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T-bet and CD11c expression in B cells is linked with IgG_{2c} isotype switching, virus-specific immune responses, and humoral autoimmunity. However, the activation requisites and regulatory cues governing T-bet and CD11c expression in B cells remain poorly defined. In this article, we reveal a relationship among TLR engagement, IL-4, IL-21, and IFN-γ that regulates T-bet expression in B cells. We find that IL-21 or IFN-γ directly promote T-bet expression in the context of TLR engagement. Further, IL-4 antagonizes T-bet induction. Finally, IL-21, but not IFN-γ, promotes CD11c expression independent of T-bet. Using influenza virus and *Heligmosomoides polygyrus* infections, we show that these interactions function in vivo to determine whether T-bet$^+$ and CD11c$^+$ B cells are formed. These findings suggest that T-bet$^+$ B cells seen in health and disease share the common initiating features of TLR-driven activation within this circumscribed cytokine milieu. ***The Journal of Immunology*****, 2016, 197: 1023–1028.**

Although initially implicated in CD4 T cell differentiation, T-bet is a key transcriptional regulator in many immune cells. Thus, as shown in the companion report (1), B cell–intrinsic T-bet expression is required to control chronic viral infections and fosters switching to IgG_{2a} (2–4), an isotype associated with both T_H1-driven Ab responses and humoral autoimmunity (5, 6). Moreover, T-bet is required for the generation of age-associated B cells, which are transcriptionally distinct from other B cell subsets and have also been associated with both viral clearance and humoral autoimmunity (7–9). Finally, many T-bet$^+$ B cells express CD11c, a phenotype associated with viral or bacterial infections, autoimmunity, and neoplasia (8, 10–13). Despite growing appreciation for the importance of T-bet–expressing B cell subsets, the signals that yield B lineage effectors characterized by T-bet expression, as well as how these regulate appropriate versus pathogenic outcomes, remain poorly defined. Candidates include cell-intrinsic cues from adaptive and innate receptors, including the BCR and TLRs, as well as signals from T follicular helper (T_{FH}) cells. In this regard, several T_H1 cytokines, including IL-12, IL-18, and IFN-γ, can induce T-bet in activated B cells (5, 6). Nonetheless, the roles and interactions of canonical T_{FH} cell cytokines, IL-21, IL-4, and IFN-γ, in regulating T-bet expression have not been systematically interrogated (14–16).

In this article, we show that mouse and human B cells integrate signals from IL-4, IL-21, and IFN-γ to regulate T-bet expression. In the context of TLR engagement, both IL-21 and IFN-γ directly drive follicular (FO) B cells to express T-bet in vitro. However, IL-4 antagonizes IL-21–driven T-bet upregulation, but enhances IFN-γ–induced T-bet expression. Moreover, IL-21, but not IFN-γ, promotes CD11c expression. Consistent with these in vitro results, the in vivo frequencies of germinal center (GC) and memory B (B_{MEM}) cells expressing T-bet or CD11c vary based on the prevailing cytokine milieu. Finally, using viral and helminthic infections in single- and double-cytokine knockout mice, we show that the relative abundance of these cytokines determines whether GC and B_{MEM} cells generated during ongoing immune responses express T-bet and CD11c. Together, these findings

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The transcriptional profiling data presented in this article have been submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77145) under accession number GSE77145.

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Abbreviations used in this article: B6, C57BL/6; BL, BioLegend; B_{MEM}, memory B; eBio, eBioscience; FO, follicular; GC, germinal center; PR8, A/Puerto Rico/8/1934; T_{FH}, T follicular helper; VCT, Violet Cell Trace; WT, wild type.

reveal a previously unappreciated interplay of IL-4, IL-21, and IFN-γ that, in concert with innate sensors, controls T-bet and CD11c expression in B cells.

Materials and Methods

Mice

Tbx21$^{-/-}$, *Stat6*$^{-/-}$, *Tbx21*$^{f/f}$*Cd19*$^{Cre/+}$, C57BL/6 (B6), and BALB/c mice were maintained and used in accordance with the University of Pennsylvania Institutional Animal Care and Use Committee guidelines. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal experiments. *Il4*$^{-/-}$ mice were a gift from Dr. Paula Oliver. *Ifng*$^{-/-}$ mice were a gift from Dr. Edward Behrens. *Il4*$^{-/-}$*Ifng*$^{-/-}$ double-deficient mice were bred in-house. *Il21r*$^{-/-}$ and *Il21*tg spleens and sera were shipped overnight on ice from Dr. Warren Leonard. All mice were 2–6 mo of age.

Infections

Mice were infected by oral gavage with 200 infectious larvae of *Heligmosomoides polygyrus* as previously described (17). Mice were infected by intranasal infection with 30 tissue culture infectious dose$_{50}$ of influenza strain A/Puerto Rico/8/1934 (PR8) (American Type Culture Collection).

In vitro cultures

Mouse CD23$^+$ splenic B cells were enriched by magnetic positive selection (Miltenyi Biotec), labeled with either Violet Cell Trace (VCT; Invitrogen) or CFSE (eBioscience [eBio]), and cultured as previously described (18). Human PBMCs were isolated from blood samples obtained from healthy donors that expressed written informed consent and after ethical approval by the University of Pennsylvania Institutional Review Board. All investigations were conducted according to the principles expressed in the Declaration of Helsinki. Human B cells were enriched by CD27 microbead negative selection followed by CD19 microbead positive selection (Miltenyi Biotec), labeled with CFSE, and cultured with indicated stimuli for 5 d. Mouse or human IL-21, IL-4, and IFN-γ were used at 25, 10, and 10 ng/ml, respectively (Shenandoah). ODN2006 was used at 1 μM (Invivogen).

Flow cytometry

FACS reagents were purchased from BioLegend (BL), BD Biosciences, or eBio: T-bet (4B10; BL), CD11c (N418; BL), IgM (R6-60.2; BD Biosciences), CD38 (90; eBio), CD138 (281-2; BL), IgD (11–26c.2a; BL), CD4 (RM4-5; BL), B220 (RA3-6B2; BL), CD62L (MEL-14; eBio), TCR-β (H57-597; BL), CD19 (6D5; BL), CXCR5 (L138D7; BL); PD-1 (RMP1-30; BL); CD8 (53-6.7; eBio), CD4 (H129.19; BL); F4/80 (BM8; eBio); Ly-6G/GR1 (RB6-8C5; eBio); CD43 (S7; BD Biosciences); CD21/CD35 (CR2/CR1; BL); CD23 (B3B4; eBio); CD93 (AA4.1; BL); peanut agglutinin–FITC (Sigma); Zombie Aqua (BL). FACS analyses were performed as described previously (18).

Serum Ab titers

ELISAs were performed as previously described (18) using anti-mouse IgG$_{2a}$, IgG$_{2b}$, IgG$_{2c}$, or IgG$_1$ HRP Abs (Southern Biotech).

Quantitative PCR analysis and transcriptional profiling

Quantitative PCR experiments were performed as previously published (18) using the following probes: *Il4* (Mm00445260_m1), *Ifng* (Mm00801778_m1), *Il21* (Mm00517640_m1), *Tbx21* (Mm00450960_m1), *Aicda* (Mm00507774_m1). Transcriptional profiling data were generated as previously described (19) and have been deposited in the Gene Expression Omnibus database for public access (accession no. GSE77145; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77145).

Statistics

Student *t* test was used to generate all *p* values: $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data are represented as box and whisker plots with mean depicted with plus sign (+).

Results and Discussion

IL-21, IL-4, and IFN-γ differentially regulate T-bet and CD11c expression

In preliminary in vitro studies, we established that IL-21 drives T-bet expression in mouse FO B cells responding to TLR9, but not BCR or CD40 signals (Fig. 1A). To explore these interactions further, we cultured FO B cells with IL-4, IL-21, or IFN-γ in the presence of TLR7 or TLR9 agonists. Both *Tbx21* transcripts and T-bet protein increased markedly in FO B cells cultured with IL-21 or IFN-γ, but IL-4 influenced these outcomes differently. IL-4 blocked IL-21–driven T-bet upregulation, but enhanced IFN-γ–mediated T-bet upregulation (Fig. 1B, Supplemental Fig. 1A).

To determine whether IL-21 and IL-4 directly regulate T-bet in B cells, either *Il21r*$^{-/-}$ or *Stat6*$^{-/-}$ B cells were cocultured with wild type (WT) B cells and stimulated as described earlier. Because IL-21R is required for IL-21 signaling and STAT6 is the key signal transducer of IL-4 (20, 21), we reasoned that coculturing these mutants with WT cells would reveal any secondary *trans* effects. To track both cell origin and division, we labeled WT or knockout cells with VCT or CFSE, respectively (Supplemental Fig. 1B). Whereas IL-21 induced T-bet expression in WT B cells, the cocultured *Il21r*$^{-/-}$ B cells remained T-bet$^-$ (Fig. 1C, top row). Analogously, although IL-21–driven T-bet upregulation in WT B cells was reversed by IL-4, cocultured *Stat6*$^{-/-}$ cells were refractory to this negative effect (Fig. 1C, bottom row). Similar results were obtained using the TLR7 agonist, CL097 (data not shown). Importantly, in all cases, IFN-γ treatment induced T-bet irrespective of *Il21r* or *Stat6* deficiency (Fig. 1C). To assess whether similar relationships exist in human

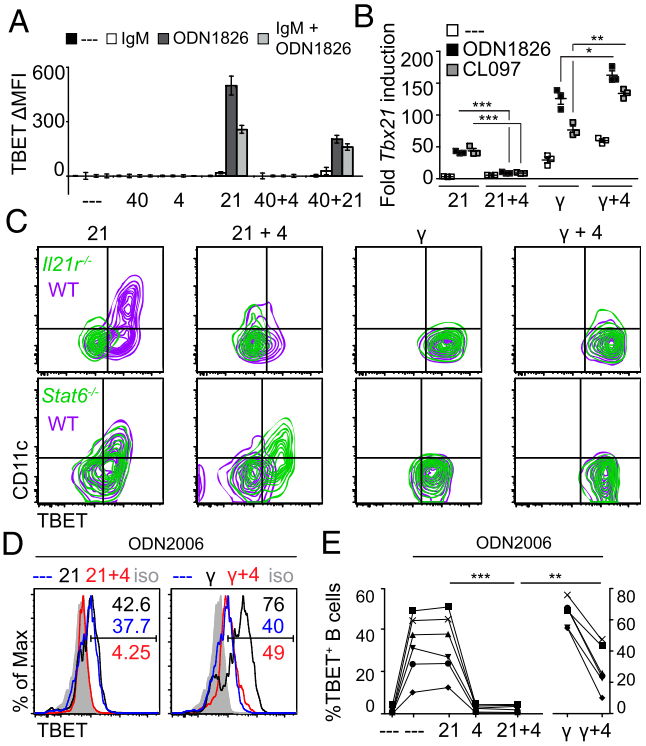

FIGURE 1. IL-4 and IL-21 act in a cell-intrinsic manner to regulate T-bet expression in vitro. Magnetically enriched CD23$^+$ splenic B cells were cultured in vitro with various combinations of anti–Ig-μ (IgM), anti-CD40 (40), IL-4 (4), IL-21 (21), and IFN-γ (γ). Mouse data are representative of three independent experiments. (**A**) WT or *Cd19*$^{cre/+}$*Tbx21*$^{f/f}$ B cells treated for 48 h and assessed for T-bet mean fluorescent intensity (ΔMFI = WT − mutant). (**B**) *Tbx21* mRNA levels in WT cells treated for 20 h. (**C**) WT, *Il21r*$^{-/-}$, or *Stat6*$^{-/-}$ B cells were labeled with either CFSE (green plots) or VCT (purple plots), treated with ODN1826 and indicated cytokines for 48 h, and then stained for CD11c and T-bet. (**D**) Magnetically enriched CD27$^-$CD19$^+$ human B cells were labeled with CFSE, treated for 108 h, and probed for T-bet on live CFSE$^-$ cells. (**E**) Frequency of T-bet$^+$ B cells from each treatment across six healthy adult donors. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

B cells, we cultured $CD27^{-}CD19^{+}$ PBMCs as described earlier. TLR9 stimulation alone upregulated T-bet in these cultures. It is not clear whether intrinsic effects of TLR signaling or *trans* effects induced by these signals underlie this observation. Nonetheless, IFN-γ significantly increased T-bet expression, and IL-4 completely blocked T-bet in all cultures except those with IFN-γ (Fig. 1D, 1E). In toto, these results show that in the context of TLR signaling, IL-4, IL-21, and IFN-γ interact to regulate T-bet expression in both mouse and human B cells.

The converse effect of IL-4 on IFN-γ– versus IL-21–induced T-bet expression suggests that unique, T-bet–associated programs are facilitated by each cytokine. We interrogated this possibility in several ways. First, because previous studies have linked T-bet with CD11c expression (8), we asked whether IFN-γ or IL-21 influence CD11c differently. The results show that IL-21 drives CD11c expression, but IFN-γ does not (Fig. 1C). Further, as with T-bet, IL-4 blocks IL-21–induced CD11c expression. Finally, IFN-γ drives T-bet expression and is not appreciably influenced by either IL-4 or IL-21 (Supplemental Fig. 1C). These findings indicate that IL-21 and IFN-γ drive T-bet and CD11c expression through distinct mediators, and that T-bet expression per se is insufficient for CD11c induction. To further interrogate differential T-bet expression driven by IL-21 versus IFN-γ, as well as to distinguish T-bet–dependent and -independent effects of each cytokine, we performed genome-wide transcriptional profiling on WT or *Tbx21*$^{-/-}$ B cells stimulated with either IFN-γ or IL-21. Principal components analysis shows that 82.7% of variance in these data was explained by the cytokine used, whereas *Tbx21* genotype accounted for 6.3% of the variance (Supplemental Fig. 1D). Further, each cytokine induces a unique transcriptional profile, including some T-bet–dependent shifts in gene expression (Supplemental Fig. 1E, Supplemental Table I). Thus, IFN-γ and IL-21 drive similar but distinct T-bet–associated phenotypes in B cells.

Together, these results show that in the context of TLR engagement, the aggregate of IFN-γ, IL-21, and IL-4 signals determines whether B cells express T-bet. TLR engagement, but not BCR cross-linking (Fig. 1A), appears necessary to position B cells for T-bet expression upon subsequent IFN-γ or IL-21 signaling. We obtained similar results with the TLR2/4 ligand LPS (not shown), suggesting pathways common to most TLRs, and perhaps other innate receptors, provide these key initial signals. We speculate that these signals alter gene loci accessibility for subsequent cytokine cues. Indeed, prior reports that $CD11c^{+}$ or $T\text{-}bet^{+}$ B cells emerge in response to a variety of viral and bacterial infections are consistent with this idea (7, 10). Moreover, the differential effects of IL-4 on IL-21 versus IFN-γ suggest a complex interplay of STAT-dependent transcriptional regulation. The clear dose–response relationship of IL-4–mediated effects is consistent with the idea that competitive relationships are involved (Supplemental Fig. 1F). Although IL-4 and IL-21 both require common γ-chain receptor to initiate STAT signal transduction (22), our *Stat6*$^{-/-}$ coculture data (Fig. 1C) indicate that competition for membrane proximal receptor components is unlikely to explain these findings. If this were the case, then *Stat6*$^{-/-}$ cells would also be subject to the repressive effects of IL-4. Instead, downstream events are more likely candidates, including differential occupation of transcriptional regulatory sites and altered stoichiometric relationships among the JAK-STAT proteins involved.

Relative abundance of IL-21, IL-4, and IFN-γ regulates T-bet expression in vivo

Our in vitro findings suggest that IFN-γ, IL-4, and IL-21 interact to modulate T-bet and CD11c expression in B cells. As an initial assessment of whether this relationship exists in vivo, we surveyed GC B and B_{MEM} cells for T-bet expression in B6 versus BALB/c mice (Supplemental Fig. 1G), because these strains display inherent T_H1 versus T_H2 skewing, respectively (23). We reasoned that if T-bet expression is promoted by milieus rich in IFN-γ, but repressed in those with plentiful IL-4 and little IFN-γ, then the frequencies of $T\text{-}bet^{+}$ B cells in these two strains should differ. In agreement with this prediction, whereas most GC B cells in B6 mice are $T\text{-}bet^{+}$, BALB/c have a lower frequency of $T\text{-}bet^{+}$ GC B cells (Fig. 2A). Importantly, CD11c protein expression was restricted to B6 B_{MEM} cells (Fig. 2B) and not GC B cells (Supplemental Fig. 1H). These findings are consistent with the notion that IFN-γ and IL-4 levels regulate T-bet expression in GC B cells. To probe the impact of IL-21 on this overall relationship, we next asked whether extraphysiological levels of IL-21 would foster accumulation of $T\text{-}bet^{+}CD11c^{+}$ B cells. Profound increases in both T-bet and CD11c expression were seen in all splenic B cells in *Il21*tg mice (Fig. 2C), which is consistent with our in vitro results suggesting that IL-21 drives both T-bet and CD11c expression. Although the partially activated state of B cells in these mice confounds conventional phenotyping strategies, nearly all mature B cells in the *Il21*tg bear a $CD23^{-}CD21^{-}$ phenotype (Supplemental Fig. 1I) identical to the T-bet–dependent age-associated B cell subset (18, 24). Finally, consistent with the role of T-bet in fostering class-switch recombination to $IgG_{2a/c}$, we observed a marked increase of

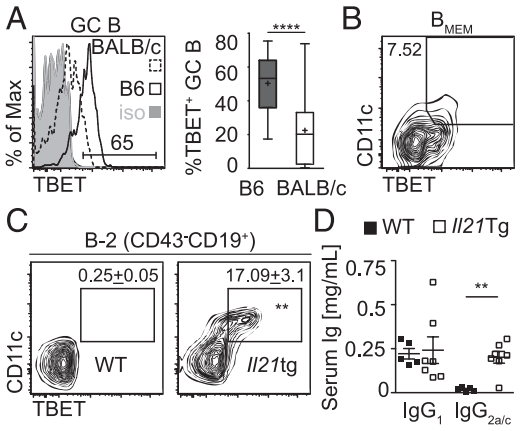

FIGURE 2. $T\text{-}bet^{+}CD11c^{+}$ cells delineate a B_{MEM} cell subset and accumulate in *Il21*tg mice. (**A** and **B**) GC B and B_{MEM} cells were analyzed for T-bet and CD11c expression by FACS. GC B and B_{MEM} cell gating strategies are in Supplemental Fig. 1G. All panels are representative of three independent experiments with ≥3 mice per strain. (**A**) T-bet staining on GC B cells from B6 ($n = 14$) or BALB/c ($n = 23$) mice with frequency enumeration. (**B**) T-bet and CD11c staining on B_{MEM} cells from B6 mice. (**C**) T-bet and CD11c staining on splenic B-2 cells from WT and *Il21*tg mice. (**D**) Serum IgG_1 or $IgG_{2a/c}$ (IgG_{2a} + IgG_{2c}) levels in WT and *Il21*tg mice were determined by ELISA. Values are means ± SEM from five WT and seven *Il21*tg mice. $^{**}p < 0.01$, $^{****}p < 0.0001$.

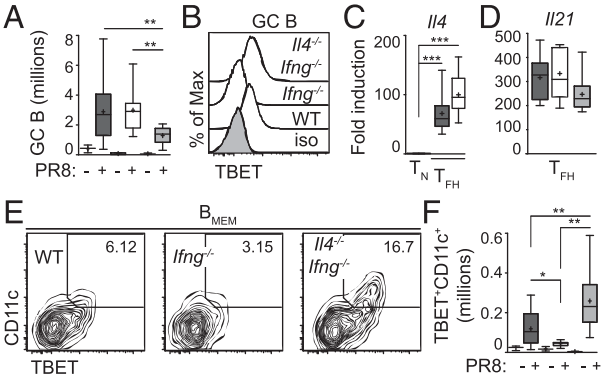

FIGURE 3. Influenza virus infection drives T-bet$^+$CD11c$^+$ B_{MEM} cell formation in the absence of both IFN-γ and IL-4. Splenocytes were harvested from noninfected (−) or day 10 after intranasal 30 tissue culture infectious $dose_{50}$ PR8 infection (+) WT (n = 21, black bars), *Ifng*$^{-/-}$ (n = 10, white bars), or *Il4*$^{-/-}$ *Ifng*$^{-/-}$ (n = 13, gray bars) mice across 3–7 experiments with ≥3 mice per group. GC B, B_{MEM}, and T_{FH} cell gating strategies are in Supplemental Fig. 1G and 1J. (**A**) Enumeration of GC B cells. (**B**) T-bet staining on GC B cells. (**C**) *Il4* and (**D**) *Il21* mRNA levels from sorted naive CD62L$^+$ CD4 T (T_N, n = 9) or T_{FH} cells. (**E**) Proportions and (**F**) numbers of T-bet$^+$CD11c$^+$ B_{MEM} cells. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

$IgG_{2a/c}$, but not IgG_1, serum Ab titers in *Il21*tg compared with WT mice (Fig. 2D).

Together, our in vitro and in vivo observations prompt a model in which the relative availability of IL-4, IL-21, and IFN-γ governs the likelihood of establishing B_{MEM} cells expressing T-bet and CD11c. Further, they suggest that abundant IFN-γ will drive a T-bet$^+$CD11c$^-$ phenotype regardless of IL-4 or IL-21 levels, but that in the absence of IFN-γ, the T-bet$^+$CD11c$^+$ phenotype is reciprocally regulated by IL-21 versus IL-4. We therefore evaluated these predictions by tracking the immune responses to either influenza virus or *H. polygyrus* in mice where cytokine availability could be experimentally manipulated.

Influenza virus infection generates T-bet$^+$CD11c$^+$ B_{MEM} in the absence of both IL-4 and IFN-γ

Influenza virus infection yields a well-characterized T-dependent and T_H1-skewed response, in which responding T_{FH} cells produce copious IFN-γ, as well as IL-21 and IL-4 (14). Thus, we reasoned that IFN-γ would induce T-bet expression in GC B and B_{MEM} cells, but in the absence of IFN-γ, IL-4 would prevent T-bet expression. Accordingly, WT or *Ifng*$^{-/-}$ mice were infected with the influenza virus strain PR8. As expected, WT animals mounted a robust GC B cell response to PR8 (Fig. 3A), and these GC B cells expressed T-bet (Fig. 3B; sort strategy and *Tbx21* expression, Supplemental Fig. 1J, 1K). In contrast, GC B cells in *Ifng*$^{-/-}$ mice failed to express T-bet even though the magnitude of the GC B cell response was similar to WT. Assuming that T_{FH} cells are the major source of cytokine, we confirmed that both WT and *Ifng*$^{-/-}$ mice made substantial numbers of T_{FH} cells (Supplemental Fig. 1J, 1L), and their capacity to make IL-4 and IL-21 was unperturbed (Fig. 3C, 3D). These results are consistent with the idea that, in the absence of IFN-γ, IL-4 blocks T-bet expression in response to IL-21. To directly test this, we infected *Il4*$^{-/-}$ *Ifng*$^{-/-}$ double-deficient mice with PR8. Although *Il4*$^{-/-}$ *Ifng*$^{-/-}$ mice mounted a blunted GC B cell response (Fig. 3A), these cells nonetheless express T-bet (Fig. 3B, Supplemental Fig. 1K).

Although the splenic plasma cell numbers were reduced in *Ifng*$^{-/-}$ mice, B_{MEM} cell numbers remained intact across genotypes (Supplemental Fig. 1M, 1N). However, the composition of the B_{MEM} cell pool differed according to genotype (Fig. 3E, 3F). Whereas WT mice generated some T-bet$^+$ CD11c$^+$ B_{MEM} cells, *Ifng*$^{-/-}$ mice produced few, if any, above noninfected control animals, likely reflecting the dominance of IL-4 in the absence of IFN-γ. Consistent with this interpretation, *Il4*$^{-/-}$ *Ifng*$^{-/-}$ mice generated the most T-bet$^+$CD11c$^+$ B_{MEM} cells. Lastly, CD11c expression was restricted to B_{MEM} cells and not GC B cells (Supplemental Fig. 1O). Overall, these findings confirm and extend our in vitro findings, because the same interplay of cytokines directs T-bet expression among B effectors in vivo. Further, our observations suggest that T-bet$^+$CD11c$^+$ B_{MEM} cells will be fostered in immune responses where IL-4 is limited.

Il4 deficiency yields T-bet$^+$CD11c$^+$ B_{MEM} independent of IFN-γ in H. polygyrus *infection*

Results with influenza virus infection are consistent with the notion that IFN-γ drives T-bet expression irrespective of concomitant IL-4 or IL-21, and that eliminating IFN-γ creates a situation where the relative levels of IL-4 and IL-21 govern the T-bet$^+$CD11c$^+$ phenotype. However, this subtractive approach does not necessarily show that, in responses where IFN-γ is normally absent, the sole determinant of T-bet expression is IL-4 availability. Accordingly, we asked whether IL-4 deficiency is sufficient to permit T-bet expression in GC B and B_{MEM} cells during a T_H2 response, using *H. polygyrus*. This intestinal helminth induces IL-4 and IL-21 production by T_{FH} cells, which drives a robust IgG_1 response (15). Thus, we hypothesized that, in the absence of IL-4, IL-21 would be sufficient to induce T-bet expression in GC B and B_{MEM} cells. To test this idea, we infected WT or *Il4*$^{-/-}$ mice with *H. polygyrus* and probed GC B cells for T-bet. As expected, WT mice mounted a GC B cell response that lacked T-bet expression, which correlated with increased serum IgG_1 titers. Conversely, although blunted in magnitude,

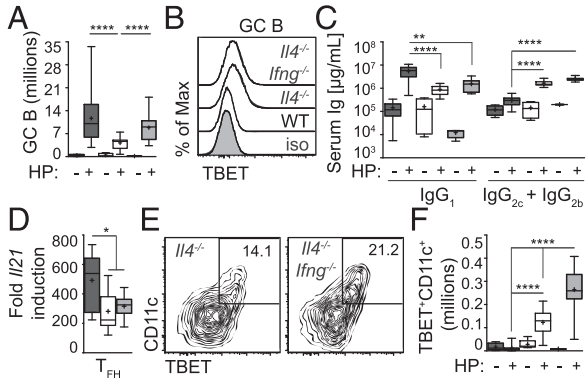

FIGURE 4. Activated B cells express T-bet independent of IFN-γ in IL-4 limiting conditions. Splenocytes and sera were harvested from noninfected (−) or day 14 after oral gavage (+) of 200 *H. polygyrus* in WT (n = 20, black bars), *Il4*$^{-/-}$ (n = 24, white bars), or *Il4*$^{-/-}$ *Ifng*$^{-/-}$ (n = 11, gray bars) mice across 3–6 experiments with ≥3 mice per group. GC B, B_{MEM}, and T_{FH} cell gating strategies are in Supplemental Fig. 1G and 1J. (**A**) Enumeration of GC B cells. (**B**) T-bet staining on GC B cells. (**C**) Serum concentrations of IgG_1 and IgG_{2c} + IgG_{2b}. (**D**) *Il21* mRNA levels from sorted T_{FH} cells. (**E**) Proportions and (**F**) numbers of T-bet$^+$CD11c$^+$ B_{MEM} cells. $^*p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$.

Il4$^{-/-}$ mice initiated a T-bet$^+$ GC B cell response with decreased serum IgG_1 titers compared with WT (Fig. 4A–C, Supplemental Fig. 1J, 1P). To eliminate the possibility that excess IFN-γ in *Il4*$^{-/-}$ mice explains these phenotypes, we infected *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice with *H. polygyrus*. The GC B cell response in *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice was similar to WT levels (Fig. 4A) but maintained T-bet expression independently of IFN-γ (Fig. 4B, Supplemental Fig. 1J, 1P). Isotype representation varied with T-bet expression: whereas WT mice produced >95% IgG_1, more than half of the serum Abs in *Il4*$^{-/-}$*Ifng*$^{-/-}$ and *Il4*$^{-/-}$ mice were IgG_{2b} and IgG_{2c} (Fig. 4C). Further, whereas *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice mounted a higher T_{FH} cell response (Supplemental Fig. 1Q), both *Il4*$^{-/-}$ and *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice produced less IL-21 (Fig. 4D). Regardless, the magnitude of the plasma cell and B_{MEM} cell response remained intact across genotypes (Supplemental Fig. 1R, 1S). However, we again observed alterations in the B_{MEM} pool according to cytokine availability. Whereas *H. polygyrus*–infected WT mice did not generate T-bet$^+$CD11c$^+$ B_{MEM} cells, both *Il4*$^{-/-}$ and *Il4*$^{-/-}$*Ifng*$^{-/-}$ mice did, again suggesting IL-21 drives a unique T-bet$^+$ phenotype (Fig. 4E, 4F). Whereas prior reports showed CD11c mRNA in GC B cells defined by CD95 and peanut agglutinin (25), we observed CD11c protein expression only in B_{MEM} cells (Supplemental Fig. 1T). This seeming disparity may indicate that CD11c transcripts in GC B cells go untranslated, as well as the further resolution of GC and B_{MEM} by CD38 in our gating strategy. Overall, the *H. polygyrus* infection data support our model, inasmuch as in the absence of IFN-γ we observe both T-bet and CD11c expression that is modulated by IL-4. Further, the consistent relationships observed in both types of infection argue that this is a feature common to most humoral immune responses.

In toto, our findings reveal a novel cytokine network that governs T-bet expression in the context of TLR stimulation. In the absence of IFN-γ, IL-4 and IL-21 reciprocally regulate T-bet and CD11c expression both in vitro and in vivo. Because immune responses are rarely monolithic with regard to these three cytokines (14, 26), distinct or multifunctional T_{FH} cells likely generate a diverse set of B effectors. Consequently, altering the cytokine milieu affects the isotypes generated (Fig. 4C) and the composition of the B_{MEM} pools (Figs. 3F, 4F) while maintaining the magnitude of the response.

It is tempting to speculate that the T-bet$^+$CD11c$^+$ B cells reported in autoimmunity, viral infections, and aging share a common underlying origin involving TLR engagement coupled with either copious IFN-γ or abundant IL-21 with little IL-4. Indeed, both TLR7 and IL-21 deficiencies ameliorate disease in humoral autoimmunity models (27, 28), and poor IL-4 production has been observed in T_{FH} cells from aged mice (29). Thus, understanding this interplay among IL-4, IL-21, and IFN-γ might better define the etiology of humoral autoimmune syndromes where such cells are implicated (8, 13, 30). Lastly, although it is clear that IFN-γ and IL-21 differentially induce CD11c expression (Fig. 1C), the functional consequences of expressing this integrin remain elusive. Importantly, the restriction of CD11c expression to B_{MEM} cells is consistent with prior B_{MEM} subsetting studies in human tonsils and may thus define a tissue-homing population (31). Accordingly, further studies are needed to assess the role of these different T-bet$^+$ B_{MEM} cells in both health and disease.

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Disclosures

The authors have no financial conflicts of interest.

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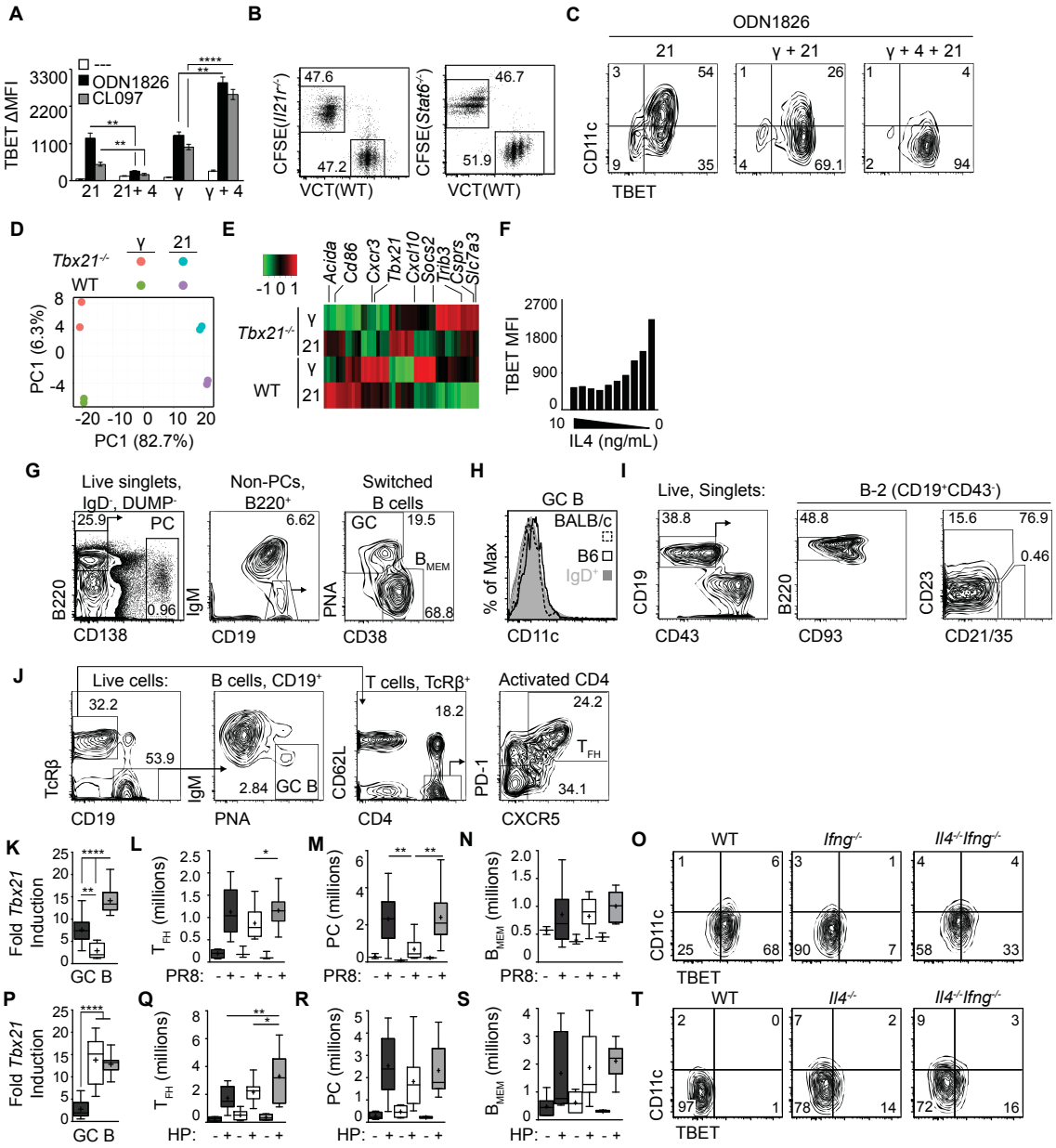

Supplemental Figure 1: Treatment abbreviation: IL4 (4), IL21 (21), IFNγ (γ). (**A**) TBET protein (Δ MFI=WT-isotype) in WT FO B cells treated for 48h. (**B**) WT, *Il21r*$^{-/-}$, or *Stat6*$^{-/-}$ FO B cells labeled with either CFSE or Violet Cell Trace (VCT), treated with ODN1826. (**C**) TBET and CD11c protein on WT FO B cells treated for 48h. (**D**) Principal component analysis and (**E**) heat map of top 50 differentially regulated genes from WT or *Tbx21*$^{-/-}$ FO B cells cultured with ODN1826 and IFNγ or IL21 for 20h; 2 biological replicates per condition from one experiment. (**F**) IL4 titration on ODN1826 and IL21 stimulated B cells probed for TBET. (**G**) Gating strategy for splenic PCs, GC B, and B_{MEM} cells. DUMP is defined as CD4, CD8, F4/80, and Gr1. (**H**) CD11c staining on GC B cells from B6 or BALB/c mice. (**I**) FACS analysis for pre-immune B cell subsets in *Il21*Tg spleens. (**J**) Representative sorting strategy for T_{FH} and GC B cells during infection for all RNA and T_{FH} cell numbers. (**K-O**) Data shown are from PR8-infected mice (WT black bars, *Ifng*$^{-/-}$ white bars, *Il4*$^{-/-}$*Ifng*$^{-/-}$ grey bars) as described in **Fig. 3**. (**P-T**) Data shown are from HP-infected mice (WT black bars, *Il4*$^{-/-}$ white bars, *Il4*$^{-/-}$*Ifng*$^{-/-}$ grey bars) as described in **Fig. 4**. (**K&P**) *Tbx21* gene expression from sorted GC B cells is shown. Splenic cell counts for (**L&Q**) T_{FH}, (**M&R**) PCs, (**N&S**) B_{MEM} cells are shown. (**O&T**) FACS stains for TBET and CD11c on GC B cells are shown.

Supplemental Table 1: Top 50 genes differentially expressed according to stimulation and genotype that generated the heatmap in **Figure 1E**.

		Tbx21 KO		WT	
diffSymbols	diffEntrez	**IFNG**	**IL21**	**IFNG**	**IL21**
Hbb-bt	101488143	8.71787854	9.96251869	9.22367706	10.7184761
Pld4	104759	9.63100389	11.8759995	10.2329291	12.2633218
Csprs	114564	10.1736638	9.78810189	9.80904596	9.12704895
Adssl1	11565	8.38037551	9.56102674	8.75866032	10.1892954
Aicda	11628	8.15876623	9.56620638	8.16116397	10.6193638
Alas2	11656	8.07791416	8.17076737	8.70427272	8.69881681
Slc7a3	11989	12.3331489	10.8922169	11.9363609	9.58431359
Camk2b	12323	8.33696042	8.46884015	9.34572705	8.76825437
Cd86	12524	10.2058114	11.4837841	11.291188	11.9359246
Cebpb	12608	11.0258621	10.2639223	10.3954173	9.79851261
Cxcr3	12766	8.02271357	7.96206013	8.89044742	8.59224553
Dapk2	13143	9.0456691	8.29162501	10.358997	8.4225753
Ddx6	13209	11.2174672	11.0245372	10.8475324	10.4293705
Dmwd	13401	8.56273345	8.78857332	9.52515528	9.25084509
Igf2bp3	140488	8.80263258	9.18672253	9.51911174	9.90179174
Gcnt1	14537	10.2827501	10.4188283	9.69190833	10.3032536
Gfi1	14581	9.03038563	8.89669405	10.0634526	8.96113555
Slc6a9	14664	10.296786	9.31732143	9.73786153	8.70447742
Gpr65	14744	9.18001479	9.65880491	9.98113174	10.0820403
Hba-a1	15122	10.6116569	11.0756745	12.8136936	12.9819337
Hhex	15242	10.9035453	11.8746337	11.4957655	12.0253224
Cxcl10	15945	9.69740321	8.04664183	11.2630435	8.07099991
Mrc1	17533	8.44177481	8.06620528	9.20057647	8.06505932
Enpp1	18605	9.43758182	9.03088817	10.1047894	8.95693649
Lgals3bp	19039	11.3456087	10.327613	10.4720993	9.52062838
Sdc3	20970	9.22445568	9.34384651	8.48282303	8.68587941
Socs2	216233	8.39402055	8.31061723	9.21300752	8.25316026
Phf11a	219131	10.6754791	10.9923491	9.91001761	10.62874
Trib3	228775	10.9374695	10.7364234	10.3685751	9.51736281
Oasl1	231655	10.0267927	9.07570675	9.41354243	8.69335125
Blvrb	233016	11.1287209	11.8152945	12.0710889	12.2488551
Rsad1	237926	8.70676248	8.35758333	9.48501461	8.97924121
Oas1g	23960	9.43862604	9.85463686	8.61867238	9.16101691
Usp18	24110	11.6695151	9.69335201	10.8173747	9.01086482
Ifnlr1	242700	10.6376498	8.60445256	10.0198364	8.45185354
Asns	27053	11.1359023	10.9173486	10.4862617	9.8579122
5031414D18Rik	271221	11.0728021	11.3432014	10.4476	11.0836712
Abi2	329165	10.767115	10.7019442	11.4634784	11.0806552
Tbx21	57765	8.50585362	8.28965615	12.0671288	10.6782958
Fkbp11	66120	9.26400071	8.93283424	9.92222723	9.10795164
Ifitm3	66141	11.6829144	10.3947567	10.5198475	9.94597162
Serpinb1a	66222	10.415432	9.24192845	9.63859227	9.25643539
Sec11c	66286	9.74339634	9.88278761	10.3682081	10.1358717
Entpd4	67464	11.9632761	11.9573035	11.2856712	11.2526164
Chac1	69065	12.0710536	11.8189737	11.3905192	10.3380145
Tmem110	69179	9.6681036	10.0680743	10.4259964	10.2240436
Endod1	71946	10.9566634	11.4212969	10.3114004	11.339
Hvcn1	74096	10.6755837	12.3965662	11.5044663	12.5150879
Ly6k	76486	8.96899275	8.66957018	9.57076498	9.26064778
Lbh	77889	12.9253357	13.7171333	12.328088	13.5347758