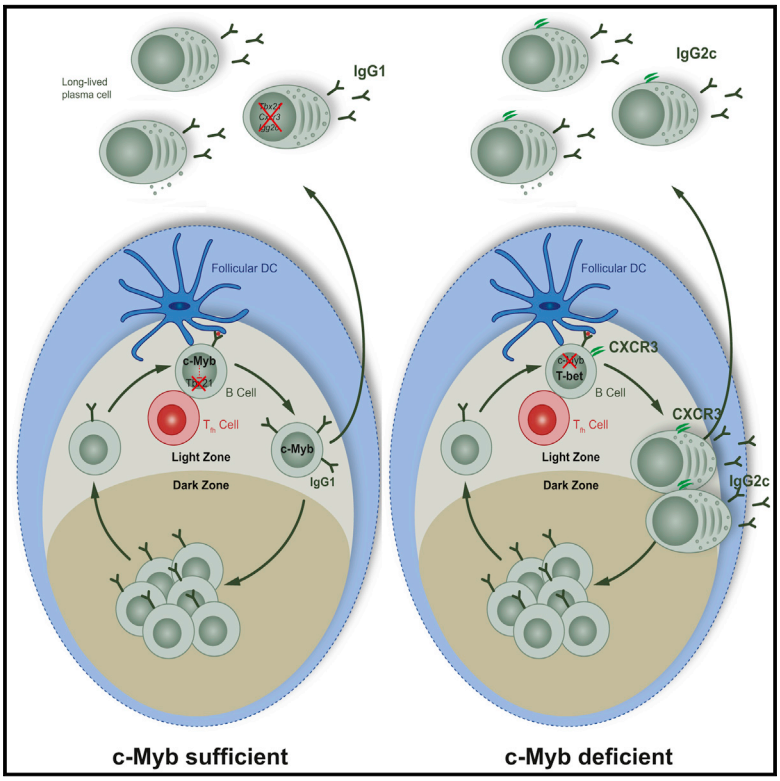
Report

Cell Reports

c-Myb Regulates the T-Bet-Dependent Differentiation Program in B Cells to Coordinate Antibody Responses

Graphical Abstract



Authors

Dana Piovesan, Jessica Tempany, Andrea Di Pietro, ..., Joanna R. Groom, David M. Tarlinton, Kim L. Good-Jacobson

Correspondence

kim.jacobson@monash.edu

In Brief

Piovesan et al. examine how B cells establish transcriptional programs that result in tailored responses to invading pathogens. The authors find that the transcription factor c-Myb represses the T-bet-mediated anti-viral program in B cells. c-Myb limits inappropriate effector responses while coordinating plasma cell differentiation with germinal center egress.

Highlights

- c-Myb deletion in mature B cells induces T-bet expression in a Th2-cell-biased response
- Enhanced T-bet causes increased serum IgG2c and expression of CXCR3
- T-bet-driven CXCR3 expression resulted in aberrant GC-PCs
- Restraint of T-bet was also important during an anti-influenza response

Accession Numbers

GSE89732

Piovesan et al., 2017, Cell Reports *19*, 461–470
April 18, 2017
http://dx.doi.org/10.1016/j.celrep.2017.03.060

Cell Reports
Report

c-Myb Regulates the T-Bet-Dependent Differentiation Program in B Cells to Coordinate Antibody Responses

Dana Piovesan,[1,2,3] Jessica Tempany,[3,4] Andrea Di Pietro,[1,2] Inge Baas,[3] Callisthenis Yiannis,[1,2] Kristy O'Donnell,[3,5] Yunshun Chen,[3,4] Victor Peperzak,[3,4] Gabrielle T. Belz,[3,4] Charles R. Mackay,[2] Gordon K. Smyth,[3,6] Joanna R. Groom,[3,4] David M. Tarlinton,[3,4,5] and Kim L. Good-Jacobson[1,2,3,4,7,*]

[1]Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC 3800, Australia
[2]Infection and Immunity Program, Biomedicine Discovery Institute, Monash University, Clayton, VIC 3800, Australia
[3]Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia
[4]Department of Medical Biology, University of Melbourne, Parkville, VIC 3010, Australia
[5]Department of Immunology and Pathology, Monash University, Prahan, VIC 3004, Australia
[6]The Department of Mathematics and Statistics, University of Melbourne, Parkville, VIC 3010, Australia
[7]Lead Contact
*Correspondence: kim.jacobson@monash.edu
http://dx.doi.org/10.1016/j.celrep.2017.03.060

SUMMARY

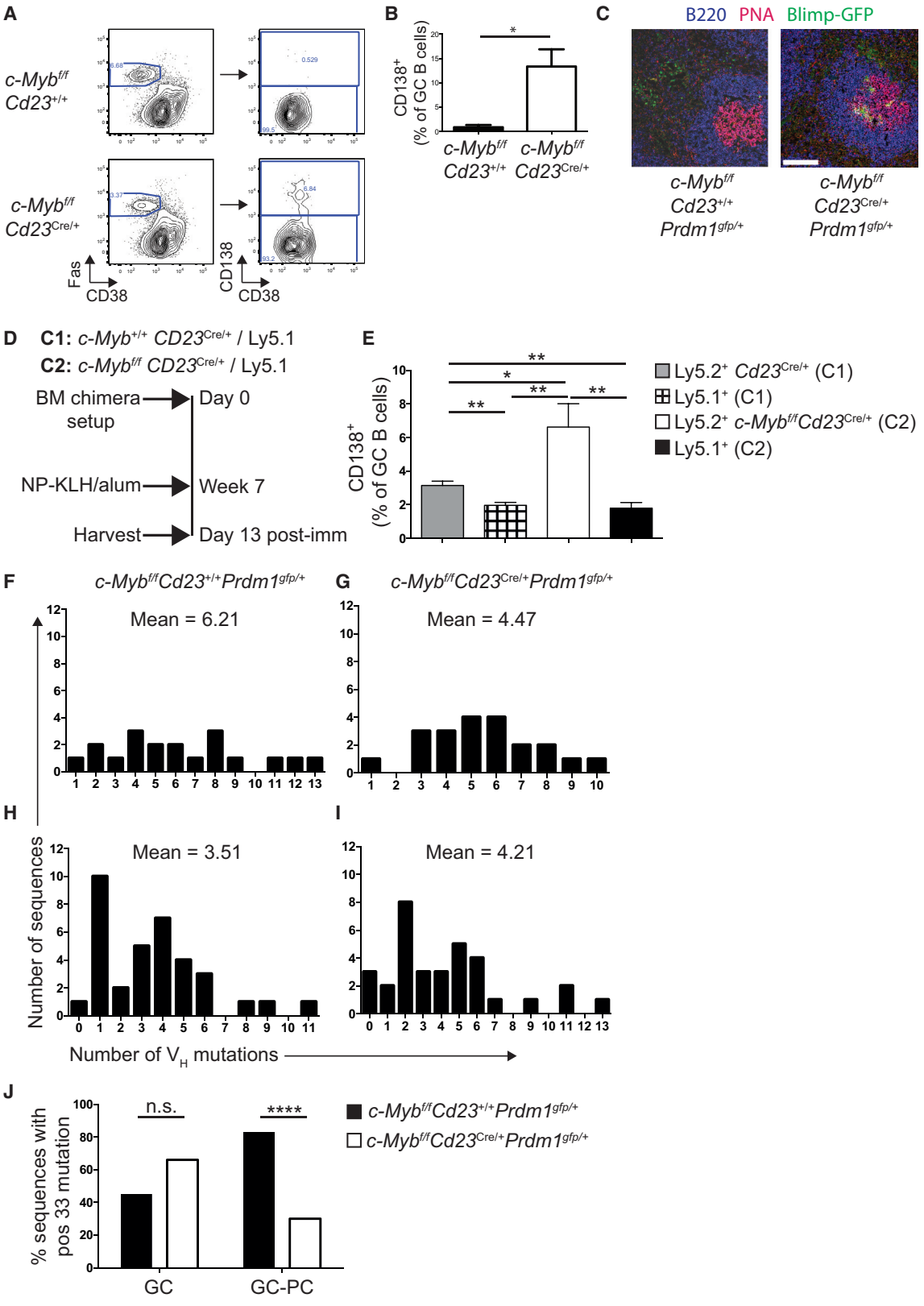
Humoral immune responses are tailored to the invading pathogen through regulation of key transcription factors and their networks. This is critical to establishing effective antibody-mediated responses, yet it is unknown how B cells integrate pathogen-induced signals to drive or suppress transcriptional programs specialized for each class of pathogen. Here, we detail the key role of the transcription factor c-Myb in regulating the T-bet-mediated anti-viral program. Deletion of c-Myb in mature B cells significantly increased serum IgG2c and CXCR3 expression by upregulating T-bet, normally suppressed during Th2-cell-mediated responses. Enhanced expression of T-bet resulted in aberrant plasma cell differentiation within the germinal center, mediated by CXCR3 expression. These findings identify a dual role for c-Myb in limiting inappropriate effector responses while coordinating plasma cell differentiation with germinal center egress. Identifying such intrinsic regulators of specialized antibody responses can assist in vaccine design and therapeutic intervention in B-cell-mediated immune disorders.

INTRODUCTION

The success of the immune system in protecting the host from a multitude of foreign pathogens is, in large part, due to its adaptability (Tarlinton and Good-Jacobson, 2013). Fine-tuning cellular function according to the type of pathogen is central to the adaptive immune response. During a humoral immune response, this refinement is typified by modulation of the B cell antigen receptor (BCR) structure to generate protective antibodies of optimal affinity and immunoglobulin (Ig) isotype matched to the infecting pathogen. Dysregulation of these processes can result in serious B cell disorders, such as autoimmunity. Thus, this specific matching is essential to generate appropriate and effective targeting of antibody to clear bacterial, viral, or parasitic infection without damaging the host.

Germinal centers (GCs) are transient structures formed during humoral immune responses that enhance the quality of memory and plasma cell (PC) populations. Tailoring of BCR effector function and affinity is achieved by two mechanisms leading up to and within the GC: somatic hypermutation (SHM) and class-switch recombination (CSR). SHM of Ig V genes drives affinity maturation due to selective expansion of cells with improved antigen binding (Victora and Nussenzweig, 2012). CSR exchanges the Ig heavy-chain constant region from that encoding IgM, expressed by naive B cells, to one of three isotypes with specialized effector functions: IgG, IgA, and IgE. IgG, comprising four subclasses, is important for clearing a wide range of infections. For instance, immunization of mice with soluble proteins induce mainly IgG1, whereas neutralizing antibodies to viruses are dominated by the IgG2a/c isotype (hereinafter referred to as IgG2c) (Coutelier et al., 1987).

Different types of pathogens induce a unique cytokine milieu during an immune response, leading to the specialization of both Th and B cell responses (Crotty, 2011). B cells integrate these extrinsic signals to produce the Ig effector phenotype optimized for each class of pathogen (Deenick et al., 2005; Stevens et al., 1988). For example, interferon gamma (IFNγ) promotes switching to IgG2c, whereas interleukin (IL)-4 promotes switching to IgG1 and IgE (Snapper and Paul, 1987). B cells responding to a pathogen will promote one class of antibody response while suppressing other classes to ensure targeted responses and to avoid Ig-mediated immune disorders. Is it likely that intrinsic molecular programs mediate this balance to allow plasticity in the responding cells tailored to the immunogen. Cytokines can activate unique transcription factors that target the switching machinery in B cells to specific isotypes in a context-specific manner. In particular, B cell-intrinsic expression



(legend on next page)

of T-bet has a central role in mediating an anti-viral B cell-specific program (Barnett et al., 2016; Rubtsova et al., 2013). Specifically, T-bet induces IgG2c production downstream of IFNγ signaling (Peng et al., 2002) and regulates survival of IgG2c memory B cells (Wang et al., 2012) and migration of PC to sites of inflammation via expression of CXCR3 (Moser et al., 2006). Conversely, how such an anti-viral program is suppressed to permit altered dominance in responses to other pathogens is not clear.

Here, we describe a crucial role of the transcription factor c-Myb in repressing the T-bet-driven anti-viral B cell program. Furthermore, inappropriate expression of T-bet and CXCR3 resulted in the dysregulation of PC differentiation within the GC. Thus, c-Myb is key to controlling inherent B cell plasticity and establishing class-specific immune responses.

RESULTS

Dysregulation of GC-to-PC Progression in the Absence of c-Myb

We recently demonstrated that c-Myb is essential for the migration of PCs to the bone marrow (BM) to form long-lived immunity (Good-Jacobson et al., 2015). c-Myb has also been shown to be expressed in human GC B cells (Basso et al., 2012). While c-Myb is not required for GC formation after immunization with the model antigen (4-hydroxy-3-nitrophenyl)-acetyl conjugated to Keyhole Limpet Hemocyanin (NP-KLH) in alum, GCs declined earlier in B cell-specific c-Myb-deficient mice than in wild-type controls (Good-Jacobson et al., 2015). In line with these data, a recent study demonstrated that Nur77$^+$ IgG1$^+$ GC B cells upregulated c-Myb in comparison to Nur77$^-$ cells (Gitlin et al., 2016). Together, these data suggest that c-Myb may regulate post-selection processes within the GC. Differentiation into PCs involves downregulation of GC and B cell markers and the upregulation of PC markers, including CD138. In B cell-specific c-Myb-deficient mice (*c-Myb$^{f/f}$Cd23$^{Cre/+}$*), a sub-population of GC B cells expressed CD138 while retaining GC B cell markers, hereinafter referred to as GC-PCs (Figures 1A and 1B). These cells were functional antibody-secreting cells (ASCs), secreting IgG as detected by ELISpot assay when sort-purified as CD138$^+$CD95^{hi}CD38^{lo} B cells (Figure S1A). To determine the location of these GC-PC phenotype cells, *c-Myb$^{f/f}$Cd23$^{Cre/+}$* mice were crossed with *Prdm1$^{gfp/+}$* (the gene encoding Blimp-1, the plasma cell transcription factor) reporter mice (Kallies et al., 2004). *c-Myb$^{f/f}$Cd23$^{Cre/+}$* or *c-Myb$^{f/f}$* littermates, both carrying one *Prdm1^{gfp}* allele, were immunized with NP-KLH in alum, and splenic GCs were assessed by histology. Consistent with the flow-cytometry results, GFP cells were detected within GCs in the absence of c-Myb (Figures 1C and S1B). While these data suggest that there was an increased frequency of cells differentiated to PCs within the GC, it does not exclude potential PCs outside the GC that still retain GC markers. In comparison to wild-type mice in which Blimp-1-expressing cells were physically and phenotypically distinct from the GC, GFP$^+$ cells in the absence of c-Myb were also positive for peptide nucleic acid (PNA) (Figure 1C). While there was increased differentiation to PCs, the memory B cell population formed but was reduced approximately 2-fold in the absence of c-Myb (Figures S2A and S2B).

It was possible that altered B cell responses affected the formation and/or function of T follicular helper cells, in turn, inducing premature differentiation to PC. However, there was no defect in T follicular helper cells in *c-Myb$^{f/f}$Cd23$^{Cre/+}$* mice (data not shown). To confirm the cell-intrinsic role of c-Myb, we utilized 50:50 BM chimeras in which both wild-type and c-Myb-deficient B cells were present (Figures 1D and 1E). Despite the presence of normal GC B cells and Tfh cells, the frequency of CD138$^+$ GC c-Myb-deficient B cells was >6%, compared to <2% for the Ly5.1$^+$ population (C2 in Figure 1E), the latter being similar to the control chimera (C1 in Figure 1E). These data demonstrate B cell-intrinsic dysregulation of GC-to-PC differentiation in the absence of c-Myb.

Affinity Maturation and Selection of CD138$^+$ GC B Cells

A major outcome of a successful T-dependent immune response is the establishment of high-affinity PC and memory B cells. We, therefore, investigated V_H mutations to assess affinity maturation of the BCR within the GC (Figures 1F–1J). The frequency distribution of mutations of GC B cells was comparable in *c-Myb$^{f/f}$Cd23$^{Cre/+}$Prdm1$^{gfp/+}$* mice and *c-Myb$^{f/f}$Cd23$^{+/+}$Prdm1$^{gfp/+}$* littermate controls (Figures 1F and 1G), although mutation frequency was decreased in c-Myb-deficient GC B cells. Similarly, the mean number and frequency distribution of V_H mutations within sort-purified CD138$^+$CD95^{hi}CD38^{lo} GC B cells was similar to the rare CD138$^+$CD95^{hi}CD38^{lo} B cells from control mice (Figures 1H and 1I). However, the representation of the affinity-enhancing W33L mutation was 30% in *c-Myb$^{f/f}$Cd23$^{Cre/+}$* mice, compared to >80% in controls (Figure 1J). In contrast, the proportion of GC B cell V_H sequences with the W33L was similar between datasets (Figure 1J). These data confirm that the CD138$^+$ GC-phenotype cells are GC derived and suggest that, while affinity maturation of GC B cells was not affected by the absence of

Figure 1. Premature Differentiation within c-Myb-Deficient GCs

c-Myb$^{f/f}$Cd23$^{Cre/+}$ and *c-Myb$^{f/f}$ Cd23$^{+/+}$* littermate wild-type mice were immunized with NP-KLH in alum.

(A and B) Flow cytometric representative plots (A) and frequency (B) of CD138$^+$ cells within the antigen-specific GC B cell population (NP^{int}Fas^{hi}CD19$^+$IgD^{lo}) 12 days post-immunization; n = 3–5 from three experiments; *p < 0.05.

(C) GFP expression in GC B cells in *c-Myb$^{f/f}$Cd23$^{Cre/+}$Prdm1$^{gfp/+}$*or *c-Myb$^{f/f}$Cd23$^{+/+}$Prdm1$^{gfp/+}$* mice. Scale bar, 100 μm.

(D and E) Mixed BM chimeras. (D) Chimera setup: BM from Ly5.1 was mixed in a 1:1 ratio with either *Cd23$^{Cre/+}$* (C1) or *c-Myb$^{f/f}$Cd23$^{Cre/+}$* (C2) BM and injected into irradiated Ly5.1 recipient mice. 7–8 weeks later, mice were immunized, and B cell responses were assessed at day 13. (E) Frequency of CD138+ cells within the GC population. n = 9 per group. *p < 0.05; **p < 0.01 (Mann-Whitney nonparametric, two-tailed test).

(F–J) V_H gene analysis: frequency distribution and mean mutation number of (F and G) CD138^{neg} GC B cells and (H and I) CD138$^+$ GC B cells; (J) Trp-to-Leu mutations in CD138^{neg} or CD138$^+$ GC B cells. Data were combined from two experiments; Fisher's exact two-tailed test. n.s., not significant; ****p < 0.0001. Data represent means ± SEM.

See also Figures S1 and S2.

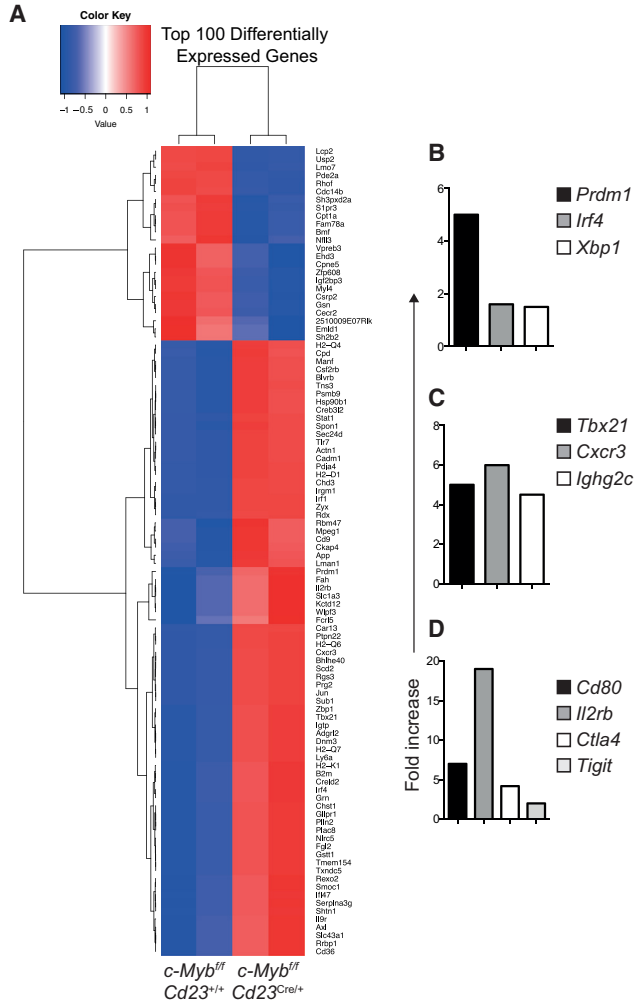


Figure 2. c-Myb Establishes a Gene Expression Program that Regulates B Cell Differentiation and the T-Bet Transcriptional Network

(A) Heatmap of RNA-seq data (Table S1); each column is an independent sample obtained from pooled, sort-purified, day-12 CD138^{neg} GC B cells from either *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ or *c-Myb*$^{f/f}$*Cd23*$^{+/+}$ mice immunized with NP-KLH in alum.

(B–D) Fold increase of mRNA for particular genes in *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ over control mice as assessed by RNA-seq: (B) PC transcription factors, (C) T-bet and T-bet-regulated genes, and (D) putative T-bet-regulated genes.

See also Table S1.

c-Myb, the CD138^{+} GC B cells in *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ mice were less stringently selected.

Regulation of a Transcriptional Program in GC B Cells by c-Myb

To understand the potential transcriptional changes being mediated by c-Myb, RNA sequencing (RNA-seq) was performed on sort-purified GC B cells, defined as CD138^{neg}CD95^{hi}CD38^{lo} (Figure 2A; Table S1). In the absence of c-Myb, ~1,150 genes were differentially expressed ($p < 0.005$), and ~300 of these had at least a 2-fold change, with the majority of these genes upregulated in c-Myb-deficient GC B cells compared to wild-type. Even though CD138^{+} cells were excluded during cell purification, PC genes were still upregulated in the absence of c-Myb (Figures 2A and 2B). *Prdm1* was upregulated ~5-fold, while other PC regulators such as *Irf4* and *Xbp1* were increased to a lesser extent (Figure 2B), suggesting that c-Myb-deficient GC B cells, or at least a subset, were poised to differentiate.

We performed C2 canonical pathway analysis from the Molecular Signatures Database (Liberzon et al., 2011) and found that IFN signaling pathways were upregulated. This was surprising, considering that NP-KLH/alum immunization is a Th2-cell-mediated response, and thus, these pathways should largely be inactive. Consistent with the pathway analysis, however, c-Myb-deficient GC B cells upregulated *Tbx21*, the gene encoding T-bet. Furthermore, the T-bet-regulated genes *Cxcr3* and *Ighg2c* were also upregulated (Figure 2C). T-bet expression in B cells has long been associated with IgG2c production (Peng et al., 2002), and regulation of CXCR3 (Barnett et al., 2016; Serre et al., 2012). However, a recent study demonstrated that the T-bet transcriptional program was important beyond mediating IgG2c production and was critical in the clearance of lymphocytic choriomeningitis virus (LCMV) (Barnett et al., 2016). We compared our RNA-seq dataset with genes associated with T-bet expression in B cells, as assessed by T-bet-positive versus T-bet-negative isotype-switched B cells from LCMV-infected mice (Barnett et al., 2016). Although some of the genes identified in the latter dataset may be the result of an unequal representation of GC B cells (e.g., *Aicda*, *Fas*, and *Cd80*), a number of genes were also differentially expressed in our dataset, such as *Cxcr3*, *Tbx21* (Figure 2C), *Cd80*, *Il2rb*, *Ctla4*, and *Tigit* (Figure 2D).

Inappropriate Expression of T-bet Disrupts GC Selection and PC Differentiation

RNA-seq analysis suggested that the T-bet-regulated gene expression program necessary for anti-viral responses was switched on in the absence of c-Myb. Accordingly, T-bet expression (Figure 3A) and NP-specific IgG2c serum antibody (Figure 3B) were increased in immunized c-Myb-deficient mice. Histological analyses confirmed an increase in IgG2c^{+} ASCs in the spleens of immunized c-Myb-deficient mice (Figures 3C and 3D). Lastly, CXCR3 expression was upregulated on c-Myb-deficient B220^{lo}CD138^{hi} cells (Figure 3E).

Together, the data thus far demonstrated that conditional deletion of c-Myb in B cells lead to an inappropriate T-bet-driven response. To confirm that this was directly downstream of the B cell-intrinsic loss of c-Myb, and not improper T cell priming such as increased IFNγ production, we again utilized 50:50 mixed BM chimeras. Similar to intact mice, c-Myb-deficient GC B cells (Figure 3F) and plasmablasts (Figures 3G and 3H) in mixed BM chimeras expressed CXCR3, while wild-type cells within the same hosts did not, confirming that upregulation of T-bet-regulated genes was not due to a change in the microenvironment. Thus, c-Myb expression appeared to be critical in repressing a T-bet-regulated, anti-viral gene expression program during a Th2-cell-biased response. To confirm, we generated *Tbx21*$^{f/f}$*c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ mice and immunized with NP-KLH in alum. As expected, deleting T-bet in c-Myb-deficient B cells (Figure 3I) restored both IgG2c (Figure 3J) and CXCR3 (Figure 3K) expression to wild-type levels. In contrast, it did not restore

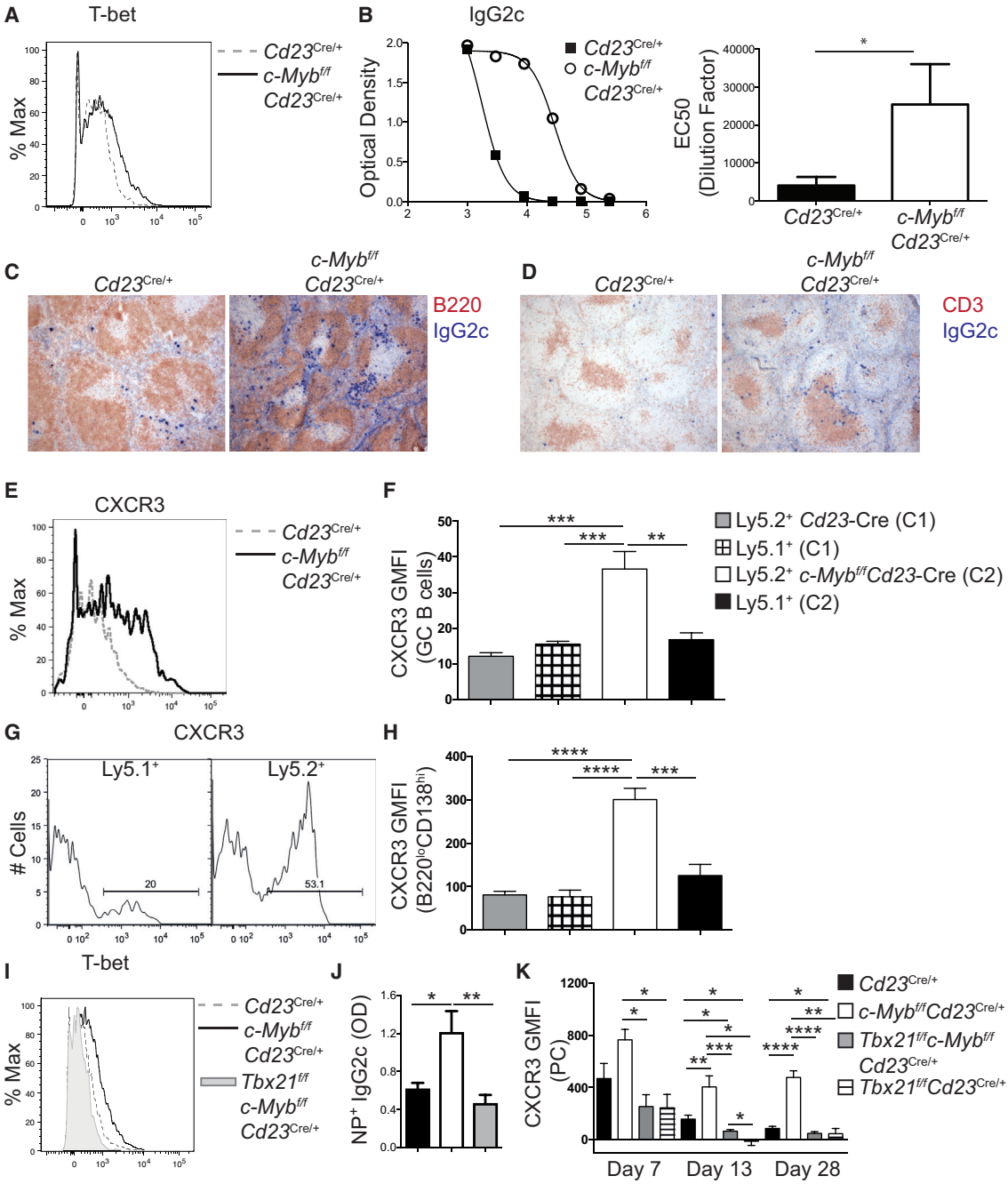


Figure 3. The T-Bet Anti-viral Program Is Turned on in the Absence of c-Myb

(A) Flow-cytometric representative histogram of T-bet expression in GC B cells from immunized *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ and *Cd23*$^{Cre/+}$ mice.

(B) ELISA analysis of NP-specific IgG2c serum antibody 7 days post-immunization; n = 4 per genotype, representative of three experiments.

(C and D) *Cd23*$^{Cre/+}$ and *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ mice were immunized with NP-KLH precipitated in alum, and spleens were harvested at day 7 post-immunization. Sections were stained with B220 (red) and IgG2c (blue) in (C) and with CD3 (red) and IgG2c (blue) in (D), representative of three spleens per genotype.

(E) Flow-cytometric representative histogram of CXCR3 expression on B220^{lo}CD138^{hi} cells.

(F–H) 50:50 mixed BM chimeras of either *Cd23*$^{Cre/+}$:Ly5.1 or *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$:Ly5.1, immunized and assessed by flow cytometry.

(F) GMFI of CXCR3 expression on GC B cells.

(G and H) Flow cytometric representative plot (G) and GMFI (H) of CXCR3 expression on PC. n = 9 mice per group, representative of three experiments.

(legend continued on next page)

migration of c-Myb-deficient PCs to the BM (Good-Jacobson et al., 2015) (Figure S3A).

We next assessed GC-PC differentiation in *Tbx21*$^{f/f}$*c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ as T-bet was also upregulated in c-Myb-deficient GC-PCs (data not shown). While immunized *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ GC B cells disproportionately upregulated CD138, deleting T-bet within these mice reduced this population to near-normal frequency (Figure 4A) but did not correct GC B cell frequency (Figure 4B). Additionally, V_H sequencing revealed that *Tbx21*$^{f/f}$*c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ CD138$^+$ GC B cells had a V_H gene mutation frequency distribution (Figure S3B) and, importantly, W33L representation (Figure 4C) equivalent to that in wild-type B cells (Figure 1J; data shown for comparison). Thus, several of the GC B cell differentiation abnormalities arising from the loss of c-Myb were corrected by the removal of T-bet, providing a causal relationship.

In contrast to the Th2-cell-biased immunization model, T-bet expression is an important driver of B cell responses to viral infections (Barnett et al., 2016; Rubtsova et al., 2013). Thus, we speculated that loss of c-Myb would not affect differentiation of GC B cells responding to influenza infection. To test this proposal, *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$, *Tbx21*$^{f/f}$*c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$, *Tbx21*$^{f/f}$*Cd23*$^{Cre/+}$, and *Cd23*$^{Cre/+}$ mice were intranasally infected with the influenza A strain HKx31, and the B cell response was assessed at day 8 (Figures 4D–4H; Figure S3). Contrary to mice immunized with NP-KLH in alum, *Tbx21*$^{f/f}$*Cd23*$^{Cre/+}$ mice infected with influenza failed to mount an adequate GC response, a feature replicated in *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ and *c-Myb*$^{f/f}$*Tbx21*$^{f/f}$*Cd23*$^{Cre/+}$ mice (Figure S3C–S3E). Influenza-infected *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ mice had a significantly increased representation of CD138$^+$ GC cells compared to that in control mice in both the spleen and mediastinal lymph node, which again was corrected by the deletion of T-bet (Figures 4D–4H). Interestingly, the wild-type frequency of CD138$^+$ GC B cells was increased at least 2-fold in flu-infected CD23-cre mice (Figure 4F), compared to NP-KLH in alum-immunized mice (Figure 4A). Together, these data demonstrate that c-Myb is an essential regulator of T-bet function, repressing the T-bet program during non-viral responses. Inappropriately lifting this repression resulted in premature differentiation of GC to PC in B cells responding to influenza.

Deleting CXCR3 Controls Aberrant GC-PC Population in c-Myb-Deficient Mice

Lastly, we asked which of a number of T-bet-regulated genes were inducing aberrant differentiation of GC B cells. Possible candidates included Blimp-1, Ig isotype switching, and CXCR3. While there have been conflicting reports of the relationship of T-bet with Blimp-1 (Cimmino et al., 2008; Xin et al., 2016), induction of Blimp-1 was recently shown to be independent of T-bet (Xin et al., 2016). Switching to secondary isotypes may induce PC differentiation, especially with respect to IgE (Yang et al., 2012). Alternatively, aberrant upregulation of CXCR3 in GC B cells may disrupt the migratory program within the GC, resulting in altered localization and subsequent receipt of signals inducing premature differentiation of PC. To investigate, we generated novel mice in which CXCR3 was specifically deleted within B cells (Figure S4). These mice were crossed with c-Myb-deficient mice, and the resultant animals were immunized with NP-KLH in alum.

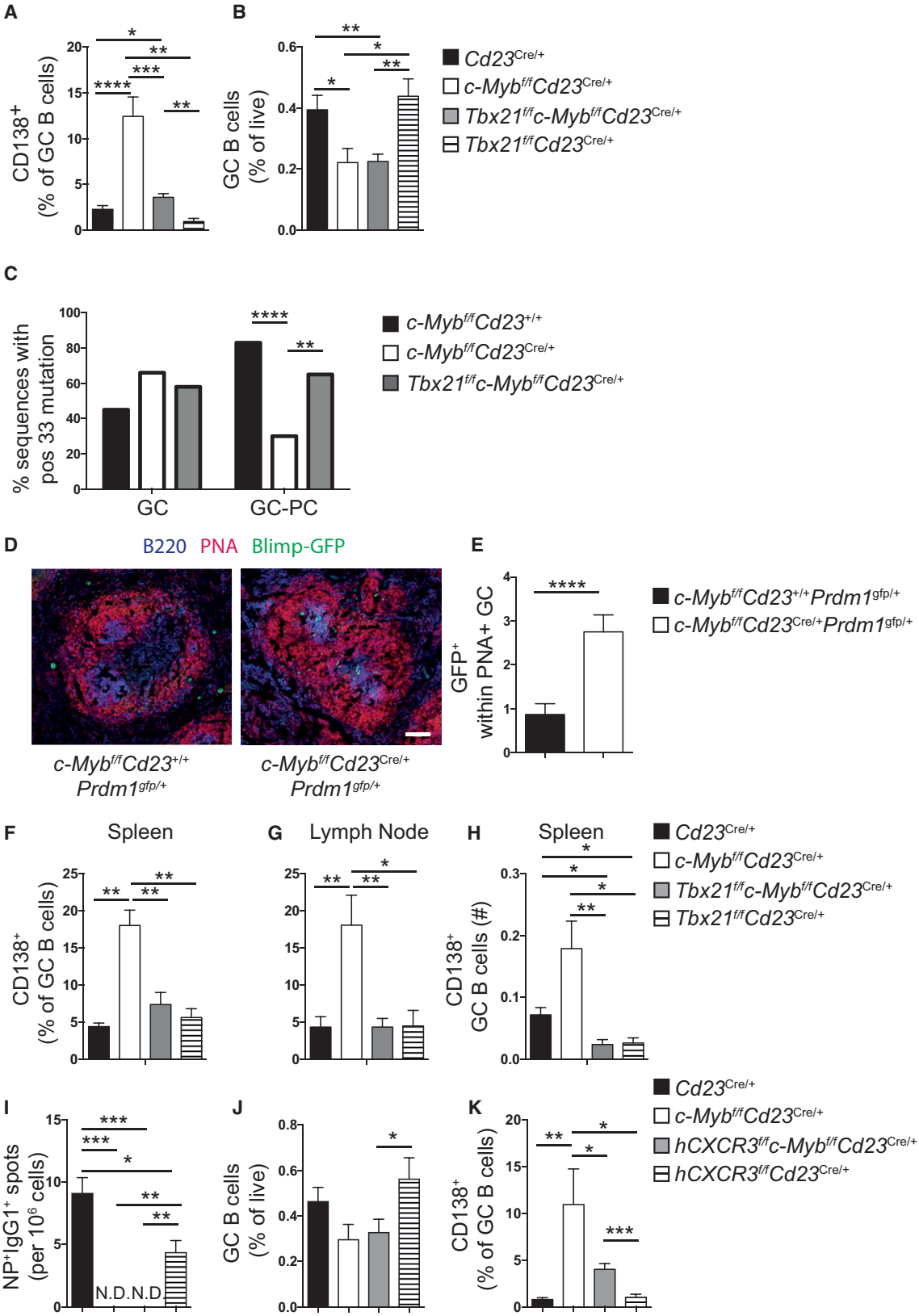
Similar to *Tbx21*$^{f/f}$*c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ mice, ELISPOT analysis demonstrated that migration of c-Myb-deficient PCs to the BM was not rescued by co-deletion of CXCR3 (Figure 4I), while ASCs were still detectable in *hCXCR3*$^{f/f}$*Cd23*$^{Cre/+}$ mice. Therefore, inappropriate T-bet and CXCR3 upregulation did not inhibit CXCR4-mediated migration of PC to the BM. Furthermore, co-deletion did not affect the frequency of cells switching to IgG1 (Figure S4B) or the frequency of GC B cells (Figure 4J), compared to *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ mice. However, it significantly reduced the frequency of CD138$^+$ cells in the GC (Figure 4K). Thus, anomalous upregulation of T-bet and CXCR3 disrupted the ordered process of affinity maturation and PC differentiation within the GC, resulting in aberrant GC products. Together, these data demonstrate a role for c-Myb in ensuring regulated protective effects of molecular programs responsible for targeted B cell responses to distinct classes of pathogens.

DISCUSSION

A key element for a successful B cell response, and that of the vast majority of vaccines, is the ability to tailor the antibody structure to suit the pathogen. In mice and humans, the heavy chain of immunoglobulin has a number of variants with different effector attributes. Typically, one of these variants will dominate an immune response, although not to the exclusion of all others. Transcriptional programs that mediate other attributes such as migration and cytokine secretion also accompany antibody adaptation. How B cells are able to integrate potentially competing signals and then drive expression of one transcriptional program at the expense of another is not well understood. Here, we have revealed a mechanism by which c-Myb is required in B cells for effective responses to immunization with protein antigens by suppressing the T-bet-driven anti-viral response.

T-bet has essential roles in multiple immune lineages. In particular, T-bet is inextricably linked with the Th1/Th2 paradigm in the CD4 T cell lineage, working in opposition to GATA3 to determine Th1 and Th2 commitment, respectively. Our data suggest that the relationship between c-Myb and T-bet in B cells does not function in the same exclusive manner for IgG1-dominated responses. While c-Myb-deficient B cells have increased IgG2c antibody and PCs, they also maintain class switching to IgG1, a canonical Th2-cell-responsive pathway in mice. Thus, c-Myb may repress transcriptional networks appropriate in other immune contexts and, thus, focus the humoral response toward

(I–K) *Cd23*$^{Cre/+}$, *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$, *Tbx21*$^{f/f}$*c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$, and *Tbx21*$^{f/f}$*Cd23*$^{Cre/+}$ mice were immunized with NP-KLH in alum. (I) Flow-cytometric representative histogram of T-bet on GC B cells. (J) Serum NP-specific IgG2c assessed by ELISA; n = 6–7, combined from two experiments. (K) Flow-cytometric analyses of CXCR3 expression on plasma cells; n = 3–15, combined from multiple experiments per time point.
Data indicate mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Mann-Whitney nonparametric, two-tailed test).
See also Figure S3.



(legend on next page)

the outcome that provides the most potent immune protection. Further work is required to determine whether c-Myb only suppresses the T-bet-driven program or whether c-Myb is also able to regulate transcriptional networks associated with responses to other types of infections.

The immune system balances protective mechanisms directed against foreign pathogens with aberrant immune responses that result in damage to the host. In the humoral system, diversity in antibody isotypes mediates the ability to clear diverse pathogens. However, immune disorders can also be mediated by particular isotypes, such as IgG2c and IgE. It is unknown how isotype switching is regulated appropriately (in response to pathogens) or inappropriately (in immune disorders). Dysregulated transcription factor expression has also been associated with immune disorders. Thus, regulatory mechanisms may resolve at two levels—normal transcriptional activation or repression during an immune response and regulation of aberrant expression. While the ability of T-bet to induce PC differentiation may be beneficial, it appears that, in the absence of c-Myb, this would be at the expense of appropriate selection of high-affinity PCs. The expression of c-Myb in the GC is targeted to Nur77^{+} GC cells (Gitlin et al., 2016), suggesting that c-Myb has an important role in regulating the selection-mediated fate decisions of isotype-switched cells. As such, inappropriate expression of T-bet within GCs in c-Myb-deficient mice resulted in increased differentiation into PC, even during a response to influenza infection in which T-bet is normally expressed. In other cell types, the importance of T-bet in regulating immune responses does not simply rely on whether it is present or absent. In T cells, T-bet facilitates different fate decisions according to the level of expression (Joshi et al., 2007; Marshall et al., 2011), thereby modulating effector or memory cell formation in accordance with the level of inflammatory mediators. The transcription factor Zeb2 has been reported to modulate differential T-bet function in CD8 T cells (Dominguez et al., 2015), but it is unclear whether it is used in B cells. c-Myb can regulate transcriptional programs through the binding of co-factors such as p300 or by forming complexes with the H3K4me3 methyltransferase MLL1 and the protein Menin to regulate gene expression (Jin et al., 2010; Nakata et al., 2010). Thus, these transcriptional networks may be regulated at multiple levels by complex formation—through the recruitment of cofactors, inhibition of binding, and chromatin structure regulated by histone modifiers. Future work will investigate the chromatin landscape of these transcriptional networks in response to type I versus type II pathogens.

T-bet, CXCR3, and IgG2c have all been linked with B cells in disease. T-bet expression is an essential regulator of age-associated B cells, which accumulate in aged female mice, autoimmune-prone murine models, and elderly female autoimmune patients (Rubtsova et al., 2013, 2017). While it is clear that T-bet drives pathogenic autoantibody production, recent studies have shown contrasting roles for T-bet in autoimmune GC (Jackson et al., 2016; Rubtsova et al., 2017). Further, in a chronic infection model, T-bet regulated B cell behavior and clearance of virus independent of antibody (Barnett et al., 2016), suggesting context-driven roles for T-bet. Here, we demonstrate that inappropriate expression of CXCR3 due to T-bet dysregulation can impact on GC-to-PC differentiation. Although deletion of CXCR3 reduced the aberrant population of CD138^{+} GC B cells, it was not completely restored to wild-type frequency. T-bet and Bcl-6 have been noted to co-repress each other, and the upregulation of T-bet may repress Bcl-6, allowing for upregulation of Blimp-1. Accordingly, GC B cells had a decrease in Bcl-6 MFI while GC-PCs did not express Bcl-6 (data not shown). Therefore, it is likely that cumulative actions of T-bet, including CXCR3 expression, repression of Bcl-6, and increased isotype switching (Gitlin et al., 2016), induce dysregulated GC B cell behavior when inappropriately expressed. Identifying the transcriptional mediators of T-bet activity will allow more targeted research into understanding the role of T-bet in B cell-mediated disease, as well as limiting aberrant production of IgG2c and PC migration to sites of immunopathology in autoimmune diseases.

Understanding the transcriptional networks regulating pathogen-specific B cell responses is essential in understanding how the balance between protection and immunopathogenesis is regulated in the immune system. Revealing these molecular relationships could potentially lead to therapeutic applications for rewiring cells away from deleterious isotypes. In sum, c-Myb brings the interplay between transcriptional regulation and cellular behavior together to regulate the critical balancing act that B cells play that allows pathogen clearance while constraining immune responses that can become damaging to the host.

EXPERIMENTAL PROCEDURES

Mice, Immunizations, and Purification of Cells

Cd23-Cre (Kwon et al., 2008) mice were provided by M. Busslinger, and *c-Myb*$^{fl/fl}$ mice provided by J. Frampton (Emambokus et al., 2003). *Prdm1*$^{gfp/+}$ reporter mice were generated as previously described (Kallies et al., 2004). *Tbx21*$^{f/f}$ mice were originally generated by S. Reiner (Wang et al., 2012). hCXCR3 conditionally deleted mice were generated as described in Figure S4.

Figure 4. Increase of GC-PCs Is Corrected by T-Bet or CXCR3 Deletion

(A and B) Frequency of CD138^{+} GC B cells (A) and GC B cells (B) in immunized mice.

(C) V_H gene analysis: Trp-to-Leu mutations in CD138^{neg} or CD138^{+} GC B cells; Fisher's exact two-tailed test.

(D–H) Mice were intranasally infected with influenza A virus (HKx31), and B cells were assessed at day 8 post-infection. (D and E) *Prdm1*-GFP expression in GC B cells. (F–H) Flow-cytometric assessment of GC-PC frequency in spleen (F), mediastinal lymph node (G), and total number of GC-PCs in the spleen (H). n = 4–6 mice per group combined from multiple experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Mann-Whitney nonparametric, two-tailed test; the white bar represents 100 μm).

(I–K) *Cd23*$^{Cre/+}$, *c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$, *hCXCR3*$^{f/f}$*c-Myb*$^{f/f}$*Cd23*$^{Cre/+}$ and *hCXCR3*$^{f/f}$*Cd23*$^{Cre/+}$ mice were assessed 11 days post-immunization with NP-KLH in alum. (I) ELISpot analysis of NP^{+}IgG1^{+} BM ASCs. (J and K) Flow-cytometric analyses of GC B cell frequency (J) and CD138^{+} GC B cell frequency (K). n = 4–7 per group, combined from two experiments.

*p < 0.05; **p < 0.01; ***p < 0.001 (Unpaired two-tailed t test; data indicate mean ± SEM).

See also Figures S3 and S4.

Ly5.1 mice were maintained at Walter and Eliza Hall Institute (WEHI). Both males and females, over 7 weeks old, were used in this study. Animal procedures were approved by both WEHI and Monash University Animal Ethics Committees. Primary responses: mice were injected intraperitoneally with 100 μg NP conjugated to KLH (molar ratio between 13 and 20), precipitated on 10% alum. Influenza infections: mice were inoculated intranasally with 10^4 plaque-forming units (PFUs) of HKx31 (H3N2) influenza virus as previously described (Belz et al., 2000; Flynn et al., 1998). Sort-purification: cells were stained with antibodies and purified by BD FACSAria or Influx (BD Biosciences), with purity >98%.

Flow Cytometry

Single cells were resuspended in PBS 2% FCS and stained for flow-cytometric analysis. Cells were analyzed on a BD FACSCanto or BD Fortessa (BD Biosciences), and data were analyzed with FlowJo software (Treestar). FcγRII/III (24G2; supernatant) was used to block non-specific binding. See Supplemental Experimental Procedures for antibody details.

BM Chimeras

50:50 chimeras: lethally irradiated Ly5.1 mice (2 × 550 rads) were reconstituted with 50% Ly5.1 BM and 50% *c-Myb*$^{fl/fl}$*Cd23*$^{Cre/+}$ or *Cd23*$^{Cre/+}$ BM. Mice were rested for 7–8 weeks and bled to test chimerism before NP-KLH in alum immunization.

Histology, ELISPOT, and ELISA

Portions of spleens were frozen in optimal cutting temperature (OCT) compound (Tissue-Tek), and 7-μM sections were cut using a microtome (Leica) and stained for immunohistochemistry, as detailed previously (Zotos et al., 2010). For immunofluorescence staining, portions of spleens were fixed in 4% paraformaldehyde and infiltrated with 30% sucrose before being embedded in OCT compound. Images were acquired on an LSM780 confocal microscope on an Axiovert 200M base (Carl Zeiss MicroImaging). Objective: 20× Plan Apochromat; NA = 0.8. Detectors are 2× PMT (photomultiplier tube) and a 32-channel GaAsP (gallium arsenide phosphide) array detector. The acquisition software used was Zen Black 2012. Images were analyzed with ImageJ (NIH). ASCs and antibody were analyzed by ELISPOT and ELISA, respectively, as previously described (Zotos et al., 2010).

V_H Sequencing

Individual cells were sort-purified on a BD FACSAria equipped with a plate sorter and sequenced as previously detailed (Zotos et al., 2010). See the Supplemental Experimental Procedures for analysis details.

RNA-Seq

CD19$^+$IgD^{lo}CD95^{hi}CD38^{lo}CD138^{neg} B cells from two independent pools of *c-Myb*$^{fl/fl}$*Cd23*$^{Cre/+}$ mice and two independent pools of c-*Myb*$^{fl/fl}$*Cd23*$^{+/+}$ littermate controls were sort-purified. RNA was isolated using the QIAGEN RNeasy Micro Kit according to the manufacturer's guidelines. RNA-seq was performed on a HiSeq2000 at the Australian Genome Research Facility (AGRF) using Illumina CASAVA pipeline, version 1.8.2. See the Supplemental Experimental Procedures for analysis details.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software, and statistical tests are stated in the figure legends. Means ± SEM are shown.

ACCESSION NUMBERS

The accession number for the RNA-seq data reported in this paper is GEO: GSE89732.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.03.060.

AUTHOR CONTRIBUTIONS

K.L.G.-J. and D.M.T. designed the research; K.L.G.-J., D.P., and J.T. performed the majority of experiments, A.D.P., C.Y., I.B., K.O., and J.R.G. performed additional experiments; J.R.G., V.P., and G.T.B. provided technical assistance, reagents, and intellectual input; K.L.G.-J., D.P., A.D.P., J.T., I.B., and J.R.G. analyzed data; Y.C. and G.K.S. analyzed RNA-seq data; C.R.M. generated the hCXCR3 mice; and K.L.G.-J. wrote the manuscript.

ACKNOWLEDGMENTS

We thank Colby Zaph, Brendan Russ, and Stephen Turner for critical reading of this manuscript; members of the Good-Jacobson, Tarlinton, and Groom labs for technical assistance; Adele Barugahare and David Powell from the Monash Bioinformatics Platform for additional bioinformatic assistance; and Remy Robert for assistance with the hCXCR3 mice. We also thank Susan Kaech for providing *Tbx21*$^{f/f}$ mice. This work was supported by a National Health and Medical Research Council (NHMRC) project grant to K.L.G.-J. (1057707), an NHMRC program grant to D.M.T. and G.T.B. (1054925), and a Leukaemia Foundation grant-in-aid to K.L.G.-J. K.L.G.-J. is supported by an NHMRC Career Development Fellowship (1108066), and D.M.T. and G.K.S. are supported by NHMRC Research Fellowships (1060675 and 1058892, respectively). V.P. was supported by fellowships from the Multiple Myeloma Research Foundation and the European Molecular Biology Organization (ALTF 1337-2010). G.T.B. and J.R.G. are supported by Australian Research Council Future Fellowships (FT110100283 and FT130100708, respectively). This work was made possible through Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS.

Received: January 6, 2017
Revised: February 24, 2017
Accepted: March 21, 2017
Published: April 18, 2017

REFERENCES

Barnett, B.E., Staupe, R.P., Odorizzi, P.M., Palko, O., Tomov, V.T., Mahan, A.E., Gunn, B., Chen, D., Paley, M.A., Alter, G., et al. (2016). Cutting edge: B cell-intrinsic T-bet expression is required to control chronic viral infection. J. Immunol. *197*, 1017–1022.

Basso, K., Schneider, C., Shen, Q., Holmes, A.B., Setty, M., Leslie, C., and Dalla-Favera, R. (2012). BCL6 positively regulates AID and germinal center gene expression via repression of miR-155. J. Exp. Med. *209*, 2455–2465.

Belz, G.T., Xie, W., Altman, J.D., and Doherty, P.C. (2000). A previously unrecognized H-2D(b)-restricted peptide prominent in the primary influenza A virus-specific CD8(+) T-cell response is much less apparent following secondary challenge. J. Virol. *74*, 3486–3493.

Cimmino, L., Martins, G.A., Liao, J., Magnusdottir, E., Grunig, G., Perez, R.K., and Calame, K.L. (2008). Blimp-1 attenuates Th1 differentiation by repression of ifng, tbx21, and bcl6 gene expression. J. Immunol. *181*, 2338–2347.

Coutelier, J.P., van der Logt, J.T., Heessen, F.W., Warnier, G., and Van Snick, J. (1987). IgG2a restriction of murine antibodies elicited by viral infections. J. Exp. Med. *165*, 64–69.

Crotty, S. (2011). Follicular helper CD4 T cells (TFH). Annu. Rev. Immunol. *29*, 621–663.

Deenick, E.K., Hasbold, J., and Hodgkin, P.D. (2005). Decision criteria for resolving isotype switching conflicts by B cells. Eur. J. Immunol. *35*, 2949–2955.

Dominguez, C.X., Amezquita, R.A., Guan, T., Marshall, H.D., Joshi, N.S., Kleinstein, S.H., and Kaech, S.M. (2015). The transcription factors ZEB2 and T-bet cooperate to program cytotoxic T cell terminal differentiation in response to LCMV viral infection. J. Exp. Med. *212*, 2041–2056.

Emambokus, N., Vegiopoulos, A., Harman, B., Jenkinson, E., Anderson, G., and Frampton, J. (2003). Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. EMBO J. *22*, 4478–4488.

Flynn, K.J., Belz, G.T., Altman, J.D., Ahmed, R., Woodland, D.L., and Doherty, P.C. (1998). Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. Immunity *8*, 683–691.

Gitlin, A.D., von Boehmer, L., Gazumyan, A., Shulman, Z., Oliveira, T.Y., and Nussenzweig, M.C. (2016). Independent roles of switching and hypermutation in the development and persistence of B lymphocyte memory. Immunity *44*, 769–781.

Good-Jacobson, K.L., O'Donnell, K., Belz, G.T., Nutt, S.L., and Tarlinton, D.M. (2015). c-Myb is required for plasma cell migration to bone marrow after immunization or infection. J. Exp. Med. *212*, 1001–1009.

Jackson, S.W., Jacobs, H.M., Arkatkar, T., Dam, E.M., Scharping, N.E., Kolhatkar, N.S., Hou, B., Buckner, J.H., and Rawlings, D.J. (2016). B cell IFN-γ receptor signaling promotes autoimmune germinal centers via cell-intrinsic induction of BCL-6. J. Exp. Med. *213*, 733–750.

Jin, S., Zhao, H., Yi, Y., Nakata, Y., Kalota, A., and Gewirtz, A.M. (2010). c-Myb binds MLL through menin in human leukemia cells and is an important driver of MLL-associated leukemogenesis. J. Clin. Invest. *120*, 593–606.

Joshi, N.S., Cui, W., Chandele, A., Lee, H.K., Urso, D.R., Hagman, J., Gapin, L., and Kaech, S.M. (2007). Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor. Immunity *27*, 281–295.

Kallies, A., Hasbold, J., Tarlinton, D.M., Dietrich, W., Corcoran, L.M., Hodgkin, P.D., and Nutt, S.L. (2004). Plasma cell ontogeny defined by quantitative changes in blimp-1 expression. J. Exp. Med. *200*, 967–977.

Kwon, K., Hutter, C., Sun, Q., Bilic, I., Cobaleda, C., Malin, S., and Busslinger, M. (2008). Instructive role of the transcription factor E2A in early B lymphopoiesis and germinal center B cell development. Immunity *28*, 751–762.

Liberzon, A., Subramanian, A., Pinchback, R., Thorvaldsdóttir, H., Tamayo, P., and Mesirov, J.P. (2011). Molecular signatures database (MSigDB) 3.0. Bioinformatics *27*, 1739–1740.

Marshall, H.D., Chandele, A., Jung, Y.W., Meng, H., Poholek, A.C., Parish, I.A., Rutishauser, R., Cui, W., Kleinstein, S.H., Craft, J., and Kaech, S.M. (2011). Differential expression of Ly6C and T-bet distinguish effector and memory Th1 CD4(+) cell properties during viral infection. Immunity *35*, 633–646.

Moser, K., Muehlinghaus, G., Manz, R., Mei, H., Voigt, C., Yoshida, T., Dörner, T., Hiepe, F., and Radbruch, A. (2006). Long-lived plasma cells in immunity and immunopathology. Immunol. Lett. *103*, 83–85.

Nakata, Y., Brignier, A.C., Jin, S., Shen, Y., Rudnick, S.I., Sugita, M., and Gewirtz, A.M. (2010). c-Myb, Menin, GATA-3, and MLL form a dynamic transcription complex that plays a pivotal role in human T helper type 2 cell development. Blood *116*, 1280–1290.

Peng, S.L., Szabo, S.J., and Glimcher, L.H. (2002). T-bet regulates IgG class switching and pathogenic autoantibody production. Proc. Natl. Acad. Sci. USA *99*, 5545–5550.

Rubtsova, K., Rubtsov, A.V., van Dyk, L.F., Kappler, J.W., and Marrack, P. (2013). T-box transcription factor T-bet, a key player in a unique type of B-cell activation essential for effective viral clearance. Proc. Natl. Acad. Sci. USA *110*, E3216–E3224.

Rubtsova, K., Rubtsov, A.V., Thurman, J.M., Mennona, J.M., Kappler, J.W., and Marrack, P. (2017). B cells expressing the transcription factor T-bet drive lupus-like autoimmunity. J. Clin. Invest. Published online February 27, 2017. http://dx.doi.org/10.1172/JCI91250.

Serre, K., Cunningham, A.F., Coughlan, R.E., Lino, A.C., Rot, A., Hub, E., Moser, K., Manz, R., Ferraro, A., Bird, R., et al. (2012). CD8 T cells induce T-bet-dependent migration toward CXCR3 ligands by differentiated B cells produced during responses to alum-protein vaccines. Blood *120*, 4552–4559.

Snapper, C.M., and Paul, W.E. (1987). Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science *236*, 944–947.

Stevens, T.L., Bossie, A., Sanders, V.M., Fernandez-Botran, R., Coffman, R.L., Mosmann, T.R., and Vitetta, E.S. (1988). Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature *334*, 255–258.

Tarlinton, D., and Good-Jacobson, K. (2013). Diversity among memory B cells: origin, consequences, and utility. Science *341*, 1205–1211.

Victora, G.D., and Nussenzweig, M.C. (2012). Germinal centers. Annu. Rev. Immunol. *30*, 429–457.

Wang, N.S., McHeyzer-Williams, L.J., Okitsu, S.L., Burris, T.P., Reiner, S.L., and McHeyzer-Williams, M.G. (2012). Divergent transcriptional programming of class-specific B cell memory by T-bet and RORα. Nat. Immunol. *13*, 604–611.

Xin, A., Masson, F., Liao, Y., Preston, S., Guan, T., Gloury, R., Olshansky, M., Lin, J.X., Li, P., Speed, T.P., et al. (2016). A molecular threshold for effector CD8(+) T cell differentiation controlled by transcription factors Blimp-1 and T-bet. Nat. Immunol. *17*, 422–432.

Yang, Z., Sullivan, B.M., and Allen, C.D. (2012). Fluorescent in vivo detection reveals that IgE(+) B cells are restrained by an intrinsic cell fate predisposition. Immunity *36*, 857–872.

Zotos, D., Coquet, J.M., Zhang, Y., Light, A., D'Costa, K., Kallies, A., Corcoran, L.M., Godfrey, D.I., Toellner, K.M., Smyth, M.J., et al. (2010). IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J. Exp. Med. *207*, 365–378.