved for use in humans. This is in contrast to current B cell specific treatments, which are expensive, and can take hours to administer. Moreover, A2aR-agonists specifically target T-bet+ B cells, whereas current anti-B cell therapies deplete the majority of B cells in the patient, harming other crucial components of humoral immunity. Because of these advantages, A2aR agonists represent an attractive, and feasible new treatment for many different autoimmune diseases.

As discussed previously, it is possible that A2aR-stimulation ameliorates disease in lupus-prone mice via a variety of mechanisms, such as by increasing the generation of T_{Reg} cells and decreasing the differentiation and proliferation of T_{eff} cells. However, given the known role for T-bet+ B cells in the pathology of SLE, and the susceptibility of these cells to A2aR-mediated depletion, the elimination of T-bet+ B cells almost certainly contributes to the amelioration of disease in MRL/lpr mice.

Finally, we show that a population of T-bet+ B cells were resistant to A2aR-mediated cell death, namely CD11c-negative T-bet^{lo} B cells. The difference in susceptibility between CD11c+ and CD11c-negative T-bet+ B cells could be due to differential expression of A2aR or differences in the metabolic states of the subsets.

These differences may also be indicative of deeper functional differences between these subsets. For example, one subset may represent a more quiescent metabolically inactive population that downregulates certain regulatory receptors such as the A2aR and as such is less susceptible to apoptosis, whereas the other population represents a more activated phenotype susceptible to A2aR-induced cell death. However, given that both of these subsets interconvert in vivo, these functional roles may be transient.

The interconversion of CD11c+ T-bet^{hi} and CD11c-neg T-bet^{lo} B cells is also likely why reappeared following the cessation of CGS-21680 treatment. The reemergence of this CD11c+ population would suggest that A2aR agonists, given for the treatment of autoimmunity, would need to be administered on a regular basis to prevent the development of new CD11c+ T-bet^{hi} B cells. Although it is likely that long-term A2aR agonist treatment would eventually eliminate all T-bet+ B cells as they interconvert and become susceptible to A2aR-mediated cell death.

The work presented here contributes to our current understanding of T-bet+ B cells in several ways. We identify the A2aR as an important signaling component in the maintenance of T-bet+ B cells and describe its utility in the depletion of these cells during autoimmunity. We also demonstrate that while CD11c+ T-bet^{hi} B cells do reappear after A2aR agonist treatment, long-term treatment will likely eliminate this population. Cumulatively, this work clarifies important questions regarding the development of T-bet+ B cells and provides new insights into the treatment of diseases characterized by the development of these cells.

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