Divergent transcriptional programming of class-specific B cell memory by T-bet and RORα

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Antibody class defines function in B cell immunity, but how class is propagated into B cell memory remains poorly understood. Here we demonstrate that memory B cell subsets unexpectedly diverged across antibody class through differences in the effects of major transcriptional regulators. Conditional genetic deletion of the gene encoding the transcription factor T-bet selectively blocked the formation and antigen-specific response of memory B cells expressing immunoglobulin G2a (IgG2a) in vivo. Cell-intrinsic expression of T-bet regulated expression of the transcription factor STAT1, steady-state cell survival and transcription of IgG2a-containing B cell antigen receptors (BCRs). In contrast, the transcription factor RORα and not T-bet was expressed in IgA+ memory B cells, with evidence that knockdown of RORα mRNA expression and chemical inhibition of transcriptional activity also resulted in lower survival and BCR expression of IgA+ memory B cells. Thus, divergent transcriptional regulators dynamically maintain subset integrity to promote specialized immune function in class-specific memory B cells.

Memory B cells are long-lived antigen-experienced B cells that typically express a high-affinity B cell antigen receptor (BCR), rapidly expand their populations and differentiate into plasma cells after antigen rechallenge1,2. Although memory B cells that express immunoglobulin M (IgM) have specialized functions3, many antigen-primed B cells switch to non-IgM isotypes under the antigen-specific regulation of follicular helper T cells4. Furthermore, non-IgM classes of membrane-bound antibody have different abilities to transduce signals through their BCR on the basis of the constant region expressed5,6. However, little is known about the molecular signals required for the survival, activation or differentiation of class-switched memory B cells. Several studies have delineated how cytokines affect transcriptional programs differently in naive B cells that culminate in class-switch recombination7,8. Interleukin 4 (IL-4) and interferon-γ (IFN-γ) reciprocally regulate class switching to IgG1 and IgG2a7, whereas the ability to express TGFβRII, the type II receptor for the cytokine TGF-β (transforming growth factor-β), in naïve B cells is required for switching to IgA8. However, it remains to be studied whether programmatic differences initiated at the time of class switching extend into memory B cell compartments to control longevity, cell fate and memory B cell function in an antibody class-specific manner.

The differentiation of effector cells of the immune system relies heavily on transcription factors that belong to the following three families: T-box, GATA and RORα10. Members of all three families share the ability to directly interact with chromatin-remodeling machinery to transactivate or repress gene targets in a cell context-dependent manner. Transcriptional regulators from each family induce molecular programs known to direct cells of the immune system into functional subsets11-13. T-bet, a member of the T-box family, has a critical role in inducing IFN-γ production by T helper type 1 (Th1) cells, natural killer (NK) cells and CD8+ cells to regulate antiviral immunity12. T-bet expression by naive B cells is sufficient to promote class switching to IgG2a and is required for IFN-γ-induced production of IgG2a in vivo14,15. In contrast, members of the GATA family are expressed by Th2 cells, basophils and mast cells and are crucial to IL-4 production and immunity to helminthes13. Although members of the ROR family are less well characterized, RORγt and RORαt are present in cells of the Th17 subset of helper T cells16 and are known to be involved in mucosal immunity to extracellular bacteria11. Although factors that belong to these families have been well characterized in several subsets of cells of the immune response, their roles in memory B cell development and the regulation of memory B cell function remain unknown.

Here we focus on IgG2a+ and IgA+ memory B cells and provide evidence for the divergent programming of memory B cell function by the major transcriptional regulators T-bet and RORα. Temporal deletion of T-bet in IgG2a+ memory B cells established a central and selective role for this regulator in the survival and antigen responsiveness of IgG2a+ memory B cells in vivo. Differences in the expression of cytokine receptors, integrins and RORαt highlighted the specialized development and unique properties of IgA+ memory B cells. Notably, a role for both T-bet and RORαt in persistent BCR transcription indicated both dynamic and ongoing class-specific requirements for each of these factors in IgG2a+ and IgA+ memory B cells, respectively. Thus, we propose that expression of any non-IgM

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antibody signifies a transcriptionally regulated commitment event across subcloned, class-specific memory B cells.

RESULTS

IgG2a memory formation requires T-bet in B cells

To look for class-specific transcriptional programs, we first focused on IgG2a+ memory B cells, given evidence that B cell–intrinsic expression of T-bet is sufficient to induce germline IgG2a transcripts15. Furthermore, IFN-γ provided by CD8+ T cells transferred from OT-I mice (which have transgenic expression of an ovalbumin-specific T cell antigen receptor) skew B cells toward IgG2a class switching in vivo in ways dependent on non-T cell expression of T-bet14. To address the B cell–intrinsic requirement for T-bet in IgG2a class switching more directly and in polyclonal mice, we used mice with germline deletion of the gene encoding T-bet (Tbx21−/−) mice17. These mice have defects in multiple cell types12, including defects in B cell–intrinsic expression of T-bet15. Consistent with those characteristics, Tbx21−/− mice had significantly fewer IgG3+, IgG2a− and IgG2b− class-switched (IgM−IgD−) CD38hi B cells (also CD19+B220+CD138+) than did wild-type mice with intact Tbx21+ B cells (Fig. 1a and Supplementary Fig. 1). Thus, the development of these putative memory B cell subsets was compromised in the absence of T-bet with no direct or indirect requirement for T-bet in IgG1 memory development.

To provide Tbx21−/+ B cells a source of wild-type polyclonal T cell help, we generated mixed peripheral chimeras deficient in components produced robust class-switched NP-specific B cell responses (Fig. 1c). Notably, the antigen-specific IgG2a B cell response was >95% lower (Fig. 1d) and extended across antigen-specific germinal-center (GC; CD38hi), memory (CD38lo) B cell (Supplementary Fig. 2a) and plasma-cell (CD138hi, Fig. 1e) compartments in the B cell–specific absence of T-bet. In contrast, slightly higher IgG1+ NP-specific B cell responses were induced in both donor populations (Fig. 1d) with similar distribution into GC, memory B cell (Supplementary Fig. 2a) and plasma-cell (Fig. 1e) compartments. We found no defects in IgG3+ or IgG2b+ NP-specific B cell compartments in the absence of T-bet (Supplementary Fig. 2b). Furthermore, total class-switched IgG2a B cells, which would contain the KLH-specific response and polyclonal reactivities in the peripheral chimeras, showed a similar selective defect in IgG2a, with numbers in IgG1, IgG3 and IgG2b B cell compartments equivalent to those of wild-type mice (Supplementary Fig. 3). Therefore, B cell–intrinsic expression of T-bet was required selectively for class switching to IgG2a and/or the survival of IgG2a+ B cells after class switching in vivo.

Survival and function of IgG2a+ memory B cells require T-bet

To enable analysis of T-bet expression in IgG2a+ B cells after class switching, we crossed mice with loxP-flanked Tbx21 alleles (Tbx21F/F mice)18 to C57BL/6 mice with tamoxifen-inducible sequence encoding Cre recombinase in the ubiquitously expressed Rosa26 locus (Rosa26CreERT2 mice). Treatment of the intact progeny (called ‘CreERT2’ mice)19) with 4-OHT induced the loss of T-bet protein in >50% of T-bet-expressing splenocytes in IgG2a+ B cells (Supplementary Fig. 2a). Treated mice had a similarly lower number of IgG2a+ B cells and/or the survival of IgG2a+ B cells after class switching in vivo.

As temporal deletion with 4-OHT in vivo targets all cells, we sought to determine whether B cell–specific loss of T-bet had caused the
selective IgG2a deficiency. In the next strategy, we induced an NP-specific B cell response in CreERT2 Tbx21+/F/F and CreERT2 Tbx21+/mice given daily injection of 4-OHT for 3 d, followed by collection of spleens on day 4, assessed after the use of a forward- and side-scatter lymphocyte gate. Middle and right, total T-bet+ splenocytes (middle) and IgG2a+ and IgG1+ B cells (right) in the treated mice at left. *P < 0.05 (Mann-Whitney test). (b) T-bet expression in B220+CD38+IgG2a+ B cells (IgG2a+; Gr-1−CD4−CD8−IgM− IgD−CD19+CD138+) or naive B cells (Naive; Gr-1−CD4−CD8−IgM+IgD+CD 19+B220+) from the treated CreERT2 Tbx21+/F/F mice in a. (c) Splenic NP gate (outlined) of IgM−IgD− B cells (Gr-1−CD4−CD8−, CD19+ or CD138+) or naive B cells (Naive; Gr-1−CD4−CD8−IgM+IgD+) from CreERT2 Tbx21+/mice 14 d after immunization with NP-KLH, before transfer into Rag1−/− recipient mice (in d). (d) Expression of CD138 and B220 (middle) on NP-specific cells (left) obtained from Rag1−/− recipient mice 7 d after transfer of a mixture of the spleen cells in c treated for 1 h in vitro with 4-OHT and mixed at a ratio of 1:1 with spleen cells from C57BL/6 mice (also 14 d after immunization with NP-KLH) for transfer, followed by boosting of recipients with soluble NP-KLH. Numbers in outlined areas indicate percent NP-specific IgM−IgD− cells (left) or CD138−B220+ cells (middle). Right, total NP-specific B220+ B cells from mice as in c (F/F) and from a separate group of Rag1−/− recipient mice together with T-bet-sufficient splenocytes (WT) mice and CreERT2 Tbx21+/mice given daily injection of 4-OHT for 3 d, followed by collection of spleens on day 4, assessed after the use of a forward- and side-scatter lymphocyte gate. Middle and right, total T-bet+ splenocytes (middle) and IgG2a+ and IgG1+ B cells (right) in the treated mice at left. *P < 0.05 (Mann-Whitney test). (f) Total NP-specific plasma cells (CD138+) after the transfer in d. *P < 0.05 (Mann-Whitney test). Data are representative of two experiments with two mice per genotype per experiment (a,b) or two experiments with three and two Rag1−/− recipient mice per group per experiment (c-f; mean and s.e.m.).

**Figure 3** T-bet expression during antigen-specific IgG2a memory development. (a) NP-specific B cell response at day 7 in lymph nodes of C57BL/6 mice immunized with adjuvant only or NP-KLH plus adjuvant, gated on Gr-1−CD4−CD8− cells, CD138+ or CD19+ B cells, and IgM−IgD− cells (left). Right, time course of the abundance of total NP-specific B cells before transfer (0), after immunization with adjuvant only (Adj) or during the primary and memory (Recall) response in mice immunized as at left with NP-KLH plus adjuvant. (b) NP-specific B cell subsets (left) and B220 expression (right) of CD38+ and IgG2a+ cells at day 7 of the response in mice immunized with NP-KLH plus adjuvant. (c) Expression of Bcl-6 mRNA in naive (IgM−IgD−CD23+/−) cells (N) at day 0 and in NP-specific CD19+B220+IgG2a+ pre-GC (CD38+/−) cells at day 7 (Pre, GC (CD38+)) cells at days 7 and 14, and memory (CD38+) cells at day 14 (M) and memory recall (CD38+) cells (Recall) at days 5 and 14 of the response (left), and Blimp-1 mRNA in IgG2a+CD138+ NP-specific plasma cells from mice immunized with NP-KLH plus adjuvant (right). (d) T-bet expression by antigen-responsive Tfh cells and populations as in c (left) and by IgG2a+ plasma cells (CD138+; right). Numbers along horizontal axes (a,c,d) indicate time in days; mRNA expression (c,d) is presented relative to that of T-bet in naive B cells, set as 1. Data are representative of at least four experiments with two groups of two to three mice at each time point (mean and s.e.m. in a,c,d).
Thus, IgG2a+ memory B cells selectively required B cell–intrinsic expression of T-bet to respond effectively to antigen rechallenge in vivo.

T-bet persists during the development of IgG2a+ memory B cells

Next we assessed changes in Tbx21 expression over the course of a primary and memory response in mice immunized with NP-KLH19,20 (Fig. 3a). Differences in the expression of CD138, B220 and CD38 on class-switched (IgM–IgD–) antigen-binding (NP+) CD19+ B cells provided direct access to IgG2a+ B cells (Fig. 3b). We used expression of the transcription factors Bcl-6 (ref. 21) and Blimp-1 (ref. 22), together with that of antibody-isotype and phenotypic markers, to further distinguish the pre-GC stage (day 7; B220+CD38hiBcl-6lo), GC stage (days 7 and 14, primary; B220hiCD38hiBcl-6hi), memory stage (day 14, primary; days 5 and 14, secondary; B220hiCD38hiBc1-6hi) and plasma-cell stage (CD138+Blimp-1hi) of the antigen-specific development of IgG2a+ memory B cells (Fig. 3c). As anticipated, primary antigen-responsive IgG2a+ B cells had abundant expression of T-bet before entry into GCs, which decreased substantially in the presence of Bcl-6 in GCs at days 7 and 14 after priming (Fig. 3d). The immunization used here, based on an agonist of Toll-like receptor 4, promoted negligible IgM+ memory B cells, with most NP-specific memory B cells expressing immunoglobulins of the IgG isotype (data not shown). After antigen rechallenge, NP-specific IgG2a+ memory B cells had higher expression of T-bet that remained high for at least 14 d after recall (Fig. 3d, right). In the presence of Blimp-1, IgG2a+ plasma cells from all stages of the primary and memory response had low but detectable expression of T-bet (Fig. 3d, left). Thus, T-bet was expressed early after the initiation of class switching to IgG2a and continued to be expressed at all stages of antigen-specific IgG2a+ memory B cell development and responses in vivo.

T-bet activity in IgG2a+ memory B cells

As evidence of T-bet activity in antigen-specific IgG2a+ CD38hi memory B cells, transcription of a series of genes known to be targets of T-bet23 was higher at day 5 of the memory response in C57BL/6 wild-type mice immunized with adjuvant and NP-KLH (Fig. 4a, top). The differences in the expression of these target genes indicated that T-bet enabled separate functions in IgG2a+ memory B cells and naive B cells. Studies have shown that Bcl-6 can directly bind to T-bet and repress the expression of T-bet target genes in T cells24. To determine whether a similar event occurs in B cells, we assayed those same T-bet target genes in GC B cells with a greater abundance of Bcl-6 and found that their expression was lower in those cells than in memory B cells (Fig. 4a, bottom). Furthermore, Blimp-1 has been shown to directly antagonize T-bet expression25. Plasma cells with a greater abundance of Blimp-1 protein had lower expression of most of the T-bet target genes assessed than did memory B cells or GC B cells (Fig. 4b). Although T-bet activity was present in IgG2a+ memory B cells, expression of Bcl-6 or Blimp-1 resulted in much less T-bet activity in IgG2a+ GC B cells or IgG2a+ plasma cells, respectively.

The BCR-driven IgG2a response requires T-bet expression

As IgM+ memory B cells were not actively excluded from the experiments described above, we purified IgG2a+ B cells from unimmunized CreERT2 Tbx21F/F mice and treated the cells with 4-OHTin vitro. We transfected those treated IgG2a+ memory B cells into Rag1−/− recipients and reactivated them through their BCR with antibody to IgG2a (anti-IgG2a). This in vivo stimulus induced robust production of IgG2a+ plasma cells with negligible numbers of CD19+ non-plasma cells remaining 4 d after transfer (Fig. 5a). Nevertheless, after treatment with 4-OHT, there were 75% fewer IgG2a+ plasma cells with no expansion of the residual CD19+ B cell compartment. Although cell recovery was low, we noted similar trends in a second series of experiments after sorting NP-specific memory B cells from immunized CreERT2 Tbx21F/F mice, treating the cells with 4-OHTin vitro, transferring the cells into Rag1−/− recipient mice and rechallenging the recipient mice with antigen (Supplementary Fig. 5). Furthermore, we noted IgG2a-selective defects in total class-switched B cells with this experimental design (data not shown).
Thus, B cell–intrinsic T-bet was required for the differentiation of plasma cells driven by the IgG2a-containing BCR in vivo.

Transcription factor STAT1, survival and IgG2a require T-bet

To examine how T-bet exerted its effect on the fate of IgG2a+ B cells, we used IgG1+ and IgG2a+ memory B cells from wild-type or CreER T2 Tbx21 F/F mice for in vitro studies. Deletion of Tbx21 with 4-OHT in vitro resulted in a significant loss of transcription of the gene encoding STAT1 (the signal transducer of IFN-γ) selectively in IgG2a+ B cells and not IgG1+ B cells, on a per-cell basis (Fig. 5b, left). In the absence of T-bet, there was also a small but significant effect on cell survival in short-term cultures in the presence of the transcription factor BAFF, with a loss of IgG2a+ B cells and more IgG1+ B cells (Fig. 5b, middle). However, over the same period, there was a loss of >75% in mature transcripts encoding the IgG2a constant region on a per-cell basis (Fig. 5b, right). Secondary culture on plates coated with anti-BCR induced a significant number of B cell blasts over 48 h in vitro, whereas IgG2a+ B cells remained small, as assessed by forward scatter, in the absence of T-bet (Fig. 5c). We selected small live cells for quantitative PCR analysis of mature transcripts encoding the IgG2a constant region and detected exaggerated losses in the absence of T-bet (Fig. 5c, right). Thus, in the absence of T-bet in vitro, downregulation of the IgG2a-containing BCR and loss of STAT1 transcription were more pronounced than was the overall loss of IgG2a+ memory B cells.

Next we investigated the IgG2a+ B cell compartment in a mouse strain with a phosphorylation defect that prevents translocation of STAT1 to the nucleus (the ‘Domino’ mutation of Stat1, induced by N-ethyl-N-nitrosourea)20. Although T-bet expression was 50% lower in those mice than in wild-type mice, there was an almost complete loss of IgG2a+ B cells (Fig. 5d) with a compensatory greater abundance of IgG1+ B cells in these mice (Fig. 5e, left). Both trends become

Figure 6 Separable programs for IgG2a+ and IgA+ memory B cells.

(a) Frequency of CD38+ and CD38− IgG2a+ B cells from spleen (left) and IgA+ B cells from Peyer’s patches (right) of unimmunized C57BL/6 mice, gated as Gr-1−CD4−CD8−CD138−, B cell–positive (CD19+ or CD138+), switched IgM+IgD− and class-specific CD138−B220−B cells. (b) Staining of the proliferation marker Ki67 on memory (Mem; CD38+), GC (CD38−) B cells and naive B cells (CD38−). (c) Expression of mRNA for cytokine receptors (top) or signal-transduction molecules (bottom) by the IgG2a+ or IgA+ splenic B cells in a, presented relative to that of IgG2a+ B cells. (d) Surface staining of CXCR3 (left) and α4β7 (right) on IgG2a+ (G2a), IgG1+ (G1), IgA+ (A) or naive splenic B cells. **P < 0.001 (unpaired t-test). Data are representative of three experiments with two mice per genotype in each (a), two experiments with three mice per genotype (b,d,e) or three experiments with two mice, two mice and one mouse per genotype per experiment (d,e; mean and s.e.m.).

*P < 0.05 (unpaired t-test).
exaggerated after adoptive transfer of spleen cells from mice with the Domino mutation of Stat1 into Rag1−/− mice without immunization (Fig. 5e, right). Thus, both T-bet expression and STAT1 activation seemed necessary for continued expression of the IgA2a-containing BCR and/or survival of IgG2a+ memory B cells in vivo.

Separable programs for IgA+ and IgG2a+ memory B cells

IgA has a dominant role at mucosal surfaces, binding to a variety of cells of the innate immune response, enhancing phagocytosis and triggering the local release of cytokines and inflammatory mediators27,28. Expression of TGFβRII by B cells is required for class switching to IgA3, with some evidence for the involvement of T-bet in the contribution of mucosal IgA responses29. Transcription factors of the Runx and Smad families have been shown to promote IgA-specific germline transcripts as precursors for class switching to IgA8,30. To compare the transcriptional programs of IgG2a+ versus IgA+ memory B cells, we isolated isotype-switched (IgM+IgD−) CD38+ B cells (also CD19+CD138−B220+ that were largely Ki67− (noncycling) as quiescent memory B cell compartments from spleen or Peyer’s patches (Fig. 6a,b). IgG2a+ memory B cells had higher expression of the interferon receptors IFN-γR1 and IFN-γR1 than did IgA+ memory B cells. In contrast, IgA+ memory B cells had higher expression of the interleukin receptors IL-17Rc and IL-22Rc than did IgG2a+ memory B cells, which suggested differences in the responsiveness of these cells to IL-17 and IL-22 (Fig. 6c). Furthermore, whereas both memory subsets had similar expression of TGFβR, IgA+ memory B cells had higher expression of TGFβ-signaling intermediates of the Smad family (Smad2, Smad3 and Smad4) and the TGF-β-associated adaptor Daxx (Fig. 6c). IgG2a+ memory B cells had the highest expression of the chemokine receptor CCR3, whereas most IgA+ memory B cells had the highest expression of integrin αβ3 (Fig. 6d). These data suggested that IgG2a+ memory B cells and IgA+ memory B cells had separable growth factor requirements, signaling propensity and tissue-homing potential (Supplementary Fig. 6).

Survival and BCR expression in IgA+ memory cells require RORγt

Among the main transcription factors downstream of TGF-β and IL-17 signaling, RORγt had higher expression in IgA+ memory cells than in other memory and naive B cells (Fig. 7a). GATA-3, Foxp3 and RORγt were detectable only in small amounts in naive B cells and IgG1+, IgG2a+ and IgA+ memory B cells. Germline deletion of the gene encoding RORγt in mice produces a complex phenotype with neurological defects and early death31. Hence, to probe the function of RORγt in IgA+ memory B cells, we silenced RORγt expression in normal IgA+ B cells through the use of small interfering RNA (siRNA) targeting RORγt. We transfected whole mesenteric lymph nodes and Peyer’s patch cells with RORγt-specific siRNA by nucleofection, which resulted in ~50% less RORγt mRNA after 4 d of culture in vitro than that in cells transfected with control siRNA (Fig. 7b, left). This degree of RORγt knockdown resulted in ~50% less mature IgA mRNA in the total cell population (Fig. 7c). IgA+ memory B cells isolated from these cultures had >80% lower mature IgA BCR transcripts on a per-cell basis than did those transfected with control siRNA (Fig. 7b). Thus, we found that IgA+ B cells expressed RORγt, along with evidence that it controlled dynamic BCR expression.

To interfere with the function of ROR proteins, we used SR1001, which inhibits the transcriptional activity of RORγt and RORα32. The presence of this functional inhibitor resulted in selectively fewer IgA+ memory B cells that survived in culture for 6 d, without affecting IgG1+ B cells (Fig. 7c). To establish the influence of this inhibitor on IgA+ memory B cells, we treated purified IgA+ memory B cells with SR1001 over short-term culture. As this drug inhibits only protein function, there was no significant effect on the expression of RORγt mRNA (Fig. 7d, right). However, there was significantly less transcription of the known RORγt target genes encoding the inositol triphosphate receptor ITPR1 and the transcription factor HIF-1α. Notably, there was a significantly lower abundance of mature transcripts encoding the IgA-containing BCR per cell (Fig. 7d). Thus, we found that RORαt affected the function of IgA+ memory B cells, along with evidence that it was required for the survival of IgA+ B cells and control of transcription of mRNA encoding the IgA-containing BCR.

DISCUSSION

Antigen-specific B cell memory is central to long-term immunoprotection and develops across many different antibody classes. Here we focused on unique properties of IgG2a+ and IgA+ memory B cells to demonstrate that divergent transcriptional regulators maintain class-specific integrity of memory B cell subsets. Both T-bet and RORγt regulated the transcription of mature BCRs, which seemed necessary for the survival of IgG2a+ and IgA+ memory B cells, respectively. In addition, we used conditional genetic models with temporal deletion of T-bet to demonstrate defects in the ability of IgG2a+ memory B cells to respond to antigen. Both IgG2a+
and IgA⁺ memory B cells expressed molecules of different transcriptional programs that reflected separate potentials for cytokine secretion, trafficking and survival that permitted flexibility in long-term antigen-specific immunoprotection. We propose that the events that lead to class switching after antigen experience lead to the ‘imprinting’ of molecular programs whose persistence is essential for maintaining the identity of memory B cell subsets.

The dynamic regulation of BCR transcription was a common component of both the RORα and T-bet programs. We found consensus binding sites for both transcription factors in the immunoglobulin 3’ regulatory region known to control class switching and antibody secretion35. Thus, separable T-bet- and RORα-dependent transcriptional programs in IgG2a⁺ and IgA⁺ memory B cells also controlled the central recognition properties of antigen-specific B cell memory.

Cognate follicular helper T cells (Tfh cells) regulate many facets of the development of antigen-specific memory B cells4. Bcl-6 programs the development of Tfh cells and their movement to B cell areas for cognate control of B cell immunity4-5. Cytokine production occurs in those follicular regions after contact with antigen-primed B cells in ways that can initiate antibody class switching35. In GCs, contact with Tfh cells regulates the clonal composition of GC B cells and export into B cell memory compartments37. In GC B cells, Bcl-6 can antagonize the function of T-bet, and this dynamic molecular interaction may underpin the fate of GC B cells and the development of IgG2a⁺ memory B cells. There is evidence of cytokine-producing GC Tfh cells that engage GC B cells in an antibody class-specific manner35. In this scenario, antibody class-specific regulation by cognate Tfh cells may streamline affinity maturation and efficiently promote the development of memory B cells.

T-bet regulates critical functions in IgG2a⁺ memory B cells. In naive T cells, signaling through the antigen receptor acts in synergy with IFN-γ-induced activation of STAT1 to promote initial T-bet expression15. We found a similar regulatory axis dependent on IFN-γ, STAT1 and T-bet in IgG2a⁺ memory B cells. Ablating T-bet expression interfered with this program, which resulted in downregulation of BCR transcription and loss of IgG2a⁺ memory B cells. Although the binding of antigens by memory BCRs is not required for survival38, we propose that memory B cells rely on tonic signaling through the BCR for survival, as do naive B cells39. Similar to antigen-specific plasma cells40, memory B cells may also retain antigen-presenting ability for extended periods in vivo. In this manner, continued BCR expression may also indirectly permit ongoing local contact with antigen-specific Tfh cells40.

T-bet activity establishes a molecular framework that governs the functions of IgG2a⁺ B cells. Although T-bet binds to target promoters regardless of cell type, its ability to transactivate and remodel loci is dependent on cell context23. The regulation of genes by T-bet is also coupled to the dynamic expression of its molecular antagonists Bcl-6 and Blimp-1 (refs. 24, 25). Notably, T-bet can arrest asymetrically in T cells at cell division41, and Bcl-6 has been shown to arrest unevenly across GC B cells42. Our studies have shown that coexpression of Bcl-6 with T-bet in GC B cells resulted in repression of the transcription of T-bet targets. However, rather than abolishing a T-bet-defined subset, Bcl-6 transiently and reversibly altered programming of IgG2a to allow productive GC activity and the development of IgG2a⁺ memory B cells.

RORα, like T-bet, is able to recruit chromatin-remodeling machinery and directly transactivate loci11. However, unlike the interactions of T-bet, the interactions of RORα with Bcl-6 and Blimp-1 have not been characterized. Although some studies have indicated Bcl-6 represses RORγt and Tfh17 differentiation33,44, its cell-intrinsic effects on RORα remain unknown. Expression of RORα is required during early development41; hence, mice deficient in RORα have severe developmental defects and die early. Studies of RORα targets in cells of the immune system have provided connections to the activation of IL-17, IL-17F, IL-22 and the receptor IL-23R (ref. 45), and the repression of IL-6 and tumor necrosis factor46. It is unknown whether activation of these targets is direct or indirect, and we found no difference in the expression of these targets in IgA⁺ memory B cells (data not shown). However, RORα is involved in several other pathways, including calcium signaling, circadian rhythm and cellular metabolism41, and there is evidence of an effect of SR1001 on ITPR1 and HIF-1α that might indicate other pathways in which IgA⁺ memory B cells may be unique relative to other classes.

Antigen-specific memory B cell responses may also require sub-specialized regulatory programs organized by antibody class. After antigen rechallenge, memory B cells need cognate T cell help to expand their populations and differentiate into plasma cells47. IgM⁺ memory B cells have functions and an ability to respond that are different from those of their IgG⁺ counterparts34,48. Downstream antibody classes engage signaling pathways different from those engaged by IgM based on expression of the constant region of the BCR5,6. Other class-specific memory B cell properties control migration; for example, T-bet drives IgG2a⁺ memory B cells to inflammatory sites via CXCRI expression, and the integrin α4β1 guides IgA⁺ memory cells to mucosal tissues27. Enhanced transcription of STAT1 and IFN-γ may uniquely sensitize IgG2a⁺ memory B cells to IFN-γ signals. Similarly, RORα can enhance the sensitivity of cells to calcium49, and the calcium-dependent kinase CamKIV can enhance RORα expression30. Those types of changes in IgA⁺ memory B cells may lower BCR-activation thresholds in a class-specific manner. Therefore, many attributes of class-specific B cell memory that are introduced after development may be further reinforced after antigen recall under the cognate guidance of memory Tfh cells40.

Memory B cells are generally considered functionally equivalent, differing only by the antibody isotype they express. Here we have unexpectedly shown that B cell memory was organized in class-specific subsets, each with separate central transcriptional regulators. Specifically, the transcriptional regulators T-bet and RORα controlled divergent IgG2a⁺ memory B cell subsets and IgA⁺ memory B cell subsets, respectively, to control separate functions in these unique class-specific memory B cell compartments. T-bet is used by many cell types in response to inflammatory stimuli, with a focus on the clearance of intracellular pathogen12. We have now shown that IgG2a⁺ B cell memory also relied selectively on a T-bet-dependent program to establish and maintain subset integrity. Similarly, IgA⁺ B cell memory is specialized to protect the mucosal surfaces27, and the selective use of transcriptional regulator RORα enhances this unique memory B cell function. Notably, these unique developmental programs can be exploited for directed immunotherapeutic applications, the future formation of class-skewing vaccines, and the treatment of cancer and autoimmunity.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
N.S.W., L.J.M.-W. and M.G.-W. conceived of and designed the project; N.S.W. did all experiments and analyzed the data for all experiments; L.J.M.-W. identified T-bet in memory B cells and contributed to the preparation of the manuscript; S.I.O. contributed to experimental design; S.I.R. provided Tbx21KO mice; T.P.B. provided SR1001; and N.S.W. and M.G.-W. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Animals. Tbx21 Fl mice (Jackson Laboratories), C57BL/6 (B6) mice, Rag1 Fl mice, B6.CD45.1 mice, Tbx21 Fl mice, mice with the Domino mutation of Stat1 and CreERT2 Tbx21 Fl mice were housed in pathogen-free conditions. All experiments were done in compliance with federal laws and institutional guidelines as approved by The Scripps Research Institutional Animal Care and Use Committee. CreERT2 Tbx21 Fl mice were generated by crossing of Tbx21 Fl mice with Rosa26-CreERT2 mice (Jackson Laboratories). For genotyping of the mutant mice, the following primers were used at a final concentration of 0.4 μM in a standard FastStart Reaction Mix according to the manufacturer's guidelines (Roche): for B6.129S6-Tbx21tm1Gliw/J (Tbx21 Fl) mice, oIMR1719 (common; 5′-TGGCGATACAGGAGGACCAAAAT A-3′), oIMR1718 (wild-type; 5′-GACTGAGCGCCCGCCACCTCTCTT AAG-3′) and oIMR1717 (mutant; 5′-GGCCGAGGCCCCACCCAGG ACGGAG-3′); and for B6.129-Gf(ROSA)26Sor tm1(creERT2)Tyj/J (Rosa26CreERT2) mice, oIMR8545 (common; 5′-AAAGTGCCTCTGATGTGTT AT-3′), oIMR8546 (wild-type reverse; 5′-GGAGCGGAGAATAGGATA TG-3′) and oIMR8547 (mutant reverse; 5′-CTCTGATCTCGGCAAT TGC-3′). For the detection of the unexcised locus in Tbx21 Fl mice and cells, primers A (sense; 5′-TATGGATTACCTGACGCTTCTCAG-3′) and B (antisense; 5′-CAGGATGGACAAATCCGTGGT-3′) were used, and for detection of deletions in the locus, ΔF (sense; 5′-AGGCCATC TCTCCAGCTCA-3′) and C2 (antisense; 5′-CTCTCGCCCTCCATC TTCAGGAC-3′) were used for amplification.

Flow cytometry. Draining lymph nodes and spleen were removed from unimmunized or immunized mice and single-cell suspensions were prepared in PBS with 5% (vol/vol) FBS. Cells (4 × 10^6 per ml) were incubated for 15 min with anti-CD16-32 (Fc block; 2.4G2) followed for 45 min at 4 °C with the following fluorochrome- or biotin-labeled monoclonal antibodies: allophyocyanin-conjugated anti-CD138 (281-2), allophycocyanin-iodotricarbocyanine–conjugated anti-CD19 (1D3), phycoerythrin-conjugated anti-CD138 (281-2), phycoerythrin–Texas red–conjugated anti-B220 (RA3-6B2), fluorescein isothiocyanate–conjugated anti-IgG1 (A85-1), anti-IgG3 (R40-8L), Horizon V500–conjugated anti-CD8 (53-6.7), Horizon V500–conjugated anti-CD4 (Rm4-5) and biotin-conjugated anti-IgG2a (5.7; all from BD Biosciences); phycoerythrin–conjugated anti-T-bet (ebio4B10), phycoerythrin–iodotricarbocyanine–conjugated anti-CD45.1 (A20), phycoerythrin–iodotricarbocyanine–conjugated anti-CXCR3 (CXCR3-173), fluorescein isothiocyanate–conjugated anti-CD45.2 (104), biotin-conjugated anti-IgG2b (RMG2-1), biotin-conjugated anti-CD45.2 (104) and biotin-conjugated anti-IgA (RMA-1; all from Biolegend); Alexa Fluor 700–conjugated anti-CD38 (90) and phycoerythrin–iodotricarbocyanine–conjugated anti-Gr-1 (Ly6C/G; RB6C5; both from eBiosciences); and allophycocyanin–conjugated–hiden, phycoerythrin–conjugated–hiden, peridinin–conjugated anti-Gr-1 (Ly6G/C; RB6C5; both from eBiosciences) and phycoerythrin–indodicarbocyanine–conjugated anti-CD19 (Rm4-5) were used for amplification.

Quantitative PCR. Cells (5 × 10^4) were sorted directly into lysis buffer (Qiagen) and mRNA was purified with RNeasy Kit (Qiagen), then cDNA was prepared as described5 with the First-Strand Synthesis System for RT-PCR (Invitrogen) with random hexamers. Platinum SYBR Green Supermix UDG reaction mix (Invitrogen) and the StepOnePlus Real-time PCR system were used for SYBR Green quantitative PCR, followed by analysis with Step One software (Applied Biosystems). Primers (Supplementary Table 1) were used at a final concentration of 0.25 mM. Gapdh (encoding glyceraldehyde phosphate dehydrogenase) was used as an endogenous control. For measurement of T-bet expression, results from naive B cells were assigned a value of 1 and relative expression was assigned accordingly. For measurement of STAT1 and IgG2a mRNA after switching, results from cells treated with 4-OHT were assigned a value of 1 and relative expression was assigned accordingly. For analysis of treatment with RORγt–specific siRNA or SR1001, results from cells treated with control siRNA or vehicle were assigned a value of 1 and relative expression was assigned accordingly. For single-cell quantitative PCR analysis of mRNA, single cells were directly sorted into 2x Reaction buffer containing SSIII RT Platinum Taq (from the SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase Kit; Invitrogen) plus outside primers for genes encoding IFN-γ, T-bet and GAPDH, at a final concentration of 0.25 μM. The 96-well plates were incubated in a thermocycler for 15 min at 50 °C, followed by 2 min at 95 °C, then for 22 cycles of 15 s at 95 °C followed by 4 min at 60 °C; 1 μl of product was used for the standard quantitative PCR described above.

Adaptive transfer. Draining lymph nodes and spleen were removed from unimmunized or immunized mice and single-cell suspensions were prepared in PBS. For the generation of chimeras with germline deletion of specific genes in peripheral cells, 3.5 × 10^7 cells from Tbx21 Fl mice or mice with the Domino mutation of Stat1 and B6.CD45.1 mice were mixed at a ratio of 1:1 (7 × 10^7 cells total) and were injected intraperitoneally into Rag1 Fl hosts. Mice were then immunized subcutaneously at the base of the tail with 400 μg NP-KLH in adjuvant based on monophosphoryl lipid A (MPL). For the generation of chimeras with conditional deletion of genes in the periphery, splenocytes from CreERT2 Tbx21 Fl mice, CreERT2 Tbx21 Fl mice and B6.CD45.1 mice were treated with 4-hydroxytamoxifen (4-OHT) (Sigma). Unfractionated CreERT2 Tbx21 Fl or CreERT2 Tbx21 Fl splenocytes (1 × 10^7) were treated for 1 h in 4-OHT-containing media (RPMI medium with 10% FBS, 2 mM l-glutamine and 1 μM 4-OHT), then were filtered and mixed at a ratio of 1:1 with 1 × 10^7 cells from B6.CD45.1 mice (2 x 10^7 total) and transferred by intraperitoneal injection into Rag1 Fl host mice. Mice were immunized 1 day later with 400 μg NP-KLH in PBS. For experiments with depletion of plasma cells and naive B cells, cells were purified by flow cytometry, and 1 × 10^6 cells from CreERT2 Tbx21 Fl mice were treated for 1 h with 4-OHT or vehicle and transferred by intraperitoneal injection into Rag1 Fl host mice. For transfer of IgG2a Fl B cells, cells were purified by flow cytometry, and 1 × 10^6 cells from CreERT2 Tbx21 Fl mice were treated for 1 h with 4-OHT or vehicle and transferred by intraperitoneal injection into Rag1 Fl host mice. Rag1 Fl hosts were given intraperitoneal injection of 75 μg anti-IgG2a (R19-15, BD Biosciences) 1 day after transfer of cells.

Immunizations. Mice were immunized subcutaneously at the base of the tail with 400 μg NP-KLH (4-hydroxy-3-nitropheny lacetyl (Biosearch) conjugated to keyhole limpet hemocyanin (Pierce)) in MPL–based adjuvant supplemented with trehalose di mycolate (1 mg per 1 mg MPL; Sigma). Antigen rechallenge was done 120 d or more after priming with 400 μg NP-KLH in MPL–based adjuvant. Soluble boost was done by mixture of 400 μg NP-KLH in PBS.

Treatment of mice with 4-OHT. After 4-OHT was dissolved in 100% ethanol at a final concentration of 20 mg/ml, it was injected intraperitoneally into unimmunized CreERT2 Fl mice or CreERT2 Tbx21 Fl mice at a dose of 0.5 mg per mouse for the first injection and 0.25 mg/ml for the two subsequent injections.

Cell culture. IgG2a Fl or IgG1 Fl cells were purified with a FACS Aria III (BD). For survival studies, 8 × 10^6 cells were placed in medium (DMEM with 10% FBS, 2 mM l-glutamine and 1 μM 4-OHT) and treated for 48 h with 200 ng/ml BAFF (R&D systems), and total cells were counted with a FACS Aria III. For gene-expression analysis, 2 × 10^6 cells were placed in medium (DMEM with 10% FBS, 2 mM l-glutamine, 50 μM β-mercaptoethanol and 1 μM 4-OHT) and treated for 48 h with 200 ng/ml BAFF (R&D systems) and live (FITC) cells were sorted directly into lysis buffer and processed as described above. For BCR-stimulation studies, 2 × 10^6 cells were placed in medium (DMEM with 10% FBS, 2 mM l-glutamine and 1 μM 4-OHT) and treated for 48 h with 200 ng/ml BAFF (R&D systems), and then were transferred into plates coated with biotin–conjugated anti-IgG2a (5.7; BD Biosciences) or biotin–conjugated anti-IgG1 (R MG1-1; Biolegend) and supplemented with an additional 200 ng/ml of BAFF, followed by culture overnight at 4 °C. Live (FITC) cells
were sorted directly into lysis buffer 48 h after transfer to coated plates and processed as described above for gene-expression analysis.

**Analysis with siRNA.** For siRNA experiments, Peyer’s patches and mesenteric lymph nodes were homogenized in medium (DMEM with 10% FBS, 2 mM l-glutamine and 50 μM β-mercaptoethanol) from unimmunized C57BL/6 mice. Cells (3 × 10^6) were transfected with an Amaxa Mouse B cell Nucleofector Kit according to the manufacturer’s protocol (Lonza). Each well was transfected by nucleofection with 300 pmol Silencer Select Pre-designed siRNA directed against RORα (Ambion) or 300 pmol control siRNA with scrambled sequence. Cells were collected whole or were sorted to purity 4 d after transfection by nucleofection, and mRNA was isolated as above.

**SR1001 treatment.** SR1001 was dissolved in 10% DMSO, 10% Tween-80 and 80% water. Whole splenocytes were homogenized and 2 × 10^7 cells were treated for 6 d with 5 μM SR1001 in medium (DMEM with 10% FBS, 2 mM l-glutamine and 50 μM β-mercaptoethanol). For IgA memory cultures, Peyer’s patches and mesenteric lymph nodes were homogenized, and IgA^+ memory cells were purified by flow cytometry and cultured for 24 h with medium and 5 μM SR1001. Live cells were collected and mRNA was obtained and analyzed as described above.

**Statistics.** Mean values, standard error of the mean, unpaired t-tests and Mann-Whitney tests were calculated with Prism software (GraphPad). A P value of less than 0.05 was considered statistically significant.