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**TITLE:**
B-Cell Activation and Tolerance Mediated by B-Cell Receptor, Toll-Like Receptor, and Survival Signal Crosstalk in SLE Pathogenesis

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**14. ABSTRACT**
We previously found that B cell receptor (BCR)-delivered TLR9 agonists initiate a response involving proliferation followed by abrupt cell death; furthermore, responding cells are rescued by survival cytokines. We posited this as a normal immune response-limiting mechanism that, if thwarted, may lead to persistence of self-reactive antibody-secreting cells. In this proposal we sought to characterize the pathways leading to post-proliferative death and rescue, and to determine how different forms of rescue lead to alternative differentiation outcomes. During the first year we showed that in the context of BCR-delivered TLR9 signals, IL-21 promotes and IL-4 opposes the T-bet+CD11c+ B cell fate. In the second and third years, we extended these findings to show that IFN-gamma also promotes the Tbet+ fate, and that B cells with this phenotype are antigen-experienced cells that emerge in normal responses to viral infections as well as in autoimmune scenarios. During a six-month no-coast extension, we completed signaling pathway analyses and analyzed B cells from additional SLE patients. We have forwarded a theoretical framework to explain the link between these activation requisites and humoral autoimmunity.

**15. SUBJECT TERMS**
regulation of B cell responses; TLR7/9 agonists

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**1. INTRODUCTION**: This grant was based on our observation that BCR-delivered TLR9 agonists initiate a self-limiting response involving proliferation and differentiation, followed by abrupt cell death, but that B cells can be rescued from death and directed toward effector fates by survival and differentiation mediators. The planned studies were thus to investigate the outcomes of this rescue and differentiation in both murine and human cells – with emphasis on how different forms of rescue lead to alternative fates associated with autoimmune disease. Following a six month no-cost extension we have successfully met all major goals as detailed under the Accomplishments, Impact, and Products sections that follow.

**2. KEYWORDS**: Tbet-postive B cell, ABC, autoimmunity, SLE

**3. ACCOMPLISHMENTS:**

**Major goals as stated in the approved SOW**

**Specific Aim 1: Detail the pathways mediating post proliferative death, rescue, and differentiation of each peripheral B cell subset in mice and humans.**

**Major Task 1.1**: Determine characteristics of death and rescue among several additional mouse and human B cell subsets. **Status: completed.**

**Major Task 1.2**: Assess the intracellular pathways that mediate cell death and rescue in each B cell subset following BCR-delivered TLR9 agonists**. Status: completed.**

**Major Task 1.3**: Further characterize the signaling systems involved in post proliferative death and rescue. **Status: completed.**

**Specific Aim 2: Assess how alternative forms of rescue mediate distinct differentiative outcomes.**

**Major Task 2.1**: Assess whether alternative exogenous signals can rescue post-proliferative cell death. **Status: completed.**

**Major Task 2.2**: Determine the signals through which BLyS versus alternative mechanisms rescue cells following BCR-delivered TLR9 agonists. **Status: completed.**

**Major Task 2.3**: Establish whether cells of each B cell subset adopt plasma cell or germinal center programs following different forms of rescue. **Status: completed.**

**Specific Aim 3: Determine whether B cells from selected SLE patients are refractory to post-proliferative death mediated by TLR9 agonists, or more responsive to plasmablast or germinal center B cell differentiation in response to rescue signals.**

**Major Task 3.1**: Assess the response of B cells from SLE patients with potential defects in TLR9 signaling**. Status: completed.**

**Major Task 3.2**: Determine whether B cells from individuals or mouse lines harboring a risk allele are refractory to post-proliferative death mediated by TLR9 agonists**. Status: completed for lupus-prone mouse lines.**

**What were the major goals of the project?** In summary, the goals of the project were to detail the pathways/phenotypic outcomes mediating post-proliferative death, rescue, and differentiation of each peripheral B cell subset in mice and humans, and to assess how alternative forms of rescue mediate distinct differentiative outcomes.

**What was accomplished under these goals?** Our progress toward these goals during the course of the project has culminated in:

- two collaborative research publications with Principal Investigator Michael Cancro as a contributing author:
  - Nundel/Green et al., J Immunol. 2015 (1)
  - Wang/Wang/Kumar et al., Nat Commun. 2018 (11) - copy appended
- three research publications with Principal Investigator Dr. Cancro as corresponding author:
  - Naradikian et al., J Immunol. 2016 (4) - copy appended
  - Russell Knode et al., J Immunol. 2017 (6) - copy appended
  - Sindhava/Oropallo et al., J Clin Invest. 2017 (7) - copy appended
- four review articles, two collaborative with Dr. Cancro as a contributing author and two with Dr. Cancro as the corresponding author:
  - Sharma et al., J Immunol. 2015 (2) - collaborative
  - Rubtsova et al., J Immunol. 2015 (3) - collaborative - copy appended
  - Naradikian et al., Immunol Rev. 2016 (5) - copy appended
  - Scholz et al., ASHI Quarterly 2017 (8) - copy appended

- one dedicated volume of Cellular Immunology edited by Dr. Cancro, containing ten collected reviews/commentaries, entitled *T-bet+ B Cells in Health and Disease*
    - Cancro (editorial, Cellular Immunol. 2017) (9) - copy appended
    - Myles & Cancro, Cellular Immunol. 2017 (10) - copy appended

**Accomplishments are organized herein according to these publications,** beginning with the research articles that resulted in part or in full from this grant.

The first collaborative research paper (P.I. is a contributing author) discussed here is entitled *Cell-Intrinsic Expression of TLR9 in Autoreactive B Cells Constrains BCR/TLR7-Dependent Responses* (Nundel/Green et al., J Immunol. 2015) (1). This paper reports distinct functional outcomes for murine B cells activated through the BCR/TLR7 vs. BCR/TLR9 pathways, thus demonstrating the capacities of TLR7 to promote and TLR9 to limit (auto)antibody production. Studies in this report were not designed to address the Major Tasks of this proposal, but nevertheless informed Specific Aim 2 of this proposal for BCR/TLR-stimulated B cells.

The first research paper with P.I. Michael Cancro as corresponding author is entitled *Cutting Edge: IL-4, IL-21, and IFN-g Interact to Govern T-bet and CD11c Expression in TLR-Activated B cells* (Naradikian et al., J Immunol. 2016) (4) **(copy appended)**. This report shows mechanisms by which protective ABCs / Tbet-positive B cells are generated.

> **Major Task 1.2: Assess the intracellular pathways that mediate cell death and rescue in each B cell subset following BCR-delivered TLR9 agonists.** Figure 1 and Supplemental Fig. 1 panels A-E of this paper show that with TLR7/9 activation, cultured murine follicular (FO) B cells and human mature naive B cells integrate IL-4, IL-21, and IFN-g signals to differentially regulate T-bet and CD11c expression. IL-21 leads to Tbet induction (Fig. 1A, C show murine protein and Fig. 1B shows mRNA) and CD11c expression (Fig. 1C), and IL-4 blocks this effect. IFN-g leads to a Tbet-positive but CD11c-negative phenotype, and IL-4 enhances this effect (Fig. 1B, C).
>
> Figure 2 and Suppl. Fig. 1 panels G-I indicate that these relationships are operational in vivo, and that Tbet+ B cells comprise a B cell memory (Bmem) subset. Experiments with wild-type and IL-21 transgenice mice indicate that the relative abundance of IL-21, IL-4, and IFN-g determine Tbet expression (Fig. 2A-C) and this is functionally significant in vivo given the high serum $IgG2_{a/c}$ observed in IL-21Tg mice (Fig. 2D).
>
> **Major Task 2.3: Establish whether cells of each B cell subset adopt plasma cell or germinal center programs following different forms of rescue.** Figure 3 and Suppl. Fig. panels J-O of this paper shows that flu virus infection of mice generates Tbet+ CD11c+ Bmem in the absence of both IL-4 and IFN-g. Figure 4 and Suppl. Fig. panels J & P-T show that nematode infection of mice generates Tbet+ CD11c+ germinal center (GC) B cells and Bmem with IL-4 deficiency and independently of IFN-g. Together, these studies indicate that Tbet+ CD11c+ Bmem that can produce $IgG2_{a/c}$ are fostered when IL-4 is limiting.
>
> **Major Task 3.1: Assess the response of B cells from SLE patients with potential defects in TLR9 signaling.** Studies of B cells from normal/healthy human donors are a necessary accompaniment to studies of B cells from SLE patients, especially given the genetic variability of humans. Figure 1 panels D & E show that human mature naive B cells upregulate Tbet with TLR9 activation alone, but nevertheless, effects of IL-21, IL-4, and IFN-g are similar to those observed in mice.

The second research paper (Michael Cancro is co-corresponding author) is entitled *Age-associated B cells express a diverse repertoire of mutated immunoglobulins and share transcriptional profiles with memory cells* (Russell Knode et al., J Immunol. 2017) (6) **(copy appended)**. This work was conducted collaboratively with the Gearhart Lab (NIA). These studies assessed the likely origins of T-bet+ B cells (ABCs) and their general properties. Overall they suggest ABCs are a form of memory B cell that emerges from germinal center (GC) reactions, and thus inform Major task 2.3.

> **Major Task 2.3: Establish whether cells of each B cell subset adopt plasma cell or germinal center programs following different forms of rescue.** Figure 1 of this paper shows that generation of T-bet-positive B cells requires MHC Class II and the CD40/CD40L axis. These are thus consistent with the notion that cognate help, in the context of appropriate TLR9 containing antigens, can rescue death. Figure 4 further suggests a germinal center origin for a significant proportion of T-bet+ B cells in aged animals. Again, understanding the origin and nature of T-bet+ B cells is important because these cells appear to be key players in autoimmune disease.

The third research paper discussed here (Michael Cancro is corresponding author) is entitled "*A TLR9 dependent checkpoint governs B cell responses to DNA-containing antigens*" (Sindhava/Oropallo et al., J Clin Invest. 2017) (7) **(copy appended)**. This details the molecular pathways mediating post-proliferative death, rescue, and (in part) differentiation of murine peripheral B cell subsets. In addition, data shown in Figure 5 suggest similar mechanisms occur in human peripheral B cells, a necessary prelude to Major Tasks 3.1 and 3.2. Specific findings (accomplishments) are summarized here by Major Task:

> **Major Task 1.1**: **Determine characteristics of death and rescue among several additional mouse and human B cell subsets.** Figure 3 of this paper (appended) shows that the response to the DNA-containing antigen STIC9 is similar for the transitional 1 (TR1), transitional 2/3 (TR2/3), follicular (FO), and marginal zone (MZ) B cell subsets of mice. Post-proliferative death is rescued by BLyS (Fig. 3A), the death mechanism is mitochondrial apoptosis and via a p38 mediated mechanism in all

subsets (Fig. 3B). Fig. 5A shows similar responses by circulating mature naïve human B cells (CD27-) to human STIC9 and human BLyS, and also shows it I via a p38-dependent mechanism.

**Major Task 1.2: Assess the intracellular pathways that mediate cell death and rescue in each B cell subset following BCR-delivered TLR9 agonists.** Figure 1 of this paper shows that the self-limiting B cell response induced by DNA immune complexes is independent of BCR specificity (AM14 vs C57BL/6), occurs 48-60 hours post-stimulation (panels C & D), can be rescued by BLyS (panels A-D & I), and requires TLR9 signaling yet does not reflect significant differences in TLR9 vs. BCR signal strengths (panels E-H).

**Major Task 1.3: Further characterize the signaling systems involved in post proliferative death and rescue.** Figure 2 of this paper extensively details the death signaling system and chronology of intracellular events for murine B cells. IN toto these data reveal that this mechanism involves p38-mediated cell cycle arrest that subsequently drives mitochondrial depolarization and caspase-9 mediated mitochondrial apoptosis/cell death. Fig. 2A and 2B show that the death is caspase 9 mediated, and not driven by necroptosis or other cell extrinsic mechanisms. Fig 2C shos that consistent with this, it is rescued in BclxL transgenic B cells. Fig 2D shows that among the MAPK pathways known to be associated with these mechanisms, only the p38K is involved, whereas ERK and JNK are not. Fig 2 E, F and G confirm this conclusion by showing that the caspacse 9 cleavage (2E) and mitochondrial depolarization (2F) and that this is blocked by multiple different p38 inhibitors (2G), ruling out off-target inhibitor effects . Figs 2H, I and J show that cell cycle arrest is the initial event, because it is blocked by the p38 inhibitor, but that blocking mitochondrial death with the BclxL transgene, prevents death but does not reverse the cell cycle arrest event (Fig 2K).

**Major Task 2.1**: **Assess whether alternative exogenous signals can rescue post-proliferative cell death.** Figure 4 of this paper shows that in addition to BLyS, CD40 costimulation and Tfh cytokines IL-21 and IFN-gamma can "rescue" murine B cells. CD40 ligation 48 hours after activation affords only partial rescue (panel B). Figure 5C shows that these relationships are also true for human naïve B cells, since CD40 ligation rescues human B cells stimulated with human STIC9 and that these rescued cells adopt the Tbet+ fate with IFN-gamma signaling.

**Major Task 2.2**: **Determine the signals through which BLyS versus alternative mechanisms rescue cells following BCR-delivered TLR9 agonists.** Figures 2 and S2 show that BLyS-mediated rescue requires BR3 but not TACI, consistent with a BCLxL-mediated anti-apoptotic signal.

**Major Task 2.3: Establish whether cells of each B cell subset adopt plasma cell or germinal center programs following different forms of rescue.** Table 1 shows that BLyS rescue allows STIC9-stimulated murine B cells to secrete antibody (a key characteristic of plasma cells). Figure 4 assesses consequences of the various forms of rescue. Briefly, rescue by BLyS alone leads to antibody secretion by STIC9-stimulated murine B cells of all subsets (Table 1, Fig. 4). CD40 costimulation with IL-21 or IFN-gamma leads to survival, expression of the transcription factor T-bet, and isotype class switching – especially to IgG2c -- in vitro. T-bet upregulation occurs in both murine (Fig. 4) and human (Fig. 5) B cells stimulated with STIC9. These are key findings because T-bet-positive B cells are associated with autoimmunity in both species. Mice immunized with amyloid (protein-DNA) complexes yielded reduced germinal center B cells and reduced class switched antibody (Fig. 4) – consistent with our hypothesis of a B cell-intrinsic self-limiting response to DNA-containing antigens, despite partial rescue by CD40 ligation.

**Major Task 3.1: Assess the response of B cells from SLE patients with potential defects in TLR9 signaling.** Figure 5 shows results for B cells from normal/healthy human donors, a necessary prelude to studies of B cells from SLE patients.

**Major Task 3.2**: **Determine whether B cells from individuals or mouse lines harboring a risk allele are refractory to post-proliferative death mediated by TLR9 agonists.** Fig. S1 of this paper shows that B cells of lupus-prone mouse strains undergo a similar post-proliferative death response to wild-type and AM-14 mice.

The second collaborative research paper (Michael Cancro is a contributing author) is entitled *IL-21 drives expansion and plasma cell differentiation of autoreactive CD11c$^{hi}$ T-bet+ B cells in SLE* (Nat Commun. 2018) (11) **(copy appended)**. This work was collaborative with the laboratory of Rachel Ettinger (MedImmune LLC).

**Major Task 3.1: Assess the response of B cells from SLE patients with potential defects in TLR9 signaling.** Figure 1 shows that T-bet+Cd11c+ B cells are associated with SLE (Fig. 1a, b) and correlate with disease status (Fig. 1c, d and Fig. 2). Further, in SLE patients, these cells are enriched for SLE-associated autoreactive specificities. This work is directly relevant to Major Tasks 3.1 and 3.2 but as it is not yet published, specific figures cannot yet be referenced here because the work is presently in submission.

**Major Task 3.2**: **Determine whether B cells from individuals or mouse lines harboring a risk allele are refractory to post-proliferative death mediated by TLR9 agonists.** SLE patients in this study were not assessed for risk alleles. Nevertheless, IL-21/IL-21R are risk alleles for SLE (and TLR9 is debatably a risk allele). This article provides evidence that IL-21 not only induces Tbet+ B cells in SLE patients, but promotes their differentiation into autoantibody-secreting plasma cells.

Figures 1 and 2 illustrate the expansion of CD11c+ B cells in SLE compared to healthy donors, and the correlation of this subset with disease status. Figures 5-8 together show that these cells are poised to become plasma cells that are the major producers of autoantibody, and indicate that IL-21 is the key co-stimulator.

**Review articles and editorial that resulted in part or in full from this grant**

The first collaborative review (P.I. is a contributing author) is entitled *Nucleic Acid-Sensing Receptors: Rheostats of Autoimmunity and Autoinflammation* (Sharma et al., J Immunol. 2015) (2).

This article provides a mechanistic model for how nucleic acid sensors (TLR7, TLR9) act as rheostats or "dimmer switches" that reduce or prevent durable immune responses to nucleic acids released during microbial infection or to self-antigens released during host-cell stress or death. This model informed all three Specific Aims of this proposal, and all of the accomplishments detailed below support it with regard to the role of TLR9.

The second collaborative review (P.I. is a contributing author) is entitled *Age-Associated B Cells: A T-bet-Dependent Effector with Roles in Protective and Pathogenic Immunity* (Rubtsova et al., J Immunol. 2015) (3) (**copy appended**).

The laboratories of the senior authors of this review, Michael Cancro and Philippa Marrack, co-discovered "ABCs" in 2011. This review summarized evidence to date that a receptor triumvirate -- nucleic acid sensors (TLR7/9), the BCR, and cytokine receptors -- governs induction of the transcription factor T-bet in B cells. T-bet expression in turn leads to the ABC fate including production of anti-viral $IgG2_{a/c}$ to combat infection, or autoreactive $IgG2_{a/c}$ which contributes to autoimmunity. This review posits that all Tbet-positive B cells -- whether protective, pathogenic, or age-associated -- share the common initiating factors of TLR7/9 activation within a specific cytokine milieu. This model informed all three Specific Aims of this proposal, and all of the accomplishments detailed below support it with regard to the roles of TLR9 and cytokines/cytokine receptors. The broader impact of this review is indicated by the fact that it has been cited by 20 research and review articles in the three years since its publication.

The first review with P.I. Michael Cancro as corresponding author is entitled *Age associated B cells: Key mediators of both protective and autoreactive humoral responses* (Naradikian et al., Immunol Rev. 2016) (5) (**copy appended**).

This review, cited by 18 other articles in the two years since its publication, summarizes mechanistic evidence that a combination of three signals -- mediated by the above-mentioned "receptor triumvirate" -- lead to ABC (Tbet+ B cell) generation. The initiating signal is provided by either pathogen-associated antigen or self-antigen; cognate help and a specific cytokine milieu lead to Tbet upregulation and the ABC fate as a long-lived antigen-experienced cell. The mechanistic model put forth in Figure 1 of this review has come to be widely accepted, and informs ongoing efforts to understand how responses to endogenous (self) vs. exogenous (pathogen-associated) nucleic acids are regulated.

The second review (Michael Cancro is corresponding author) is entitled *The ABCs of a new memory B cell subset* (ASHI Quarterly, 2017 (8) (**copy appended**). This review reaches an important audience, the American Society for Histocompatibility and Immunogenetics. The notable congruence between characteristics of graft rejection and requisites for ABC generation is summarized. A model for ABC formation in response to alloantigens is incorporated into the above-referenced model of ABC formation in response to pathogen-associated and self-antigens.

**Dedicated issue of Cellular Immunology: T-bet Expressing B Cells**

The introductory editorial is entitled *Expanding roles for the Tbet+ B cell subset in health and disease* (Cancro MP, Cellular Immunol. 2017) (9) (**copy appended**). This timely issue, conceived, organized and edited by P.I. Michael Cancro, compiles articles from ten of the laboratories whose research has contributed to current understanding of the origin, nature, and function of Tbet-expressing B cells in the decade since their discovery.

The first review in this volume (Michael Cancro is corresponding author) is entitled *Signals that drive T-bet expression in B cells* (Myles et al., Cellular Immunol. 2017) (10) (**copy appended**). This review focuses on the signals that lead to T-bet expression, as well as signals that poise B cells for Tbet expression including innate receptors (like TLRs) and the likely importance of the Jak/STAT axis in cytokine receptor signaling.

**A 6-mo. no-cost project extension** was granted in August 2017. During the requested extension we extended analyses of phosphoproteomics involved in our recently described TLR9-dependent B cell checkpoint (Naradikian et al., J Immunol. 2016; Sindhava/Oropallo, J Clin Invest. 2017) (4, 7). In addition, we enlarged the sample sizes of our analyses of B cells from SLE patients, based on our collaborative findings showing that Tbet+ B cell levels correlate with increasing SLEDAI score in SLE patients (Ettinger et al., Nat Commun. 2018) (11). We feel the project extension yielded further valuable, albeit preliminary, information pertinent to both basic mechanisms of tolerance and to potential therapeutic molecular targets.

Specifically, this extension allowed us to better address **Major Task 1.3: Further characterize the signaling systems involved in post proliferative death and rescue.** In 2016, we demonstrated that in the context of TLR9 stimulation, IL-4 antagonizes or "blocks" IL-21-driven Tbet expression but enhances IFNg-driven Tbet expression (Naradikian et al., 2016) (4). Because STAT6 is the primary membrane-proximal signal transducer of IL-4, we conducted co-culture experiments with STAT6 knockout and wild-type B cells. Results in Figs. 1 and S1 (4) indicated that the IL-4 "blocking" effect on IL-21-driven Tbet expression was STAT6-dependent, whereas IFNg-driven Tbet expression was not.

As noted above, STAT6 is the primary signal transducer of IL-4. STAT3 is the primary signal transducer of IL-21, and STAT1 the primary signal transducer of IFNg. Additional STATs are likely in play for all of these cytokines. During the 6-mo. project extension we began studies of STAT signaling by culturing C57BL/6 (wild-type, WT) murine CD23+ (follicular, FO) B cells with the TLR9 agonist CpG and various combinations of IL-21, IFNg, and IL-4. Phosphorylation/activation status of STAT6, STAT3, or STAT1 was determined using both phospho-flow and Western blotting. Preliminary results, all pending validation, are summarized here.

STAT6 phospho-flow (sample provided in **Fig. A**) and Western blotting (not shown) together indicate that B cells stimulated with CpG + IL-21 (or IFNg) + IL-4 show higher levels of STAT6 phosphorylation than those stimulated with CpG + IL-21 (or IFNg). This is consistent with our prior results indicating a STAT6-dependent IL-4 "blocking" effect (Naradikian et al., J Immunol. 2016) (4).

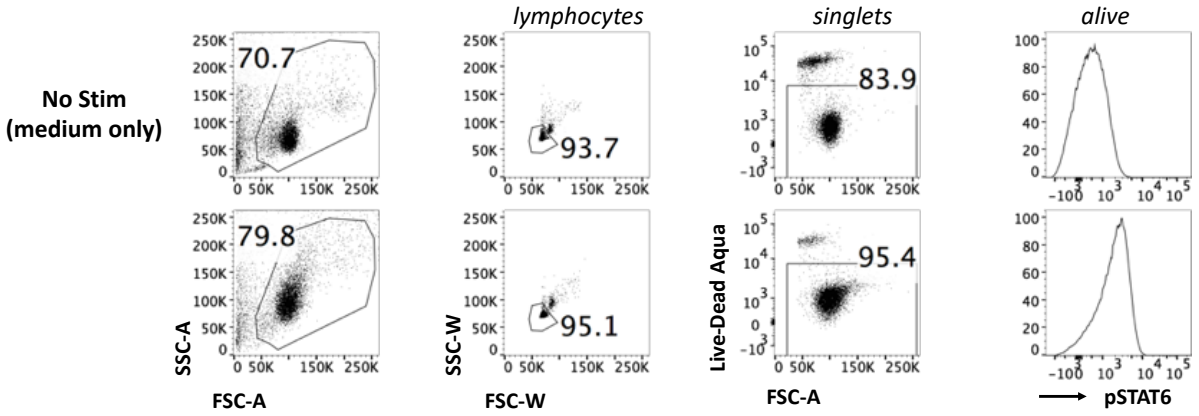


**Fig. A: Sample gating for phospho-flow.** WT CD23+ FO B cells were cultured 12-16 hrs without stimulation (medium only) or with various stimuli, then harvested and stained with anti-pSTAT6 (this example) or other pSTAT antibody. Cells were first gated on lymphocytes (left panel), then singlets and live cells; histograms show pSTAT6 in the live lymphocyte singlet gate. Numbers next to each gate indicate percentages.

STAT3 is phosphorylated to the same degree in B cells stimulated with CpG + IL-21 and those stimulated with CpG + IL-21 + IL-4 (**Fig. B, green and orange lines**). Thus, these preliminary results leave several avenues open for future investigation: The IL-4 antagonistic effect may be independent of STAT3 signaling. It may occur in downstream signaling events. Another possibility is that STAT3-STAT6 heterodimers form upon IL-21 + IL-4 stimulation, and this is the molecular basis for the IL-4 antagonism of IL-21-driven Tbet expression.

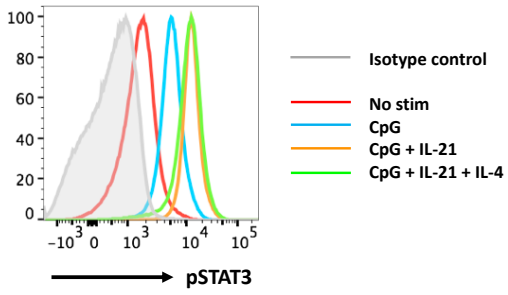


**Fig. B:** STAT3 activation/phosphorylation in WT CD23+ FO B cells were cultured with stimuli shown before staining with anti-pSTAT3. Isotype control-stained cells were cultured with CpG + IL-21 + IL-4 before staining with isotype control antibody.

STAT1 phospho-flow (not shown) and Western blotting (sample shown in **Fig. C**) have not yet been validated, but one example of the latter is presented here. This indicates that B cells stimulated with CpG+IFNg or CpG+IFNg+IL-4 may show similar levels of STAT1 phosphorylation by Western blotting (**Fig. C lanes 3, 4**). In contrast, B cells stimulated with CpG+IL-21 appear to have low pSTAT1, while cells stimulated with CpG+IL-21+IL-4 show no STAT1 phosphorylation (**Fig. C lanes 5, 6**). If these results are repeatable and validated, including experiments with STAT1-deficient B cells, it will be tempting to speculate that a threshold of IL-21-induced STAT1 phosphorylation may be required to induce Tbet.

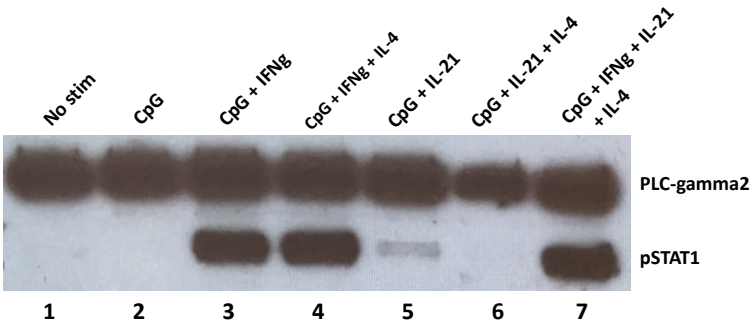


**Fig. C: Sample Western blot for pSTAT1.** WT CD23+ FO B cells were cultured for 30 minutes without stimulation (medium only) or with various stimuli before lysis and Western blotting. Upper bands are protein loading control PLC-gamma2 and lower bands indicate phosphorylated STAT1.

**What opportunities for training and professional development has the project provided?** Although training was not a goal of the project per se, these studies helped to serve as a research training and professional development vehicle for several individuals in the course of the project, including postdoctoral trainees Arpita Myles, Lauren E. Higdon, and James J. Knox; Research Associates Vishal J. Sindhava and Jean L. Scholz; and graduate students Martin S. Naradikian and Rebecca L. Rosenthal.

**How were the results disseminated to communities of interest?** The work under this award has contributed to 11 peer-reviewed research papers and reviews (see PUBLICATIONS list below). In addition, key aspects of the work were presented by MP Cancro at several international meetings or invited seminars (see PRODUCTS list below), or in posters or talks by Dr. Cancro's trainees (see PRODUCTS).

## 4. IMPACT

**What was the impact on the development of the principal discipline(s) of the project?**

The work supported by this grant has been highly productive, as evidenced by the publications and products enumerated below. In particular, the J Clin Invest publication (7) represents a comprehensive assessment of the signals and pathways involved in generating T-bet+ B cells. Moreover, our description of a TLR9-dependent tolerance checkpoint in B cell responses to DNA-containing antigens, as well as their relationship to the Tbet+ B cell subset ('ABC subset') we previously described, have been largely accepted by the community at large and have established avenues of inquiry now being pursued by multiple labs interested in SLE in particular and humoral autoimmunity in general -- as further illustrated by the special issue of Cellular Immunology edited by the P.I. (9).

**What was the impact on other disciplines?** Nothing to report.

**What was the impact on technology transfer?** Nothing to report.

**What was the impact on society beyond science and technology?** Nothing to report.

**5. CHANGES/PROBLEMS**: Nothing to report.

## 6. PRODUCTS

**PUBLICATIONS:** The following publications resulted in full or in part from this grant support.


1. Nündel K, Green NM, Shaffer AL, Moody KL, Busto P, Eilat D, Miyake K, Oropallo MA, **Cancro MP,** Marshak-Rothstein A. Cell-intrinsic expression of TLR9 in autoreactive B cells constrains BCR/TLR7-dependent responses. *J Immunol*. 2015 Mar 15;194(6):2504-12. doi: 10.4049/jimmunol.1402425. Epub 2015 Feb 13. PubMed PMID: 25681333; PubMed Central PMCID: PMC4382804.

2. Sharma S, Fitzgerald KA, **Cancro MP,** Marshak-Rothstein A. Nucleic Acid-Sensing Receptors: Rheostats of Autoimmunity and Autoinflammation. *J Immunol*. 2015 Oct 15;195(8):3507-12. doi: 10.4049/jimmunol.1500964. Review. PubMed PMID: 26432899; PubMed Central PMCID: PMC4593056.

3. Rubtsova K, Rubtsov AV, **Cancro MP,** Marrack P. Age-Associated B Cells: A T-bet-Dependent Effector with Roles in Protective and Pathogenic Immunity. *J Immunol.* 2015 Sep 1;195(5):1933-7. doi: 10.4049/jimmunol.1501209. Review. PubMed PMID: 26297793; PubMed Central PMCID: PMC4548292. **APPENDED**

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5. Naradikian MS, Hao Y, **Cancro MP.** Age Associated B cells: Key mediators of both protective and autoreactive humoral responses. *Immunol Rev.* 2016 Jan;269(1):118-29. doi: 10.1111/imr.12380. PMID: 26683149 **APPENDED**

6. Russell Knode LM, Naradikian MS, Scholz JL, Hao Y, Liu D, Ford ML, Tobias JW, Ca**ncro MP,** Gearhart PJ. Age-associated B cells express a diverse repertoire of mutated immunoglobulins and share transcriptional profiles with memory cells. *J Immunol.* 2017 Mar 1;198(5):1921-1927. doi: 10.4049/jimmunol.1601106. Epub 2017 Jan 16. PMID:28093524 **APPENDED**

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8. Scholz JL, Sindhava V, **Cancro MP.** The ABCs of a new memory B cell subset. *ASHI Quarterly*. 41(2):20-22. **APPENDED**

9. **Cancro MP.** Editorial: Expanding roles for T-bet+ B cells in immunological health and disease. *Cell Immunol*. 2017 Nov;321:1-2. doi: 10.1016/j.cellimm.2017.10.004. Epub 2017 Oct 14. PMID:29224845 **APPENDED**

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11. Wang S, Wang J, Kumar V, Karnell JL, Naiman B, Gross PS, Rahman S, Zerrouki K, Hanna R, Morehouse C, Holoweckyj N, Liu H; Autoimmunity Molecular Medicine Team, Manna Z, Goldbach-Mansky R, Hasni S, Siegel R, Sanjuan M[1], Streicher K, **Cancro MP**, Kolbeck R, Ettinger R. IL-21 drives expansion and plasma cell differentiation of autoreactive CD11c$^{hi}$ Tbet$^{+}$ B cells in SLE. *Nat Commun.* 2018 May 1;9(1):1758. doi: 10.1038/s41467-018-03750-7. **APPENDED**


**P.I. PRESENTATIONS:** The following presentations by P.I. Michael Cancro resulted in full or in part from this grant support.

| **Year 1** |
|---|
| <u>International</u><br>• Sep, 2014 "Innate, Adaptive, and Survival Receptors: A Molecular Trio Governing B Cell Selection and Survival." Guest speaker, Immunology Seminar series; University of Gottingen, Gottingen, Germany<br>• Sep, 2014 "A New Route to T-bet." Guest speaker, University of Erlangen Immunology Retreat; Lohr, Germany<br>• Nov, 2014 "Innate, Adaptive, and Survival Receptors: The Triple Threat of B Cell Selection." Charles Gould Easton Seminar, University of Toronto; Toronto, Ontario, Canada<br>• Mar, 2015 "A TLR9 dependent peripheral B cell tolerance mechanism" Keystone Joint Symposia Meetings on B Cell Biology and Function" and "Prophylactic and Therapeutic Antibodies"; Banff, Alberta, Canada<br><br><u>National</u> |

- Oct, 2014 "B Cell Development and the Role of BLyS." Scientific session, Blood Bank Association of America annual meeting; Philadelphia, PA
- Nov, 2014 "Homeostasis, Tolerance, and Age: A Space and Time Problem for B Cells." Immunobiology Seminar Series, Yale University School of Medicine; New Haven, CT
- Feb, 2015 "Homeostasis, tolerance, and age: A space & time puzzle for B cells." Department of Microbiology and Immunology Guest Seminar Series, University of Texas School of Medicine, UT Health Science Center; San Antonio, TX
- Feb, 2015 "Autoimmunity and B-cell Biology: Back to Basics." Invited speaker, Bone Marrow Transplant Tandem Meeting; San Diego, CA
- Apr, 2015 "B cell tolerance and homeostasis: A confluence of innate, adaptive, and survival signals" Stanford Immunology Seminar Series; Stanford University, Palo Alto, CA
- May, 2015 "B cell tolerance and homeostasis: A confluence of innate, adaptive, and survival signals." Guest speaker; Immunology and Microbiology Seminar Series, University of Arkansas School of Medicine; Little Rock, AR
- Jul, 2015 "Germinal center reactions and B cells." MedImmune Visiting Scientist Seminar series; Gaithersburg, MD
- Aug, 2015 "Relaxing B cell selection checkpoints" DAIDS/NIAID Workshop on Germinal Center Dynamics and Antibody Affinity Maturation for Protective Immunity. Rockville, MD

**Year 2**

International

- May, 2016 "Age associated B Cells: T-bet driven effectors of protective and autoimmune responses" University of British Columbia and Simon Fraser University (Vancouver, Canada)
- May, 2016 "Age associated B Cells: T-bet driven effectors of protective and autoimmune responses" Keystone Symposium: B Cells at the Intersection between Innate and Adaptive Immunity, Stockholm, Sweden

**Year 3**

International

- Sep, 2016 "Now we know our ABCs: T-bet driven effectors of protective and autoimmune responses" 5th international GK Symposium on Regulators of Adaptive Immunity, Erlangen, Germany
- Nov, 2016 "Innate, adaptive, and & survival signals: Targets for modulating B cell tolerance & selection" Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Peoples Republic of China
- Dec, 2016 "Now we know our ABCs: Expanding roles for T-bet+ B cells in health and disease" Shanghai Immunology institute, Shanghai, Peoples Republic of China
- Dec, 2016 "Integrating innate, adaptive, & survival signals to control B cell selection, homeostasis and tolerance" Pasteur Institute of Shanghai, Shanghai, Peoples Republic of China

National

- Sep, 2016 "Memory B cell subsets" ASHI 42nd Annual Meeting. Symposium on "Targeting B, Plasma Cells and Complement for the Treatment of Allograft Rejections" St. Louis, MO
- Nov, 2016 "Now we know our ABCs? Expanding roles for T-bet+ B cells in health and disease." Immunology and Microbiology guest seminar series, Univ. of Miami Miller School of Medicine, Miami, FL
- Feb, 2017 "Now we know our ABCs: T-bet driven effectors of protective and autoimmune responses" Genomics Institute of the Novartis Research Foundation, La Jolla, CA
- Apr, 2017 "Now we know our ABC's Tbet driven effectors of protective and autoimmune responses" Immunology Seminar Series; Cincinnati Children's Hospital Medical Center, Cincinnati OH
- May, 2017 "Now we know our ABCs: Expanding roles for T-bet+ B cells in health and disease." Guest Seminar Series, Department of Microbiology and Immunology, University of Alabama Birmingham, Birmingham, AL
- Jul, 2017 "Now we know our ABCs: Expanding roles for T-bet+ B cells in health and disease" Benaroya Research Institute Seminar Series, Benaroya Research Institute, Seattle, WA
- Aug, 2017 "New Subsets, New checkpoints: T-bet+ B cells in health and disease" Uniformed Services University of the Health Sciences Molecular Biology Program Guest Seminar Series. Bethesda, MD

**Extension (Aug. 2017 - Feb. 2018)**

International

- Sep, 2017 "T-bet+ B cells in health and disease" EMBO international meeting 'To B or Not to B: B cells in health and disease' San Filieu, Spain

National

- Nov, 2017 "New subsets, new checkpoints: Expanding roles for Tbet+ B cells in health and disease" Western University of Michigan Stryker

- Nov, 2017 "Now we know our ABCs, Tbet+ B cells in health and disease" Feinstein Visiting Professor Seminar Series, Feinstein Institute for Medical Research, Manhasset, New York
- Dec, 2017 "New subsets, new checkpoints: Expanding roles for Tbet+ B cells in health and disease" University of Pittsburgh Immunology Seminar Series; Pittsburgh, PA
- Feb, 2018 "Mechanisms that regulate Age-Associated B cell formation and survival" Keystone Symposium on Aging Inflammation and Immunity, Austin TX

**MEETING ABSTRACTS:** The following meeting abstracts resulted in full or in part from this grant support; presenters who are/were Dr. Cancro's trainees are underlined.

| **Year 1** |
|---|
| None |
| **Year 2** |
| Martin S. Naradikian, Susanne Linderman, Lucas Woods, Daniel Beiting, Rosanne Spolski, E. John Wherry, Christopher Hunter, Scott Hensley, Warren J. Leonard, and Michael P. Cancro. "IL-4 and IL-21 reciprocally regulate a unique T-BET-driven phenotype in B cells. (January 2016, Midwinter Conference of Immunologists, Asilomar, CA) (talk and poster) |
| **Year 3** |
| Arpita Myles and Michael P Cancro. "Co-stimulation and cytokines differentially regulate metabolic profiles in activated B cells" Asilomar Midwinter conference of Immunologists. (January 2017, Midwinter Conference of Immunologists, Asilomar CA) (talk and poster)<br><br>Shu Wang*, Jingya Wang*, Brian Naiman*, Jodi Karnell*, Phil Gross*, Saifur Rahman*, Molecular Medicine Group, Richard Siegel†, Sarfaraz Hasni†, Michael P. Cancro^, Roland Kolbeck* and Rachel Ettinger* "CD11c expression in B cells is driven by IL-21 and associates with autoimmune disease manifestations in SLE." 2017 American Assoc. of Immunologists Annual Meeting (May 2017, Washington, D.C.)<br><br>Patricia J. Gearhart, Lisa M. Russell Knode, Martin S. Naradikian, and Michael P. Cancro. Age-associated B cells express a diverse repertoire of mutated immunoglobulins and share transcriptional profiles with memory cells NIH Symposium on Immune dysregulation in Aging. (September 2016, Arlington VA)<br><br>Shu Wang, Jingya Wang, Brian Naiman*, Jodi Karnell*, Phil Gross*, Saifur Rahman*, Molecular Medicine Group, Richard Siegel†, Sarfaraz Hasni†, Michael P. Cancro^, Roland Kolbeck* and Rachel Ettinger* Role and regulation of CD11c+Tbet+ B cells in SLE. FOCIS Annual Meeting (June 2017, Chicago, IL) |
| **Extension (Aug. 2017 - Feb. 2018)** |
| Rebecca Rosenthal, Arpita Myles (Co-First Authors), Elinor Willis, Scott Hensley, Mike Cancro. Age Associated B Cells Represent a Tissue Resident Memory B Cell Subset. (January 2018, Midwinter Conference of Immunologists, Asilomar, CA) (poster) |

**Website(s) or other Internet site(s):** Nothing to report.

**Technologies or techniques:** Nothing to report.

**Inventions, patent applications, and/or licenses:** Nothing to report.

**Other Products:** Nothing to report.

**7. PARTICIPANTS AND COLLABORATING ORGANIZATIONS:**

| **Name** | **Michael P. Cancro** |
|---|---|
| **Project Role** | P.I. |
| **Researcher Identifier** | N/A |
| **Nearest person month worked** | 2 |
| **Contribution to project** | Principal Investigator; oversee all research |
| **Funding support** | This award; R01-AI-118691 (NIAID) |

| Name | Jean L. Scholz |
|---|---|
| Project Role | Research Associate |
| Researcher Identifier | N/A |
| Nearest person month worked | 3 |
| Contribution to project | Perform experiments and oversee logistics |
| Funding support | This award; R01-AI-118691 (NIAID) |

| Name | Arpita Myles |
|---|---|
| Project Role | Postdoc |
| Researcher Identifier | N/A |
| Nearest person month worked | 6 |
| Contribution to project | Perform experiments related to Tbet+ B cells in autoimmunity and infection; cell signaling mechanisms; analyze and present data |
| Funding support | This award; R01-AI-118691 (NIAID) |

| Name | James J. Knox |
|---|---|
| Project Role | Postdoc |
| Researcher Identifier | N/A |
| Nearest person month worked | 2 |
| Contribution to project | Perform experiments related to Tbet+ B cells, cell signaling mechanisms |
| Funding support | This award; R01-AI-118691 (NIAID); T32 HL07954 (NHLBI) |

| Name | Rebecca L. Rosenthal |
|---|---|
| Project Role | VMD/PhD Candidate |
| Researcher Identifier | N/A |
| Nearest person month worked | 2 |
| Contribution to project | Perform experiments related to Tbet+ B cells, cell signaling mechanisms |
| Funding support | This award; R01-AI-118691 (NIAID); T32 AI055428 (NIAID) |

| Name | Mariya Kostiv |
|---|---|
| Project Role | Technician |
| Researcher Identifier | N/A |
| Nearest person month worked | 2 |
| Contribution to project | Prepare reagents, run assays, organize animal colony |
| Funding support | This award; R01-AI-118691 (NIAID) |

| Name | Joanna B Madej |
|---|---|
| Project Role | Undergraduate work-study student (Lab Aide) |
| Researcher Identifier | N/A |
| Nearest person month worked | 1 |
| Contribution to project | Prepare reagents, run assays, organize animal colony |
| Funding support | This award; R01-AI-118691 (NIAID); work-study award, Univ. of Pennsylvania |

Prior lab members who contributed to this project

| Name | Vishal J. Sindhava |
|---|---|
| Project Role | Research Associate |
| Researcher Identifier | N/A |
| Nearest person month worked | 2 |
| Contribution to project | Perform experiments related to Tbet+ B cells, cell cycle / cell signaling |
| Funding support | This award; R01-AI-118691 (NIAID) |

| Name | Lauren E. Higdon |
|---|---|
| Project Role | Postdoc |
| Researcher Identifier | N/A |
| Nearest person month worked | 1 |
| Contribution | Perform experiments related to Tbet+ B cells in autoimmunity and infection; cell signaling mechanisms; analyze and present data |
| Funding support | 50% this award |

| Name | Martin Naradikian |
|---|---|
| Project Role | Graduate student |
| Researcher Identifier | N/A |
| Nearest person month worked | 6 |
| Contribution | Perform experiments related to Tbet+ B cells in autoimmunity and infection; cell signaling mechanisms; analyze and present data |
| Funding support | This award; T32 AI055428 (NIAID) |

| Name | Kenneth J. Roberts |
|---|---|
| Project Role | Technician |
| Researcher Identifier | N/A |
| Nearest person month worked | 6 |
| Contribution to project | Prepare reagents, run assays, organize animal colony |
| Funding support | This award; R01-AI-118691 (NIAID) |

**Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
No change.

**The following grants were received or renewed during the past year:**

R01 AI118691; Cancro, Michael (PI); 02/10/15-01/31/20
Mechanistic studies of BLyS-mediated modulation in HIV-1 Env-specific antibody responses

T32 AI055428; Cancro, Michael (PI); 06/01/03-07/31/19
Training Program in Immune System Development and Regulation

1 R21 AI133998 (D. Allman and MP Cancro multi PI); 06/01/2017-05/31/2019
Plasma Cell Priming

**What other organizations were involved as partners?** Nothing to report.

**8. SPECIAL REPORTING REQUIREMENTS:** Does not apply.

**9. APPENDICES:** Nine publications, indicated above under sections 3 and 6 by yellow highlight, are appended in order of publication date.

**APPENDIX:** Rubtsova et al., J Immunol. 2015 (review)

Rubtsova K, Rubtsov AV, **Cancro MP,** Marrack P. Age-Associated B Cells: A T-bet-Dependent Effector with Roles in Protective and Pathogenic Immunity. *J Immunol.* 2015 Sep 1;195(5):1933-7. doi: 10.4049/jimmunol.1501209. Review. PubMed PMID: 26297793; PubMed Central PMCID: PMC4548292.

Brief Reviews

The Journal of Immunology

# Age-Associated B Cells: A T-bet–Dependent Effector with Roles in Protective and Pathogenic Immunity

Kira Rubtsova,[*,†,‡] Anatoly V. Rubtsov,[*,†,‡] Michael P. Cancro,[§] and Philippa Marrack[*,†,‡,¶,‖]

**A newly discovered B cell subset, age-associated B cells, expresses the transcription factor T-bet, has a unique surface phenotype, and accumulates progressively with age. Moreover, B cells with these general features are associated with viral infections and autoimmunity in both mice and humans. In this article, we review current understanding of the characteristics, origins, and functions of these cells. We also suggest that the protective versus pathogenic actions of these cells reflect appropriate versus aberrant engagement of regulatory mechanisms that control the Ab responses to nucleic acid–containing Ags.** ***The Journal of Immunology*****, 2015, 195: 1933–1937.**

Advancing age is accompanied by shifts in many qualitative and quantitative aspects of immune function. These changes, collectively termed immune senescence (1, 2), include blunted primary and memory immune responses, reduced vaccine efficacy, and increases in the prevalence of inflammatory and autoimmune pathologies (2–6). Although the underlying mechanisms remain unclear, a growing literature documents contributions from age-associated changes at the systemic, molecular, and cellular levels. Systemically, serum and local concentrations of inflammatory cytokines are progressively elevated in both mice and humans, yielding an overall phenomenon described as inflammaging (7, 8). In addition, monoclonal gammopathies, as well as Abs reactive with chromatin and dsDNA, frequently emerge with increasing age (9–12). Finally, with a few exceptions, such as type 1 diabetes and juvenile rheumatoid arthritis, the frequency of autoimmune disease increases with age. These pathologies may reflect causal relationships with overall changes in the immune system, the cumulative impact of environmental insults, or combinations of these factors. Alternatively, some of these features may instead initiate in young individuals and stem from normal immune activity, but their pathogenic actions may only become manifest once the underlying effectors reach a minimum threshold with advancing age.

At a cellular level, the output of primary lymphoid organs wanes, reflecting a shift toward myeloid lineage preference in hematopoietic stem cell specification (13, 14), reductions in key developmental gene expression (15–17), and altered microenvironmental and homeostatic feedback mechanisms (18, 19). Despite this dwindling lymphocyte production, total numbers of mature B and T cells remain relatively unchanged. Nevertheless, nearly all peripheral lymphoid pools exhibit altered dynamics, shifts in functional subset representation, and changes in clonal composition. Thus, the renewal rates of both T and B cell pools decline (15, 20, 21), in part explaining how overall numbers can be maintained in the absence of newly generated cells. Further, inordinate clonal expansions are observed in both T and B cell compartments. Although some of these age-associated changes may result from immune dysregulation, others may simply reflect the cumulative influence of antigenic experiences and normal homeostatic processes. In accord with this notion, the ratio of T cells displaying naive versus memory phenotype inverts with age. Similarly, a substantial shift in the composition of peripheral B cell pools accompanies advancing age, reflecting the gradual appearance of a novel B cell subset whose properties and origins are the focus of this review.

### *The emergence and characteristics of age-associated B cells*

Recently, our laboratories described a phenotypically and functionally unique B cell subset that accumulates with age that we named age-associated B cells (ABCs) (22, 23). These cells display a characteristic transcriptional profile, compete homeostatically with naive follicular (FO) and marginal zone (MZ) B cells, and bear hallmark features of Ag-experienced cells. ABCs are detected in the spleen, blood, and bone marrow and less frequently in the peritoneal cavity or lymph nodes. Detailed understanding of their locale relative to splenic follicles and MZs is lacking, but recently reported age-associated changes in the cells occupying MZs make these sites a potential candidate (24). Finally, ABCs are associated with appropriate humoral responses to certain classes of infectious and inflammatory stimuli, arise prematurely in autoimmune-prone mouse strains,

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Abbreviations used in this article: ABC, age-associated B cell; FO, follicular; MZ, marginal zone; $T_{FH,}$ T follicular helper, .



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and may be enriched for autoreactive Ab specificities (23, 25). The origins and roles of ABCs in normal immune responses, as well as in immune senescence and autoimmunity, remain areas of intense investigation.

Although sharing many features, some heterogeneity exists among ABCs. Hao et al. (22) identified ABCs by the lack of both CD21 and CD23 expression. The frequency and the numbers of these B cells increased with the age, accounting for as much as 30% of splenic B cells in 22-mo-old mice. Further phenotypic analysis of this $CD23^-/CD21^-$ ABC population revealed that they differ from MZ, FO, or B1 B cells and showed that they express several markers shared with exhausted memory B cells (26). Simultaneously, Rubstov et al. (23) reported a population of $CD11c^+/CD11b^+$ B cells that appears in healthy aged female mice and in autoimmune-prone animals (23). These cells clearly overlapped with those reported by Hao et al. (22), because they expressed low levels of CD21 and CD23 and elevated levels of CD5, Fas, and CD138. However, in contrast to the more broadly defined cells described by Hao et al. (22), the $CD11c^+/CD11b^+$ B cells described by Rubstov et al. (23) uniformly expressed high levels of the activation markers CD80, CD86, and MHC class II. A comparison of surface markers among the ABCs defined by Hao et al. (22) and Rubtsov et al. (23) is shown in Table I. Importantly, both groups found that ABCs accumulate with age and tend to arise earlier and more consistently in female mice. Although this surface phenotype heterogeneity remains to be fully resolved, it likely reflects alternative routes of ABC generation.

A key feature of ABCs is that they express and depend upon B cell–intrinsic expression of the transcription factor T-bet (25). Consistent with this notion, T-bet overexpression induces acquisition of the ABC phenotype (25), indicating that it acts as a master regulator of ABC character. The exact mechanism whereby T-bet promotes and maintains the ABC phenotype remains unclear, but ongoing chromatin immunoprecipitation and deep sequencing studies will likely reveal both direct and indirect effects of T-bet on characteristic ABC gene expression patterns.

As might be anticipated from their unique T-bet driven transcriptional program, ABCs differ substantially from other B cell subsets in their activation requisites, functional capacities, and survival requirements. In contrast to FO or MZ B cells, ABCs survive but respond poorly to BCR engagement. However, they proliferate robustly to simulation with either TLR9 or TLR7 agonists, either alone or in combination with BCR ligation. Moreover, following TLR stimulation in vitro ABCs elaborate a unique spectrum of regulatory cytokines, with notably robust production of both IL-10 and IFN-γ. Recent in vivo studies have suggested that they are also an abundant source of TNF-α in vivo (27).

Table I. Comparison of the expression of surface markers by mouse and human ABCs and exhausted human B cells

| | Mice (Ref. 22) | Mice (Ref. 23) | Humans (Ref. 23) | Exhausted Human B Cells |
|---|---|---|---|---|
| CD19 | N/A | High | + | + |
| B220 | + | + | + | N/A |
| CD11c | +/− | + | + | + |
| CD11b | N/A | + | N/A | + |
| CD21 | − | − | − | Low |
| CD23 | − | − | − | + |
| Fas | N/A | + | + | N/A |
| CD138 | N/A | Int | N/A | Low |
| CD5 | − | Int | + | N/A |
| CD80/86 | Low | High | High | High |
| MHC class II | Low | High | N/A | N/A |
| T-bet | N/A | + | + | N/A |
| Surface IgM | + | +/− | − | N/A |
| Surface IgD | Low | +/− | − | − |

Int, intermediate; N/A, not available; −, negative; +, positive.

While most murine ABCs express IgM, they rapidly switch to IgG production after stimulation with TLR ligands (23, 25). Regardless of their source – autoimmunity, age or viral infection – ABCs are prone to IgG2a/c production (23, 25), consistent with the established role of T-bet in switching to this IgH isotype (28–32). However, the specificity of the IgG produced by ABCs differs depending on their source; ABCs obtained from autoimmune or aged mice produce autoreactive IgG, whereas ABCs from virally infected mice produce predominantly antiviral IgG (Fig. 1) (23, 25). Together, these observations imply involvement of BCR signaling during the differentiation and recruitment of B cells into the ABC subset, despite their apparently dampened response to BCR ligation alone.

In addition to Ab secretion, ABCs can serve as Ag presenters; following activation, they can produce regulatory cytokines capable of skewing the differentiation of other adaptive and innate cell subsets. For example, early studies showed that ABCs obtained from aged animals can present Ag and tend to induce Th17 polarization (22). More recent findings extend this idea and suggest that ABCs obtained from aged or autoimmune mice process and present Ag more efficiently than do other B cells (33).

The accumulation of ABCs has profound effects on the dynamics and homeostasis of peripheral B cell pools. Interestingly, ABCs express the canonical BAFF receptors BR3 and TACI, but unlike FO and MZ B cells, they do not rely on BAFF for survival. Thus, as ABCs accumulate they engender reciprocal decreases in FO B cell numbers through competition for BAFF (22). Moreover, recent studies from Riley and colleagues (27) suggest that ABCs negatively influence B-lineage commitment or development of bone marrow progenitors, implying a causal role for ABCs in the decline of B cell lymphopoiesis with age. These observations may bear on reports that B lymphocyte ablation can rejuvenate B lymphopoiesis in aged individuals (34), in as much as ABCs do not reappear quickly during self-reconstitution.

It is tempting to speculate that the progressive dominance of ABCs at the expense of FO B cells impacts adaptive humoral responses, and a growing body of evidence suggests that this may be the case. For example, adoptive-transfer experiments showed that multiple aspects of T follicular helper ($T_{FH}$) cell differentiation—including those that depend upon B cell Ag presentation, such as the upregulation of IL-4 and IL-21 production—are profoundly compromised in aged mice, regardless of T cell donor age (35, 36). Thus, the outcome of cognate presentation by ABCs may differ from other APCs, failing to reinforce the $T_{FH}$ cell program or directing pre-$T_{FH}$ cells to alternative effector fates. In agreement with this idea, ABC presenters skew primed T cells to a Th17 fate in vitro (22).

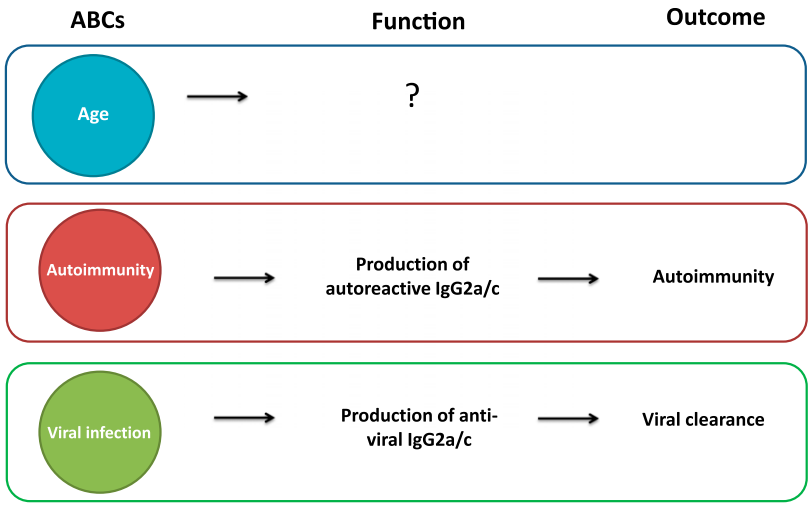


**FIGURE 1.** ABCs in age, autoimmunity, and infection. The function and outcome of the appearance of ABCs in aged animals are still unknown. In autoimmune animals, ABCs produce high titers of autoantibodies (mostly of IgG2a/c isotype) upon stimulation, which may be the cause of autoimmunity. During the infection, ABCs produce antiviral IgG (mostly IgG2a/c), which is required for efficient viral clearance.

*ABC generation in health and disease*

ABCs probably arise from activation-driven differentiation. Early work ruled out the possibility that ABCs represent the product of B cell genesis in the aged microenvironment, because they do not reappear after irradiation and autoreconstitution. Instead, multiple lines of evidence now suggest that they are a normal differentiative alternative taken by naive B cells when responding to certain classes of exogenous and endogenous stimuli. Initial evidence that ABCs can arise from naive B cells was suggested by experiments in which FO B cells from young donors were transferred to replete young or old congenic hosts. One month later, the recovered donor cells that had undergone extensive division had adopted an ABC phenotype, regardless of host age (22). Although these findings showed that ABC-like cells could be derived from quiescent preimmune B cells, the activating stimuli were unclear, and the paucity of recovered cells prevented detailed functional analyses.

The early descriptions of ABCs also indicated that TLR7 and MyD88, but not IFN-αR, were required for the accumulation of ABCs (23), consistent with their being derived from events driven by activating receptors. Subsequent in vitro analyses revealed that T-bet upregulation, the hallmark of ABC generation, was induced most effectively by concomitant receipt of BCR ligation, TLR7 stimulation, and IFN-γ (25) (Fig. 2).

Taken together, these observations suggest that ABCs originate under circumstances involving Ags that engage the BCR and also contain ligands for endosomal nucleic acid sensors, which also induce a promoting cytokine milieu. If this is the case, ABCs would be expected to arise during normal adaptive responses to microbial pathogens, as well as during potentially autoreactive responses to self components, as long as this tripartite set of conditions is established. Indeed, several lines of evidence now indicate that ABCs arise and play key roles in both situations, providing clues about their emergence with age and connection with humoral autoimmunity.

*ABCs in infection and immunity.* B cells closely resembling ABCs arise during antiviral immune responses (25). These T-bet$^+$ CD11c$^+$ B cells appear at the peak of the humoral immune response during infection with mouse gammaherpesvirus 68, mouse CMV, lymphocyte choriomeningitis virus, and vaccinia. B cells with very similar phenotypic and functional characteristics also were described recently in *Ehrlichia muris* infection (37). Importantly, ABCs derived during these responses secrete pathogen-specific IgG upon restimulation in vitro more efficiently than FO B cells from the same host, indicating recruitment of Ag-specific B cells into the ABC pool rather than nonspecific enlargement of a bystander ABC pool. Further, ABC differentiation is a critical element of the successful immune response to viral infection. Mixed bone marrow chimeras in which the B cell compartment was T-bet deficient and unable to initiate ABC differentiation displayed dramatically reduced viral-specific IgG2a/c titers, less efficient viral clearance, and higher viral burden (Fig. 1) (25). This is in agreement with prior studies indicating that IgG2a/c most effectively drives viral clearance due to its efficiency in Ab-dependent cell–mediated cytotoxicity and high affinity for activating FcRs (38–41).

These findings also strengthen the idea that ABCs arise via BCR-mediated activation in the context of TLR stimulation and appropriate cytokine milieus; BCR engagement affords virus uptake and trafficking to endosomal nucleic acid sensors, whereas NK cells and T cells secrete abundant IFN-γ in response to the virus to provide the appropriate cytokine microenvironment (Fig. 2).

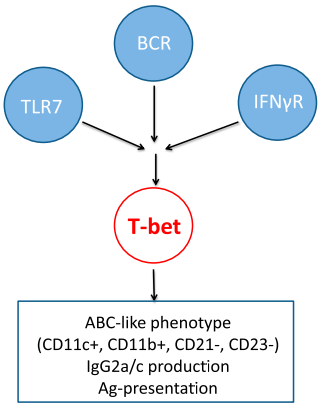


**FIGURE 2.** Model for T-bet induction in B cells and its role in B cell fate. Synergistic signaling via BCR, TLR7, and IFN-γR in B cells leads to the induction of high levels of T-bet expression, which, in turn, drives the expression of an ABC phenotype and class-switching to the production of IgG2a Abs.

**APPENDIX:** Naradikian et al., J Immunol. 2016

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Cutting Edge

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# Cutting Edge: IL-4, IL-21, and IFN-γ Interact To Govern T-bet and CD11c Expression in TLR-Activated B Cells

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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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The online version of this article contains supplemental material.

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

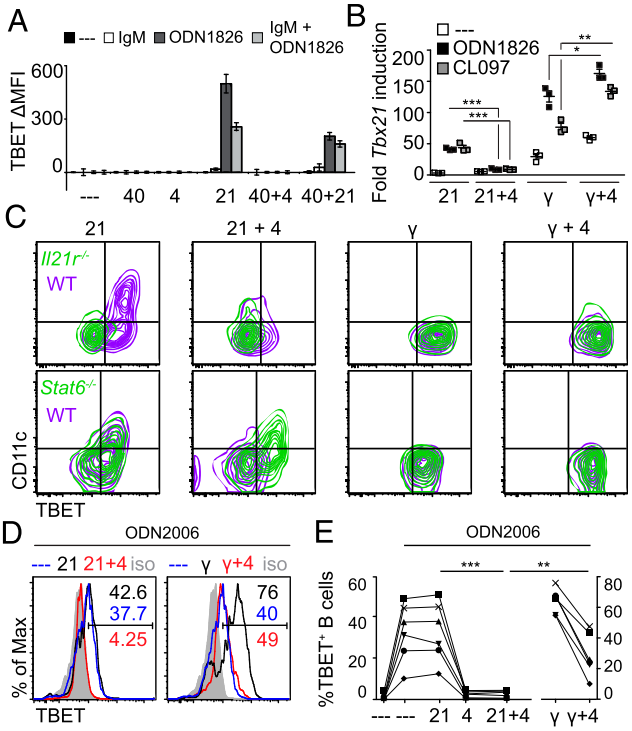


**FIGURE 1.** IL-4 and IL-21 act in a cell-intrinsic manner to regulate T-bet expression in vitro. Magnetically enriched CD23$^+$ splenic B cells were cultured in vitro with various combinations of anti–Ig-μ (IgM), anti-CD40 (40), IL-4 (4), IL-21 (21), and IFN-γ (γ). Mouse data are representative of 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-$bet^{+}$ 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-$bet^{+}$ B cells in these two strains should differ. In agreement with this prediction, whereas most GC B cells in B6 mice are T-$bet^{+}$, BALB/c have a lower frequency of T-$bet^{+}$ 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-$bet^{+}CD11c^{+}$ 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

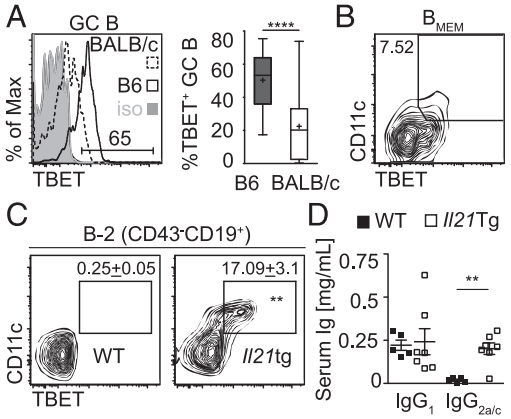


**FIGURE 2.** T-$bet^{+}CD11c^{+}$ cells delineate a $B_{MEM}$ cell subset and accumulate in *Il21*tg mice. (**A** and **B**) GC B and $B_{MEM}$ cells were analyzed for T-bet and CD11c expression by FACS. GC B and $B_{MEM}$ cell gating strategies are in Supplemental Fig. 1G. All panels are representative 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. $^{**}p < 0.01$, $^{****}p < 0.0001$.

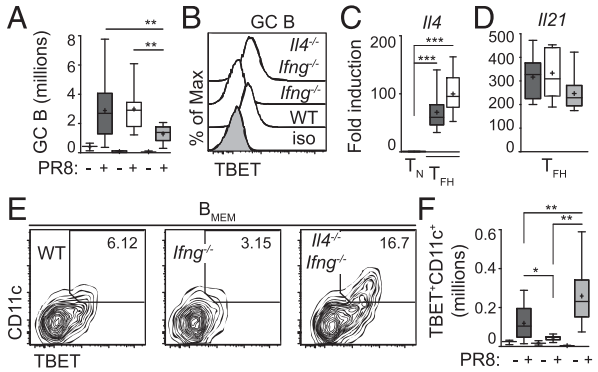


**FIGURE 3.** Influenza virus infection drives T-bet$^+$CD11c$^+$ $B_{MEM}$ cell formation in the absence of both IFN-γ and IL-4. Splenocytes were harvested from noninfected (−) or day 10 after intranasal 30 tissue culture infectious $dose_{50}$ PR8 infection (+) WT ($n$ = 21, black bars), *Ifng*$^{-/-}$ ($n$ = 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,

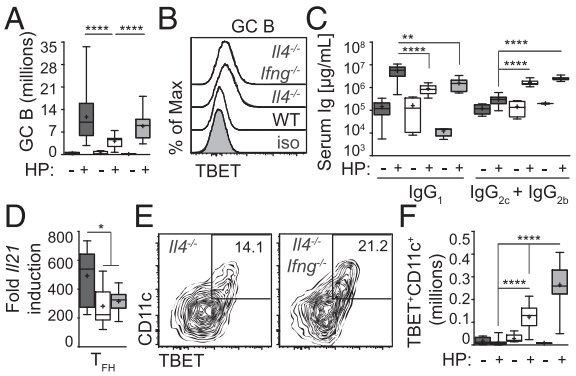


**FIGURE 4.** Activated B cells express T-bet independent of IFN-γ in IL-4 limiting conditions. Splenocytes and sera were harvested from noninfected (−) or day 14 after oral gavage (+) of 200 *H. polygyrus* in WT ($n$ = 20, black bars), *Il4*$^{-/-}$ ($n$ = 24, white bars), or *Il4*$^{-/-}$*Ifng*$^{-/-}$ ($n$ = 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

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**APPENDIX:** Naradikian et al., Immunol Rev. 2016 (review)

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Immunological Reviews

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# Age-associated B cells: key mediators of both protective and autoreactive humoral responses

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**Abstract:** A subset of B cells with unique phenotypic and functional features—termed Age-associated B cells (ABCs)—has recently been identified in both mice and humans. These cells are characterized by a T-BET driven transcriptional program, robust responsiveness to TLR7 and TLR9 ligands, and a propensity for $IgG_{2a/c}$ production. Beyond their age-related accumulation, these cells play roles in both normal and pathogenic humoral immune responses regardless of host age. Thus, B cells with the ABC phenotype and transcriptional signature appear during viral, bacterial, and parasitic infections, but also arise during humoral autoimmune disease in both mouse models and humans. These observations suggest that both autoantigens and certain classes of pathogens provide the signals required for ABC differentiation. Herein, we review the discovery and features of ABCs, and propose that they are a memory subset generated by nucleic acid-containing antigens in the context of a promoting inflammatory cytokine milieu.

Keywords: *Age-associated B cell, autoimmunity, T-BET, TLR7, TLR9, CD11c*

## Introduction

Protective immunity relies on establishing appropriate lymphocyte effector functions during primary responses, then sustaining these qualities in effector and memory lymphocyte pools. While correctly tailored effector functions promote lifelong immunity, inappropriate effector choices can yield failed pathogen clearance, chronic inflammation, or autoimmunity. Importantly, although the specificity of adaptive immune responses is achieved via the T- and B-cell antigen receptors (TCRs and BCRs), the quality of subsequently generated effectors is guided by further cues that reflect the context of ongoing host–pathogen interactions. For example, conserved pathogen-associated molecular patterns (PAMPs) engage various pattern recognition receptors (PRRs), whose signals initiate differentiative programs that direct particular effector choices (reviewed in 1). Similarly, cytokines and costimulatory molecules induced through these interactions can further focus and modulate effector differentiation. Master transcriptional regulators that reinforce particular fates

frequently characterize these effector differentiation patterns. Thus, pre-immune T cells adopt alternative effector fates based on TCR signal strength (2, 3), costimulatory cues (4, 5), and cytokine milieu. Multiple T-cell effector and memory lineages are now well established, with a corresponding appreciation of the master transcriptional regulators fostering each fate (reviewed in 6–8).

Analogously, B cells can adopt a variety of distinct effector fates that are guided by BCR signal strength coupled with additional cues that foster key transcriptional programs. These include antibody production, antigen presentation and costimulatory interactions with CD4 T cells, and cytokine secretion. Among these, the nature, levels, and persistence of immunoglobulin heavy chain isotypes that are provoked have profound implications for response quality, inasmuch as these features largely dictate the spectrum of effector functions that will be established. Indeed, nearly all heavy chain isotypes link adaptive humoral responses with a circumscribed group of innate inflammatory cells and systems important for pathogen clearance, such as complement fixation, opsonization, degranulation, and antibody-dependent cell-mediated cytotoxicity (ADCC). Many of these effector processes rely on isotype-specific Fc receptors that are differentially distributed among innate, myeloid, and other cell types, which can either enhance or limit the activation and behavior of these cells (reviewed in 9). While marshaling these potent effector systems is critical to effective humoral immunity, the same mechanisms mediate autoimmune and chronic inflammatory pathologies. Accordingly, the importance of understanding the signals and pathways that control adoption of various effector and memory B-cell fates is twofold: it will afford the potential to manipulate response quality in the elimination or control of pathogens, as well as reveal the regulatory circuits that avert errant or overzealous responses that lead to autoimmunity or chronic inflammation.

The recently discovered Age-associated B-cell (ABC) subset exemplifies this dichotomy. Mounting evidence indicates ABCs and their accompanying effector functions are crucial for effective humoral immunity to certain classes of infectious agents, but ABCs are also associated with autoimmunity in both humans and mouse models. These cells display unique phenotypic and functional properties, and make up a continuously increasing proportion of peripheral B cells with advancing age (10–12). Herein, we discuss current understanding of the characteristics and origins of ABCs, contrast them with other major B-cell subsets, and review their roles in both health and disease. Overall, we propose that in all of these contexts, ABCs represent antigen-experienced B cells whose unifying feature is a T-BET driven transcriptional program elicited by antigens that engage endosomal nucleic acid sensors in the context of a promoting cytokine milieu. Finally, we propose a model whereby these features and activation requisites—while enabling effective immunity to infectious agents meeting these criteria—align ABCs with responses to self ligands bearing similar properties, suggesting that responses to these types of ligands must be tightly regulated in order to maintain tolerance to nucleic acid-containing self antigens.

## Overview of B-cell development and homeostasis

In order to compare and contrast ABCs with other B-cell pools, the characteristic features of major B-cell subsets are briefly discussed here. Further, as the proportional representation of ABCs progressively increases at the expense of other B-cell pools, a succinct overview of how peripheral B-cell numbers are homeostatically controlled is provided.

### Pre-immune B-cell pools

B-cell subsets are defined by surface marker criteria that are correlated with developmental stage, activation status, recirculation properties, and anatomic locale. *Table 1* summarizes these criteria, features, and transcriptional regulators for developing and pre-immune B-cell subsets. In healthy adults, B cells are generated continuously from hematopoietic stem cells in the bone marrow (BM), where immunoglobulin (Ig) heavy and light chain gene rearrangements are completed during the pro- and pre-B-cell stages, respectively (13) (reviewed in 14, 15). Commitment to the B lineage involves both the acquisition of a B-cell transcriptional program and the suppression of programs leading to other hematopoietic fates (16). Accordingly, adoption of B lineage fate involves the expression of PAX5 (17–19), EBF1 (20–23), and E2A proteins (24, 25), which establish a transcriptional circuit that not only induces and reinforces B lineage genes but also represses transcription factors driving alternate lineages (26). Upon acquisition of surface IgM, developing B cells enter the immature (IMM) subset. These cells migrate to the periphery via the blood and pass through the so-called transitional (TR) developmental stages before entering the comparatively long-lived mature follicular (FO) or marginal zone (MZ) B-cell pools (27–32). The cues required to adopt the MZ instead of FO B cell fate include tonic BCR signal strength and transcriptional programs driven by Notch2 (33) interactions with Delta-Like-1 (34, 35) reviewed in (36).

***Table 1.*** **Features of developing and pre-immune B-cell subsets**

| Locale | B cell subset | Surface phenotype | Transcriptional regulators | BLyS receptors | Survival cytokine dependence |
|---|---|---|---|---|---|
| BM | pro-B | $CD19^+$, $CD43^+$, $CD93^+$, $IgM^-$, $IgD^-$ | PAX5 | None | IL7 |
| | pre-B | $CD19^+$, $CD43^-$, $CD93^+$, $IgM^-$, $IgD^-$ | PAX5 | None | unknown |
| | Imm | $CD19^+$, $CD93^+$, $IgM^{hi}$, $IgD^{lo/-}$ | PAX5 | BR3/TACI | unknown |
| Spleen/ Blood | TR | $CD19^+$, $CD93^+$, $CD43^-$, $IgM^{hi}$, $IgD^{lo/-}$, $CD21^-$, $CD23^-$ | PAX5 | TACI/BR3 | BLyS |
| Blood, lymph, spleen | FO | $CD19^+$, $CD93^-$, $CD43^-$, $IgM^{lo}$, $IgD^{hi}$, $CD21^{lo}$, $CD23^+$ | PAX5 | TACI/BR3 | BLyS |
| | MZ | $CD19^+$, $CD93^-$, $CD43^-$, $IgM^{hi}$, $IgD^{lo}$, $CD21^+$, $CD23^{lo}$ | Notch targets | TACI/BR3 | BLyS |
| Lymph nodes, Spleen | GC (DZ) | $CD19^+$, $IgM^-$, $IgD^-$, $PNA^+$, $CD95^+$, $CXCR4^+$, $CD83^-$ | BCL6 | BR3 | BLyS |
| | GC (LZ) | $CD19^+$, $IgM^-$, $IgD^-$, $CD23^+$, $PNA^+$, $CD95^+$, $CXCR4^-$, $CD83^+$ | BCL6 | BR3 | BLyS |
| Spleen | SLPC | $CD19^+$, $B220^+$, $IgM^{+/-}$, $IgD^-$, $CD138^{hi}$ | BLIMP1 | TACI/BCMA | APRIL? BLyS? IL6 |
| Blood, BM | LLPC | $CD19^-$, $B220^-$, $IgM^-$, $IgD^-$, $CD138^{hi}$ | BLIMP1 | BCMA | APRIL? |
| | Sw Bmem | $CD19^+$, $IgM^-$, $IgD^-$, $CD38^+$, $PDL2^{+/-}$, $CD73^{+/-}$, $CD80^{+/-}$ | unknown | BR3 | unknown |
| | IgM Bmem | $CD19^+$, $IgM^+$, $IgD^-$, $CD38^+$, $PDL2^{+/-}$, $CD73^{+/-}$, $CD80^{+/-}$ | unknown | BR3 | unknown |
| Spleen Blood, BM | ABC | $CD19^+$, $CD93^-$, $CD43^-$, $IgM^{+/-}$, $IgD^{+/-}$,$CD21^-$, $CD23^-$, $CD11c^{+/-}$ | T-BET | BR3/TACI | unknown |

Stringent selection based on BCR specificity occurs during both the IMM and TR stages: only 10% of IMM cells produced survive to exit the marrow, and only 30% of the TR cells thus generated survive to join the FO or MZ pools (28). These profound cell losses reflect both negative and positive selection based on BCR signal strength (37–43), presumably reducing the frequency of polyreactive and self-reactive specificities (38, 40, 44), as well as selecting for cells with optimal subthreshold signal strength (39, 43, 45, 46). Indeed, multiple studies now indicate that the representation of frankly autoreactive B-cell specificities in each pool decreases at each successive differentiation stage (47, 48). Thus, among peripheral B cells, the TR pool is a rich source of autoreactive BCR specificities compared to most other B-cell subsets (49).

The steady-state sizes of pre-immune FO and MZ B-cell pools are controlled by the TNF family member, B Lymphocyte Stimulator (BLyS, a.k.a. BAFF) (50, 51) (reviewed in 52). This cytokine sustains FO and MZ B-cell survival by signals delivered through BLyS receptor 3 (BR3, a.k.a. BAFFR) (53–57). All TR, FO, and MZ B cells express BR3 (58, 59) and compete continuously for BLyS to survive, such that available systemic BLyS determines their lifespan and thus overall FO and MZ pool sizes [(54, 55), reviewed in (60)].

### Antigen-experienced B-cell pools

B cells enter functionally distinct subsets following antigen-mediated activation (*Table 1*). In general, antigen-driven B-cell responses involve a rapidly generated burst of short-lived plasma cell (SLPC) formation, followed by the expansion of activated FO B cells that initiate germinal centers (GCs) upon receiving CD40 costimulation delivered during cognate interactions with CD4 T cells. B cells within GCs undergo antibody class switch recombination (CSR) to isotypes other than IgM, mediated by the deaminase enzyme AID (61, 62). While these processes are thought to occur most efficiently and frequently in GCs, affinity maturation and CSR in extrafollicular sites have been reported (63, 64). GC B cells are also the targets of AID-mediated somatic hypermutation (SHM) and affinity-based selection, such that mutated clones with higher affinity for the immunizing antigen are selectively preserved. As GCs progress and resolve, they yield B memory ($B_{mem}$) and long-lived plasma cells (LLPCs). Circulating antibodies have biological half-lives in the range of days to weeks depending on heavy chain isotype; thus, long-term protective immunity must rely on either LLPCs that persist indefinitely without seeding from $B_{mem}$ (65–67), or upon the sustained generation of SLPCs from $B_{mem}$ precursors driven by persistent antigen.

To adopt activated and effector fates, pre-immune B cells must integrate instructional cues from a variety of signals, sustain the mutational stress of CSR and SHM, and undergo profound morphological changes to produce antibody. To support these functions, activated B cells undergo significant transcriptional reprogramming based on the cues they receive. Thus, BCR-mediated cell activation in the context of CD40 costimulation induces translation of the transcription

factor BCL6, which is critical for adoption of GC B-cell characteristics (68–70). For example, despite the DNA breaks incurred during CSR and SHM, BCL6-mediated repression of p53 renders GC B cells resistant to apoptosis (71). Differentiation to antibody secretion and the plasma cell fate requires further transcriptional reprogramming. Though initially thought to be required for PC commitment (72), the transcription factor BLIMP1 is necessary for *bona fide* PC differentiation (73). This likely stems from the ability of BLIMP1 to extinguish PAX5 expression, which affords the profound shifts in functional and morphological characteristics associated with PC differentiation (74, 75). In this regard, these cross-competing transcription factors facilitate a gene expression program to direct mature B cells through activation, selection, and terminal effector function. No master regulator of the $B_{mem}$ cell lineage is yet identified; however, T-BET and RORα are associated with $IgG_{2a/c}$ and IgA respectively, and are critical for the formation and maintenance of some $B_{mem}$ subsets (76).

The homeostatic regulation of antigen experienced B lineage pools is less well understood, and likely involves a more complex set of players than the comprehensive role assumed by BLyS for pre-immune pools. Indeed, the heterogeneity in lifespan and composition of B memory and PC subsets has only begun to be appreciated over the last several years, and will likely expand further. For example, recent conceptual advances challenge the notion that PCs are a monolithic population. Instead, T-cell-independent PCs live longer than previously thought (77); new subset markers have revealed phenotypic heterogeneity and complex turnover kinetics in BM PC pools (78, 79); and some PCs secrete cytokines and antimicrobial agents in addition to antibodies (80). Despite this growing complexity, several factors that govern PC longevity and homeostasis have been identified. LLPC survival depends, at least in part, on BLyS and/or APRIL signaling via the BCMA and/or TACI receptors (81, 82). In this regard, LLPCs are thought to occupy survival niches independent of the primary pool. Homing to and occupation of these longevity-fostering niches is still an active area of research especially given newly discovered subsets and isotype-associated functional differences (83).

Similarly, several $B_{mem}$ subsets have been defined, based on the presence of different isotypes and further markers (*Table 1*). The immunological role of $B_{mem}$ cells may appear redundant with simultaneous, clonally similar, and elevated antibody titers; however, recent evidence highlights a distinct $B_{mem}$ cell role in response to the pathogenic variants that have escaped the neutralizing capacity of the primary antibody response (84). Moreover, delineating which $B_{mem}$ subsets rapidly differentiate into PCs or engage in further GC formation upon secondary antigen challenge is an area of intense investigation. Although some evidence suggests that isotype determines $B_{mem}$ cell subset and properties (85, 86), more recent work shows that surface markers such as PD-L2 and CD80 predict functional outcomes irrespective of isotype (87). Furthermore, the maintenance of these pools is independent of BLyS, antigen, or T cells (88–90). *In toto*, these observations indicate that PCs and $B_{mem}$ play non-redundant roles in immunity and occupy distinct homeostatic niches.

## The discovery and features of ABCs

Advancing age is accompanied by far-reaching shifts in immune system development and function, including reduced lymphopoiesis (91–94), blunted primary and recall immune responses (95–110), the appearance of autoantibodies (111–116), and increased frequencies of autoimmune and autoinflammatory conditions (117). Accordingly, shifts in the generation and relative representation of lymphocyte subsets have been scrutinized as potential mechanisms underlying these features. Within the T lymphocyte lineage, thymic involution, reduced T lineage specification, and an inversion in the ratio of naive to memory T lymphocytes are established age-associated phenomena (92, 105, 113, 118–124), and more recent studies have linked these shifts to compromised vaccine and disease responses. Within the B lineage, similar reductions in early lineage specification and shifts in the sizes and kinetics of developing B-cell pools have also been appreciated for some time (91, 93, 94, 99, 101, 103, 125–130). However, despite numerous clues indicating changes in the functional attributes of peripheral B cells with age (99, 100, 112, 129, 131–134), whether shifts in naive and antigen experienced B-cell subsets occur with age, as well as the functional consequences of such changes, remained unclear until recently. Within this context, a B-cell subset with unique functional properties was identified and characterized.

### ABCs emerge with age and have a unique surface marker phenotype

ABCs were first described in simultaneous reports from Hao *et al.* and Rubtsov *et al.*, using distinct, but largely complimentary phenotypic and functional criteria (10, 11) (reviewed in 12). As their name implies, the splenic ABC

pool emerges in mid-life and continuously enlarges with advancing age. Thus, in healthy adult mice, ABCs are either absent or comprise a negligible proportion of peripheral B cells until about 12 months of age, then increase steadily in both proportion and number (10–12, 135). ABCs are observed in multiple inbred strains and F1 combinations, suggesting they are a common feature of the aging B-cell pool (10). Although the tempo with which ABCs emerge varies considerably among individuals, they generally comprise 30–40% of mature recirculating B cells by 24–30 months of age (135). Moreover, ABCs tend to appear sooner and achieve higher numbers and representation in females (11), although the exact basis for this sex-associated dichotomy is unclear [discussed in (12)].

ABCs display a distinct surface phenotype in mice (*Table 1*). They are mature B cells, as evidenced by their lack of CD93. Further, while positive for both B220 and CD19, they lack the canonical FO, MZ, or B1 B-cell markers CD23, CD21, and CD43, respectively. Detailed phenotypic, functional, and transcriptome analyses confirmed that ABCs differ from all previously defined B-cell subsets. Although Hao *et al.* (10) used this combination of features to define the population, Rubtsov *et al.* (11) employed CD11c as a singularly characteristic marker. Thus, while the B-cell population circumscribed by the criteria in Hao *et al.* includes the $CD11c^+$ cells of Rubtsov *et al.*, it also captures additional cells that lack CD11c. This distinction may underlie some apparent differences in functional attributes discussed below.

Anatomically, ABCs are found in the blood, spleen, and BM, but are rarely observed in lymph nodes (10). While these qualities suggest they are circulating cells, multiple observations suggest their trafficking and homing patterns differ from FO B cells and other recirculating subsets. For example, expression of the follicle homing factor CXCR5 is reduced on ABCs (10), and more recent studies reveal they are enriched at the splenic T:B border, presumably reflecting migratory differences imparted by increased CCR7 expression (136). Based on these observations, it is tempting to speculate that ABCs may be involved in the age-associated alterations in MZ composition previously reported by Birjandi *et al.* (137). Further, based upon their absence from the lymphatics, coupled with the lack of formal studies probing their circulatory properties, it remains possible that some ABCs are sessile tissue-resident cells. In this regard, further phenotypic analysis of ABCs may reveal their origins and function in health and disease.

### ABC activation and survival requirements differ from those of other B-cell subsets

In addition to their distinct phenotype and localization characteristics, ABCs display unique activation requisites and functional attributes. Initial *in vitro* studies revealed that, unlike TR, FO, or MZ B cells, ABCs fail to proliferate after BCR cross-linking, but nonetheless survive (10). In contrast, they exhibit robust proliferative responses to stimulation via TLR9 or TLR7, and despite being refractory to BCR cross-linking alone, concomitant BCR engagement potentiates their proliferative responses to TLR ligands (10, 11). Consistent with these observations, both TLR9 and TLR7 expression are elevated in ABCs (10). There is probably heterogeneity among ABCs in terms of relative responsiveness to TLR7 versus TLR9 ligation; cells identified by the surface marker criteria of Hao *et al.* show more extensive proliferation to TLR9 than to TL7 agonists, whereas cells defined by the criteria of Rubtsov *et al.* display the reverse. Whether this reflects alternative routes of generation, different stages of the ABC differentiation pathway, or other distinctions remains unclear.

ABCs are unusual—and again unlike other B-cell subsets—because they express both BR3 and TACI but are independent of BLyS for their survival (10). Thus, BLyS neutralization *in vivo* spares ABCs, despite eliminating FO and MZ B cells. Because ABCs display the same spectrum of BLyS receptors as FO and MZ B cells, they bind and sequester BLyS equivalently and act as super-competitors for this limiting cytokine, inasmuch as they consume BLyS but do not need it to survive. Accordingly, as ABCs are generated and accumulate, they capture a progressively larger proportion of the BLyS-dependent niche at the expense of the FO compartment. Moreover, this capacity for BLyS-independent survival is similar to what has been reported for $B_{mem}$ cells, although currently defined $B_{mem}$ pools do not express BR3 (138). Given the recent advances in delineating murine $B_{mem}$ subsets (87, 139), exploring potential heterogeneity within the ABC pool may elucidate how ABCs fit into the broader immunological context.

### ABCs exhibit a characteristic set of effector attributes

The unique functional attributes of ABCs extend to virtually all B-cell effector mechanisms, including antigen presentation, cytokine secretion, kinetics of PC differentiation, and isotype-switching preferences. Several studies have established that ABCs are effective antigen presenting cells (APCs). Initial findings *in vitro* indicated that ABCs can serve as APCs, in accord with their comparatively high levels of

MHC II, CD80, and CD86 (10). Further, ABC-mediated antigen presentation *in vitro* tends to skew naive CD4 T cells to a $T_H17$ fate, although this was not exclusive of other CD4 T cell cytokine profiles (10). More recent findings indicate that ABCs are also potent APCs *in vivo* (136). When activated by either TLR7 or TLR9 agonists, ABCs secrete a variety of cytokines, including IFNγ, IL-4, IL-6, and IL-10. Finally, upon activation, ABCs rapidly differentiate to antibody secreting PCs and tend toward $IgG_{2a/c}$ class switching (136, 140), despite their broad surface IgM and IgD expression (10). Given their propensity to become antibody-secreting cells, ABCs may constitute a population of slowly accumulating $B_{mem}$ cells generated in response to nucleic acid containing antigens. While it is unclear whether isotype or costimulatory molecule expression governs $B_{mem}$-cell responses (85–87), most ABCs are unswitched and remain uncharacterized for CD80, PD-L2, and CD73 expression. Lastly, ABCs also express CD95 and CD138 based on microarray analysis (11), which are markers associated with the GC and PC cell fates, respectively (see *Table 1*). Further characterization should distinguish the possibility of distinct ABC subsets or an atypical state of activation. *In toto*, ABC effector mechanisms seem to be largely normal, although skewed toward a type-I response.

### ABCs in aging—causes or effects?

Inasmuch as ABCs were first detected by virtue of their progressively increasing representation with age, the question arises as to whether they contribute to age-associated alterations in immune activity and immune responsiveness. This seems likely based on their unique spectrum of activation requirements, antigen presenting capacity, and effector cytokine profile, and several recent findings support this notion.

First, recent studies suggest that ABCs may be instrumental in the decreased B lymphopoiesis associated with age. Ratliff *et al*. (135) assessed the proportion of ABCs in mice at different ages, and found that the degree of B lymphopoiesis depression was proportional to ABC representation with age. Moreover, they showed that ABCs are a potent source of TNFα, and that this cytokine was in part responsible for inhibiting the survival of B lineage precursors *in vitro* and *in vivo*. These observations, coupled with the ability of ABCs to occupy homeostatic space to the detriment of pre-immune FO B cells, are particularly intriguing in light of reports that B lymphopoiesis and robust humoral responses can be rejuvenated in aged individuals by B ablative regimes (141–145).

Second, recent studies have revealed aspects of $T_{FH}$ generation that rely on cognate B-cell interactions differ in young and aged mice. For example, several laboratories have shown that optimal IL-21 and IL-4 production among dendritic cell-primed $T_{FH}$ cells only occurs following antigen presentation by B cells (146–148). However, under *in vivo* immunization conditions that normally foster this $T_{FH}$ profile, aged individuals fail to generate $T_{FH}$ cells with the characteristic upregulation of IL-4 (149). While causal links have not yet been forged, it is tempting to speculate that ABC antigen presentation may foster a $T_{FH}$ cytokine profile that lacks IL-4. Alternatively, $T_{FH}$ cells generated in aged individuals may intrinsically skew toward a cytokine profile lacking IL-4, which might be instrumental in driving ABC generation.

Third, in addition to the decline of B lymphopoiesis and functional T-cell alterations, an increase in anti-dsDNA, antinuclear, and other autoantibodies occurs in both mice and humans (111, 114, 150, 151). Indeed, ABC accumulation correlates with serum autoantibody levels, and ABCs isolated from aged mice and stimulated with a TLR7 agonist produced anti-chromatin antibodies (11). While not themselves PCs, ABCs could constitute a pool of $B_{mem}$ cells that feed a population of autoantibody secreting PCs. Thus, while ABC accumulation has a number of correlative associations with B-cell development, altered T-cell functionality, and autoantibody production, causality is yet to be established.

## ABCs in health and disease

The differentiative routes to an ABC phenotype, the signals prompting this program, and the progenitor-successor relationships involved, have been the subjects of ongoing investigations for several years. Early studies ruled out the possibility that ABCs represent the product of aberrant or skewed B-cell development in the aged BM, inasmuch as ABCs do not reappear during autoreconstitution after B-cell depletion by sublethal irradiation or other regimes (10). Instead, accumulating evidence suggests that ABCs are antigen-experienced B cells arising from activation with nucleic acid-containing antigens in the context of a promoting cytokine milieu. Further, they can likely arise from most pre-immune peripheral B-cell subsets, including the FO and TR pools. The antigens that engender an ABC-like response are intracellular pathogens such as viruses. In this setting, viruses provide three key signals that foster ABC identity: BCR ligation, nucleic acid sensing TLR engagement, and an inflammatory cytokine milieu. However, B cells with some degree of specificity for nucleic acid

containing self-ligands may also receive the requisite three signals leading to ABC characteristics (Fig. 1).

### ABCs are products of a T-BET driven transcriptional program

Transcription factors guide and reinforce cell fate decisions for proper biological functions. In accord with this general developmental principal, early transcriptional profiling revealed that ABCs express the transcription factor T-Box Expressed in T cells (T-BET) encoded by the *Tbx21* gene (11). Initially, Glimcher's group described the role of T-BET in CD4 helper T cells as a regulator of IFNγ production and $T_H1$ cell fate (152). Despite this moniker, several other cell types including CD8 T cells, Natural Killer cells, and dendritic cells express and require T-BET for their specific immunological function (reviewed in 153). In the B lineage, T-BET expression fosters switching to $IgG_{2a/c}$ (76, 154, 155) and is necessary for controlling viral titers (140). Indeed, early studies across multiple mouse strains infected with both RNA and DNA viruses representative of 11 different genera elicited $IgG_{2a/c}$ isotypes, which have subsequently been shown to be protective (156, 157).

Given T-BET's association with humoral autoimmunity and role in protective anti-viral humoral responses, the factors responsible for its upregulation are of particular interest. While the external cues driving B-cell T-BET expression were initially reported as concomitant IL-12 and IL-18 stimulation (152), subsequent studies have also demonstrated that IFNγ shares this capacity (158, 159). Under most circumstances, these cytokine signals are derived from $T_{FH}$ cells. In order for both GC B cells and $T_{FH}$ cells to fully differentiate, they must engage in intimate and reciprocal interactions beyond the initial T-cell priming event with a dendritic cell (146). $T_{FH}$ cells are transcriptionally and anatomically distinct from other T-helper lineages (160), yet they still harbor the capacity to make IFNγ and IL-4 against viruses and helminthes, respectively (161–163). In addition to the effects of the hallmark cytokine of the $T_{FH}$ lineage, IL-21 (164, 165), how all of these cytokine signals are integrated to instruct appropriate isotype and B-cell fate decisions remains unclear. Thus, the induction of T-BET in the B lineage relies on particular classes of antigenic stimuli—including those provided by DNA and RNA viral infections—in the context of inflammatory cytokines. *In toto*, the emergence, functional attributes, and T-BET driven nature of ABCs suggest they may reflect the sum of accumulated responses to antigens fulfilling these activation criteria over the lifespan of the host organism. Accordingly, while ABCs will increase steadily within an individual, the rate will vary depending on exposures to appropriate stimulatory cues, and will thus vary substantially across a population. This strikes a cautionary note for assessments of ABC accumulation in humans, in as much as they are both generically diverse and are heterogeneous with respect to antigen exposure history.

### ABCs are generated in response to intracellular infections

Early adoptive transfer studies revealed that FO B cells give rise to ABCs. In these studies, CFSE labeled FO B cells were

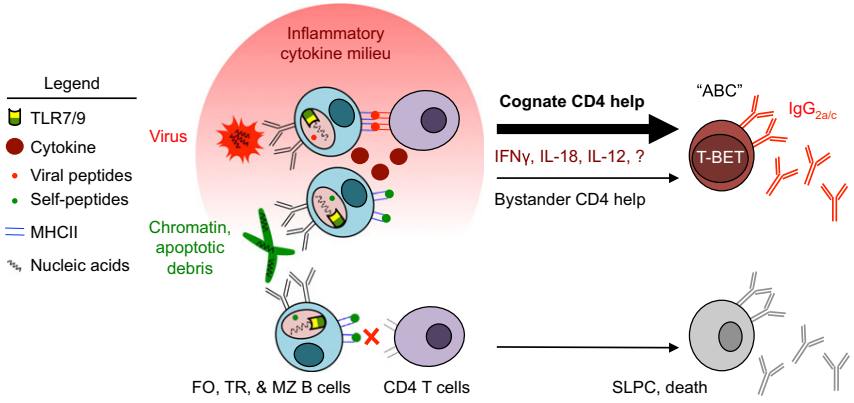


**Fig. 1. Both beneficial and pathogenic Age-associated B cells (ABCs) arise via a common triad of signals.** Pre-immune FO, MZ, and/or TR B cells bind, internalize, and traffic antigens to endocytic compartments via the BCR. Pathogen degradation leads to viral nucleic acids engaging TLR7 or TLR9. Processing and loading of viral peptides onto MHCII molecules results in cognate CD4 T-cell interactions and T-cell-derived inflammatory cytokine secretion. Currently, IFN-gamma, IL-18, or IL-12 are known to serve this role, but additional, yet to be interrogated, cytokines (depicted as "?") may also positively or negatively regulate ABC fate. The combination of these signals leads to T-BET$^+$ memory and effectors, and to $IgG_{2a/c}$ isotype switching. Normally, B cells that internalize nucleic acid containing self-antigens fail to survive because they lack the cognate T-cell interactions and appropriate cytokine signals to further differentiate and survive. However, inadvertent or aberrant receipt of these signals can afford survival of these self reactive cell and enable their recruitment into a long-lived memory and effector ABC pool.

transferred to congenic hosts and harvested 30 days afterward, revealing that transferred cells which had extensively proliferated had acquired the ABC phenotype (10). This observation linked the generation of ABCs with extensive division, although the stimulating conditions driving the phenotype were unclear, and further functional characterization of these recently formed ABCs was hampered by the small cell numbers recovered in such experiments. Nonetheless, these findings suggest that ABCs are likely an antigen-experienced pool. Since B-cell responses against viruses result in a protective, T-BET-dependent $IgG_{2a/c}$ response, Marrack's group hypothesized that a viral infection would drive T-BET *in vivo*. Indeed, mice infected with gamma herpes virus, vaccinia, or LCMV all produced T-BET$^+$CD11c$^+$ B cells (140). Of the TLR agonists used, the authors found TLR7 stimulation to be the most effective at driving T-BET expression in the context of BCR and IFNγ stimulation. Lastly, B-cell intrinsic T-BET expression played a non-redundant role in controlling viral and anti-viral $IgG_{2a/c}$ production. These findings have been extended to human studies where a subset of B cells in HIV-viremic individuals also display an ABC surface phenotype (166), but are yet to be fully characterized for T-BET and CD11c. *In toto*, these data support the notion that ABCs are indeed antigen experience cells arising from immune responses characterized by nucleic acid-containing antigens in the context of pro-inflammatory cytokines. Whether other inflammatory cytokines harbor the capacity to induce an ABC-like phenotype and effector function remains unclear. Nonetheless, given the similar nature of the antigens, nucleic acid-containing self-antigens, like viruses, may drive a similar ABC-like program.

### ABCs are associated with humoral autoimmune and autoinflammatory diseases

Beyond their initial identification in aged mice, ABCs prematurely accumulate in a variety of autoimmune- prone mouse strains. A considerable ABC population accumulates as early as 6 months of age in mice lacking the tyrosine kinase, Mer, as well as in $NZB/WF_1$ animals (11). In accord with these findings, ABCs constitute a higher proportion of total B cells in female RA patients (11). Moreover, before their detailed characterization in mice, ABC-like cells were also observed in common variable immunodeficiency (167) and Sjögren's syndrome patients (168). It is unclear if the CD21$^{-/lo}$ cells in these individuals constituted a *bona fide* ABC population, since they were not characterized for CD11c, T-BET, or other subsequently identified ABC makers. In contrast, a more recent investigation of an early-onset Evans syndrome patient exhibited premature senescence and an increased number of CD11c$^+$T-BET$^+$ B cells that correlated with antinuclear antibodies (169). Interestingly, duplicating the tripeptidyl peptidase II (TPP2) frameshift mutation in mice recapitulated both the disease and ABC phenotype. Thus, ABCs are implicated in both human autoimmune syndromes and in murine models of humoral autoimmunity. These finding prompt the question of how the dichotomous roles of ABCs—providing beneficial and appropriate effector functions during intracellular infection but also being associated with humoral autoimmunity—can be mechanistically reconciled.

### A unique signaling triad links appropriate and pathogenic ABC generation

ABCs in aged, virally infected, and autoimmune-prone mice and humans share a common triumvirate of stimuli for their origin: BCR signals, endosomal nucleic acid sensor ligation, and a type-I inflammatory cytokine milieu. This commonality is likely the link that connects beneficial and pathogenic ABC formation. In particular, the role of endosomal nucleic acid sensors driven by BCR-acquired antigens may prove the most important feature. Over the past decade the contribution of nucleic acid-sensing PRRs, particularly TLR7 and TLR9, to the etiology of autoimmune disease have come to the forefront. Beginning with the seminal observation that BCR delivered nucleic acid ligands have distinct survival and proliferative potentials for B cells (170), it has become increasingly apparent that TLR7 and TLR9 both promote and regulate the progression of humoral autoimmunity. To test this idea, Shlomchik's group bred SLE-prone mice to either TLR7 or TLR9-deficient mice (171, 172). Because extra *Tlr7* gene copies result in an SLE-like disease (173), it was expected that both TLR7 and TLR9 deficiency would ameliorate disease in autoimmune-prone mice. While TLR7 deficiency yielded the expected result, TLR9 deficiency unexpectedly exacerbated disease (171, 174). This suggests not only a regulatory role for TLR9 but also differential disease outcomes based on whether autoreactive B cells can sense RNA or DNA moieties. These relationships are further complicated by the fact that TLR9 deficiency in other models of autoimmunity ameliorates disease (175). Though the mechanism by which TLR9 limits disease remains unclear, recent findings suggest a role for type-I interferon signaling in disease etiology (176). Whether ABC formation occurs in these

models of autoimmunity and whether TLR7 and TLR9 modulate ABC accumulation is yet to be established. Thus, the signals from the BCR, nucleic acid sensing TLRs, and inflammatory cytokines—while involved in appropriate ABC-mediated immunity—are exactly those that, when dysregulated, foster humoral autoimmunity.

These ideas lead to the model schematized in *Fig. 1*. In general, any nucleic acid-containing antigen harbors the capacity to drive an ABC phenotype. However, growing evidence suggests that T-cell derived pro-inflammatory cytokine signals, as well as cognate help, may be required to engender a long-lived ABC fate. In this regard, an activated B-cell presenting viral peptides will efficiently receive all three signals required for recruitment into long-lived effector subsets. In contrast, autoreactive B-cells binding nucleic acid-containing self molecules—such as apoptotic debris or other sources—while receiving BCR and TLR signals, should fail to receive further cues for GC formation and survival, resulting in short-lived responses or death. However, inadvertent or aberrant recruitment of such short-lived autoreactive cells into long-lived effectors would thwart this peripheral tolerance system, and yield sustained autoantibody and auto inflammation driven by $IgG_{2a/c}$ production. Multiple routes to such dysregulation could be envisioned, including overabundance of autoantigen or deficiencies in the molecules that mediate these regulatory circuits.

## Perspective

ABCs impact a broad spectrum of immunological phenomena, including protective immunity to some pathogen classes, immunosenescence, and autoimmunity. In mice, ABCs constitute an accumulating population of B cells with unique surface phenotype, signaling properties, and effector status. While initially described in aged mice, phenotypically and transcriptionally identical cells are observed in humoral autoimmunity and immune responses against intracellular pathogens in both mice and humans. These observations lead us to propose that ABCs are a memory B cell population, and that the signals and interactions that yield ABCs in both normal and autoreactive humoral responses are comparable; these consist of concomitant signals from the BCR, nucleic acid sensing TLRs, and inflammatory cytokines. Thus, understanding how responses to endogenous versus exogenous nucleic acid-bearing ligands are regulated in order to maintain tolerance yet allow appropriate responses to pathogens should yield insights relevant to both protective and autospecific humoral immunity.

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**APPENDIX:** Russell Knode et al., J Immunol. 2017

Russell Knode LM, Naradikian MS, Scholz JL, Hao Y, Liu D, Ford ML, Tobias JW, Ca**ncro MP,** Gearhart PJ. Age-associated B cells express a diverse repertoire of mutated immunoglobulins and share transcriptional profiles with memory cells. *J Immunol.* 2017 Mar 1;198(5):1921-1927. doi: 10.4049/jimmunol.1601106. Epub 2017 Jan 16. PMID:28093524

# Age-Associated B Cells Express a Diverse Repertoire of $V_H$ and Vκ Genes with Somatic Hypermutation

**Lisa M. Russell Knode,* Martin S. Naradikian,† Arpita Myles,† Jean L. Scholz,† Yi Hao,†,[1] Danya Liu,‡ Mandy L. Ford,‡ John W. Tobias,§ Michael P. Cancro,† and Patricia J. Gearhart***

**The origin and nature of age-associated B cells (ABCs) in mice are poorly understood. In this article, we show that their emergence required MHC class II and CD40/CD40L interactions. Young donor B cells were adoptively transferred into congenic recipients and allowed to remain for 1 mo in the absence of external Ag. B cells expressing the T-bet transcription factor, a marker for ABCs, were generated after multiple cell divisions from C57BL/6 donors but not from MHC class II– or CD40-deficient donors. Furthermore, old CD154 (CD40L)-deficient mice did not accrue ABCs, confirming that they arise primarily through T-dependent interactions. To determine what Igs ABCs express, we sequenced $V_H$ and Vκ rearranged genes from unimmunized 22-mo-old C57BL/6 mice and showed that they had a heterogeneous repertoire, which was comparable to that seen in old follicular and marginal zone B cell subsets. However, in contrast to the follicular and marginal zone cells, ABCs displayed significant somatic hypermutation. The mutation frequency was lower than found in germinal center cells after deliberate immunization, suggesting that ABCs have undergone mild stimulation from endogenous Ags over time. These observations show that quiescent ABCs are Ag-experienced cells that accumulate during T cell–dependent responses to diverse Ags during the life of an individual.** ***The Journal of Immunology*****, 2017, 198: 1921–1927.**

Profound changes in the composition and dynamics of lymphoid populations occur with age, likely contributing to the decline in immune status, collectively termed immune senescence. For example, B cell production from bone marrow steadily decreases with age, yet the numbers of peripheral B cells remain relatively constant as a result of slowed turnover and altered representation of naive and Ag-experienced B cell subsets (1–8). A novel B cell subset that accumulates with age, termed age-associated B cells (ABCs), was identified recently (9–12). These cells have unique features that include preferential responsiveness to TLR7 and TLR9 ligands, surface markers consistent with prior Ag activation, and expression of the T-box transcription factor, Tbx21 (T-bet), which is required for their accumulation (13). Some ABCs also express Itgax (CD11c), an integrin that potentiates their ability to present Ag to T cells (14). ABCs are associated with the onset and severity of humoral autoimmunity in animal models and humans (10, 15, 16). Further, these cells play roles in age-associated immune dysfunctions, including elevated inflammatory cytokine levels and reduced B cell generation rates (11). Finally, a growing literature suggests that B cells with similar characteristics arise during some viral, bacterial, and parasitic infections (13, 17–21), implying a role for ABCs in normal immune function.

Despite these observations, the origin and nature of ABCs remain poorly understood. In this study, we investigated their formation, Ig repertoire, and level of somatic hypermutation. The results indicate a polyclonal, Ag-experienced B cell population that arises primarily through T-dependent immune responses to diverse endogenous Ags.

## Materials and Methods

### *Mice*

All mice used for experiments were females on a C57BL/6 background. Old mice were obtained from the Charles River aged mouse colony at 18 mo of age and used at 22 mo. *Cd154*$^{-/-}$ (B6.129S2-Cd40lg$^{tm1Imx}$/J) mice were purchased from the Jackson Laboratory and kept until 22 mo of age. Young (2–4 mo) CD45.1 and CD45.2 mice were obtained from the Jackson Laboratory. Young *I-Ab*$^{-/-}$ mice were from Terri Laufer (University of Pennsylvania), and *Cd40*$^{-/-}$ spleens from young mice were sent from M. Ford's colony. *Aid*$^{-/-}$ mice were bred in the National Institute on Aging colony. Animal protocols were reviewed and approved by the Animal Care and Use Committees at the National Institute on Aging and the University of Pennsylvania.

### *Adoptive transfers*

CD23$^{+}$ splenic B cells from 2-mo-old CD45.2 mice were enriched by positive selection using the MACS bead system (Miltenyi Biotec). Cells

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The online version of this article contains supplemental material.

Abbreviations used in this article: ABC, age-associated B cell; AID, activation-induced deaminase; FO, follicular; HA, hemagglutinin; HA-PE, PE-labeled probe that recognizes HA of PR8; MZ, marginal zone; PR8, influenza strain A/Puerto Rico/8/1934.

were labeled with CFSE (eBioscience), according to the manufacturer's instructions, and 8 million cells were transferred into each CD45.1 congenic host by retro-orbital injection.

### *Flow cytometry and FACS sorting*

Single-cell suspensions were prepared from spleens and stained with fluorochrome-conjugated Abs. For flow cytometry of the adoptive-transfer and influenza experiments, we used Live/Dead Zombie Aqua, anti-CD45.1-AF700 (A20), anti-CD45.2-BV421 (104), anti-CD19-BV785 (6D5), anti-CD23 biotin (B3B4), and anti-CD11c (N418) (BioLegend). Anti-CD43–PE (S7) was from BD Biosciences. Cells were analyzed on an LSRII, and data were analyzed using FlowJo software (TreeStar). Intracellular stains for T-bet were performed with anti–T-bet–allophycocyanin (4B10; BioLegend) and a Foxp3 transcription factor kit (eBioscience), according to the manufacturer's instructions. For FACS sorting to isolate subsets, we used anti-CD43–allophycocyanin (S7; BD Biosciences). Anti-CD23–PE Cy7 (B3B4), anti-CD21/CD35–eFluor 450 (4E3), anti-CD45R–FITC (B220, RA3-6B2), and anti-CD93 (AA4.1)-allophycocyanin were from eBioscience. Stained splenocytes were analyzed with a BD FACSCanto II or sorted using a BD FACSAria III, BD FACSAria Fusion, iCyt Reflection (Sony Biotechnology), or Beckman Coulter MoFlo. Follicular (FO) B cells were isolated as CD93 (AA4.1)$^-$ CD43$^-$ B220$^+$ CD21/35$^+$ CD23$^+$. Marginal zone (MZ) B cells were isolated as CD93 (AA4.1)$^-$ CD43$^-$ B220$^+$ CD21/35$^+$ CD23$^{lo}$. ABCs were isolated as CD93 (AA4.1)$^-$ CD43$^-$ B220$^+$ CD21/35$^-$ CD23$^-$. Analyses were done using FlowJo software.

### *V gene identification and mutation analyses*

Sorted cells were lysed in TRIzol reagent, and RNA was prepared. cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Ig H chain VDJ genes and Ig κ light (Igκ)-chain VJ genes were amplified using Taq polymerase (TaKaRa; Clontech) with 5′ degenerate primers specific to framework 1 of V genes and 3′ primers located in IgM or Igκ constant regions, as previously described (22). PCR products were cloned into StrataClone TA cloning vector (Agilent Technologies) and sequenced. Only sequences with unique VDJ or VJ joins were counted. The sequences were blasted against the mouse Ig loci using IgBLAST from the National Center for Biotechnology Information to identify V, D, and J gene segment usage and mutations. For mutational analysis of the $J_H4$ intron, DNA was prepared, and a 492-bp intronic region downstream of $J_H4$ from rearranged $V_HJ558$ genes was amplified using nested PCR. The first round used forward primer J558 5′-AGCCTGACATCTGAGGAC-3′ and reverse primer V1.8NR4R 5′-TCCATACACATACTTCTGTGTTCCT-3′, and the second round used the same J558 forward primer listed above and reverse primer JH2827Bam 5′-CGCGGATCCGATGCCTTTCTCCCTTGACTC-3′. DNA was amplified using Herculase II Fusion DNA Polymerase (Agilent Technologies). The amplified PCR products were cloned into a StrataClone Blunt PCR Cloning vector (Agilent Technologies) and sequenced.

### *Influenza virus infection and analysis*

Four-month-old C57BL/6 mice were left uninfected or infected intranasally with 30 tissue culture infectious $dose_{50}$ of influenza strain A/Puerto Rico/8/1934 (PR8), which was provided by Dr. S. Hensley (University of Pennsylvania). Both uninfected and infected mice were sacrificed 100 d later. To detect hemagglutinin (HA)-reactive B cells, we used a PE-labeled probe that recognizes HA of PR8 (HA-PE) (23). The probe was used at a concentration of 1:500, and data acquisition and analysis were performed as described (23).

## Results

### *ABC generation requires B cell expression of MHC class II and CD40*

We showed previously that ABCs could arise from FO B cells after in vivo expansion in adoptive hosts (9). This extensive division may reflect homeostatic expansion or could implicate Ag-driven activation involving T cell help and costimulation. To distinguish between these possibilities, we modified our adoptive-transfer model with CFSE-labeled donor B cells to use MHC class II– or CD40-deficient donor B cells. The rationale was that homeostatic expansion should be independent of Ag presentation and costimulation, whereas Ag-driven events should not. As shown in Fig. 1A, after 1 mo in the absence of immunization, a small proportion (~0.2%) of C57BL/6 donor B cells underwent five to eight rounds of division, likely in response to stimulation by endogenous Ags. These extensively divided CFSE$^{lo}$ cells were CD23$^-$ and T-bet$^+$, which are markers for ABCs. Although the events occurred in only a month, they represent a snapshot of the slow accumulation of ABCs with time. In contrast, B cells from MHC class II–deficient (*I-Ab*$^{-/-}$) and CD40-deficient (*Cd40*$^{-/-}$) mice underwent fewer divisions with far less T-bet expression than

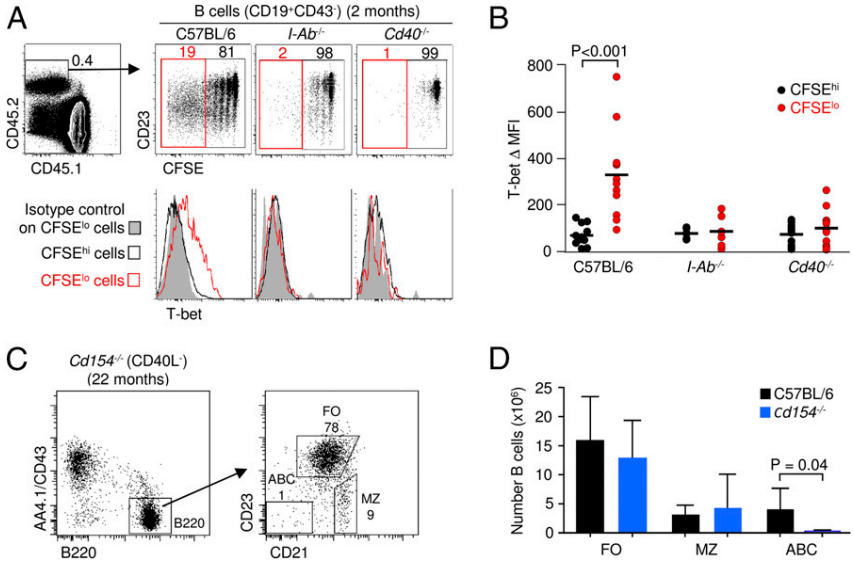


**FIGURE 1.** Interactions with MHC class II and CD40 drive the accumulation of ABCs. (**A**) CD23$^+$ FO B cells from 2-mo-old donor mice (CD45.2) were labeled with CFSE and adoptively transferred into young congenic CD45.1 hosts. Recipient mice were analyzed 1 mo later. Shown are a representative plot of the gating strategy and representative dot plots of CFSE dilution in C57BL/6, *I-Ab*$^{-/-}$, and *Cd40*$^{-/-}$ cells. Numbers depict the percentage of cells in each box. Cells with multiple rounds of proliferation (CFSE$^{lo}$) are boxed in red. Line graphs show intracellular staining for T-bet in CFSE$^{lo}$ cells. (**B**) Analyses of T-bet change in mean fluorescence intensity (ΔMFI) are summarized in three independent experiments for a total of 12 mice for C57BL/6, 6 mice for *I-Ab*$^{-/-}$, and 12 mice for *Cd40*$^{-/-}$. (**C**) Spleen cells from 22-mo-old *Cd154*$^{-/-}$ mice were gated on live B220$^+$ cells. A representative dot plot shows the absence of ABCs (CD23$^-$CD21$^-$). Numbers represent the percentage of B cells in each population. (**D**) Absolute B cell numbers of the indicated cell subset from old C57BL/6 and *Cd154*$^{-/-}$ mice. Error bars signify the SD of values from 31 C57BL/6 mice and 5 *Cd154*$^{-/-}$ mice. The *p* values were calculated by an unpaired, equal variance Student *t* test.

did C57BL/6 cells of the same division cohort. Analyses of multiple mice (Fig. 1B) confirmed a significant increase in T-bet mean fluorescence intensity in $CFSE^{lo}$ cells compared with $CFSE^{hi}$ cells from C57BL/6 donors, whereas cells from *I-Ab*$^{-/-}$ and *Cd40*$^{-/-}$ donors had no increase. The data suggest that ABCs arise from B cells involved in immune responses to T-dependent Ags, because cognate Ag-presenting capacity and competence to receive CD40 costimulation are required. This interpretation further predicts that CD154-deficient mice, which lack CD40L, should have reduced ABC accumulation. Consistent with this expectation, analysis of splenic B cells from 22-mo-old CD154-deficient mice revealed a paucity of ABCs (Fig. 1C), despite no change in FO and MZ compartments compared with controls (Fig. 1D). Collectively, these results show that ABCs are generated slowly after endogenous Ag presentation via MHC class II and costimulation via the CD40 receptor with CD40L on T cells. The notion that ABCs are derived from T-dependent immune responses raises questions about the breadth and nature of potential Ags involved in their generation and whether they bear hallmarks of germinal center participation. Accordingly, we interrogated Ig variable (V) gene usage and levels of somatic hypermutation among quiescent, naturally occurring ABCs from old mice.

### *ABCs exhibit a diverse V gene segment repertoire*

ABC accumulation may reflect the aggregate of immune responses to a large and diverse class of endogenous Ags and, thus, involve a broad array of clonotypic specificities. Alternatively, accumulation could be mediated by common exposure to a limited array of self- or environmental ligands that generate oligoclonal expansions with limited repertoire diversity. To differentiate between these possibilities, we sorted FO, MZ, and ABC B cell subsets from 22-mo-old mice and compared $V_H$ and Vκ gene segment usage. Because the majority of ABCs express IgM (9), sequencing analyses for H chain genes were done on cDNA amplified with a Cμ 3′ primer and degenerate $V_H$ 5′ primers. Likewise, κ L chain genes were identified by amplifying cDNA with a Cκ 3′ primer and degenerate Vκ 5′ primers. Some 2400 unique sequences for both loci were collected and analyzed. Overall, the usage of $V_H$ and Vκ gene segments was similar among all three subsets. For $V_H$ genes, 85 genes from 12 families were identified, and their frequencies were measured within the subsets. ABCs were compared separately with FO (Fig. 2A) and MZ (Fig. 2B) cells, and significant differences in over- or underutilization were seen in only two to four individual genes. For Vκ genes, 69 genes from 15 families were found; when ABCs were compared with FO (Fig. 3A) or MZ (Fig. 3B) cells, only three to five genes were significantly over- or underused. Thus, there was no evidence for strong repertoire skewing, arguing against a restricted Ag-driven response. We also did not observe significant selection for amino acid replacement changes in CDRs for Ig H chain and Igκ-chains from the ABC population (data not shown). These results suggest that ABCs develop in response to a broad range of Ags.

### *ABC V genes have undergone somatic hypermutation*

The requirement for CD40–CD154 interactions in ABC accumulation suggests that most ABCs are products of activation involving cognate T cell help. If so, the V genes of ABCs should contain increased frequencies of mutations compared with other subsets. To

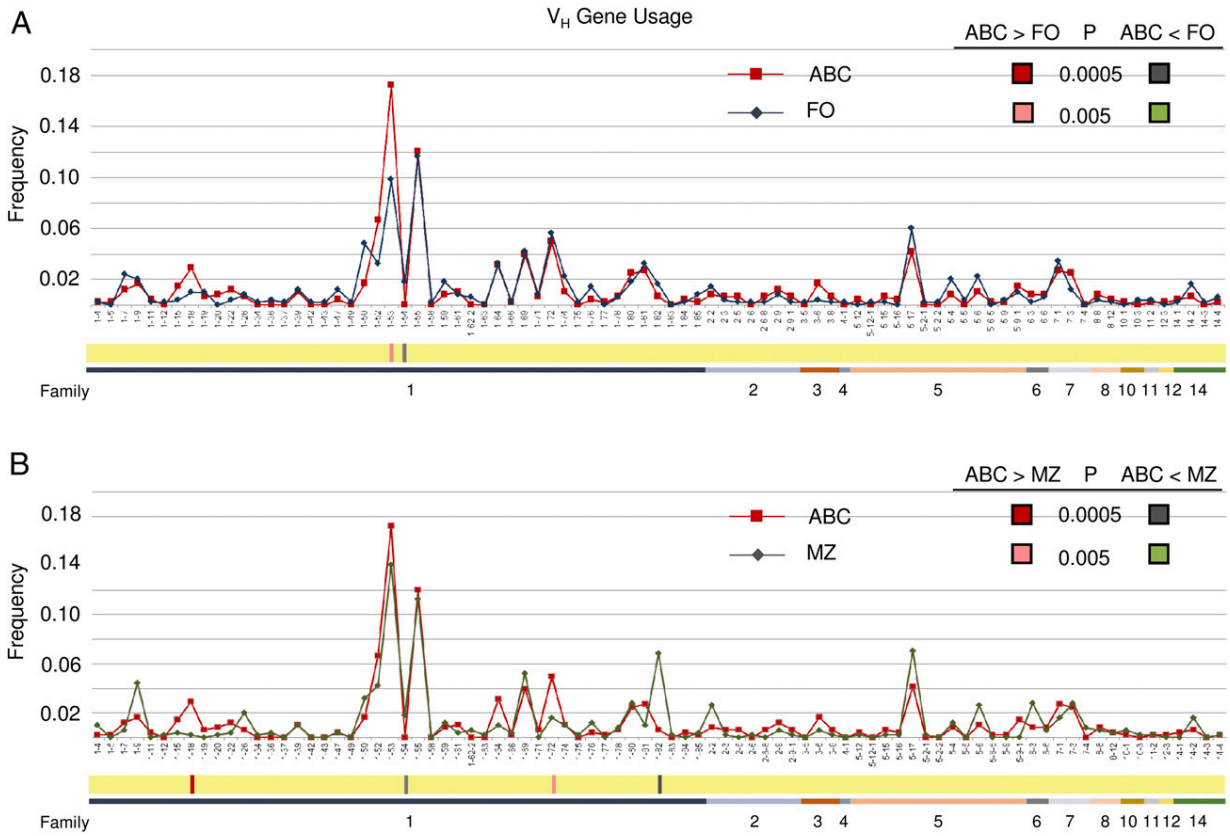


**FIGURE 2.** Diverse $V_H$ gene segment usage in ABCs. The frequency of gene expression within the indicated B cell population in old mice ($n$ = 18–24 mice for FO, MZ, and ABC subsets) was determined using RT-PCR. For each subset, ~400 $V_H$ sequences were analyzed. V genes were grouped by family, which is indicated numerically below the graph. Significant differences in V gene usage between ABC and FO (**A**) or between ABC and MZ (**B**) subsets were calculated using the Fisher exact test and are shown below the gene name in the yellow bar. The $p$ value heat map scale is shown.

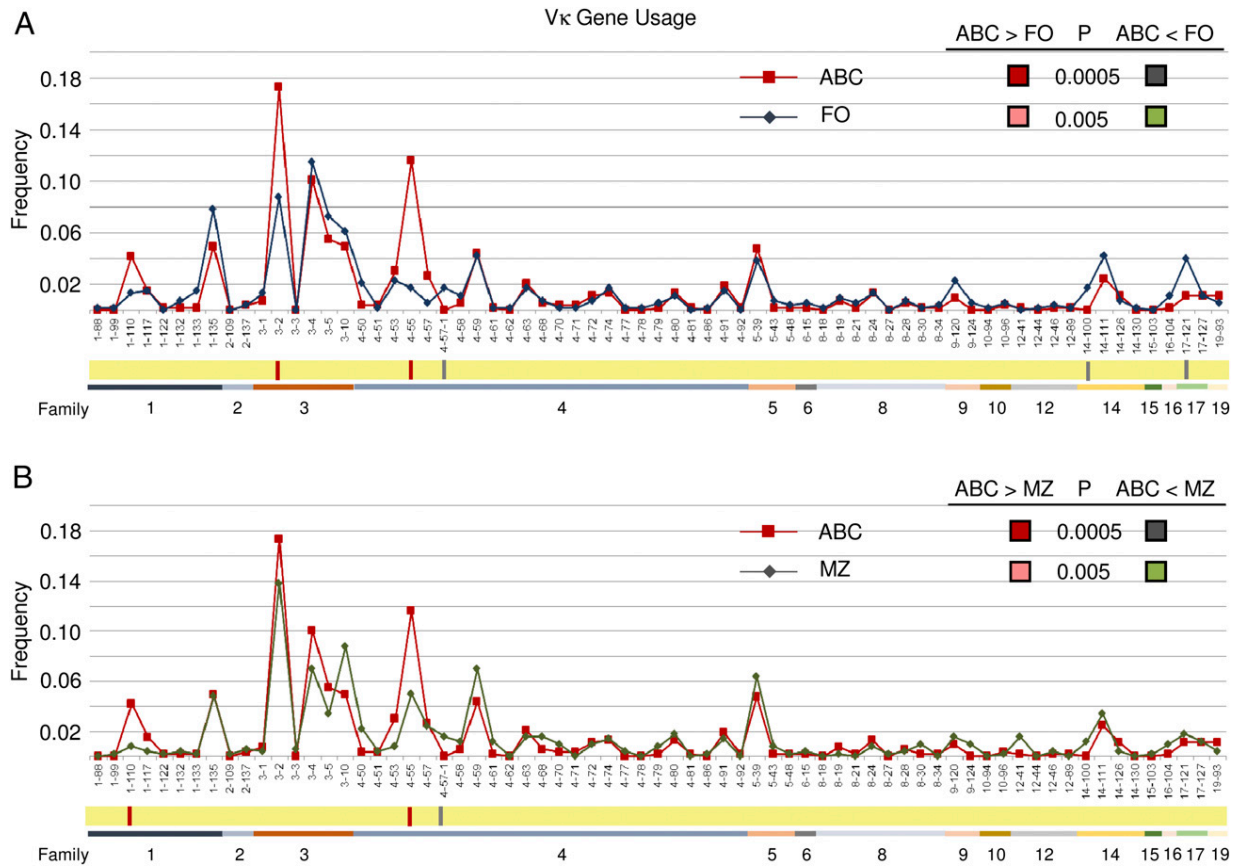


**FIGURE 3.** Diverse Vκ gene segment usage in ABCs. (**A**) ABC vs. FO subsets. (**B**) ABC vs. MZ subsets. Details are similar to Fig. 2 legend.

address this, we counted the number of mutations in VDJ heavy and VJ κ light exons amplified from FO, MZ, and ABC B cell subsets from 22-mo-old mice used for the repertoire analysis. Sequences of V, D, and J gene segments were compared with their germline counterparts to identify mutations. VDJ and VJ genes from ABCs had a significant 4-fold increase in mutations compared with FO cells and a significant 2-fold increase compared with MZ cells (Fig. 4A, 4B). As a control, V exons were sequenced from FO and MZ cells from young *Aid*$^{-/-}$ mice, which cannot undergo hypermutation because the activation-induced deaminase protein is absent. The mutation frequency was $\sim 2 \times 10^{-3}$ mutations per base pair for activation-induced deaminase–deficient cells, which represents the background frequency of errors produced during cDNA synthesis and PCR amplification. The distribution of mutations per sequence is shown in Fig. 4C, which shows that two thirds of sequences from ABCs had mutations, indicating that most of these B cells have encountered some type of Ag during their existence. An examination of the types of nucleotide substitutions in the cadre of >2700 mutations from VDJ and VJ genes from the ABC sequences showed no difference compared with FO and MZ substitutions (data not shown). Because the error rate for sequencing cDNA clones from RNA is elevated due to errors from the low-fidelity reverse transcriptase used to make cDNA, we also analyzed mutations in the 492-bp $J_H4$ intronic region directly from DNA, using a high-fidelity polymerase. FO, MZ, and ABC B cell subsets were sorted as described above, and the $J_H4$ region was amplified from genomic DNA. As shown in Fig. 4D–F, there was a significant increase in mutation frequency from ABCs compared with those from FO and MZ cells, confirming that ABCs have undergone somatic hypermutation. As a control, introns were sequenced from germinal center B cells of young mice taken 4 wk after immunization with (4-hydroxy-3-nitrophenyl) acetyl–chicken γ globulin (24), and the frequency was 5-fold higher than in ABCs. This comparison places ABCs in the middle between naive and germinal center cells, suggesting that they undergo mild chronic stimulation by endogenous Ags versus acute stimulation by immunization.

### *Some Ag-specific B cells express T-bet and CD11c*

Our results suggest an Ag-driven origin for ABCs that, coupled with their continuous accumulation, BLyS independence, and resting state (9, 25), lends credence to the idea that they are an unusual subset of B cells. To further interrogate the provenance of ABCs, we compared their gene-expression profiles with those from FO B cells sorted from old and young mice. ABC uniqueness is shown by a subset of 70 genes with ≥5-fold higher expression in old ABCs compared with old and young FO B cells (Supplemental Fig. 1A, Supplemental Table I). T-bet and CD11c were overexpressed in ABCs, confirming previous reports (13, 14). Principal component analysis was used to visualize intersample variation among all of the genes from sorted subsets and illustrated that old ABCs have distinct gene-expression profiles compared with FO B cells from old and young mice (Supplemental Fig. 1B).

Based on their accumulation of somatic hypermutation, we hypothesized that ABCs represent a subset of Ag-experienced B cells whose accretion reflects the cumulative aggregate of challenges that drive their formation. To demonstrate that another subset of Ag-experienced B cells arising from deliberate infection also expresses T-bet and CD11c, we infected young mice with influenza. HA-specific B cells were tracked by binding to fluorescent labeled HA-PE. Prior to infection, the frequency of HA-reactive B cells was low (Fig. 5A), consistent with prior estimates of ~1/50,000 splenic B cells (26). Following infection, mice displayed the expected

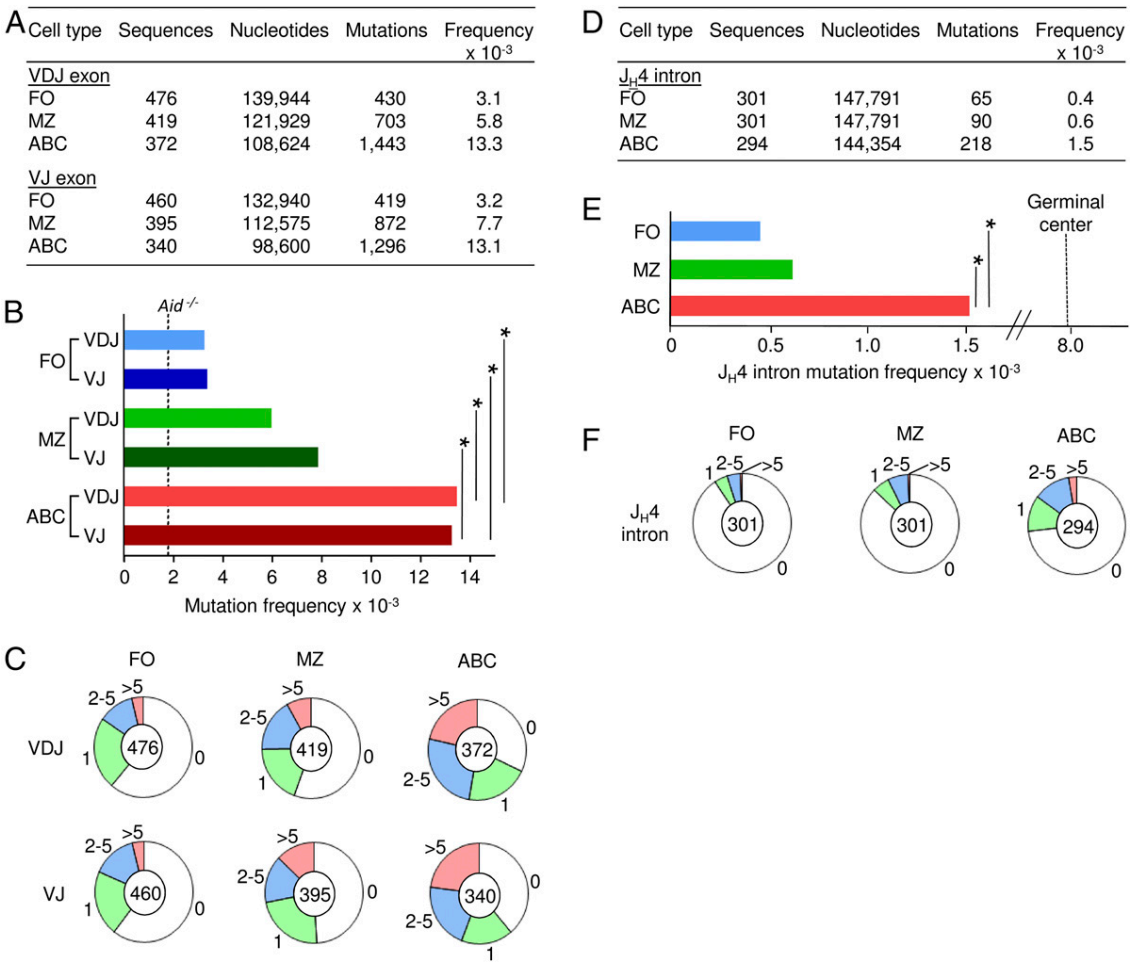


**FIGURE 4.** ABCs have increased somatic hypermutation. (**A**–**C**) Exon sequences from Figs. 2 and 3 were analyzed for mutations in rearranged VDJ genes for the H chain and in rearranged VJ genes for the κ L chain. (A) Numbers of unique sequences, nucleotides, mutations, and frequencies. (B) Mean mutation frequencies (mutations/nucleotides) in the exons of each B cell subset were calculated. The dotted line indicates the mutation frequency in $Aid^{-/-}$ FO and MZ B cells from young mice. (C) Distribution of mutations per sequence. The number of sequences is shown in the center of each circle. Segments represent the proportion of sequences that contain the indicated number of mutations. (**D**–**F**) $J_H4$ intron sequences were analyzed from genomic DNA of 11–13 mice for each subset. (D). Numbers of sequences, nucleotides, mutations, and frequencies. (E) Mean mutation frequencies; the dotted line represents the frequency in germinal centers from young immunized mice. (F) Distribution of mutations per sequence. $^*p < 0.0001$, $\chi^2$ test.

weight loss and fully recovered 30 d later (Fig. 5B), indicating that the virus was cleared. At day 100 postinfection, HA-reactive B cells increased, and ~25% of these were T-bet$^+$CD11c$^+$ (Fig. 5C, 5D). Collectively, these observations on influenza-infected mice support the analogy that some long-lived Ag-experienced cells express the phenotype associated with ABCs.

## Discussion

These studies probe the origin and nature of ABCs, a B cell subset that steadily accumulates with age and whose surface phenotype and transcriptional signature were associated with humoral autoimmunity and antipathogen immune responses. We provide three lines of evidence that ABCs are a unique B cell subset. First, adoptive-transfer studies using MHC class II and CD40-deficient cells confirm our prior report that young FO B cells can give rise to ABCs after extensive division (9) and extend this observation in several ways. Notably, they demonstrate that ABCs can be generated with cognate T cell help, which is substantiated by the lack of ABCs in old *CD154*$^{-/-}$ mice. These findings also significantly connect T-bet expression with these extensively divided cells, a feature that is well established in ABC genesis (12, 13). In addition, our results indicate that homeostatic expansion is unlikely to be the major source of ABCs. The transfer experiments involved replete hosts, with minimal space for donor cells to fill by division, and the relatively few transferred cells from MHC class II– and CD40-deficient donors that divided did not express T-bet. Overall, our data support the hypothesis that most ABCs are the cumulative result of enduring environmental Ag stimulation through T-dependent mechanisms. However, the results do not exclude a TLR-mediated origin for some ABCs that may respond to viral or autoimmune stimuli (27).

Second, analyses of V gene segment use and somatic hypermutation indicate encounters with multiple Ags. The breadth of V-gene usage speaks against a monolithic origin in terms of Ag or epitope recognition and demonstrates that ABCs represent a cross-section of responses to a broad array of Ags. Numerous early reports suggested that the total B cell repertoire was restricted in old mice (5, 28–31). However, our extensive analysis of 85 $V_H$ gene segments and 69 Vκ gene segments revealed a rich spectrum of V-gene usage by FO, MZ, and ABCs from old mice, indicating that global sequencing generates a more comprehensive view of the repertoire than do limited studies of Ag-specific cells. Only a handful of genes were over- or underused by ABCs; overall, there was no significant difference when ABCs were compared with FO

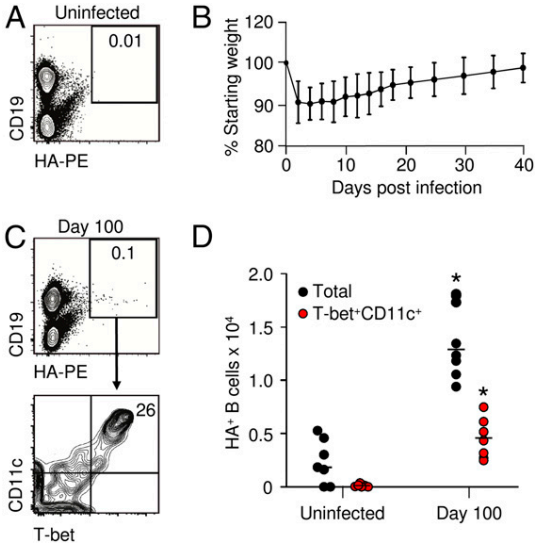


**FIGURE 5.** B cells infected with influenza HA express T-bet and CD11c. Mice were infected intranasally with PR8, and spleens were harvested 100 d postinfection. (**A**) Representative profile of HA-PE–binding B cells ($CD19^+$) in an uninfected spleen. Number shows the percentage of cells in highlighted box. (**B**) Weight loss and recovery postinfection. Error bars signify SD from eight mice. (**C**) Gating profile for HA-binding B cells in spleens at day 100 (upper panel). Number in box reflects the percentage of cells. A portion of HA-binding B cells express T-bet and CD11c (lower panel). (**D**) Number of B cells that bind HA. Total $T\text{-bet}^-CD11c^-$ and $T\text{-bet}^+CD11c^+$ cells (black circles) compared with only $T\text{-bet}^+CD11c^+$ cells (red circles). Data are from seven uninfected and eight d-100 infected mice collected in three independent experiments. $*p < 0.01$, uninfected versus infected groups, unpaired equal-variance Student *t* test.

or MZ repertoires. Such results would be expected if multiple heterogeneous Ags generated the diverse repertoire. Possible candidates are self-antigens or Ags of the microbiota environment. With regard to self-antigens, a previous report found that mice stimulated chronically with TLR7 agonists developed ABCs expressing high titers of anti-Smith autoimmune Abs (10). However, the few genes that were overused in ABCs in this study did not possess positively charged CDR3 regions in their rearranged sequences, which are common in self-reactive Abs (32). It appears that healthy old mice without deliberate immunization develop a heterogeneous repertoire, without propensity for autoimmune Abs. Although the repertoires of ABCs, FO cells, and MZ cells were similar, the results of the mutation analyses were strikingly different. ABCs showed clear evidence of mutation in the $V_H$ and Vκ exons compared with FO and MZ B cells. Although FO B cells had the lowest mutation frequency, consistent with their preimmune status, the frequency was 2-fold higher in MZ cells, which have likely encountered microbial Ags during circulation, and 4-fold higher in ABCs. In ABCs, mutations did not accumulate in CDRs, consistent with the lack of selection of certain V genes in the repertoire analysis. Furthermore, there was a significant increase in mutation frequency in ABCs in the noncoding $J_H4$ intron, which is a broad substrate for hypermutation in the absence of selection (33). However, it remains uncertain whether all ABCs are the products of germinal center reactions. For example, most ABCs have IgM receptors and continue to express the surface receptor TACI (9), both of which are inconsistent with the germinal center B cell phenotypes (34). Moreover, the mutation frequency in ABCs was lower compared with germinal center B cells from immunized mice (24). There is precedent for mutated IgM-bearing cells occurring in the absence of germinal centers (35, 36). Alternatively, ABCs may represent early germinal center emigrants that exit before concerted selection (37, 38).

Third, microarray analyses of gene expression show that ABCs from old mice are unique in relation to FO cells from young and old mice. Transcription analysis was also performed by Rubtsov et al. (10) to compare old ABCs with old FO, old B1, and young B1 cells. However, the two analyses profile different sets of genes because the cells were isolated under different conditions. The ABCs in the study from Rubtsov et al. (10) were sorted for $CD11b^+$ expression, and the ABCs analyzed in this study were sorted as $CD21^-$ $CD23^-$. Nonetheless, both analyses show that T-bet and CD11c are greatly increased in old ABCs relative to old FO cells. We found that this signature was also present in some long-lived B cells following influenza infection 100 d later, confirming our previous report (39). By analogy, ABCs are Ag experienced, because they have increased somatic hypermutation, and they require T cell interactions for their generation. The cells presumably arise from chronic stimulation by endogenous Ags, but it is important to note that ABCs are resting cells that persist over time. Whether they can undergo recall responses when they encounter cognate Ags remains to be determined.

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## Disclosures

The authors have no financial conflicts of interest.

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**APPENDIX:** Sindhava/Oropallo et al., J Clin Invest. 2017

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# A TLR9-dependent checkpoint governs B cell responses to DNA-containing antigens

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**Mature B cell pools retain a substantial proportion of polyreactive and self-reactive clonotypes, suggesting that activation checkpoints exist to reduce the initiation of autoreactive B cell responses. Here, we have described a relationship among the B cell receptor (BCR), TLR9, and cytokine signals that regulate B cell responses to DNA-containing antigens. In both mouse and human B cells, BCR ligands that deliver a TLR9 agonist induce an initial proliferative burst that is followed by apoptotic death. The latter mechanism involves p38-dependent $G_1$ cell-cycle arrest and subsequent intrinsic mitochondrial apoptosis and is shared by all preimmune murine B cell subsets and $CD27^-$ human B cells. Survival or costimulatory signals rescue B cells from this fate, but the outcome varies depending on the signals involved. B lymphocyte stimulator (BLyS) engenders survival and antibody secretion, whereas CD40 costimulation with IL-21 or IFN-γ promotes a $T\text{-}bet^+$ B cell phenotype. Finally, in vivo immunization studies revealed that when protein antigens are conjugated with DNA, the humoral immune response is blunted and acquires features associated with $T\text{-}bet^+$ B cell differentiation. We propose that this mechanism integrating BCR, TLR9, and cytokine signals provides a peripheral checkpoint for DNA-containing antigens that, if circumvented by survival and differentiative cues, yields B cells with the autoimmune-associated $T\text{-}bet^+$ phenotype.**

## Introduction

Despite the elimination of many autoreactive B cells during development (1, 2), mature B cell pools include a substantial proportion of polyreactive and self-reactive clonotypes (3–5). This observation suggests that later, activation-associated checkpoints exist to minimize the likelihood that such cells will engage in antibody production, memory B cell formation, or affinity maturation focused on self-antigens. Several recent observations bear directly on this possibility. First, mounting evidence indicates that neither the presence nor the activation of these autoreactive clones is sufficient to engender autoantibody production; instead, additional signals are needed to overcome regulatory constraints that prevent frank autoimmunity (6–14). Cognate T cell help, B lymphocyte stimulator (BLyS, also known as BAFF), IFN-γ, and IL-21 have been implicated as possible second signals (15–25). BLyS overexpression yields humoral autoimmunity (13), and both IFN-γ and IL-21 play roles in systemic autoimmune diseases (26–29). Second, many autoantibodies bind DNA- or RNA-containing complexes, and numerous studies link the endosomal nucleic acid–sensing receptors TLR9 and TLR7 to autoimmune diseases (12, 13, 15, 18, 30–34). Surprisingly, TLR9 deficiency exacerbates autoimmune symptoms in several mouse models, indicating that TLR9 may play a role in limiting the activation of autoreactive B cells. Finally, recent evidence ties this signaling triad — B cell receptor (BCR), TLR7/9, and IL-21 or IFN-γ — to the generation of $T\text{-}bet^+CD11c^+$ B cells (35), which are associated with autoimmunity in both mice and humans (36, 37). Together, these observations suggest a relationship among the BCR, TLR9, and cytokines that govern both normal and self-reactive antibody responses to nucleic acid–containing antigens, but the nature of this tripartite interaction remains unclear.

Herein, we show that in both mouse and human B cells, TLR9 agonists linked to BCR ligands induce apoptotic death after an initial proliferative burst. The underlying mechanism involves p38 MAPK–dependent cell-cycle arrest, followed by intrinsic mitochondrial apoptosis. However, B cells undergoing this program can be rescued, and the mode of rescue determines subsequent B cell fate. Whereas BLyS affords differentiation to antibody secretion, CD40 costimulation with either IFN-γ or IL-21 yields the $T\text{-}bet^+$ B cell phenotype. Finally, we show in vivo that when antigens are complexed with DNA, the magnitude and quality of humoral responses are altered. Together, these findings reveal a cell-intrinsic, TLR9-dependent mechanism that governs the initiation, quality, and extent of B cell responses to DNA-associated antigens. Further, our data suggest that breaching this checkpoint may provide a route to autoimmunity in the context of DNA-containing self-antigens.

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## Results

*DNA immune complexes induce self-limiting B cell responses that are rescued by BLyS*. Prior studies showed that rheumatoid factor–transgenic (RF-transgenic) B cells from AM14 mice proliferate in a TLR9-dependent manner when stimulated with chromatin immune complexes (ICs) formed by the monoclonal antibody PL2-3 (38). To reconcile these findings with exacerbated autoimmune disease in *Tlr9*$^{-/-}$ mice, we performed analyses of cell division and survival under varying conditions. In these experiments, we used CD23$^{+}$ splenic B cells, which are 95% or more quiescent follicular (FO) B cells. Either BCR cross-linking with F(ab′)$_2$ fragments of rabbit anti-mouse IgM (anti-μ) or TLR9 stimulation with the oligodeoxynucleotide 1826 (ODN 1826) induced several rounds of division, with the majority of cells remaining alive (Figure 1A). We observed similar results in cells stimulated with a combination of ODN 1826 and anti-μ. In contrast, proliferation induced by PL2-3 ICs was followed by overwhelming cell death (Figure 1A). This did not reflect nutrient exhaustion, since replenishing chromatin-IC–stimulated cultures with fresh medium had no ameliorating effect. Strikingly, BLyS rescued the chromatin-IC–stimulated B cells, restoring viability at all time points (Figure 1, A and C).

To establish whether this response is characteristic of all B cells, regardless of BCR specificity, we synthesized a stimulatory TLR9 immune complex (STIC9) consisting of a biotinylated CpG-rich dsDNA fragment of approximately 600 bp derived from a murine genomic CpG island sequence, termed clone 11 (39), linked to biotinylated Fab fragments of rabbit anti-mouse IgM via streptavidin (SA) (Figure 1B, inset). In contrast to smaller, thioester-linked CpG oligonucleotides like ODN 1826, clone 11 cannot freely enter B cells but is transported to a TLR9 compartment by BCR-mediated internalization. Thus, STIC9 mirrors PL2-3 IC stimulation, but is independent of BCR specificity, involves only TLR9, and eliminates potential Fc-γ receptor engagement.

Splenic CD23$^{+}$ B cells stimulated with STIC9 recapitulated the post-proliferative death and BLyS-mediated rescue seen with PL2-3–stimulated RF B cells (Figure 1B). A CpG-negative dsDNA IC (CGNEG) synthesized in a manner identical to our synthesis of STIC9 mimicked anti-μ alone, reflecting BCR cross-linking without concomitant TLR9 engagement (Figure 1B). We investigated the timing of post-proliferative death to allow the appropriate design of sampling points in subsequent mechanistic studies. The kinetics of post-proliferative death were similar in both PL2-3–stimulated RF-transgenic AM14 or STIC9-stimulated C57BL/6 B cells, commencing by 48 hours after stimulation and being virtually completed by 60 hours (Figure 1, C and D). Importantly, STIC9 and PL2-3 induced similar degrees of post-proliferative death in B cells from AM14 mice, indicating that STIC9 engages the key elements triggered by the natural autoantigen (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI89931DS1). Moreover, B cells from several lupus-prone strains — NZB/W F1, Sle1, Sle2, and Sle3 — showed similar post-proliferative death responses (Supplemental Figure 1, B and C). Thus, the breakthrough of autoantibody production in these models probably reflects inappropriate rescue and differentiation rather than an intrinsic defect in this mechanism per se.

The post-proliferative death response depends on TLR9 signals, since the proliferation and survival of *Tlr9*$^{-/-}$ B cells treated with STIC9 resembled anti-μ–stimulated cells, reflecting BCR cross-linking in the absence of a TLR9 signal, despite internalization of the DNA CpG motif (Figure 1, E and F). Importantly, the death response induced by STIC9 does not reflect substantial differences in BCR or TLR9 signal strengths, as the degrees of spleen-associated tyrosine kinase (SYK) phosphorylation and downstream nerve growth factor IB (Nur77) induction were similar in cultures stimulated with either STIC9 or anti-μ plus ODN 1826 (Figure 1, G and H). Moreover, ODN 1826 directly coupled to anti-μ mimicked STIC9, ruling out the possibility that differences in TLR9 binding valency or receptor avidity were responsible (Figure 1I). Thus, both natural and defined BCR ligands containing TLR9 agonists drive a unique program that abruptly terminates B cell activation and expansion. Further, this self-limiting response is extended by survival-promoting signals like BLyS.

*Post-proliferative death involves intrinsic mitochondrial cell death following p38-dependent cell-cycle arrest*. We next interrogated the mechanisms of STIC9-induced B cell death. Post-proliferative death did not reflect *trans* effects, since PL2-3 had no effect on the survival of B6.SJL B cells cocultured with PL2-3–stimulated AM14 B cells (data not shown). Instead, we found that STIC9 directly induced apoptotic cell death, as revealed by caspase 9 and caspase 3 cleavage, which was blocked by BLyS (Figure 2A). Since apoptosis involves either a caspase 8–dependent extrinsic pathway or a caspase 8–independent intrinsic pathway, we asked which is initiated by STIC9. Because caspase 8–KO mice are embryonically lethal unless receptor-interacting protein kinase 3 (RIP3) is also absent (40), we compared STIC9-activated B cells from C57BL/6, *RIP3*$^{-/-}$, and caspase 8$^{-/-}$ *RIP3*$^{-/-}$ mice. STIC9 induced equivalent cell death in the double-KO and control cell populations (Figure 2B), implicating the intrinsic apoptotic death pathway and attendant mitochondrial depolarization. BLyS-mediated rescue, as evidenced by blocked caspase 9 and 3 cleavage (Figure 2A), is consistent with death via this pathway, since BLyS sustains mitochondrial stability (41–43). Two BLyS receptors, BLyS receptor 3 (BR3, also known as BAFFR) and transmembrane activator and CAML interactor (TACI), are expressed on mature naive B cells, and either could account for the BLyS-mediated rescue (44, 45). STIC9 stimulation upregulated both BR3 and TACI (Supplemental Figure 2, A and B), but while rescue was intact in *Taci*$^{-/-}$ B cells, BLyS failed to rescue STIC9-stimulated BR3-deficient B cells (Supplemental Figure 2, C and D). Thus, BLyS-mediated rescue of post-proliferative death requires BR3, whereas TACI is dispensable. Consistent with the well-established ability of BR3 to induce BCL-XL and other antiapoptotic BCL-2 family members (43), B cells from BCL-XL–transgenic mice, which overexpress BCL-XL in the B cell lineage, resisted STIC9-mediated cell death, even in the absence of BLyS (Figure 2C). Finally, mitochondrial depolarization following STIC9 stimulation was confirmed by flow cytometric analyses and was prevented by BCL-XL overexpression (Figure 2C).

While these observations showed that intrinsic mitochondrial death is the ultimate route to TLR9-dependent post-proliferative death, the upstream initiating events remained unclear. Three MAPK mediators, the JNK, ERK, and p38 kinases, are integral to both BCR and TLR9 signaling (46, 47). MAPK signaling differs in AM14 B cells stimulated by PL2-3 versus those stimulated by anti-μ or ODN 1826 (48). Moreover, DNA-containing antigens

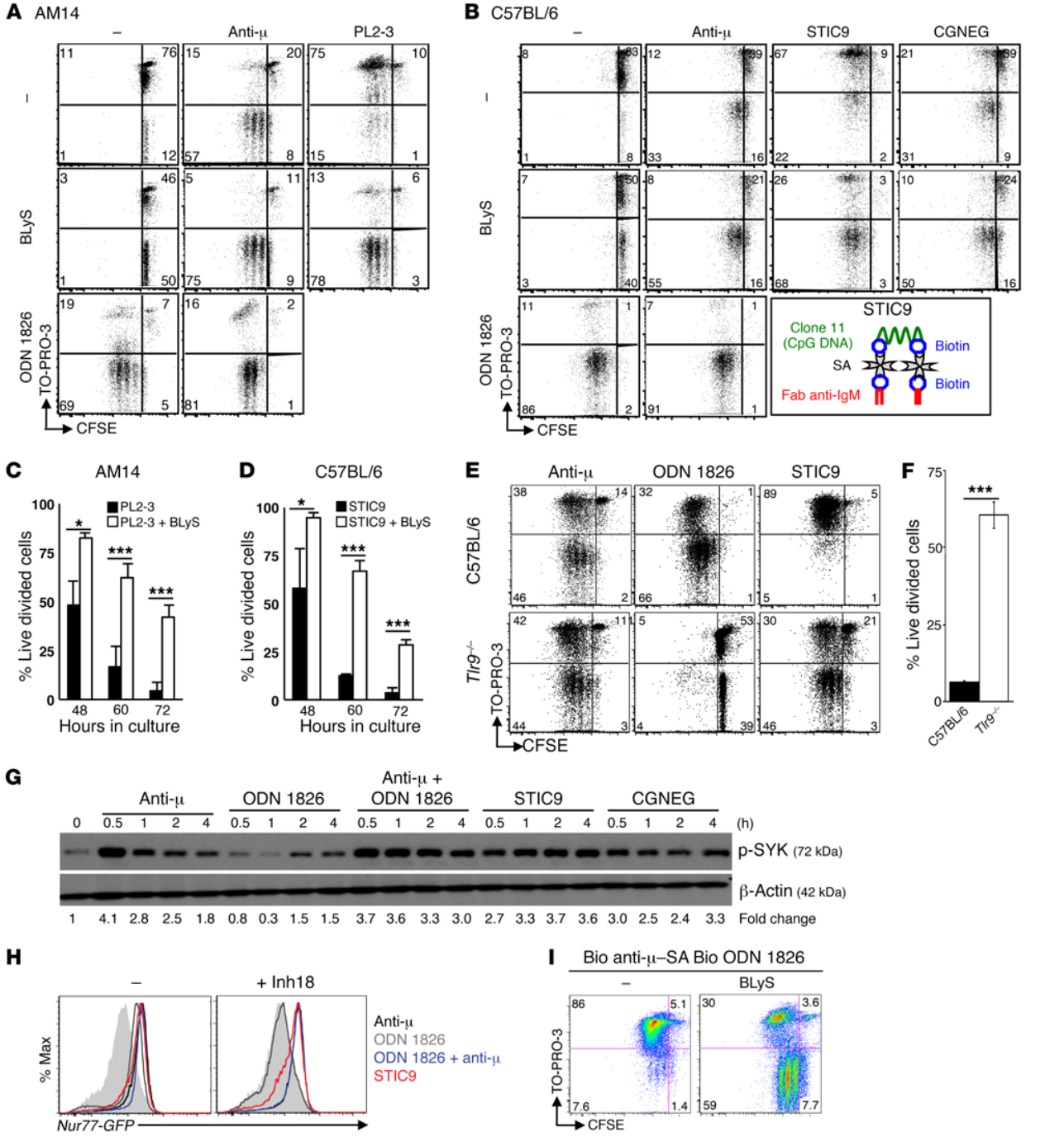
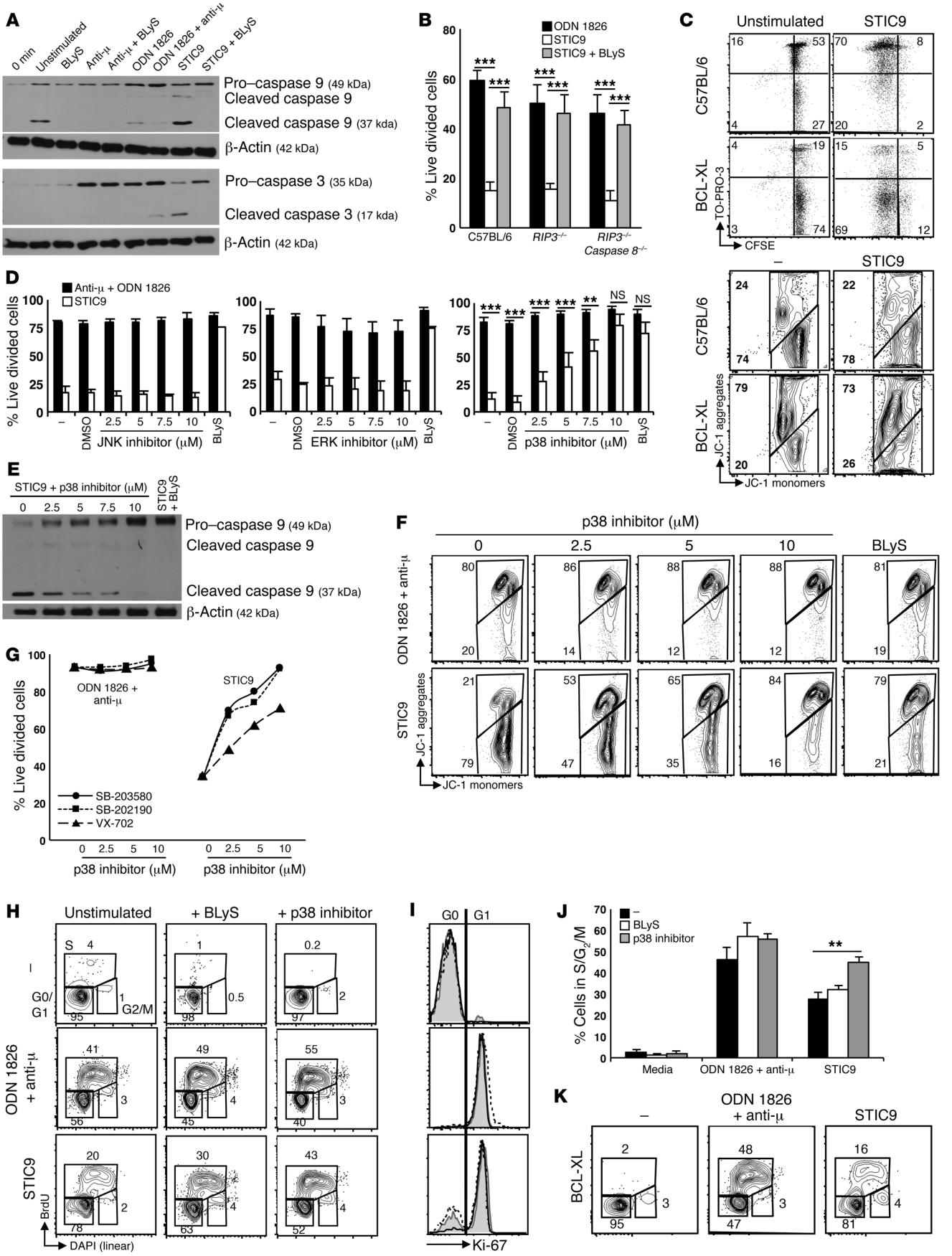



**Figure 1. Addition of BLyS prevents AM14 and WT B cells from undergoing proliferation-associated cell death following stimulation with BCR-delivered TLR9 ligands**. Representative FACS analysis at 60 hours (**A**) and percentage of live divided cells at 48, 60, and 72 hours (**C**) in AM14 CD23$^+$ splenocytes cultured with the indicated stimuli in the presence or absence of BLyS. Dead cells were stained with TO-PRO-3, while CFSE dilution indicates proliferation. (**B** and **D**) Representative FACS analysis at 60 hours (**B**) and percentage of live divided cells at 48, 60, and 72 hours (**D**) in C57BL/6 CD23$^+$ splenocytes cultured with the indicated stimuli in the presence or absence of BLyS. Diagram in **B** (inset) depicts the structure of STIC9. (**E** and **F**) Representative FACS analysis at 60 hours (**E**) and percentage of live divided cells 60 hours after STIC9 stimulation (**F**) in C57BL/6 and *Tlr9*$^{-/-}$ CD23$^+$ B cells. (**G**) Immunoblot analysis of p-SYK in protein extracts isolated from CD23$^+$ C57BL/6 splenocytes cultured with the indicated stimuli. Fold-change differences in expression are shown compared with unstimulated cells. Values in parentheses indicate the molecular weight. (**H**) FACS analysis of B cells from *Nur77-GFP* reporter mice at 5 hours, cultured with the indicated stimuli, with or without TLR9 inhibitor (Inh18) as described previously (84). Gray-filled area represents no stimulation; black line represents F(ab')$_2$ fragments of anti-IgM; gray line represents ODN 1826; blue line represents F(ab')$_2$ fragments of anti-IgM plus ODN 1826; and red line represents STIC9. Max, maximum. (**I**) Representative FACS plots show the proliferation and survival of C57BL/6 CD23$^+$ splenocytes cultured for 60 hours with SA-linked biotinylated (Bio) ODN 1826 and biotinylated F(ab)$_2$, in the presence or absence of BLyS. Data represent a minimum of 3 independent experiments with 3 mice each. Error bars indicate the mean ± SEM. $^*P < 0.05$ and $^{***}P < 0.001$, by 2-tailed Student's *t* test. "–" signifies unstimulated cells.


A
0 min
Unstimulated
BLyS
Anti-μ
Anti-μ + BLyS
ODN 1826
ODN 1826 + anti-μ
STIC9
STIC9 + BLyS
Pro–caspase 9 (49 kDa)
Cleaved caspase 9
Cleaved caspase 9 (37 kda)
β-Actin (42 kDa)
Pro–caspase 3 (35 kDa)
Cleaved caspase 3 (17 kda)
β-Actin (42 kDa)
B
ODN 1826
STIC9
STIC9 + BLyS
% Live divided cells
C57BL/6
RIP3−/−
RIP3−/− Caspase 8−/−
C
Unstimulated
STIC9
C57BL/6
BCL-XL
TO-PRO-3
CFSE
JC-1 aggregates
JC-1 monomers
D
Anti-μ + ODN 1826
STIC9
% Live divided cells
JNK inhibitor (μM)
ERK inhibitor (μM)
p38 inhibitor (μM)
DMSO
BLyS
NS
E
STIC9 + p38 inhibitor (μM)
STIC9 + BLyS
Pro–caspase 9 (49 kDa)
Cleaved caspase 9
Cleaved caspase 9 (37 kDa)
β-Actin (42 kDa)
F
p38 inhibitor (μM)
BLyS
ODN 1826 + anti-μ
STIC9
JC-1 aggregates
JC-1 monomers
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% Live divided cells
ODN 1826 + anti-μ
STIC9
SB-203580
SB-202190
VX-702
p38 inhibitor (μM)
H
Unstimulated
+ BLyS
+ p38 inhibitor
G0/G1
G2/M
ODN 1826 + anti-μ
STIC9
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DAPI (linear)
I
G0
G1
Ki-67
J
BLyS
p38 inhibitor
% Cells in S/G2/M
Media
ODN 1826 + anti-μ
STIC9
K
ODN 1826 + anti-μ
STIC9
BCL-XL

**Figure 2. Cell death in response to STIC9 stimulation follows p38 MAPK–mediated cell-cycle arrest and mitochondrial apoptosis.** (**A**) Immunoblot analysis of caspase 9 and caspase 3 cleavage in protein extracts from CD23$^+$ C57BL/6 splenocytes cultured for 60 hours with the indicated stimuli. Values in parentheses indicate the molecular weight. (**B**) Percentage of live divided CD23$^+$ splenocytes from C57BL/6, *RIP3*$^{-/-}$, and *RIP3*$^{-/-}$ caspase 8$^{-/-}$ mice following culture with the indicated stimuli. (**C**) Representative FACS plots of C57BL/6 and BCL-XL CD23$^+$ splenocytes cultured for 60 hours with no stimulation or with STIC9 either loaded with CFSE and stained with TO-PRO-3 or stained with the mitochondrial stability–assessing dye JC-1. (**D**) Percentage of live divided C57BL/6 CD23$^+$ splenocytes following stimulation with either anti-μ or STIC9 in the presence of various concentrations of the JNK inhibitor SP600125, the MEK1/2 inhibitor U0126, or the p38 inhibitor SB203580. Since vehicle and non-vehicle control groups showed no differences, the latter was used for controls in subsequent experiments. (**E**) Immunoblot analysis of caspase 9 cleavage as described in **A**. (**F**) Representative FACS plots assessing the mitochondrial stability of C57BL/6 CD23$^+$ cells cultured for 60 hours with the indicated stimuli. (**G**) Percentage of live divided C57BL/6 CD23$^+$ splenocytes following culture as in **D** with various p38 inhibitors. (**H** and **I**) FACS analysis measuring the cell-cycle status of C57BL/6 CD23$^+$ splenocytes cultured for 48 hours with the indicated stimuli. (**I**) $G_0$ and $G_1$ phases were distinguished through Ki-67 staining. The gray area represents STIC9 alone; the dashed line represents STIC9 plus BLyS; and the solid black line represents STIC9 plus the p38 inhibitor SB203580. (**J**) Percentage of cells in the S/$G_2$/M phase treated as in **H**. (**J** and **K**) FACS analysis measuring the cell-cycle status of BCL-XL–transgenic CD23$^+$ splenocytes cultured for 48 hours. Error bars indicate the mean ± SEM; $n \geq 3$ replicate analyses, and results are representative of 2 (**B** and **E**) or a minimum of 3 (**A**, **C**, **D**, and **F**–**K**) independent experiments. $^{**}P < 0.005$ and $^{***}P < 0.001$, by 2-tailed Student's *t* test. NS, not significant.

have been shown to affect B cell responses by modulating subcellular compartmentalization of TLR9 and MAPK signaling (49). Accordingly, we reasoned that STIC9 signals probably involve the MAPK pathways and asked whether inhibition of JNK, ERK, or p38 could block STIC9-induced apoptosis. Neither JNK nor ERK inhibition altered the patterns of response to ODN 1826 plus anti-μ or STIC9, despite both inducing the expected decrease in viability and proliferation with anti-μ (Figure 2D and Supplemental Figure 2E). In contrast, p38 inhibition abrogated STIC9-induced cell death, and at 10 μM, the inhibitor rescued cells to the same extent as did BLyS (Figure 2D). Further, p38 inhibition prevented the caspase 9 cleavage and mitochondrial depolarization that otherwise follow STIC9 stimulation (Figure 2, E and F). To exclude off-target effects, we used 2 additional p38 inhibitors — SB202190 and VX702 — and both rescued STIC9-driven death (Figure 2G). Thus, BCR-delivered TLR9 ligands, unlike independent BCR or TLR9 stimulation, trigger a p38-dependent mechanism that leads to intrinsic mitochondrial apoptosis.

Following activation, B cell survival requires successful cell-cycle transit, and p38 has been implicated in stress-induced cell-cycle arrest (50). To determine the effect of STIC9 on cell-cycle status, B cells were stimulated and harvested after 48 hours, a time point at which viability in all cultures is comparable (Figure 1D). As expected, 44% of B cells stimulated with ODN 1826 plus anti-μ were in the S/$G_2$/M phase. In contrast, the majority of cells stimulated by STIC9 were in $G_1$, with only 22% of the cells in S/$G_2$/M (Figure 2, H–J), in spite of the fact that most underwent several rounds of division (Figure 1B). Together, these findings suggest that STIC9–stimulated cells experience cell-cycle arrest at the $G_1$-S transition.

The addition of a p38 inhibitor reduced the proportion of cells in $G_1$, with a corresponding elevation of the proportion of cells in S/$G_2$/M (Figure 2, H–J), suggesting that STIC9 stimulation induces $G_1$/S cell-cycle arrest prior to mitochondrial apoptosis. However, it remained possible that cells undergoing mitochondrial apoptosis simply accumulate in the $G_1$ phase of the cell cycle. To address this possibility, we cultured B cells from BCL-XL mice with STIC9. Like C57BL/6 B cells, the majority of STIC9-stimulated BCL-XL transgene–positive cells were in $G_0$/$G_1$, despite their resistance to apoptosis (Figure 2K), consistent with the view that cell-cycle arrest precedes initiation of the intrinsic mitochondrial death pathway. Overall, these findings show that BCR-delivered TLR9 agonists terminate B cell activation through a p38-dependent cell-cycle arrest mechanism that subsequently drives mitochondrial cell death.

*All preimmune B cell subsets undergo TLR9-dependent post-proliferative death that can be rescued by BLyS.* Both transitional (TR) and marginal zone (MZ) B cells express TLR9, include autoreactive or polyreactive clonotypes, and have been implicated in humoral autoimmune disease (51–53). Therefore, to examine how BCR-delivered TLR9 ligands affect these B cell subsets, we FACS sorted FO, MZ, TR type 2/3 (TR2/3), and TR type 1 (TR1) B cells as previously defined (54, 55). In accord with prior studies, anti-μ induced death among TR and MZ B cells (Figure 3A). In contrast, all subsets divided following stimulation with ODN 1826 plus anti-μ and were alive after 60 hours in culture. Mirroring our results with magnetic cell-sorting–enriched (MACS-enriched) CD23$^+$ B cells, all subsets examined had proliferated following STIC9 stimulation and then died by 60 hours in culture (Figure 3A). Cell death in all subsets was most likely via mitochondrial intrinsic apoptosis, as cleavage of caspase 9 was observed in sorted FO, MZ, TR2/3, and TR1 B cells following STIC9 stimulation (Figure 3B). These data provide a previously unappreciated intrinsic role for TLR9 in limiting the responses of all preimmune B cell subsets to DNA-containing antigens.

*BLyS allows STIC9-stimulated B cells to become antibody-secreting cells.* Since B cells can be rescued from post-proliferative death by BLyS, we asked whether this or other signals enable progression to either antibody secretion or other differentiative fates. Accordingly, we first asked whether B cells stimulated with STIC9 and kept alive by BLyS could become antibody-secreting cells. As expected, CD23$^+$ B cells cultured with BLyS alone secreted minimal antibody, however, both TR and FO subsets secreted 10-fold more antibody following stimulation with either ODN 1826 plus anti-μ or STIC9 plus BLyS (Table 1 and Figure 4A, upper panel). Supernatants from MZ B cells revealed a similar pattern of Ig secretion, albeit at a greater magnitude, consistent with their vigorous response to TLR stimuli and propensity to undergo rapid plasma cell differentiation (56). Moreover, enzyme-linked immunosorbent spot (ELISPOT) analyses confirmed that ODN 1826 plus anti-μ or STIC9 plus BLyS induced antibody-secreting cell (ASC) formation (Figure 4A, lower panel). Importantly, lambda$^+$ B cells from 3H9 BCR–transgenic mice, which are dsDNA specific and normally eliminated at the TR stage (17, 57–60), were able to differentiate into ASCs following stimulation with STIC9 plus BLyS (Supplemental Figure 3C). These data reveal that TLR9 ligands delivered via the BCR yield a similar, self-limiting response by all major peripheral B cell subsets and that concomitant survival signals permit continued differentiation into ASCs.

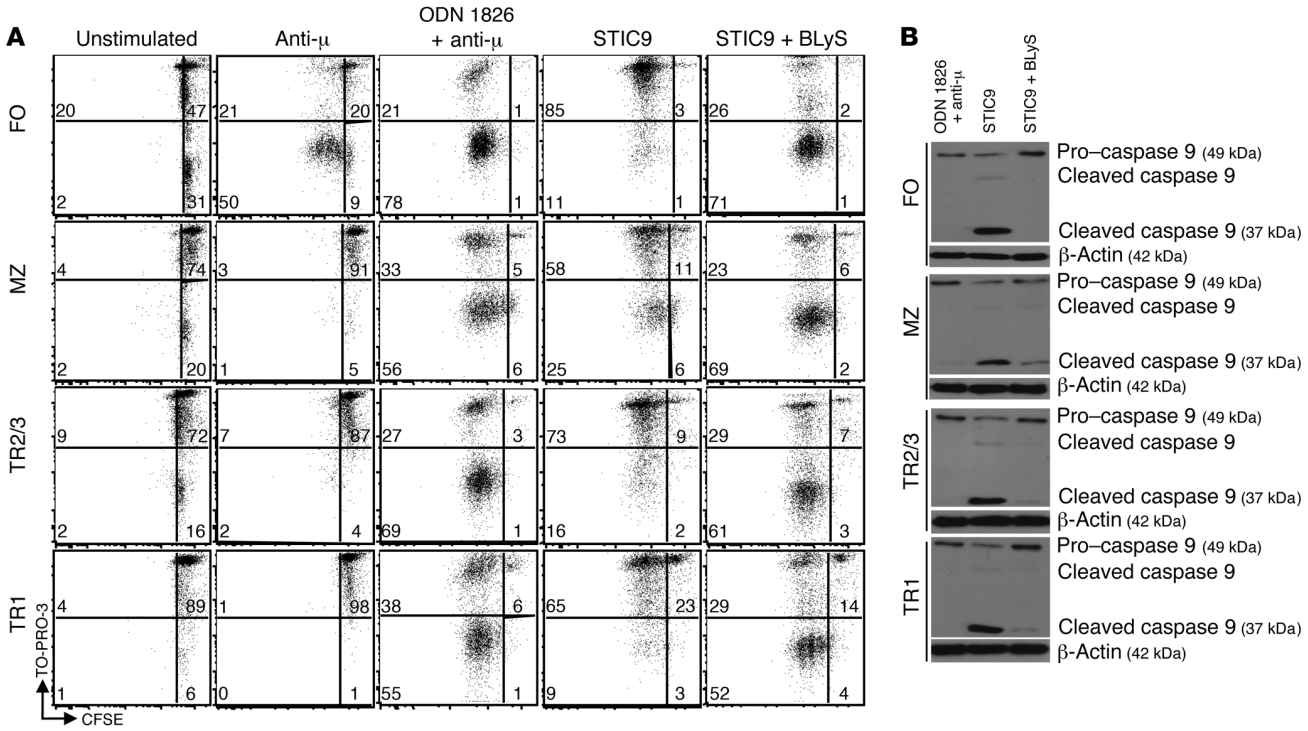


**Figure 3. FO, MZ, and TR B cells behave similarly following STIC9 stimulation.** (**A**) FACS analysis of proliferation and survival in sorted B220$^+$AA4.1$^-$CD23$^+$CD21/35$^-$ FO, B220$^+$AA4.1$^-$CD23$^-$CD21/35$^+$ MZ, B220$^+$AA4.1$^+$CD23$^+$ TR2/3, and B220$^+$AA4.1$^+$CD23$^-$ TR1 splenic B cells cultured for 60 hours with no stimulation, with F(ab′)$_2$ fragments of anti-IgM, with ODN 1826 plus F(ab′)$_2$ fragments of anti-IgM, STIC9, or with STIC9 plus BLyS. Dead cells were stained by TO-PRO-3, while CFSE dilution indicates proliferation. (**B**) Immunoblot analysis of caspase 9 cleavage in protein extracts isolated from FO, MZ, TR2/3, and TR1 B cells cultured for 60 hours with ODN 1826 plus F(ab′)$_2$ fragments of anti-IgM, STIC9, or STIC9 plus BLyS. Protein (10 μg) was loaded into each well, and β-actin was used as a loading control. Values in parentheses indicate the molecular weight. All data are representative of 3 (**A**) or 2 (**B**) independent experiments.

*CD40 costimulation and FO helper T cell cytokines rescue TLR9-dependent post-proliferative death and foster a T-bet$^+$ fate*. Although BLyS allows STIC9-stimulated B cells to survive and differentiate into ASCs, we reasoned that alternative second signals — such as CD40 costimulation and instructive cytokines characteristic of T cell-dependent immune responses — might foster alternative differentiative fates. Moreover, because T-bet expression and IgG$_{2a/c}$ isotype switching are regulated by IL-21, IFN-γ, and IL-4 in the context of TLR9 signals (35), we speculated that these features might extend to STIC9-stimulated cells. Accordingly, we assessed the effects of CD40 costimulation in the context of IL-21, IFN-γ, and IL-4 on the survival and differentiative outcomes of STIC9-stimulated B cells.

CD40 signaling alone mirrored the findings with BLyS, rescuing the cells and enabling antibody secretion (Figure 4, A and B). The added presence of IFN-γ, IL-21, or IL-4 did not affect CD40-mediated rescue (Figure 4B, upper panel). We reasoned that during B cell responses in vivo, CD40 signals would be delayed with respect to BCR-mediated activation, reflecting the need for B cell antigen processing and presentation to receive cognate T cell help. We therefore assessed rescue by CD40 signals delivered 24 or 48 hours after STIC9 stimulation. The results indicated that CD40 signals received within 24 hours of STIC9 stimulation yield equivalent rescue when these signals are received simultaneously and that even 48 hours after STIC9 stimulation, CD40 ligation affords partial rescue (Figure 4B, lower panel).

In accord with previous reports, both T-bet expression and IgG$_{2c}$ class switching increased markedly when either IFN-γ or IL-21 (Figure 4, C–G) was added in conjunction with CD40 ligation; whereas, IL-4 fostered neither T-bet expression nor IgG$_{2c}$ switching but instead yielded IgG$_1$ production. As shown previously in the context of TLR9 signaling (35), the induction of T-bet

**Table 1. Antibody production by cells cultured with STIC9 and BLyS**

| Splenic B cell subset | Culture conditions | | |
|---|---|---|---|
| | BLyS | ODN 1826 + anti-μ | STIC9 + BLyS |
| FO | 4 ± 1 | 113 ± 27$^B$ | 66 ± 25$^A$ |
| MZ | 20 ± 4 | 2,905 ± 2,392 | 4,230 ± 336$^B$ |
| TR2/3 | 10 ± 5 | 199 ± 45$^B$ | 134 ± 45$^B$ |
| TR1 | 11 ± 3 | 159.3 ± 40$^B$ | 167 ± 44$^B$ |

Mean ± SD of total Ig (ng/ml) in supernatants from sorted subsets cultured for 60 hours. $n$ = 3 independent experiments. A 2-tailed Student's $t$ test was used for the statistical analysis. $^A$Denotes significant differences at $P < 0.05$ for stimulation with BLyS alone. $^B$Denotes significant differences at $P < 0.01$ for stimulation with BLyS alone.

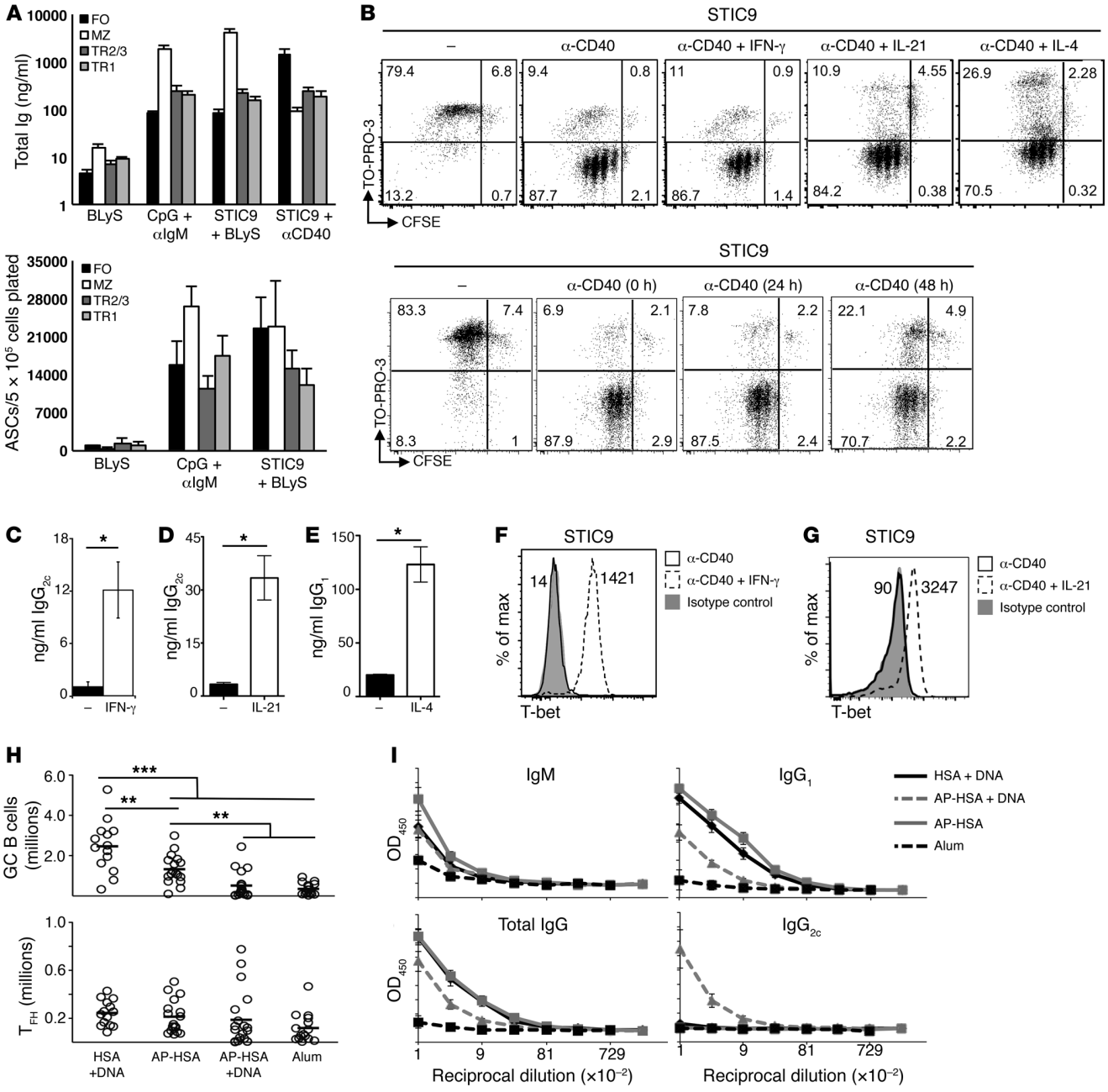


**Figure 4. CD40 and Tfh cytokines promote survival, T-bet expression, and $IgG_{2c}$ class-switching in STIC9-stimulated cells, and DNA conjugation modulates T cell-dependent B cell responses in vivo.** (**A**) Sort-purified FO, MZ, TR2/3, and TR1 B cells cultured for 60 hours with the indicated stimuli. Total Ig was measured in the supernatants by ELISA, and total ASCs were measured by ELISPOT. Each stimulation group (CpG plus anti-IgM, STIC9 plus BLyS, and STIC9 plus anti-CD40) induced significantly more ($P < 0.05$) total Ig or ASCs compared with BLyS stimulation alone when compared with the respective B cell subsets. (**B**) FACS analysis of the proliferation and survival of FO B cells cultured for 60 hours with the indicated cytokines and anti-CD40 added simultaneously with STIC9 stimulation (upper panels), or with anti-CD40 added at different time points after STIC9 stimulation (lower panels). (**C**–**E**) FO B cells were cultured with STIC9 plus anti-CD40, with or without (**C**) IFN-γ, (**D**) IL-21, or (**E**) IL-4 for 60 hours, following which (**C** and **D**) $IgG_{2c}$ and (**E**) IgG1 were measured by ELISA. (**F** and **G**) FO B cells were cultured with STIC9 plus anti-CD40, with or without (**F**) IFN-γ or (**G**) IL-21 for 60 hours, and cells were probed for T-bet by intracellular flow staining. Plots show T-bet expression in live cells. Numbers inside the plots indicate the Δ mean fluorescence intensity (MFI) (experimental minus isotype control). (**H**) Total number of splenic GC B cells [DUMP(CD4, CD8, GR-1, F4/80)$^-$IgD$^{lo/-}$CD19$^+$CD138$^-$CD38$^-$GL7$^+$FAS$^+$] and Tfh cells (CD19$^-$CD4$^+$CD62L$^{lo/-}$CXCR5$^{hi}$PD-1$^{hi}$) present in the indicated immunized C57BL/6 mice on day 14 after immunization. Each symbol represents an individual mouse. ANOVA with Bonferroni's correction was used for multiple comparisons. (**I**) HSA-specific serum titer in the indicated immunized mice on day 14 after immunization. All data are representative of 2 (**A**) or 3 (**B**–**I**) independent experiments. (**C**–**E**) Error bars indicate the mean ± SEM. *$P$ <0.05, **$P < 0.005$, and ***$P < 0.001$, by 2-tailed Student's $t$ test.

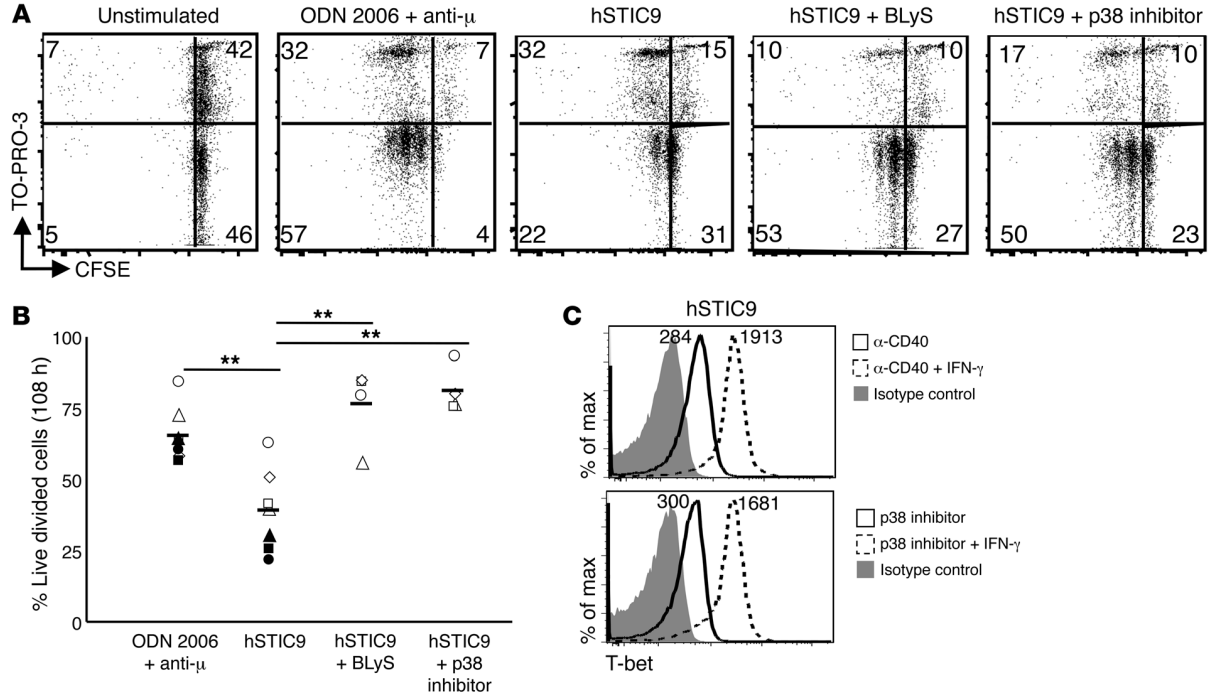


**Figure 5. Naive human B cells undergo post-proliferation apoptosis following stimulation with BCR-internalized TLR9 ligands.** (**A**) Representative FACS plots showing proliferation and survival in human $CD27^-CD19^+$ PBMCs cultured for 108 hours with no stimulation, ODN 2006 plus $F(ab')_2$ fragments of anti-IgM, hSTIC9, hSTIC9 plus BLyS, or hSTIC9 plus SB203580. Dead cells were stained by TO-PRO-3, while CFSE dilution indicates proliferation. (**B**) Percentage of live divided cells from multiple donors treated as in **A**. Each symbol represents a single donor. Black symbols ($n = 3$) indicate cells that were cultured with ODN 2006 plus $F(ab')_2$ fragments of anti-IgM and hSTIC9, while white symbols ($n = 4$) indicate cells that were additionally cultured with hSTIC9 plus BLyS or hSTIC9 plus the p38 inhibitor SB203580. (**C**) Human $CD27^-CD19^+$ PBMCs were cultured with STIC9 plus p38 inhibitor (10 μM) or STIC9 plus anti-CD40, with or without IFN-γ, for 108 hours. At the end of the culture, cells were stained for T-bet. Plots show T-bet expression in live cells. Numbers inside the plot indicate Δ MFI (experimental minus isotype control). $n = 3$ for all data, and results are representative of at least 3 independent experiments. $^{**}P < 0.005$, by 2-tailed Student's $t$ test.

expression by IFN-γ and IL-21 occurs within the first 24 hours of activation and is independent of the extent to which B cells have divided after STIC9 plus anti-CD40 stimulation (Supplemental Figure 3, A and B).

*Antigen-complexed DNA yields blunted humoral responses with T-bet⁺ B cell–associated characteristics.* Considered together, these in vitro analyses predicted that TLR9 agonists delivered by the BCR should limit B cell responses. We reasoned that in the absence of additional signals, incipient responses to DNA-containing antigens should prematurely terminate, whereas the presence of additional signals such as cognate CD4 help and cytokines should modulate the response and determine its quality. We used immunization with amyloid proteins to test this hypothesis in vivo. These misfolded proteins form fibrous structures termed amyloid aggregates, sometimes including cofactors such as DNA (61), and we have previously reported a method to produce amyloid proteins with or without DNA (61, 62). Using this approach, we immunized mice with amyloid precursor HSA (AP-HSA), AP-HSA linked to DNA, or native HSA mixed with, but not cross-linked to, DNA. Alum was used as an adjuvant in all conditions, and we also included an adjuvant-only control. On day 14 after immunization, mice receiving HSA plus DNA or AP-HSA had mounted an immune response with germinal center (GC) B cells, FO helper T cells (Tfh) (Figure 4H), and $IgG_1$ antibody (Figure 4I). In contrast, immunization with AP-HSA plus DNA yielded reduced GC B cell numbers that were equivalent to those detected with adjuvant only, as well as reduced titers of class-switched HSA-specific antibodies that were skewed toward $IgG_{2c}$ (Figure 4, H and I). Interestingly, Tfh numbers were normal with this immunization (Figure 4H). Immunization with AP-HSA plus DNA ICs yielded a reduction in GC B cell numbers, but not complete ablation of the response. This finding is in agreement with our in vitro observations (Figure 4, A and B) suggesting that CD40 ligation affords rescue even several days after B cell activation by BCR ligands that contain a TLR9 agonist. Together, these findings are consistent with the overall relationships established by our in vitro analyses, since complexed DNA modulates the humoral response and, in the presence of cognate help, engenders the $IgG_{2c}$ isotype–switching characteristic of T-bet induction.

*BCR-delivered TLR9 ligands limit human $CD27^-$ B cell responses.* Finally, we questioned whether human B cells are similarly regulated by DNA-containing antigens. Human TLR9 signals optimally to a different CpG motif than does murine TLR9, but this motif appears in the clone 11 sequence that we used in STIC9. Therefore, we designed a human stimulatory TLR9 complex (hSTIC9) by linking biotinylated clone 11 to biotinylated Fab anti-human IgM with SA. Naive ($CD27^-CD19^+$) human B cells were isolated and cultured for 108 hours, when proliferative responses most closely resembled those seen after 60 hours with murine $CD23^+$ B cells (Figure

5A). As expected, the majority of human B cells cultured with ODN 2006 plus $F(ab')_2$ anti-human IgM were alive and had proliferated (Figure 5, A and B). Mirroring the response of murine B cells, human B cells stimulated with hSTIC9 underwent a proliferative burst followed by apoptosis, and either BLyS addition or p38 inhibition rescued this death (Figure 5, A and B). Moreover, when human B cells were rescued from death, they expressed T-bet, which was further enhanced in the presence of IFN-γ (Figure 5C). These findings strongly suggest that human and murine preimmune B cells undergo similar responses to BCR-delivered TLR9 ligands and that these probably reflect similar molecular mechanisms.

## Discussion

This study reveals a relationship among BCR, TLR9, and cytokine signals that regulate B cell responses to DNA-containing antigens. BCR ligands that deliver a TLR9 agonist yield a brief proliferation that is terminated by cell-cycle arrest and intrinsic apoptotic death. This self-limiting response is characteristic of all major preimmune B cell subsets in mice and humans and requires MAPK p38 activation. BLyS prolongs this otherwise short-lived response, enabling the activated B cells to survive and differentiate into ASCs, whereas CD40 costimulation and Tfh cytokines yield both antibody secretion and differentiation into the autoimmune-associated T-bet$^+$ B cell phenotype. Together, these observations disclose a mechanism that prevents or limits responses to DNA-associated BCR ligands and may help explain how TLR9 deficiency promotes humoral autoimmunity.

The proapoptotic role of TLR9 shown here is seemingly at odds with an extensive literature showing that TLR9 agonists engender B cell division without cell death and are effective vaccine adjuvants (63). However, TLR9 signaling in these contexts likely differs from the BCR-mediated internalization of natural ICs or STIC9, as such small molecules do not involve BCR engagement in their uptake (64, 65). In addition, TLRs can signal from a range of endolysosomal compartments with varying functional outcomes (66, 67). Studies of simultaneous engagement of BCR and TLR9, similar to STIC9, showed that BCR and TLR9 colocalize in autophagosomes and initiate the hyperphosphorylation of p38 MAPK after such activation. Results of these studies also demonstrated the unique signaling pathways by which B cells regulate responses to DNA-containing antigens, namely, by governing the subcellular location of TLR9 and MAPK signaling (49, 68). Whether free CpG ODNs reach the same compartment as BCR-delivered TLR9 ligands is unclear, but CpG ODN and ICs elicit different cytokines from AM14 B cells (48), consistent with fundamentally different signaling outcomes. Further, while the affinity of TLR9 for DNA components of different ligands is a potentially confounding factor, ODN 1826 engenders post-proliferative death when conjugated to $F(ab')_2$ anti-μ, favoring the interpretation that BCR-mediated delivery is the key distinction. Similarly, BCR engagement and signal strength might also impact outcome. However, multiple measures of BCR signal strength indicate that BCR signals induced by each reagent used herein are comparable and further support the notion that BCR-associated delivery of the TLR9 agonist underlies this unique response.

The STIC9 reagent affords the separation of BCR-mediated proliferative signals from TLR9-dependent cell death, thus enabling interrogation of the downstream pathways. While one might argue that a dominant, TLR9-mediated death pathway is involved, this seems unlikely, because simultaneous but independent ligation of TLR9 and the BCR does not result in cell death. Instead, we favor the notion that crosstalk between these systems is responsible, whereby TLR9 ligation impedes or redirects survival signals that otherwise accompany BCR signaling. The exact points of intersection remain unclear, but engagement of both the BCR and TLR9 initiates the NF-κB and MAPK pathways, so crosstalk between these, including the documented hyperactivation of p38 (49), likely plays a role in the post-proliferative death mechanism reported herein. Indeed, our findings reveal a central role for p38 in STIC9-induced cell-cycle arrest and mitochondrial apoptosis, functions that have previously been associated with the p38 pathway (69–71). Cell-cycle arrest and mitochondrial apoptosis may reflect parallel but independent consequences of p38 signaling following stimulation with BCR-delivered TLR9 ligands. Alternatively, as cell-cycle arrest occurs even when apoptosis is blocked, it may be a proximal event that leads directly to mitochondrial destabilization. Regardless, both reflect potential limiting mechanisms that prevent continued expansion or prolonged survival of autoreactive B cell clones.

The TLR9-mediated post-proliferative death response that we observed is likely relevant to the etiology of humoral autoimmunity. For example, it may help explain why TLR9-deficient autoimmune strains develop more severe disease than do their TLR9-sufficient counterparts (15, 16, 18–20). Consistent with this notion, autoimmune disease is similarly exacerbated when TLR9 deficiency is limited to B cells (72). Nevertheless, in addition to its negative regulatory role, TLR9 is also required for B cell activation, ASC differentiation, and the production of isotype-switched, DNA-reactive autoantibodies in autoimmune-prone mice (15, 32). Further, this unique response — an initial proliferative burst followed by rapid elimination — is common to all major preimmune subsets in mice and humans. This suggests that TR and MZ B cells, which contain polyreactive and autoreactive clones, could be recruited to clear TLR9-containing BCR ligands such as apoptotic debris, but are quickly eliminated to avoid sustained activity against self-components. Alternatively, this mechanism might be important for purging self-reactive B cells at the immature and TR stages. In fact, in 3H9 mice, either exogenous BLyS or TLR9 deficiency extends the lifespan of dsDNA-specific B cells normally lost at the TR stage (17, 59), and mutations impacting TLR9 function are associated with a more polyreactive B cell repertoire (73). These possibilities are not mutually exclusive and could both serve as checkpoints in the prevention of autoimmune disease.

Impeding the TLR9-mediated post-proliferative death response through either intrinsic failure in the relevant pathways or misdirected survival and differentiation signals could increase the propensity for autoimmune pathogenesis, and it is tempting to speculate that these differences might lead to distinct risk and disease features. For example, circumventing this mechanism through excess BLyS might afford the formation of short-lived autoreactive plasmablasts. Indeed, BLyS depletion therapy has had mixed success in ameliorating systemic lupus erythematosus (SLE) flares, perhaps reflecting restoration of this pathway in a subset of patients (24). In contrast to BLyS, T cell–mediated rescue of BCR-TLR9 coengagement might

foster GC differentiation, allowing somatic hypermutation, affinity maturation, and the generation of long-lived plasma or memory B cells. This is supported by our observations that addition of anti-CD40 with the IL-21 and IFN-γ cytokines engenders survival and induces T-bet expression and class-switching to $IgG_{2a/c}$. Since T-bet$^+$ B cells have been reported in the context of aging, autoimmunity, and infections (74), these cells may arise in response to TLR9 ligands delivered via the BCR, and while such signals would ordinarily trigger apoptosis, cognate T cell help and additional cytokines could result in the breakthrough of autoantibody production. Additionally, in vivo and in vitro data suggest that RNA-sensing receptors, such as TLR7, do not promote post-proliferative cell death, but instead foster plasma cell differentiation (75). Thus, activation via these pathways may require concomitant TLR9 signals to control the overall response (30, 33, 76). Indeed, in mouse models in which TLR7 plays a critical role, TLR9 haploinsufficiency exacerbates disease (15, 16, 18–20). Finally, some B cell subsets may resist this mechanism. Our preliminary observations indicate that CD27$^+$ human B cells may resist hSTIC9-driven post-proliferative death, suggesting that some memory B cell subsets are refractory. Thus, TLR9 may play a unique role in limiting the duration of potentially autoreactive responses, and alternative routes for bypassing this regulatory system may underlie some of the variability in the clinical features and therapeutic outcomes observed in humoral autoimmunity.

## Methods

*Mice*. C57BL/6 and *Nur77-GFP* reporter mice (77) were purchased from the The Jackson Laboratory. AM14 mice were maintained at the University of Massachusetts. *Tlr9*$^{-/-}$ mice were provided by P. Scott (University of Pennsylvania). *Taci*$^{-/-}$ (78), 3H9-transgenic, and B cell–specific BCL-XL–transgenic (79) mice were maintained at the University of Pennsylvania. BR3-deficient mice were maintained at the University of Southern California (Los Angeles, California, USA). Caspase 8$^{-/-}$*RIP3*$^{-/-}$ and *RIP3*$^{-/-}$ mice were provided by William Kaiser and Edward Mocarski (Emory University, Atlanta, Georgia, USA).

*B cell cultures*. B cells were collected from 8- to 16-week-old male and female mice. Splenic B cells were isolated by positive selection and cultured as described previously (38, 39, 44, 76, 80). Briefly, B cells were stimulated with 10 micrograms/ml $F(ab)'_2$ fragments of goat anti-IgM (Jackson ImmunoResearch Laboratories); 1 μg/ml anti-CD40 (clone HM40-3; BD); and 1 μM CpG DNA (ODN 1826; InvivoGen). STIC9 ICs were formed by combining a biotinylated CG-rich dsDNA fragment (39) with SA and Fab anti-mouse IgM at a final concentration of 0.5 μg/ml dsDNA, 0.13 μg/ml SA, and 0.5 μg/ml Fab anti-mouse IgM. Murine IL-21, IL-4, and IFN-γ (Shenandoah Biotechnology Inc.) were used at 25, 10, and 10 ng/ml, respectively. Human peripheral blood mononuclear cells (PBMCs) were isolated through negative selection with magnetic anti-human CD27 beads, followed by positive selection with anti-human CD19 beads (Miltenyi Biotec) and cultured in round-bottomed plates for 108 hours. Cells were loaded with CFSE (Invitrogen, Thermo Fisher Scientific) as described previously (44). Human PBMCs were collected from both healthy men and women, aged 28–52 years. Inhibitor studies used 2.5–10 μM SB203580 (InvivoGen); SB202190 (Cell Signaling Technology); VX-702 (Cayman Chemical); U0126 (Cell Signaling Technology); or SP600125 (InvivoGen). Stimulations had 500 ng/ml recombinant human BLyS (rhBLyS) (Human Genome Sciences Inc. or R&D Systems); 10 μg/ml $F(ab')_2$ anti–human IgM (Jackson ImmunoResearch Laboratories); 1 μM CpG DNA (ODN 2006; InvivoGen); or hSTIC9, formed by combining 1 μg/ml biotinylated clone 11 DNA; 0.5 μg/ml SA (New England Biolabs); and 0.25 μg/ml Fab anti-human IgM, $FC_{5\mu}$ fragment specific (Jackson ImmunoResearch Laboratories) for 1 hour at 4°C.

*Flow cytometry*. For all analyses, live/dead discrimination was assessed using either a LIVE/DEAD Fixable Aqua Stain Kit, DAPI, or TO-PRO-3 (Invitrogen, Thermo Fisher Scientific). Spleens were disrupted to single-cell suspensions and red blood cells lysed using ammonium-chloride-potassium (ACK) buffer (Lonza). Fluorochrome-conjugated or biotinylated antibodies against mouse CD19 (clone 6D5), B220 (clone RA3-6B2), CD21/CD35 (clone CR2/CR1), AA4.1/CD93 (clone AA4.1), IgD (clone 11–26c.2a), CD138 (clone 281-2), CXCR5 (clone L138D7), PD-1 (clone RMP1-30), TCRβ (clone H57-597), and T-bet (clone 4B10) were purchased from BioLegend; CD23 (clone B3B4), IgM (clone R6-60.2), CD95 (clone Jo2), and CD62L (clone MEL-14) from BD Biosciences; and CD4 (clone RM4-5), CD8 (clone 53-6.7), F4/80 (clone BM8), GR-1 (clone RB6-8C5), and CD38 (clone 90) from eBioscience. Cells were stained with antibodies in PBS/1% BSA containing mouse IgG Fc fragments (Jackson ImmunoResearch Laboratories). Mouse IgG1, κ antibody (clone MOPC-21; BioLegend) was used as an isotype control for T-bet staining, as described previously (35). BR3 and TACI were detected with anti-mouse BR3 (clone eBio7H22-E16; eBioscience) or anti-mouse TACI (clone 8F10; R&D Systems), with rat IgG1, κ antibody (clone eBRG1; eBioscience) and rat IgG2a, κ antibody (clone RTK2758; R&D Systems), respectively, as isotype controls. All stains were incubated for 30 minutes at 4°C, except for CXCR5, which was added first and incubated at room temperature for 1 hour. Staining with biotinylated antibodies was followed by staining with Brilliant Violet 650–conjugated SA (BioLegend). Data were collected on a BD LSR II Flow Cytometer and analyzed with FlowJo software (Tree Star).

*Mitochondrial membrane potential*. Mitochondrial membrane potential was analyzed by staining with JC-1 (BD Biosciences) according to the manufacturer's protocol.

*Cell-cycle analysis*. Cells were pulsed with 50 μg/ml BrdU for the last 90 minutes of cell culture; fixed and permeabilized using BD Cytofix Solutions A and B (BD Bioscience); incubated with 225 μg/ml DNase solution in 150 mM NaCl and 400 mM $MgCl_2$ for 35 minutes at room temperature; and stained with FITC anti-BrdU antibody (B44; BD Bioscience) and DAPI. $G_0/G_1$ was differentiated with anti–Ki-67 (16A8; BioLegend).

*Immunoblot analysis*. Lysates were prepared and separated by SDS-PAGE as described previously (81, 82). Proteins were quantified by a Bio-Rad assay (Bio-Rad). Protein (10 μg) was loaded into each well, and β-actin was used as a loading control. Immunoblots were performed using rabbit anti-mouse caspase 9, rabbit anti-mouse caspase 3, or rabbit anti-mouse phosphorylated SYK (p-SYK) (all from Cell Signaling Technology). Peroxidase-conjugated donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch) was used as a secondary detection antibody. Peroxidase-conjugated mAb against mouse β-actin (AC-15; Sigma-Aldrich) was used as a loading control. Quantity One 1-D Analysis Software (Bio-Rad) was used for gel densitometry.

*ELISA and ELISPOT analyses*. Plates were coated with 10 μg/ml anti-Ig (H+L) (SouthernBiotech) as previously described (83). For ELISA, culture supernates were plated and incubated with secondary HRP-conjugated goat anti-mouse Igκ plus anti-mouse Igλ antibodies

(SouthernBiotech). Plates were analyzed as previously described (83). For ELISPOTs, cells that had been cultured for 48 hours were replated and cultured for 8 hours at 37°C, then developed with biotin-conjugated anti-mouse Igκ plus anti-mouse Igλ (SouthernBiotech) as previously described (83). Plates (Corning) were coated overnight with 1 μg/ml native HSA and blocked for 2 hours in PBS/2% BSA. Sera were added to the first row of plates at a 1:100 dilution, with serial 3-fold dilutions down the rows. Anti-HSA antibodies were detected with goat anti-mouse IgG, $IgG_1$, $IgG_{2c}$, and IgM conjugated with HRP (SouthernBiotech). TMB Substrate Reagent (BD) was used to detect HRP activity, and 2 M sulfuric acid (J.T. Baker) was used to stop the reaction. Plates were read at $OD_{450}$ on an EMax Microplate Reader (Molecular Devices).

*Preparation of amyloid and immunization*. AP-HSA was prepared as previously described (62). Briefly, HSA (Sigma-Aldrich) was incubated for 2 hours in EDC (1-ethyl-3-[3-dimethyl-aminopropyl] carbodiimide hydrochloride) (Sigma-Aldrich) at a ratio of 1:5 (w/w). The mixture was neutralized by adding 10% (v/v) of 1 M Tris-HCl (Invitrogen, Thermo Fisher Scientific) at pH 10.5 and then dialyzed overnight into sterile PBS. To produce AP-HSA plus DNA, AP-HSA was incubated for more than 2 hours with *E. coli* K12 genomic DNA (InvivoGen) at a 7:3 (w/w) ratio of protein to DNA. As a control for an immunogen containing unlinked DNA and protein, native HSA was mixed with DNA at a 7:3 (w/w) ratio. Each preparation was precipitated in aluminum potassium sulfate (Thermo Fisher Scientific) at 0.25 mg/ml 10% alum solution. The pH was adjusted to 6.5, and precipitates were resuspended in sterile PBS. Mice were immunized i.p. with 70 μg protein, and spleens and sera were collected upon sacrifice 14 days later.

*Statistics*. All data are represented as the mean ± SEM. Statistical analysis was performed using a 2-tailed, equal-variance Student's *t* test. ANOVA analysis with Bonferroni's correction was used for multigroup comparisons in Figure 4H. A *P* value of less than 0.05 was considered statistically significant.

*Study approval*. Mice were handled according to a protocol approved by the IACUC of the University of Pennsylvania and the Animal Care and Use Review Office of the US Army Medical Research and Materiel Command (AMRMC). Human samples were obtained from the University of Pennsylvania Human Immunology Core, which maintains an IRB-approved protocol for secondary use of deidentified human donor specimens. Use of these cells in this study did not constitute human subjects research as defined by the NIH, and exempt determinations were made by the IRB of the University of Pennsylvania and the US AMRMC Office of Research Protections, Human Research Protection Office.

## Author contributions

VJS, MAO, KM, MN, LEH, LZ, NG, KN, AMS, SDE, and AM performed experiments; MPC, AMR, WS, WC, and TK helped design experiments and provided reagents; and MAO, VJS, MPC, and AMR wrote the manuscript.

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**APPENDIX:** Scholz et al., ASHI Quarterly 2017 (review)

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# The ABCs of a New Memory B Cell Subset

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## Introduction

The concept of immunological memory has been appreciated for at least two millennia; during a pandemic sweeping through the armies fighting the Peloponnesian wars in BC 430, Thucydides observed that individuals who had survived disease "had now no fear for themselves; for the same man was never attacked twice — never at least fatally."[1] Nonetheless, as implied by our continued use of an anthropomorphic metaphor, "memory," a mechanistic understanding of the cellular and molecular basis for this phenomenon remains incomplete. While indirect evidence for specific cellular differentiated states that mediate immunological memory has existed for some time, the identities and origins of so-called "memory cells" in both the T and B lymphoid lineages have only recently been appreciated. For example, markers among antigen-experienced subsets within the T lineage were introduced in the past two decades,[2] and only during the last several years have clear functional attributes of various memory T cell subsets been revealed.[3] Cells constituting the B lineage memory pool were similarly inferred, yet direct demonstrations of tractable markers corresponding to memory B cell subsets have only recently emerged.[4–6] Nonetheless, it is generally accepted that memory B cells play central roles in both health and disease — affording protection to subsequent exposures after natural immunization or vaccination and serving as the source of persistent autoantibody production in humoral autoimmunity, chronic inflammatory conditions and chronic rejection phenomena.[7–13] Accordingly, refining our understanding of memory B cell subsets in terms of the signals that drive their formation and the ramifications of their functional attributes should allow memory subset phenotyping as a prognostic or diagnostic indicator in each of these circumstances. Herein, we overview general characteristics of memory B cells in mice and humans and detail the characteristics of a newly discovered memory B cell subset that appears to play key roles in both health and pathogenesis.

*The concept of immunological memory has been appreciated for at least two millennia…*

## Memory B Cells Look, Feel and Act Different

Minimally, memory cells are defined as cells generated during an immune response that persist indefinitely and participate in maintaining protective immunity. Accordingly, memory B cells in both mice and humans arise during primary and secondary antigen exposures and display unique surface markers and functional characteristics that distinguish them from their pre-immune counterparts. Although memory B cells can presumably arise during any form of immune response, they seem most efficiently generated by the germinal center (GC) reaction initiated during T cell-dependent immune responses. Indeed, in models where the surface phenotypes of antigen-specific B cells can be tracked during an immune response, memory B cells emerge following GC formation, whereas responses that fail to engender GCs also fail to produce high numbers of persistent antigen-specific cells. Accordingly, most memory B cells exhibit characteristics associated with GC B cell differentiation. Most display somatically mutated Ig genes, and some — but not all — have undergone class switch recombination from IgM/IgD to downstream heavy-chain isotypes.

Several surface differentiation markers have been proposed as general features for memory B cells, but increasing evidence suggests that multiple memory B cell subsets exist, some of which either lack or vary in their spectrum of these canonical characteristics. Thus, in the mouse, several markers have been associated with memory B cell subsets, particularly CD38, CD73, CD80 and PDL-1. In the human, CD27 expression has historically been used to identify memory B cells, although it is now clear that CD27 is not expressed on all antigen-experienced subsets.[11] Finally, memory B cells do not have the same survival and activation requisites as B cells in primary pools; while the follicular (FO) and marginal zone (MZ) B cell pools rely on the cytokine BLyS for their survival and lifespan, many memory B cells do not.[14] In addition, memory B cells differ in their requisites for reactivation, in terms of both BCR and cytokine signaling. Finally, some memory B cells are more likely to proceed directly to antibody-forming plasma cell differentiation than their pre-immune counterparts.

## ABCs Are a T-bet+ B Cell Subset Associated with Both Normal Immune Responses and Humoral Autoimmunity

A novel B cell subset termed "age-associated B cells" (ABCs) was recently described in mice and was so named because it enlarges steadily with advancing age.[15,16] In fact, in mice ≥22 months of age, ABCs can comprise 30% or more of the peripheral B cell pool.[15,17] The cells within this quiescent subset display unique characteristics in terms of phenotype, transcriptional profile and functional attributes.[15,16,18,19] While maintaining the canonical B lineage markers of CD19 and B220, mouse ABCs lack CD93, CD21, CD23 and CD43, thereby distinguishing them from transitional (TR), FO, MZ and B1 B cells. Moreover, roughly a third of ABCs express CD11c (integrin alpha-x, encoded by the ITGAX gene) and essentially all express the transcription factor, T-bet ("T-box expressed in T cells," encoded by the TBX21 gene).[20] Key functional attributes of ABCs include a failure to proliferate after BCR cross-linking, robust responsiveness to TLR9 or TLR7 agonists, BAFF-independent survival and rapid differentiation to antibody secretion upon activation.[9,10,15,16,21]

In humans, recent studies have identified novel memory B cell subsets — all of which lack the "canonical" CD27 marker — in several infectious and autoimmune diseases. Similar to mouse ABCs, these CD27 – CD19+ cells are often, if not always, T-bet+ and lack markers of FO or TR subsets. Moreover, many are also CD11c+, and some bear additional markers unique to human B cells, such as FCRL4. While all probably represent activated or antigen-experienced B cell pools, they have been given various monikers based on disease context, surface phenotype and functional attributes. These include "exhausted" B cells first reported by the Moir and Fauci labs in HIV-infected individuals,[22] "atypical memory" B cells in individuals from malaria endemic regions noted by the Pierce and Crompton group[23,24] and the "double-negative" B cells or CD11c+ B cells reported in humoral autoimmune disease patients by Anolik and Sanz[25] and others.[26–28] While it is not yet clear whether these subsets are fully congruent with one another, the shared characteristic of T-bet expression implies a common origin and also suggests that they may be the human equivalent of murine ABCs. Consistent with this possibility, several of the functional characteristics attributed to murine ABCs are shared by one or more of these human T-bet+ B cell subsets, including poor response to BCR ligation,[22,23] strong responses to TLR7 or TLR9 agonists and apparent associations with viral infections or humoral autoimmunity.[15]

## ABCs Are a Memory B Cell Subset That Arise Under Certain Activating Conditions

Recent work reveals that both TLR7/9 engagement and an inflammatory cytokine milieu with abundant IFN-g, IL21 or IL27 are required to generate T-bet+, ABC-like cells.[16,20,29] Thus, cells whose BCRs engage antigens that contain TLR9 or TLR7 agonists during responses that create this cytokine milieu assume the ABC fate.[9,10] Obvious candidates for such antigens are infectious microbes, including viruses, bacteria and parasites, as well as self-components, including nucleic acids such as apoptotic debris and chromatin. ABCs are clearly generated in response to viral antigens; T-bet+ B cells arise and persist following influenza infection or immunization in mice[20,21] and are required to clear or control LCMV and MHV infections.[19,30] In humans, they have been observed in both HIV- and HCV-infected patients.[31] Further, they have been reported in *Erlichia muris* infection in mice[32] and are also observed during parasitic infections such as *Heligmosomoides polygyrus*. Similarly, the notion that nucleic acid containing self-antigens will yield ABCs is supported by the growing appreciation for correlations between T-bet+ CD11c+ B cells and humoral autoimmunity in both mice and humans. In lupus-prone mice, T-bet+ CD11c+ B cells emerge in large numbers at an abnormally early age, correlate with disease severity and gender, and are the source of pathogenic IgG2a/c autoantibody.[16,33] In humans, elevated numbers of cells with ABC characteristics are seen in the circulation of rheumatoid arthritis, systemic lupus erythematosus and Sjögren's syndrome patients.[16,25–28,34] It is likely that this association with humoral autoimmune syndromes reflects breaches in the tolerance mechanisms that normally regulate responses to nucleic acid associated antigens,[29,35,36] thereby allowing responses to apoptotic or necrotic cellular debris in the context of an inflammatory microenvironment.[9,10] Figure 1A presents our previously proposed model schematizing these relationships. Importantly, this mechanistic model for ABC generation in humoral autoimmune responses raises the broader question of whether all forms of tissue destruction that yield nucleic acid associated debris and inflammation, including tissue graft rejection, will result in emergence of ABCs.

## Are ABCs Correlated with Transplant Rejection?

B cells have been associated with the etiology and prognosis of both acute and chronic graft rejection, and intragraft B cell infiltrates are associated with poor graft performance. In the broadest sense, B cells mediate rejection phenomena through antigen presentation that enables activation of alloreactive T cells, as well as through the formation of alloantibodies. These general roles are supported by the observation that acute rejection is attenuated when host B cells are MHC II deficient, leading to impaired alloreactive CD4 and CD8 memory T cell responses. Further, B cell depletion therapy in non-human primates and humans can reduce the incidence of chronic graft rejection in some scenarios.[37] These and related observations have been considered extensively in collected review volumes elsewhere.[38] Finally, it is well established that alloantibody responses once established persist, suggesting that memory B cell reservoirs have been generated. Accordingly, identifying the features of alloreactive memory B cell pools may yield diagnostic, prognostic or therapeutic promise.

There is striking congruency between the features of graft rejection and the generative requisites for ABCs. Thus, B cells responding to nucleic-acid complexed antigens acquire the ABC phenotype, and rejection is accompanied by ongoing tissue damage that yields apoptotic and necrotic cell debris likely to contain such complexes. Further, a prevalence of inflammatory cytokines

such as IFN-γ, IL-21 or IL-27, which skew activated B cells toward the ABC fate, is consistent with the cytokine profiles associated with active rejection processes. Finally, ABCs are potent antigen presenting cells; they express high levels of MHC class II, CD80 and CD86 and can enable T cell activation and differentiation both in vitro and in vivo.[15,18] Inasmuch as apoptotic and necrotic debris coupled with the associated inflammatory cytokine milieu mirror the criteria needed to generate ABCs, it is tempting to speculate that graft-specific self- and alloantigens, particularly those that are aggregated with ligands for nucleic acid-sensing TLRs, will prompt activated B cells to adopt the ABC fate. Moreover, by virtue of their strong antigen presenting capacity, ABCs derived from initial events associated with graft destruction may amplify the response in a "feed-forward" fashion, through increased availability of apoptotic debris and a local or systemic inflammatory milieu. A hypothetical model for this possibility is presented in Figure 1B.

Accordingly, it may prove worthwhile to assess the presence and levels of ABC-like cells in peripheral blood of graft recipients, based on generic ABC markers such as T-bet or CD11c, as well as in conjunction with probes for alloantigen specificity. If fruitful, the detection and characterization of ABCs in graft recipients has the potential to yield novel diagnostic or prognostic tools and might eventually identify targets for precision depletion strategies.

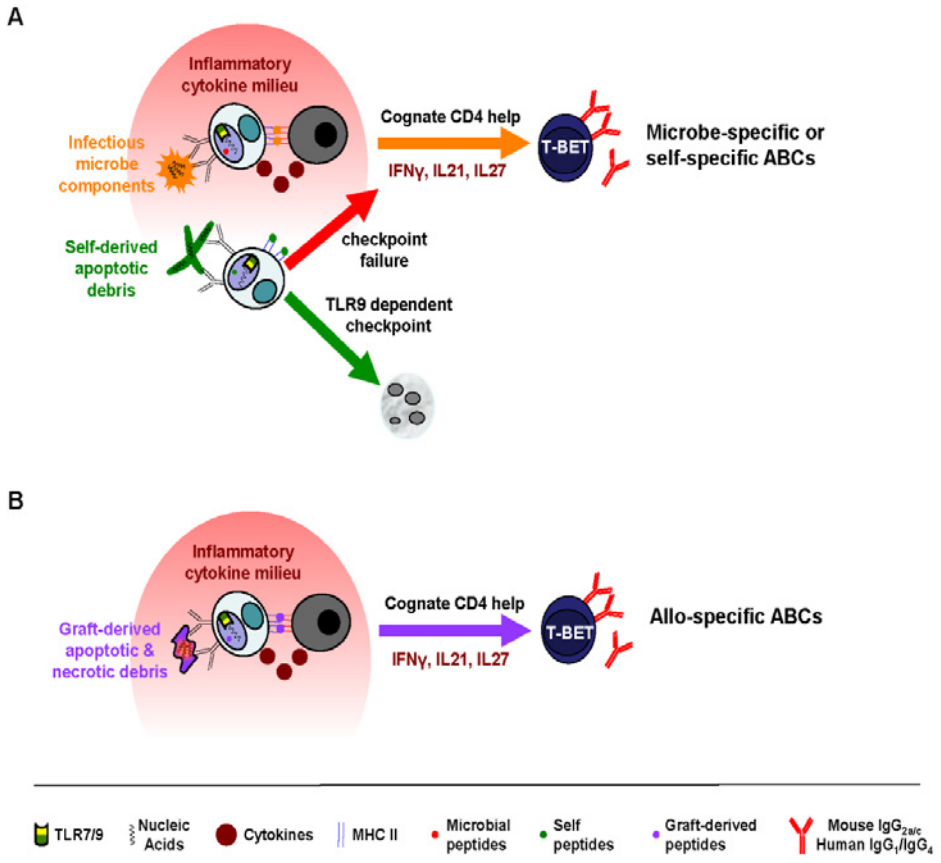


**Figure 1. Hypothetical Models for ABC Formation in Response to Nominal, Self- and Alloantigens.**
(A) Pathogen-specific and autoreactive ABCs arise through a common triad of signals. In the upper pathway shown in this panel, pre-immune FO, MZ or TR B cells bind, internalize and traffic pathogen-derived nominal antigens (orange) to endosomal compartments via the BCR (first signal). Pathogen-derived nucleic acids engage TLR7 or TLR9 (second signal), and processing and loading of pathogen-derived peptides onto MHC II molecules results in cognate CD4 T cell interactions and T cell-derived inflammatory cytokine secretion (third signal). The combination of these signals leads to T-bet+ B cell memory and effectors (orange arrow), with preferential class switching to mouse IgG2a/c or human IgG1 or IgG4. The lower pathway depicted in this panel shows that normally, B cells internalizing nucleic-acid-containing self-antigens such as chromatin or apoptotic debris (green) die at a recently described TLR9-dependent checkpoint[29] because they fail to receive the cognate help and/or appropriate cytokine signals to survive and further differentiate (green arrow). However, checkpoint failure or aberrant receipt of these third signals can enable recruitment of autoreactive cells into memory and effector ABC pools (red arrow). (B) Graft rejection may drive alloantigen-specific ABCs via the same signaling triad. This panel schematizes a hypothetical pathway to ABC generation driven by alloantigens associated with graft destruction. Apoptotic or necrotic debris containing graft-derived alloantigens (purple) and nucleic acids are engaged by B cells then internalized, thus providing BCR and TLR signals similar to those delivered by the nominal or self-antigens depicted in panel A. Alloantigen-specific T cells provide the cognate help and instructive cytokine signals for survival and differentiation, yielding recruitment of alloreactive B cells into memory and effector ABC pools (purple arrow).

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**APPENDIX:** Cancro, Cell Immunol. 2018 (editorial)

**Cancro MP.** Editorial: Expanding roles for T-bet+ B cells in immunological health and disease. *Cell Immunol*. 2017 Nov;321:1-2. doi: 10.1016/j.cellimm.2017.10.004. Epub 2017 Oct 14. PMID:29224845 **APPENDED**

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Editorial

# Expanding roles for the Tbet+ B cell subset in health and disease

The past decade has witnessed escalating scrutiny of a previously unappreciated Tbet+ B cell subset that arises during chronic and acute microbial infections, accrues with normal aging, and is associated with humoral autoimmunity. Although independently described in each of these settings, accumulating observations suggest common features in terms of phenotype, transcriptional signatures, and underlying mechanisms governing their formation. This volume provides a forum where the different manifestations of B cells with these characteristics are considered collectively. Hopefully, this will highlight their common and unique attributes, as well as the key questions remaining about their roles in immune system function.

The earliest investigations of Tbet expression in B cells revolved around the role of this transcription factor in promoting IFN-γ production and class switch recombination to IgG2a/c [1,2]. While these studies did not use Tbet expression to define a stable B lineage differentiation subset, they nonetheless revealed a role for this transcription factor in B cell physiology and function. Credit for initial accounts identifying Tbet+ B cells as a discrete functional subset is shared by several groups, but each described them in different contexts and thus conferred different monikers. Using *Erlicha muris* infection, the Winslow lab in 2008 identified CD11c+Tbet+ B cells that they termed plasmablasts, due to a propensity for spontaneous and rapid differentiation to antibody secretion [3]. In the same year, Moir et al. reported a novel, atypical ($CD27^-$) human memory B cell subset in HIV patients, representing the first report of such cells a viral infection [4]. These cells were refractory to BCR ligation and hence were termed "exhausted." The age-associated B cell ("ABC") – a naturally occurring subset that enlarged with advancing age – was subsequently reported in companion papers by Rubtsov et al. and Hao et al. [5,6]. These ABCs bore similarities to the cells in the prior reports; they were refractory to BCR stimulation but responded to TLR7 and TLR9 ligands, many were Tbet+, and many also expressed the marker CD11c [5,6]. In addition, Rubtsov et al. drew an important connection with humoral autoimmunity, showing that ABCs appeared earlier in autoimmune-prone mouse strains, and were elevated in some autoimmune diseases [6]. Parallel to these reports, the Blomberg and Riley groups reported a similar pool of cells in aged mice, and inferred roles for these cells in the age-associated dampening of B lymphopoiesis, as well as the overall process of 'inflammaging' [7].

In the years following these initial observations, a steady litany of papers probing the emergence and functional roles of this B cell subset in these and other venues has emerged. Their appearance and persistence following microbial challenges – particularly viral infections – have now been firmly established. These include human infections with HIV, HCV, and influenza, as well as mouse infection models of influenza, LCMV, and MHV, and *E. muris* [4,8–12]. Articles in this volume from the Betts, Winslow, and Swain labs, as well as a jointly penned contribution from the Pierce and Moir groups, provide strong overviews of established and emerging views of their relationship to infectious diseases.

Similarly, the steady accumulation of these cells with normal aging is now well documented, although opinions vary regarding the basis for this phenomenon. Contributions to this volume from the Blomberg, Frasca and Riley groups detail the role of these cells in generating an overall inflammatory milieu that impacts diverse immune functions, and may also presage connections with other features of aging such as shifts in lipid metabolism. In terms of origin, alternative views are presented in articles from the Swain group and the Cancro and Gearhart labs. These revolve around whether ABCs are an accumulating naïve pool that achieves prominence with advancing age, or are instead antigen-experienced cells arising from life-long antigenic exposures, whose slow turnover accounts for the continuous enlargement of this pool. Importantly, these views are not mutually exclusive, especially considering the phenotypic heterogeneity within this population.

The connection of Tbet+ B cells with autoimmunity, originally noticed by Rubtsova et al., has strengthened in the last few years, with associations in rheumatoid arthritis, systemic lupus erythematosus, and Sjogren's syndrome now reported in the literature. Similarly, this association is also well established in several mouse models of autoimmunity. Together, these studies yield strong evidence that ABCs are commonly associated with humoral autoimmunity, and are enriched for autoreactive specificities in these diseases. Contributions herein from the Pernis, Ettinger, and Rubtsova groups consider these observations in detail and speculate on their basis.

Our understanding of the cellular and molecular requisites for Tbet+ B cell differentiation has also steadily advanced. The emerging picture is one in which innate and inflammatory cytokine signals play central roles, with endosomal nucleic acid sensing TLRs, IFN-γ and IL21 prominent [13,14]. These signals and the downstream transcriptional consequences are overviewed here in the article from Myles et al.

While a great deal has been learned about these B cells to date, a host of fundamental questions remain. The origin of these cells, as well as how this question might be informed by closer scrutiny of the phenotypic heterogeneity, are basic questions whose resolution may lie in applying advanced genetic tools. For example, what are the lineal relationships – if any – between CD11c+ versus CD11c− Tbet+ B cells? Are these different activation states or circulating versus sessile examples of the same cells, or do they arise independently of one another in different activating circumstances? Similarly, the preimmune B cell compartments that give rise to Tbet+ B cells under physiological circumstances has not been rigorously addressed. Although some in vitro and in vivo studies suggest follicular B cells can give rise to cells with these characteristics [5,12–14], it

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may be that other B cells subsets – such as the marginal zone subset – are most predisposed to adopt this fate. Alternatively, perhaps – as suggested by Swain et al. in this volume – progenitors with these characteristics are generated during normal B lymphopoiesis, and are thus themselves a preimmune pool that is expanded following appropriate stimuli.

Fertile ground for future investigation is, of course, determining the exact roles played by these cells in the various venues where they have been described. Thus, even while it is clear that Tbet expression in the B lineage is essential for control in some infections, the full range of effector or regulatory roles played by these cells has not been interrogated in detail. In addition, how they contribute to protection, or perhaps failed protection, after subsequent pathogen challenge by reinfection or recrudescence awaits study. Particularly intriguing in this regard – given their prominence in relapsing and successive viral infections like HIV and influenza – is their possible role in previously intractable phenomena such as "original antigenic sin." Similarly, our understanding of their role in the etiology and progression of autoimmune disease has just begun to be probed. While it is evident that they are often enriched for characteristic autoreactive antibodies, the basis for skewing such specificities towards this subset in humoral autoimmunity, how and whether this is related to their roles in infectious disease, and whether they might be useful prognostic, diagnostic or therapeutic targets, are exciting prospects. Finally, the relationship of these B cells to different plasma cell compartments, especially in the maintenance of either protective or detrimental antibody titers – is a critical question for future investigation. Several observations suggest Tbet +CD11c+ B cells are poised to undergo plasma cell differentiation quickly, but the requisites for plasma cell induction from Tbet+ cells, as well as their relative contribution to long-versus short-lived plasma cell pools, is unknown.

Unraveling these and further arising questions about the origin, nature, and function of Tbet+ B cells has strong potential to reveal translational opportunities in vaccine design, as well as diagnostic, prognostic and therapeutic strategies for autoimmunity and autoinflammatory disease.

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**APPENDIX:** Myles et al., Cell Immunol. 2017 (review)

Myles A, Gearhart PJ, **Cancro, MP.** Signals that drive T-bet expression in B cells. *Cell Immunol. 2017 Nov;321:3-7.* doi: 10.1016/j.cellimm.2017.09.004. Epub 2017 Sep 11. PMID:28923237

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Research paper

# Signals that drive T-bet expression in B cells

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ABSTRACT

Transcription factors regulate various developmental and functional aspects of B cells. T-bet is a recently appreciated transcription factor associated with "Age-associated B cells" or ABCs, the development of autoimmunity, and viral infections. T-bet expression is favored by nucleic acid-containing antigens and immune complexes and is regulated by interplay between various cytokines, notably, the TFH cytokines IL-21, IL-4 and IFNγ. Adaptive signals by themselves cannot upregulate T-bet; however, they have a synergistic effect on induction of T-bet by innate receptors. The functional role of T-bet + B cells is unclear, although it is known that T-bet promotes class switching to IgG2a/c. It is likely T-bet serves dichotomous roles in B cells, promoting pathogenic autoreactive antibodies on one hand but mediating microbial immunity on the other, making it a target of interest in both therapeutic and prophylactic settings.

Host-pathogen interactions and environmental cues collectively shape the quality of primary adaptive immune responses by initiating circuits that enable effector and memory lymphocytes to provide protective immunity and react effectively to subsequent challenges. Inappropriate differentiation can result in a failure to protect the host, and can engender immune pathologies associated with autoimmunity, allergy, and chronic inflammatory disorders. Accordingly, a complete understanding of the signaling networks underlying the establishment of discrete effector and memory cell pools is key to developing effective vaccines and therapeutic strategies.

Shifts in transcriptional programs are fundamental to the direction of cell fate, and these shifts are determined by the aggregate of extrinsic signals received during activation. Herein, we briefly summarize key aspects of transcriptional regulation within the B lineage, followed by a more detailed consideration of the signals that drive antigen-experienced B cells to adopt fates associated with T-bet expression.

## 1. Transcription factors guide B cell differentiation and function

As in all cell lineages, B cell genesis and differentiation require turning on appropriate developmental programs and silencing those that foster other fates. Detailed reviews about the nature and interactions of transcription factors that orchestrate late B cell development can be found elsewhere [1-4], and are thus treated briefly here. The Pax5, EBF1 and E2A proteins are some of the earliest controllers that establish the transcriptional network responsible for promoting B cell development and suppressing other lineages. For example, Pax5 expression is critical for commitment to B cell fate; Pax5-deficient pro-B cells retain the potential to develop into non-B cell lineages [5,6]. Pax5 regulates the expression of many B cell surface molecules and receptors, including CD19, CD21, CD79a, as well as other relevant transcription factors like IRF4/8 and BACH2. All mature B cells continue to express Pax5, and deletion even at these mature stages yields reversion to a multipotent progenitor-like state, highlighting the role of this transcription factor in maintaining B cell character [7]. Exogenous signals that activate key transcriptional regulatory pathways also govern triage into different pre-immune B cell pools; for example, Notch-2 transcriptional activities are required for marginal zone B cell differentiation. Once a B cell is within the quiescent mature follicular (FO) or marginal zone (MZ) pools, these transcriptional programs are maintained at steady state unless activating signals are received.

## 2. Activation initiates transcriptional program shifts

Analogous to the differentiation of pre-immune B cells, the fates of activated B cells are also guided by shifts in transcription factor representation. In accord with the tenets of clonal selection, B cells require engagement of their antigen receptor – the BCR – to initiate activation. The immediate consequences of BCR signaling involve modification or further activation of pre-existing transcriptional regulatory systems, such as NF-κB, NFAT, and AP-1. The strength and duration of the BCR signal per se can impact eventual cell fate. For example, strong BCR signaling is associated with a higher propensity to rapidly adopt a plasma cell fate [8,9]. Despite the influence of BCR

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ligation on these relatively short-term outcomes, the ultimate fate of BCR-activated cells is also strongly influenced by additional exogenous signals. These signals include co-stimulation received during cognate T helper interactions, cytokines within the activating milieu, and signals from Pathogen Associated and Danger Associated Molecular Patterns (PAMPs and DAMPs) via innate receptors such as toll-like receptors (TLRs). The permutations, kinetics, and downstream integration of these signaling cues prompt the establishment of distinct transcription factor landscapes, which in turn drive fate choice and effector function. Two archetypical examples of this in antigen-activated B cells are Bcl6 and BLIMP1, transcription factors required for germinal center (GC) formation and GC B cell proliferation versus plasma cell (PC) differentiation, respectively. Thus, BCL6 expression is upregulated in response to cognate helper T cell interactions, and represses the activity of cell cycle regulators and molecules involved in DNA damage response. As a result, GC B cells are able to proliferate rapidly and undergo somatic hypermutation. In contrast, BLIMP1 promotes the development of plasma cells. BCL6 and BLIMP1 are reciprocally antagonistic – so BCL6/Blimp1 mutual repression is essential for B cells to commit exclusively to either GC or PC fate. These functions are clearly evidenced by the phenotype of Bcl6-deficient mice; GC development is blocked but plasma cells secreting low-affinity antibodies still develop [10].

These examples illustrate how fundamental and master transcriptional regulators act to govern major fate and differentiation choices within the pre-immune and antigen-experienced B cell pools, based on the aggregate of initiating upstream signals. While the existence of broad categories – such as GC versus plasma cell fates – have been appreciated for some time, recent findings indicate that further functional subsets exist among antigen-experienced B cells – and this diversification is similarly established through engagement of key transcriptional regulators. One such example is T-bet, encoded by the *tbx21* gene. This transcription factor was first described in helper T cells – hence the moniker "T-Box Expressed in T cells" – in studies that showed T-bet promotes IFNγ production, but suppresses IL-4 and IL-5. Thus, T-bet skews the differentiation of naive CD4 cells to a Th1 profile while repressing the Th2 program [11,12]. Subsequent studies revealed that T-bet is required for differentiation and function of effector CD8+ T cells [13], and interactions between T-bet and other transcription factors play key roles in the development immune cell subsets. For example, the T-bet versus Eomes axis is critical to CD8 effector versus memory differentiation [14-16].

It is now clear that T-bet expression defines a unique, antigen-experienced B cell subset. Early studies suggested that T-bet played a role in inflammatory cytokine production and immunoglobulin isotype switching [17,18], and more recent observations have expanded these findings to show that T-bet is a key player in determining the nature and quality of effector and memory B cell subsets. Studies from Szabo et al. established a link between T-bet expression and IFNγ production in B cells. While these authors did not examine the exact signals driving T-bet expression, they laid the groundwork for other studies that went on to identify activation requirements and cytokine circuits that are instrumental in inducing T-bet expression in B cells. Subsequent work revealed that immunoglobulin isotype switching to $IgG_{2\,a/c}$ is facilitated by T-bet [18–21], as are some instances of anti-viral and anti-bacterial immunity which, incidentally, rely on $IgG_{2a/c}$-mediated protection [22–24]. More recently, T-bet was found to be important for the emergence of age-associated B cells (ABCs), and T-bet expressing B cells have been described in a variety of infections and autoimmune scenarios. While these at first glance these may seem disparate and poorly connected phenomena, they likely provide clues to common signals that initiate the T-bet transcriptional program in activated B cells.

## 3. Age-associated B cells, a T-bet driven subset

The discovery of a B cell subset that accumulates with age, and also arises in infection and autoimmunity, led to questions about what transcriptional programs direct its differentiation. Phenotypically, these naturally occurring ABCs express B220, CD19, and are negative for CD43 and CD93, indicating that they are mature B2 cells. However, they lack CD23 and CD21/35, canonical pre-immune B cell markers that discriminate between FO and MZ B cell subsets. Also distinct from FO and MZ subsets, roughly half of all ABCs defined by these criteria express T-bet, and among these, about one-third also express CD11c. ABCs do not proliferate (but still survive) in response to BCR ligation in vitro. Instead, ABCs proliferate in response to endosomal TLR signals, particularly from TLR7 and TLR9. In accord with increased T-bet expression, they tend to secrete antibodies of the $IgG_{2a/c}$ isotype when activated [25,26]. Since T-bet positive B cells are a subset of ABCs, it is pertinent to summarize what is known about development of ABCs prior to addressing factors inducing T-bet expression

## 4. The genesis of ABCs

The origin of naturally arising ABCs remains incompletely understood, although increasing evidence suggests that most, if not all, are the result of antigen-driven activation. It remains possible that age-related alterations in B cell lymphopoiesis foster the generation of a pre-immune B cell subset with these characteristics. However, sublethal irradiation and autoreconstitution of aged mice resulted in a splenic B cell profile similar to young mice, with a marked absence of ABCs [25]. Thus, the aged bone marrow microenvironment is not fundamentally predisposed to generating ABC-like cells. Nonetheless, increasing evidence suggests that ABCs themselves may dampen overall B lymphopoiesis ([27], Riley et al. this volume). Cell cycle analysis revealed that ABCs themselves are quiescent, leading to the conclusion that they accumulate with age, rather than self-renew [25]. To explore whether ABCs can be derived from existing mature B cell subsets, FO B cells were CFSE-labelled and adoptively transferred into young congenic hosts. A month later, some of the transferred cells had divided, and those that had undergone the most exhaustive division had also acquired an ABC phenotype. Thus, ABCs can arise from pre-immune pools such as FO B cells, consistent with the notion that they reflect antigen-driven differentiation, and accumulate over time. The observation that FO B cells underwent several divisions before giving rise to ABCs led to the question of what cell intrinsic and microenvironmental requisites were necessary for this process. To address this, Russell Knode et al. modified the adoptive transfer system described above, and used donor CD23+ B cells from either MHC $II^{-/-}$ or $CD154^{-/-}$ mice. While WT donor cells proliferated, and adopted ABC characteristics ($CD23^{-}$ and $T\text{-}bet^{+}$), the knockout cells failed to do either. Additionally, aging $CD154^{-/-}$ mice did not develop ABCs [28]. Together, these observations indicate that the development of T-bet expressing ABCs from pre-immune B cells requires antigen presentation and cognate help.

These observations make it tempting to speculate that most ABCs are derived from antigen-driven events, and several further observations favor this possibility. First, our recent analyses of the Ig heavy and light chains from sorted, naturally occurring ABCs revealed a largely stochastic representation of VL and VH gene segment usage, suggesting that these ABCs reflect an aggregate of immune experiences over the life of the individual, thus drawing from the full repertoire of BCRs. Second, these analyses revealed clear evidence of somatic hypermutation among ABCs, strengthening the case for a germinal center origin [29]. Nonetheless, it is worth remembering that T-bet expression is a characteristic of only about half of $CD23^{-}CD21^{-}$ B cells. It is as yet unknown what prompts the dichotomy of T-bet expression in the mature $CD23^{-}CD21^{-}$ pool. Perhaps the overall 'natural' $CD23^{-}CD21^{-}$ ABC population includes both naïve and antigen experienced cells, the latter being characterized by T-bet expression (see Swain et al., this volume, for a discussion of this idea). Little is known about whether and how the T-bet positive and negative fractions differ functionally, or if T-bet expression is more easily induced in the T-bet negative fraction of

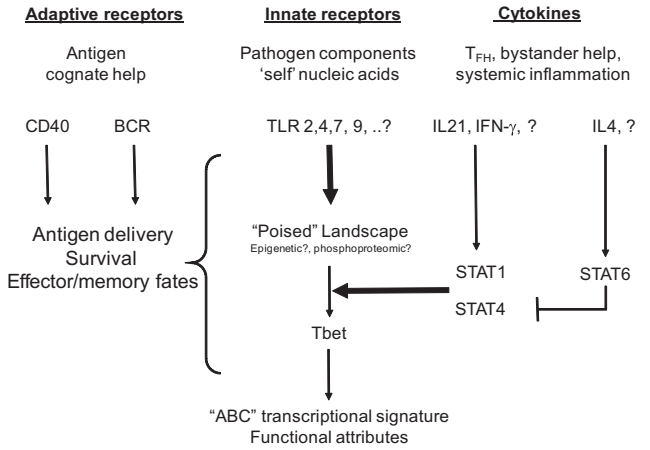


**Fig. 1.** Signals driving T-bet expression in B cells. The three signaling systems that govern the initiation of Tbet expression and subsequent differentiative fates in B cells are shown. Necessary signals are shown with bold arrows. Question marks indicate that further analogous signals may remain undiscovered. Pathogen-associated or self ligands that engage innate receptors, like TLRs, are required to poise B cells for T-bet expression. TLRs that have been directly implicated in T-bet expression are listed, although other DAMPs and PAMPs may also provide such signals. Actual transcription of T-bet requires the appropriate cytokine milieu and associated STAT signaling. To date, IL-21 and IFN-γ singly and synergistically induce T-bet expression in poised cells. In contrast, IL-4 can block IL21-driven T-bet expression. Other cytokines signaling through similar Jak/STAT pathways have not been investigated; they could shed light on how T-bet expression is regulated in different infections, autoimmunity or inflammation. Adaptive signaling- BCR and co-stimulation – are not sufficient to induce T-bet expression. However, BCR-mediated delivery of nucleic acids containing antigens amplifies TLR-driven T-bet expression. Cognate CD4, while not sufficient, appears to be essential for T-bet expression, but can foster a GC versus plasma cell fate decisions among T-bet+ cells.

these cells as compared to FO or MZ subsets. As more insights are gained, we will have a better picture of how the T-bet positive ABC fraction differs from the negative one in terms of antigen experience and downstream signals.

## 5. Signals driving T-bet in B cells

Observations to date suggest that T-bet expression in B cells is governed primarily by innate receptor signals and prevailing cytokine milieu, either alone or superimposed on activation via adaptive receptors and co-stimulation. This triumvirate of signaling systems, as well as their downstream impact on T-bet expression, are schematized in Fig. 1.

T-bet expression in murine FO B cells is driven by TLR but not BCR signaling. Liu et al. were the first to show that CpG and LPS could induce T-bet at the mRNA level as early as three hours after stimulation [20]. Rubtsova et al. subsequently determined that TLR7 signaling was crucial for the emergence of CD11c$^+$ ABCs, most of which also have high T-bet gene expression [22]. These observations were confirmed and extended by Naradikian et al., where T-bet expression was induced in response to TLR9 or TLR7 signaling, but not to BCR and/or anti-CD40 stimulation alone [30]. These data bear interesting parallels to the original findings of Hao et al. and Rubtsova et al., who reported that ABCs proliferate only when stimulated via TLR9 or TLR7. Nonetheless, several observations suggest that BCR signals and co-stimulation via CD40 are often associated with the generation of Tbet$^+$ ABC-like cells. First, simultaneous ligation of the BCR and TLR has a synergistic effect on of ABC proliferation [25], suggesting that while BCR ligation is dispensable for ABC activation, it is nonetheless capable of amplifying the response once TLR signals are received. Moreover, Russel-Knode et al. recently reported that ABCs fail to arise in aged CD154-deficient mice, and that neither MHC II-deficient nor CD40 knockout B cells acquire the ABC phenotype following adoptive transfer [28]. Thus, T-bet expression in B cells seems to require TLR-mediated signals, but – at least in the case of naturally arising T-bet$^+$ ABCs – probably occurs in the context of cognate CD4T cell help. This led to the question of whether canonical T follicular helper ($T_{FH}$) cytokines – which would be received concomitant with cognate helper interactions – also play roles in adoption of the T-bet$^+$ fate.

Cytokine milieu modulates transcriptional profile of immune cells, and T-bet expression is regulated by interplay between the $T_{FH}$ cytokines IL-21, IL-4 and IFNγ. Both IFNγ and IL-21 drive T-bet expression, but while IL-4 enhances IFNγ-induced T-bet, it has a suppressive effect on IL-21-induced expression. This points towards a complex regulatory circuit that modulates immune responses in the context of cytokine availability. This can be observed even in uninfected/unimmunized mice: the prototypical TH2-skewed mouse strain BALB/c lacks spontaneous T-bet$^+$ GC B cells and has reduced T-bet expression in memory B cells (Bmem) compared to C57BL/6, which is TH1-skewed [30]. Pathways downstream of cytokine signaling that lead to T-bet expression in ABCs are as yet unknown. Nonetheless, as might be predicted by the roles for common gamma-chain cytokines, the existing literature points to a role for the JAK-STAT pathways. Thus, STAT1$^{-/-}$ knockout splenocytes do not upregulate T-bet when stimulated in vitro with IFNγ, whereas STAT4$^{-/-}$ splenocytes have T-bet levels comparable to wild type controls [31]. Earlier studies reported a reduction in T-bet expression in STAT4$^{-/-}$ mice [32], which was presumably due to a deficiency of IFNγ. These reports show that a signaling circuitry involving STATs and cytokines stimulates T-bet expression in an antigen driven manner. Similar STAT-mediated regulatory networks apparently exist in B cells. Naradikian et al., showed that IL-4 suppressed T-bet expression induced by IL-21 in a STAT6 dependent manner. Interestingly, IL-4 synergistically enhanced IFNγ driven T-bet expression, indicating that STAT-mediated signaling is complex and context-dependent [30]. There is evidence of a feedback loop amongst T-bet, STAT and cytokines. Targeted deletion of T-bet in B cells leads to reduction in STAT1 gene transcription [18]. Since STAT1 regulates IFNγ signaling, T-bet's impact on STAT-1 transcription could be a positive regulatory mechanism to maintain its own transcription (via IFNγ).

The association between STAT signaling and T-bet expression in ABCs requires deeper research. Furthermore, only a few cytokines have been tested for their ability to induce/suppress T-bet. As T-bet expression is examined in various pathological conditions, more cytokines of interest will be revealed, hopefully providing a tool to interrogate the STAT pathway in the context of T-bet expression and function.

Cytokine-mediated regulation of T-bet expression is secondary to whether the B cell was stimulated via TLRs. In *in vitro* experiments, IL-21 induced T-bet only in B cells activated via TLR9 or TLR7, and not in those activated via BCR and CD40 ligation alone. This indicates that TLR signaling must poise B cells to express T-bet upon receiving appropriate cytokine cues. Thus, mode of activation by an antigen, coupled with available cytokines yields a tripartite regulatory network that modulates T-bet expression. For instance, a Th2 type pathogen (e.g.; Helminth infection) would lead to increased IL-4, which would suppress IL-21's ability to direct B cells towards a T-bet- driven transcriptional profile. Similarly, viral infections, or nucleic acid-based antigens would elicit a Th1 response, where IFNγ and IL-21 together would promote increased T-bet expression in a TLR-dependent manner, enabling class switching to $IgG_{2a/c}$ and other T-bet-associated characteristics. Indeed, an increasing body of literature implicates T-bet expressing B cells in a variety of infections, including influenza [30], LCMV [33], HCV [34], and HIV [35] [36].

It is also important to address the role of cognate T cell help in the context of infections. CD154-deficient mice do not acquire naturally arising ABCs with age, leading to the conclusion that cognate help is important for T-bet expression. However, we do not know if the effects are direct or indirect: is CD40-CD40L signaling inducing T-bet expression at a cell intrinsic level, or is it merely necessary for GC formation, which in turn leads to the development of T-bet expressing B cells? The

latter possibility would imply a bystander effect of CD4 help. Based on existing data, one would propose a model wherein cognate help following TCR activation would lead to GC formation and T-bet expression. However, cognate help might well be an event which is parallel – not precursor- to induction of T-bet expression. To address this, one needs to closely monitor when GC and T-bet expression programs are switched on in a cell, and whether T-bet$^+$ B cells always arise from GC B cells. Further insights can be gained from a carefully controlled experiment in which GC reaction is blocked but cognate interactions occur; would T-bet$^+$ B cells still be induced? Deconstructing the events leading to induction of T-bet expression is an important next step in ABC research.

Simultaneous ligation of BCR and TLR dampens cytokine-mediated T-bet expression [30]. On the other hand, delivery of TLR agonist via BCR has a synergistic effect, and this axis may be relevant to autoimmunity. Leadbetter et al., showed that BCR-mediated delivery of TLR antigens is a potential mechanism for delivery of autoantigens (chromatin) that can activate autoreactive B cells [37]. Data from our lab provides further mechanistic insights into this phenomenon; when a B cell is stimulated via this route and survives, it expresses T-bet in the presence of IL-21 or IFNγ and promotes class-switching to antibodies of the $IgG_{2a/c}$ isotype [38]. This points to the existence of a TLR-driven checkpoint to regulate survival of autoreactive clones and the implication of T-bet in promoting detrimental autoimmune responses.

The need for TLR signaling in promoting T-bet expression, and that of BCR (along with co-stimulation) in repressing the same, points to distinct signaling outcomes downstream of NF-KB and MYD88. Moreover, the observation that relevant cytokines upregulate T-bet in an exclusively TLR-dependent manner adds a further layer of complexity of involving Jak/STAT signaling. Is TLR signaling facilitating STAT binding to the T*bx21* locus? How these signals integrate is still a mystery- its resolution will likely shed some more light on B cell differentiation.

## 6. Outcome of T-bet driven signaling in B cells

While the complexity of signals driving T-bet expression in B cells is still being probed, a parallel avenue of investigation is the implication and functional consequence of signaling downstream of T-bet. Viral infections provide an excellent model to track the development and properties of antigen-specific ABCs, since they are nucleic-acid associated antigens that can trigger TLR signaling. Indeed, T-bet expression in B cells can be critical for effective pathogen clearance, since B lineage-restricted T-bet deficiency yielded inability to control LCMV infection, which could be ameliorated by adoptive transfer of immune serum [33]. Whether this solely reflects the effector function of IgG2a/c, or involves other T-bet driven mediators produced by B cells, such as IFN-γ, for full effect remains unclear. In addition to these incisive findings, reports in several different viral infection systems are consistent with a central role for Tbet+ B cells in immune protection [22,29,33,34,36]. In influenza infection, antigen-specific T-bet$^+$ B cells can be detected as late as 100 days post infection where they make up about 20–30% of the total antigen-specific B cell pool [28]. Does this mean that T-bet$^+$ B cells are a subset of memory B cells? A recent report by Chang et al. describes T-bet B cells in HCV-infected patients [34]. They found that T-bet$^+$ B cells were increased only in patients with HCV infection, whereas the non-HCV cirrhotic cohort was similar to healthy controls. This supports the notion that T-bet expression requires nucleic-acid containing antigen. This population also expressed markers of tissue-resident memory as well as naturally arising murine ABCs ($CD27^-CD21^-CD95^+CD11c^+CXCR3^+$). Viral clearance was accompanied by reduction in circulating T-bet$^+$ B cells, whereas in vitro stimulation of B cells (already exposed to HCV once) with HCV antigens lead to increased T-bet expression. Thus, T-bet$^+$ expression could be a hallmark of certain memory B cells, which are most likely tissue resident.

There is a growing appreciation for specialization even amongst effector and memory immune subsets. T lymphocytes, for example, can diverge into central, effector, stem cell memory and tissue resident memory depending on antigen, cytokine and even metabolic cues. These subsets differ not just phenotypically but also functionally. This would lead one to question, if T-bet expressing B cells are indeed a novel variety of memory B cells, what is their functional relevance? Clearly, memory subsets can impact the nature of secondary responses.

Their association with autoimmunity would indicate a pathological role. Clues regarding T-bet expressing B cells also come to us from autoimmune models. An abundance of nucleic acid containing autoantigens, accompanied by cytokine imbalance makes for a conducing environment for increased T-bet expression. Indeed, ABCs accumulated in young mice prone to autoimmunity and T-bet$^+$ B cells have been described in autoimmune patients. These cells secrete autoantibodies upon in vitro stimulation, pointing towards a role in autoimmune pathology [22,26]. B cell-specific conditional deletion of T-bet in a spontaneous murine model of SLE resulted in reduced kidney damage, mortality and lowered serum levels of anti-chromatin autoantibodies [39]. Interestingly, the authors report that the difference in serum autoantibody levels between conditional knockout mice, and their T-bet$^-$sufficient littermates decreased over time, suggesting that T-bet may not be the only factor regulating development of autoimmune phenotype. How T-bet interacts with other B cell-intrinsic factors to shape favorable and detrimental immune responses is an avenue for future research.

The role of T-bet$^+$ B cells in models of infection is a bit less clear, particularly with reference to secondary immune responses. ABCs are critical components of the primary immune response [30,33] and persist long after infection has cleared [28]. They may rapidly produce antibodies in response to a secondary infection by the same pathogen, which is definitely an advantage to the host. However, it is unknown how these cells react to infection with a heterosubtypic virus. Lung-resident memory cells could mediate original antigenic sin by rapidly producing $IgG_{2a/c}$ antibodies specific to the primary antigen and reducing the efficacy of response to the secondary virus. It is also vital to understand difference between T-bet positive and negative cells. T-bet is a transcriptional regulator; hence it would be expected to fundamentally alter downstream signaling outcomes, with likely changes in function as well. Since T-bet$^+$ B cells are detected several weeks post infection, their survival requirements are definitely different from most B cells. However, are they the same or different from the surviving T-bet negative pool?

T-bet expression is critical for isotype switching to $IgG_{2a/c}$, which connects ABCs to plasma cell development and differentiation. Their role in humoral immunity could be associated with formation of plasma cells. In this regard, it is necessary to investigate the relationship of T-bet expressing B cells with different plasma cell populations. Switching to IgA, for example, is enabled by RORα, not T-bet [18]. Whether T-bet and RORα exert their effects independently is yet unknown. T-bet, being a regulator of lineage determination, could be guiding the development of one kind of plasma cell subset over another, perhaps modulating the process by interacting with classical plasma cell transcription factors. If this is indeed the case, then it would mean that T-bet expression is playing a major role in shaping secondary immune responses to infections, response to vaccines and development of autoimmunity. Addressing these questions requires deeper investigation into nuances of signals driving T-bet expression in B cells.

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**APPENDIX:** Wang/Wang/Kumar et al., Nat Commun. 2018

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# IL-21 drives expansion and plasma cell differentiation of autoreactive CD11c$^{hi}$T-bet$^{+}$ B cells in SLE

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Although the aetiology of systemic lupus erythematosus (SLE) is unclear, dysregulated B cell responses have been implicated. Here we show that an unusual CD11c$^{hi}$T-bet$^{+}$ B cell subset, with a unique expression profile including chemokine receptors consistent with migration to target tissues, is expanded in SLE patients, present in nephrotic kidney, enriched for autoreactive specificities and correlates with defined clinical manifestations. IL-21 can potently induce CD11c$^{hi}$T-bet$^{+}$ B cells and promote the differentiation of these cells into Ig-secreting autoreactive plasma cells. While murine studies have identified a role for T-bet-expressing B cells in autoimmunity, this study describes and exemplifies the importance of CD11c$^{hi}$T-bet$^{+}$ B cells in human SLE.

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Systemic lupus erythematosus (SLE) is an autoimmune disease of which the aetiology is unclear, although dysregulation of B cell function is believed to have a key role in disease pathogenesis[1]. There are several reports of altered B cell phenotypes in individuals with SLE. Conventionally, CD27 is used as a marker of memory B cells, where CD27/CD70 interactions are involved in the regulation of B cell activation and plasma cell differentiation[2,3]. However, a growing body of literature suggests that subsets of $CD27^-$ memory B cells also exist. Memory B cells that lack CD27 expression and co-express CD11c and FcR-like antigen (FcRL4/IRTA-1) are described in the tonsil[4]. In SLE, Sanz and colleagues report a population of autoreactive memory B cells that lacks CD27 expression and is associated with clinical manifestations of lupus[5]. Others also observe $CD27^-$ memory-like B cells in SLE that are defined by high spleen tyrosine kinase (SYK) or CD95 expression, that similarly correlate with disease activity[6,7]. Furthermore, other B cell populations are described in SLE including $CD19^{hi}CXCR3^{hi}$ B cells that associate with poor clinical outcomes after rituximab treatment[8], or $CD24^-$-activated naive B cells that may be precursors of plasma cells[9].

Another population of B cells described to be increased in autoimmune disease is a B cell subset that expresses CD11c, a marker traditionally associated with dendritic cells. In rheumatoid arthritis (RA), Sjögren's Syndrome and common variable immunodeficiency disorder, $CD11c^+$ B cells are expanded[10–12]. Moreover $CD11c^+IgD^-CD27^-$ 'double-negative' B cells in multiple sclerosis are reported to be present in both the peripheral blood as well as the cerebrospinal fluid[13]. B cells that express CD11c are also observed in parasitic disease, after malarial infection[14]. In mice, and in women with RA, $CD11c^+$ B cells accumulate with age, thus termed age-associated B cells (ABC) [11,15]. These ABCs express the T-box transcription factor (T-bet), and require both Toll-like receptor (TLR) signalling and T-bet for their generation[11,15–17]. Furthermore, the complementarity-determining regions of murine ABCs contain a substantial number of somatic hypermutations and require both CD40L and MHC class II for their development, suggesting that interactions with activated T cells in the germinal centre may be required for their generation[18].

In murine B cells, T-bet expression drives class switch recombination (CSR) to IgG2a[11,19,20], while inhibiting CSR to IgG1 and IgE, resulting in protection from allergic inflammation[21]. In vivo, T-bet is critical for maintaining antigen specific memory of IgG2a B cells[20]. In autoimmunity, loss of T-bet in murine models of lupus results in greatly reduced B cell-driven disease manifestations[19,22]. Furthermore, removal of $CD11c^+$ B cells from mice immunised with TLR7 agonist markedly reduces anti-Smith (Sm) titres[11]. Taken together, these studies suggest that in pre-clinical murine models, the majority of B cells with autoreactive specificity originate from $CD11c^+$T-$bet^+$ B cells. Additionally, in the context of influenza immunisation, $CD21^{lo}$ B cells with a phenotype similar to ABCs are described to be post-germinal centre memory B cells[23].

In humans, T-bet expression can be induced in B cells by IL-27, IFNγ[24] or IL-21[16,25,26] and can be expressed by $CD11c^+$ B cells of healthy individuals[27]. In both humans and mice, B cell receptor (BCR)/TLR9 co-engagement results in cell cycle arrest and subsequent cell death of B cells; when rescued from this TLR9-dependent checkpoint by co-stimulation with anti-CD40 and IL-21, B cells adopt the T-$bet^+$ cell fate[28]. However, neither T-bet expression in B cells from SLE patients, nor the potential contribution of these cells to disease manifestations has been systematically investigated.

Clearly B cells are dysregulated in lupus. One of the most potent cytokines that regulates B cell function is IL-21[29,30]. IL-21 belongs to the common γ receptor family of cytokines and plays a non-redundant role in driving plasma cell differentiation[31–35]. Depending on the signals that B cells receive, IL-21 can induce a range of responses including B cell activation, proliferation, differentiation or death[29]. IL-21 can also influence the ability of B cells to act as suppressor cells either by directly upregulating granzyme B[36], or by driving differentiation of IL-10-producing regulatory B cells[37]. Activation of B cells from healthy donors with IL-21 co-stimulation can upregulate SYK expression associated with hyporesponsiveness of BCR signalling[38]. Single-nucleotide polymorphisms in both IL-21 and the IL-21 receptor (IL-21R) have also been associated with susceptibility to SLE[39,40]. Moreover, both soluble IL-21 and IL-21 producing T cells are elevated in blood of lupus patients[41–44], where $IL\text{-}21^+$ T cells correlate with frequency of memory B cells[43] and thus, overexpression of IL-21 may contribute to clinical manifestations of disease through propagation of pathogenic autoantibodies.

In this study, we examine a large cohort of SLE patients in order to further define altered B cell subpopulations in SLE and possible links to IL-21. Here, we describe a population of $CD11c^{hi}$ B cells that are poised to differentiate into Ig-producing plasma cells with autoreactive specificities. These findings may suggest that $CD11c^{hi}$ B cells contribute to the pathogenesis of SLE through the generation of autoreactive plasma cells and indicate that targeting $CD11c^{hi}$ B cells may have therapeutic benefit.

## Results

**$CD11c^{hi}$ B cells correlate to disease manifestations in SLE**. B cell phenotype was evaluated by flow cytometry from blood samples of over 200 SLE patients and 147 matching healthy donors (Supplementary Table 1), and compared to disease severity scores and other measures of disease activity. Strikingly, a high proportion of circulating B cells isolated from SLE patients displayed greatly increased density of CD11c, comparable to the density observed on dendritic cells isolated from the same individual (Fig. 1a). The frequency of these $CD11c^{hi}$ B cells was increased in SLE patients compared to healthy donors (Fig. 1b). These B cells were present in tonsil and spleen, but at very low frequencies, suggesting that CD11c expression did not simply denote an activated phenotype (Fig. 1b). To evaluate the relationship with disease activity, the percentage of $CD11c^{hi}$ B cells was compared to SLE patients grouped according to the SLEDAI disease activity index. Notably, the frequency of $CD11c^{hi}$ B cells was increased in patient groups with higher SLEDAI and was highest in the most severe patients with SLEDAI of 9 or above (Fig. 1c). We next determined if $CD11c^{hi}$ B cells were preferentially expanded in SLE with distinct clinical manifestations. $CD11c^{hi}$ B cells were found at the highest frequencies in SLE patients with coincident active nephritis and malar rash (Fig. 1d), suggesting that this B cell subset has the potential to associate with specific clinical disease manifestations. Notably, the SLEDAI of patients with these specific disease manifestations was not different from those with other symptoms, such as arthritis, where $CD11c^{hi}$ B cells were not enriched (Fig. 1d, Supplementary Fig. 1a), illustrating the specificity of this clinical association. When patients were stratified based on immunomodulatory drug treatment, such as CellCept (MMF) or cyclophosphamide (CTX), no association was found between MMF and percent $CD11c^{hi}$ B cells, whereas an association was found for CTX (Supplementary Fig. 1b). As the more severe patients were not necessarily on CTX, the correlation we observed between CD11c frequency and SLEDAI is unlikely due to associations with CTX treatment.

Other measures of disease activity were also compared to $CD11c^{hi}$ B cells where a significant negative correlation with C3 and C4 concentrations was found ($r = -0.38$ and $-0.32$,

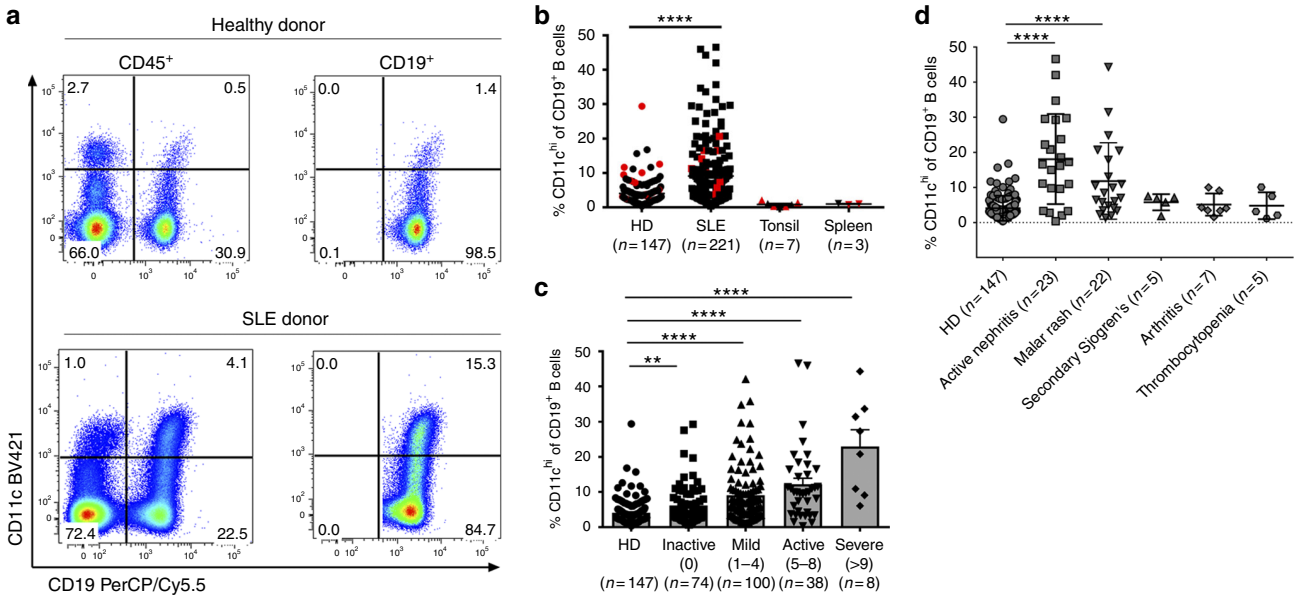


**Fig. 1** CD11c$^{hi}$ B cells are expanded in SLE and correlate to SLEDAI and specific clinical manifestations. **a**–**d** % CD11c$^{hi}$ B cells (of CD19$^{+}$ cells) was determined as shown in **a**. **b** Distribution of %CD11c$^{hi}$ B cells (of CD19$^{+}$ cells) (healthy donors $n = 147$ (123 unique donors, 24 of which are repeats) or SLE patients $n = 221$ (112 unique donors, 109 of which are repeats), tonsil or spleen. Male donors labelled in red. **c** %CD11c$^{hi}$ B cells (of CD19$^{+}$cells) isolated from blood of healthy donors or SLE patients grouped by SLEDAI range. **d** %CD11c$^{hi}$ B cells (of CD19$^{+}$cells) from healthy donors was compared to SLE with different clinical manifestations. Patients with more than one manifestation were excluded ($n = 10$). **b**–**d** (Data are represented as mean ± SEM, non-parametric Mann–Whitney test, $^{**}p < 0.01$, $^{****}p < 0.0001$)

respectively, $p < 0.0001$, Pearson correlation test), a weak positive correlation with erythrocyte sedimentation rate was obtained ($r = 0.17$, $p = 0.01$, Pearson correlation test), and no association with C-reactive protein noted (Supplementary Fig. 1c).

Tubulointerstitial inflammation in lupus nephritis (LN) has been shown to be a predictor of progression to renal failure[45]. As the highest association of CD11c$^{hi}$ B cells was found in lupus patients with active nephritis, we examined if CD11c$^{hi}$ B cells could be found in the target tissues of nephritic kidney. Thus, we examined kidney biopsies of active LN patients for the presence of CD20$^{+}$CD11c$^{+}$ B cells. Notably, CD20$^{+}$CD11c$^{+}$ B cells were noted in all biopsies that contained B cell infiltrate (Fig. 2a–c), either as a diffuse staining pattern (Fig. 2a), or often contained within an ectopic-like follicle (Fig. 2b). These data demonstrate that CD11c$^{+}$ B cells are not only present in the circulation, but have the capacity to migrate into target tissues.

**Phenotypes of CD11c$^{hi}$ B cells from SLE patients**. Next, we determined if these CD11c$^{hi}$ B cells from this SLE cohort expressed the transcription factor T-bet as T-bet has been described in B cells from both humans and mice[10,17,21,25,26,46]. As shown in Fig. 3, nearly all CD11c$^{hi}$ B cells present in SLE patients express T-bet (Fig. 3a, Supplementary Fig. 2a). The CD11c$^{hi}$ T-bet$^{+}$ B cells were largely found not to express CD38 and express low levels of the memory B cell antigen CD27, with a portion expressing IgD (Fig. 3b). While a fraction of the CD11c$^{hi}$ B cells expressed IgD, an equal portion expressed IgG or IgA, suggesting that these post-switched CD11c$^{hi}$ B cells are antigen experienced, and may represent post-germinal centre B cells (Fig. 3c). CD11c$^{hi}$ B cells present in healthy individuals also expressed T-bet but were reduced compared to SLE patients (Supplementary Fig. 2b, c).

In order to further characterise CD11c$^{hi}$ B cells, these cells were examined for cell surface expression of both conventional and unconventional B cell antigens. In the majority of B cells examined from over 200 SLE patients, CD11c$^{hi}$ B cells were found to be largely CD27$^{lo}$CD38$^{lo}$ (Fig. 4a). Further characterisation of these cells revealed that CD11c$^{hi}$ B cells expressed higher density of CD19, CD20, CD32 (FcγR), the sialyltransferase antigen CD75[47], and similar densities of HLA-DR as compared to the CD11c$^{-}$ B cells (Fig. 4a, b). Although in mice, CD11c$^{+}$ ABC express CD5, CD11b and the plasma cell antigen, syndecan1/CD138[11], in SLE, these CD11c$^{hi}$ B cells were largely negative or expressed low densities of these antigens, and were largely negative for CD21 and CD23. Expression of CD24 was also evaluated where high density of CD24 is observed on the majority of CD27$^{+}$ memory B cells[48]. While CD11c$^{-}$ B cells, as expected, expressed CD24, the CD11c$^{hi}$ B cells did not appear to express this antigen (Fig. 4c).

Upon further analysis of TNF receptor family members, it was revealed that these CD11c$^{hi}$ B cells expressed a high density of BAFF receptor (BAFFR), intermediate densities of transmembrane activator and CAML interactor (TACI) and minimal B-cell maturation antigen (BCMA) that is expressed by plasma cells (Fig. 4d). Unexpectedly, these B cells expressed a low density of CD40, while expressing high density of the apoptotic antigen Fas/CD95. The phenotype of these CD11c$^{hi}$ B cells was found to be similar to that of ABC's described previously in mice that express CD11c, and are CD21$^{-}$CD23$^{-}$CD95$^{+}$ and largely IgD$^{lo}$BAFFR$^{+}$, TACI$^{+}$ and BCMA[−15].

A population of CD27$^{-}$CD11c$^{+}$ B cells has been described in the tonsil that express FcRL4[4], or in the peripheral blood of healthy individuals that express FcRL5[27]. As shown in Fig. 4e, blood CD11c$^{hi}$ B cells expressed low densities of FcRL3 and FcRL4, although at higher levels than the CD11c$^{-}$ B cells (Fig. 4e, g). Strikingly, however, these cells were found to uniformly express very high densities of FcRL5 (Fig. 4e, g). IL-21R is expressed at high densities on naive B cells, but the expression is downregulated on CD27$^{+}$IgD$^{-}$ or IgD$^{+}$ memory B cells[49] (Fig. 4f). Notably, similar to naive B cells, CD11c$^{hi}$IgD$^{-}$ and CD11c$^{hi}$IgD$^{+}$ B cells were found to express high densities of IL-21R (Fig. 4f).

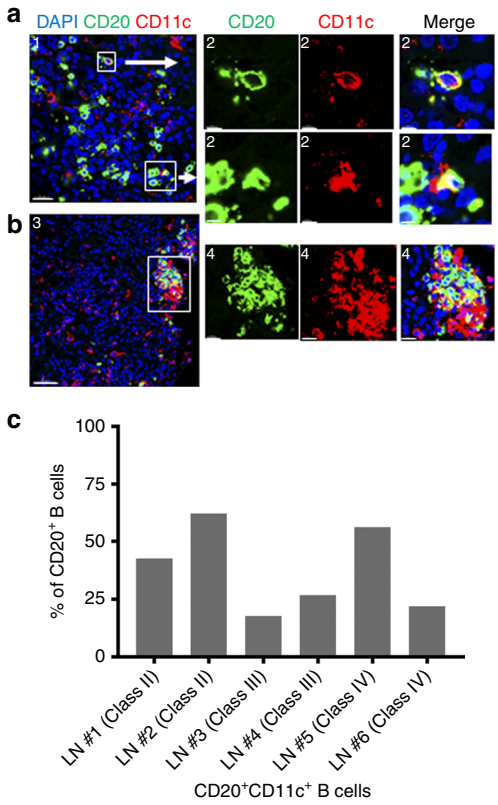


**Fig. 2** CD11c$^{hi}$ B cells are present in nephritic kidney in SLE. Nephritic kidneys from SLE patients were examined for the presence of CD11c$^{hi}$ B cells. A total of 11 kidney biopsies were examined, of which 6 contained B cell infiltrate as defined in methods where all 6 showed presence of CD20$^{+}$CD11c$^{+}$ double positive B cells. Green shows CD20, red shows CD11c, and yellow indicates double positive cells. **a** Kidney section of lupus nephritis Class II. **b** Kidney section of lupus nephritis Class IV-b. Space bars show length as indicated. Scale bars = 1, 20 μM; 2, 3 μM; 3, 50 μM; 4, 10 μM. **c** Frequencies of CD20$^{+}$CD11c$^{+}$ double positive B cells present in nephritic kidneys as indicated compared to CD20$^{+}$ B cells from six unique lupus nephritis donors

In order to understand how CD11c$^{hi}$ B cells from healthy donors (albeit lower in number) compared to those present in SLE, cell surface phenotype was examined. As shown in Supplementary Fig. 3 expression patterns of all antigens examined by flow cytometry was similar between healthy donors and SLE patients, suggesting similarities of these CD11c$^{hi}$ B cells between healthy and autoimmune individuals. It is important to point out that although CD27 is ~10 fold increased in CD11c$^{hi}$ compared to CD11c$^{-}$ B cells, CD27 expression of memory B cells is 223.7-fold increased (healthy donors) and 168.5-fold increased (SLE) compared to naive B cells (Supplementary Fig. 3b). Thus, although CD11c$^{hi}$ B cells do express greater CD27 then CD11c$^{-}$ B cells, it is greatly downregulated compared to memory B cells.

To more fully understand the differentiation stage of these B cells, we examined the telomere length of CD11c$^{hi}$ B cells and compared this to the telomere length of other B cell populations from the same donor. Previously, it has been reported that CD27$^{+}$ memory B cells have a longer telomere length than naive B cells, which is believed to be important in the generation of long-lived memory[50]. As shown in Fig. 4h, the relative telomere length (RTL) of CD27$^{-}$CD38$^{+}$ naive B cells was found to be shorter than that of CD27$^{+}$ memory B cells or plasma cells. Notably, the RTL of CD27$^{-}$CD11c$^{hi}$ B cells was comparable to memory B cells and plasma cells, in contrast to naive B cells (Fig. 4h). Taken together, these data suggest that these CD11c$^{hi}$ B cells represent a subpopulation of antigen experienced B cells, despite low expression of CD24, CD27 and high expression of IL-21R.

To better characterise how these cells relate to naive vs. memory B cells, gene expression profiling was performed using RNA sequencing (RNAseq). CD19$^{+}$ B cells from SLE patients were sorted into naive (CD11c$^{-}$IgD$^{+}$CD27$^{-}$), memory (CD11c$^{-}$IgD$^{-}$CD27$^{+}$), pre-switched CD11c$^{hi}$ (CD11c$^{hi}$IgD$^{+}$), or post-switched CD11c$^{hi}$ (CD11c$^{hi}$IgD$^{-}$) B cells. Genes that showed significant up or downregulation were evaluated. Strikingly, CD11c$^{hi}$ B cells showed very different gene expression pattern compared to both naive and memory B cells (Fig. 5a, b). Overall, 1103 genes were significantly upregulated and 819 genes were significantly downregulated in CD11c$^{hi}$ B cells compared to naive and memory B cells, respectively [fold change (FC) >2, false discovery rate (FDR) <0.05; Fig. 5a]. For highly expressed genes with counts per million (CPM) of 50 or above, 256 and 153 genes were significantly up or downregulated, in CD11c$^{hi}$ B cells compared to both naive and memory B cells. (Supplementary Fig. 4a). IgD$^{+}$CD11c$^{hi}$ were remarkably similar to IgD$^{-}$CD11c$^{hi}$ B cells (Supplementary Fig. 4a, b). Venn diagram shows that for the highly expressed genes of over 50 CPM only 17 and 61 genes were up, or downregulated, respectively, comparing IgD$^{-}$CD11$^{hi}$ to IgD$^{+}$CD11$^{hi}$ B cells.

**CD11c$^{hi}$ B cells transcriptome from SLE and RA patients.** Further inspection of the genes that were differentially expressed showed that the key phenotypic surface markers of CD11c$^{hi}$ B cells are corroborated by mRNA expression such as *CD19*, *MS4A1* (*CD20*), *ITGAX* (*CD11c*), *TBX21* (*T-bet*), *CR2* (*CD21*), *FCER2* (*CD23*), *FCGR2B* (*CD32B*), *CD24*, *CD27*, *CD38*, *CD40* and *IL-21R* (Fig. 5c, Supplementary Note 1). Notably, RNAseq revealed that CD11c$^{hi}$ B cells appeared related to plasma blasts with upregulation of *PRDM1* (*BLIMP1*), *AICDA* (*AID*), and *XBP1*, as well as genes upregulated in SLE plasma cells[51], such as *BMP6*, *EMP3* and *S100A4* (Supplementary Note 1). As CD11c$^{hi}$ B cells express low densities of CD27, CD38, and CD138, this suggests that these cells may be precursors of plasmablasts. Presence of CD11c$^{hi}$ B cells in the tissue led us to examine chemokine receptors to better understand their migration capabilities. Our data show that inflammatory chemokine receptors, *CXCR3* and *CCR9*, used by plasmablasts and IgA$^{+}$ antibody secreting cells to enter sites of inflammation and mucosa respectively[52], were upregulated by CD11c$^{hi}$ B cells compared to naive B cells. Moreover, homoeostatic chemokine receptors necessary for migration into lymphoid organ (*CXCR4*, *CXCR5* and *CCR7*) were downregulated on CD11c$^{hi}$ B cells, although abundantly expressed by naive B cells[52] (Fig. 5c, Supplementary Note 1). Additional genes and pathways associated with CD11c$^{hi}$ B cells include: BCR signalling (increased *SYK* and *CD79B*), immunomodulation and activation (increased *TLR9*, *CD80*, *CD86* and *CD72*, capable of downregulating CD27[53]), Fc receptor family members involved in Ig and immune complex binding (increased *FcRL2/3/4/5* and *CD32A/B/C*), and immune inhibitors (increased *PD1*, *PDL1*, and *CTLA4*). Furthermore, our data show that cytokine and cytokine receptors are uniquely expressed in these B cells, including gamma-c family receptors (low *IL4R*, *IL2RA* and high *IL2RB*, *IL21R*), and TNF family members (low *LTA*, *LTB*, and high *TNFRSF1B* (*TNFR2*), *TNFSF14* (*LIGHT*), *TNFRSF19* (*TROY*)), as well as unusual expression of cytokines involved in plasma cell differentiation and survival (low *IL6* and high *IL10*) (Fig. 5c and Supplementary Note 1). Lastly, CD11c$^{hi}$ B cells express minimal levels of *CD5* or *CD138*, consistent with cell surface phenotype,

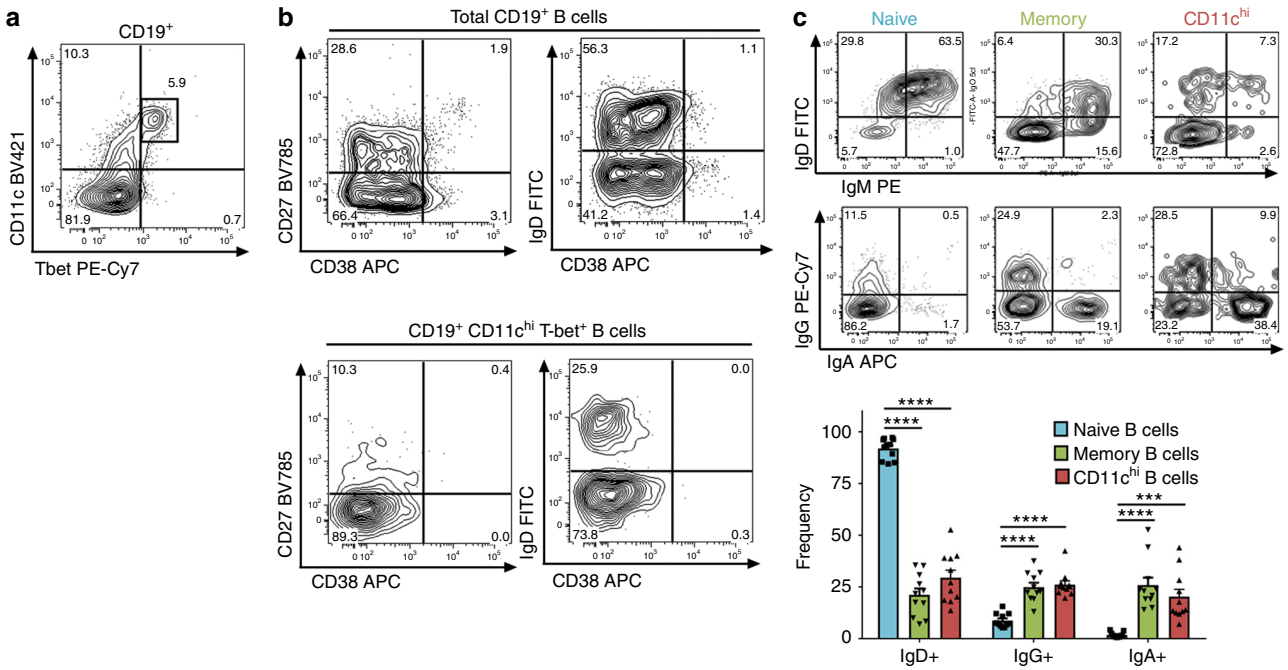


**Fig. 3** CD11c$^{hi}$ B cells express T-bet and largely do not express CD27 or CD38 and contain switched B cell receptors. **a**, **b** %CD11c$^{hi}$ T-bet$^{+}$ B cells (of CD19$^{+}$cells) in blood was determined in SLE and cell surface phenotype determined. Data representative of 21 unique healthy donors and 39 unique SLE with four repeat SLE donors. **c** Cell surface expression of IgD by IgM or IgG by IgA was determined on CD19$^{+}$CD11c$^{-}$CD27$^{-}$ naive (blue), CD11c$^{-}$CD27$^{+}$ memory (green), or CD11c$^{hi}$ (red) B cells from SLE (Data are represented as mean ± SEM, unpaired *t* test with Welch's correction, ***$p < 0.001$, ****$p < 0.0001$, $n = 11$)

but do express higher level of *CD11b*, compared to naive B cells, as described in murine ABCs[11] (Supplementary Note 1).

To assess the biological processes and signalling pathways of CD11c$^{hi}$ B cells, gene set enrichment analysis (GSEA) was performed. Importantly, pathway enrichment showed that IL-21-inducible genes were found to be upregulated in CD11c$^{hi}$ B cells compared to naive B cells, suggesting these cells were activated by IL-21 in vivo, as well as genes associated with cell adhesion (Fig. 5d).

CD11c$^{+}$CD27$^{hi}$CD5$^{+}$ "ABCs" have been reported in RA[11], which appear to express a distinct B cell phenotype to that which we described here. In order to address if cells of similar phenotype to the CD11c$^{hi}$ B cells present in SLE are also found in patients with RA, we examined blood B cells from RA patients. As shown in Supplementary Fig. 4c–d, an increase of CD11c$^{hi}$ B cells was noted in RA patients compared to healthy controls, whose demographics are shown (Supplementary Table 2). The CD11c$^{hi}$ B cells in this RA cohort was not found to associate with age (Supplementary Fig. 4e), contrary to previous reports[11]. Further studies of RNAseq analysis revealed that these CD11c$^{hi}$ B cells from RA patients share mRNA expression profiles similar to that found in CD11c$^{hi}$ B cells of SLE, including altered expression of *CD19*, *CD11c*, *T-bet*, *FcRL5*, *CD32B*, *CD21*, *CD23*, *CD24*, *CD27*, *CD38* and *CD40* compared to other B cell subsets (Supplementary Fig. 4f, g). Of interest, CD11c$^{hi}$ B cells sorted from both SLE and RA patients also shared unique transcriptome profile in regard to mRNA expression of cytokine/cytokine receptors, Fc receptors, migration molecules, transcription factors, signalling factors and other pathways (Supplementary Note 2). RNAseq analysis of CD11c$^{hi}$ B cells from healthy donors revealed that while some key genes such as *ITGAX*, *TBX21*, *CD24*, *CD38*, *FCRLs* are regulated comparable to RA and SLE patients, the majority of genes did not share similar transcriptome pattern (Supplementary Note 3). Taken together, these data suggest that although CD11c$^{hi}$ B cells of healthy donors and SLE patients share commonalities in surface antigen expression the unique transcriptome patterns may suggest that CD11c$^{hi}$ B cells from SLE have distinct functionalities.

**Links of CD11c$^{hi}$ B cells with plasma cells and autoantibody.** We often noted that SLE patients with a high frequency of CD11c$^{hi}$ B cells also displayed large percentage of CD19$^{+}$CD38$^{hi}$CD27$^{hi}$ plasma cells. Correlations of CD11c$^{hi}$ B cells to frequency of plasma cells present in SLE blood showed an association of these two B cell subsets (Fig. 6a). Thus, we next addressed whether the frequencies of CD11c$^{hi}$ B cells were also associated with lupus-associated autoantibodies. The autoantibody profile of this cohort of lupus patients was examined by assessing reactivity against a panel of 95 autoantigens and compared to frequencies of CD11c$^{hi}$ B cells present in the blood as well as compared to the level of reactivity from sera of healthy donors. Out of all the autoantigens tested, a distinct subset of over half (55) of the autoantibodies showed a significant correlation (Pearson correlation test, FDR ≤0.05) to CD11c$^{hi}$ B cells (compare Supplementary Table 3 to Table 4). Levels of several antinuclear autoantibodies associated with SLE[54] correlated with CD11c$^{hi}$ B cells, including antibodies to dsDNA, nucleosome, several histones, RNP, Smith, La, and chromatin (Fig. 6b, Supplementary Table 3). Additional autoantibodies that correlated with these cells, including antibodies to alpha-actinin, C1q, collagen X, histone H1 and aggrecan, have been reported to be present in LN[55,56] (Supplementary Table 3). Importantly, the majority of the SLE-associated autoantibodies fell above the levels noted in healthy donors. Further, no significant correlation (as defined by Pearson correlation test *p*-value <0.05) of CD11c$^{hi}$ B cell frequency was found to any of the 95 autoantibodies in healthy donors (Fig. 6b, Supplementary Fig. 5). Taken together,

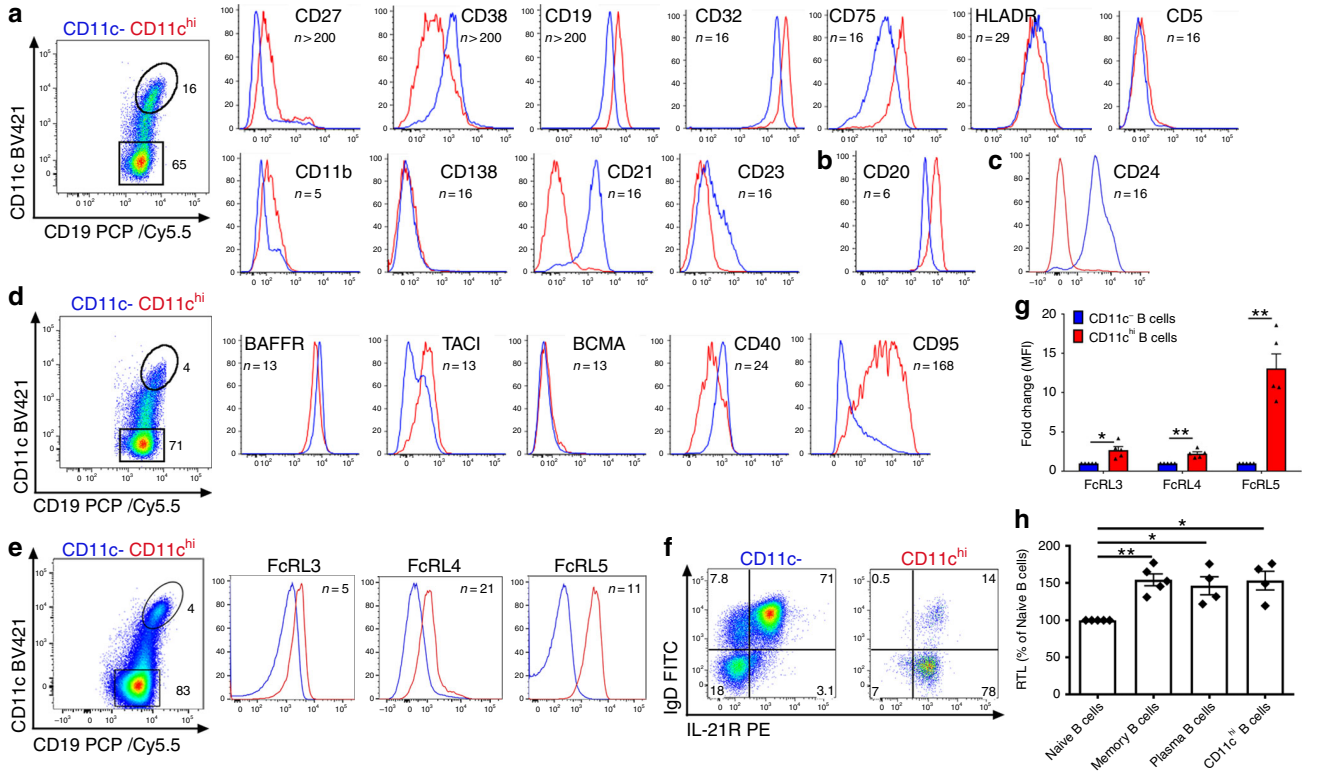


**Fig. 4** Phenotype of CD11c$^{hi}$ B cells from SLE patients. **a**–**e** Phenotype of CD11c$^{-}$ (blue) or CD11c$^{hi}$ (red) CD19$^{+}$ B cell subsets as indicated was determined in SLE where a unique donor is shown for each panel. **a** Representative phenotype of B cell subsets from the same SLE donor. **b**, **c** CD20 or CD24 expression in B cell subsets from separate SLE donors. **d** Expression of TNF family members was determined on SLE B cell populations as indicated. **e** FcRL family members were assessed on SLE B cell populations as indicated from the same donor. **f** Expression of IgD by IL-21R was determined on SLE B cell populations as indicated. **g** Fold change of FcRL measured as Mean Fluorescence intensity (MFI) of B cell subsets compared to CD11c$^{-}$ B cells as indicated. (Data are represented as mean ± SEM, unpaired *t* test with Welch's correction $*p < 0.05$, $**p < 0.01$, $n = 5$ SLE). **h** Relative telomere length (RTL) was determined in B cell populations as indicated and plotted as % naive B cells. Sorting strategy shown in Supplementary Fig. 11a (Data are represented as mean ± SEM, unpaired *t* test with Welch's correction, $*p < 0.05$; $**p < 0.01$, $n = 5$ unique donors in independent experiments

our data suggest a link between CD11c$^{hi}$ B cells with blood plasma cells and a defined set of autoantibody specificities in SLE. However, these data do not rule out other potential B cell populations in SLE patients that could show similar correlations.

**CD11c$^{hi}$ B cells are poised to become plasma cells**. Based on the positive association of CD11c$^{hi}$ B cells with plasma cells and serum autoantibodies, we next asked if these B cells were poised to differentiate into plasma cells, or were anergic as described of B cells with a similar phenotype in RA[10]. Previously, we have shown in vitro, that activated T cells induce plasma cell differentiation of total human blood B cells[34]. Thus, CD19$^{+}$ B cells from SLE patients were sorted into CD11c$^{-}$CD27$^{-}$ naive, CD11c$^{-}$CD27$^{+}$ memory, or CD11c$^{hi}$ B cells and cultured with activated T cells. As shown in Fig. 7a, activated T cells were much more effective at inducing plasma cell differentiation (as defined by CD19$^{+}$CD27$^{hi}$CD38$^{hi}$ cells) from CD11c$^{-}$CD27$^{+}$ memory B cells compared to CD11c$^{-}$CD27$^{-}$ naive B cells. Notably, CD11c$^{hi}$ B cells responded efficiently to the activated T cells, with 45 and 70% of B cells displaying a plasma cell phenotype after day 7 or 11 days of culture, respectively (Fig. 7a). Consistent with this, sorted naive, memory and CD11c$^{hi}$ B cells were found to produce IgG after activation with T cells, where total IgG levels increased with length of culture (Fig. 7b). Similar to plasma cell phenotype, IgG levels from cultures of CD11c$^{hi}$ B cells with activated T cells was similar to memory, rather than naive B cells. Importantly, unlike plasma cells, these CD11c$^{hi}$ B cells freshly isolated from SLE donors did not spontaneously produce IgG as determined by EliSpot, nor in culture in the absence of activated T cells (Supplementary Fig. 6).

The next question we addressed was whether these antibodies contained autoreactive specificities. To this end, supernatant from the above co-cultures were tested for reactivity against a panel of 95 autoantigens. Strikingly, plasma cells that were differentiated from the CD11c$^{hi}$ B cells produced significantly higher levels of autoantibodies compared to those differentiated from naive or memory B cells. In the cohort of SLE patients examined, out of the 95 specificities tested, 11 autoantibodies were found to be significantly increased ($p$-value <0.05, as determined by Linear model based group comparison as described in Methods) in the CD11c$^{hi}$ B cell group, compared to those from the naive B cell group (Fig. 7c). Moreover, seven autoantibody specificities were found to be significantly increased ($p$-value <0.05, as determined by Linear model-based group comparison as described in Methods) in the CD11c$^{hi}$ B cell group compared to the memory B cell group (Supplementary Fig. 7). Importantly, no autoantigen specificities were significantly increased in the memory B cell group compared to CD11c$^{hi}$ B cells. The increased level of autoantibodies noted in the CD11c$^{hi}$ B cell group did not appear to be simply due to augmented production of total IgG, as both memory and CD11c$^{hi}$ B cells were found to secrete similar amounts of total IgG on day 7 or day 11 of culture (Fig. 7b).

We also addressed if CD11c$^{hi}$ B cells isolated from healthy donors had the capacity to differentiate into plasma cells. As shown in Supplementary Fig. 8, both purified memory and CD11c$^{hi}$ B cells efficiently differentiated into plasma cells and

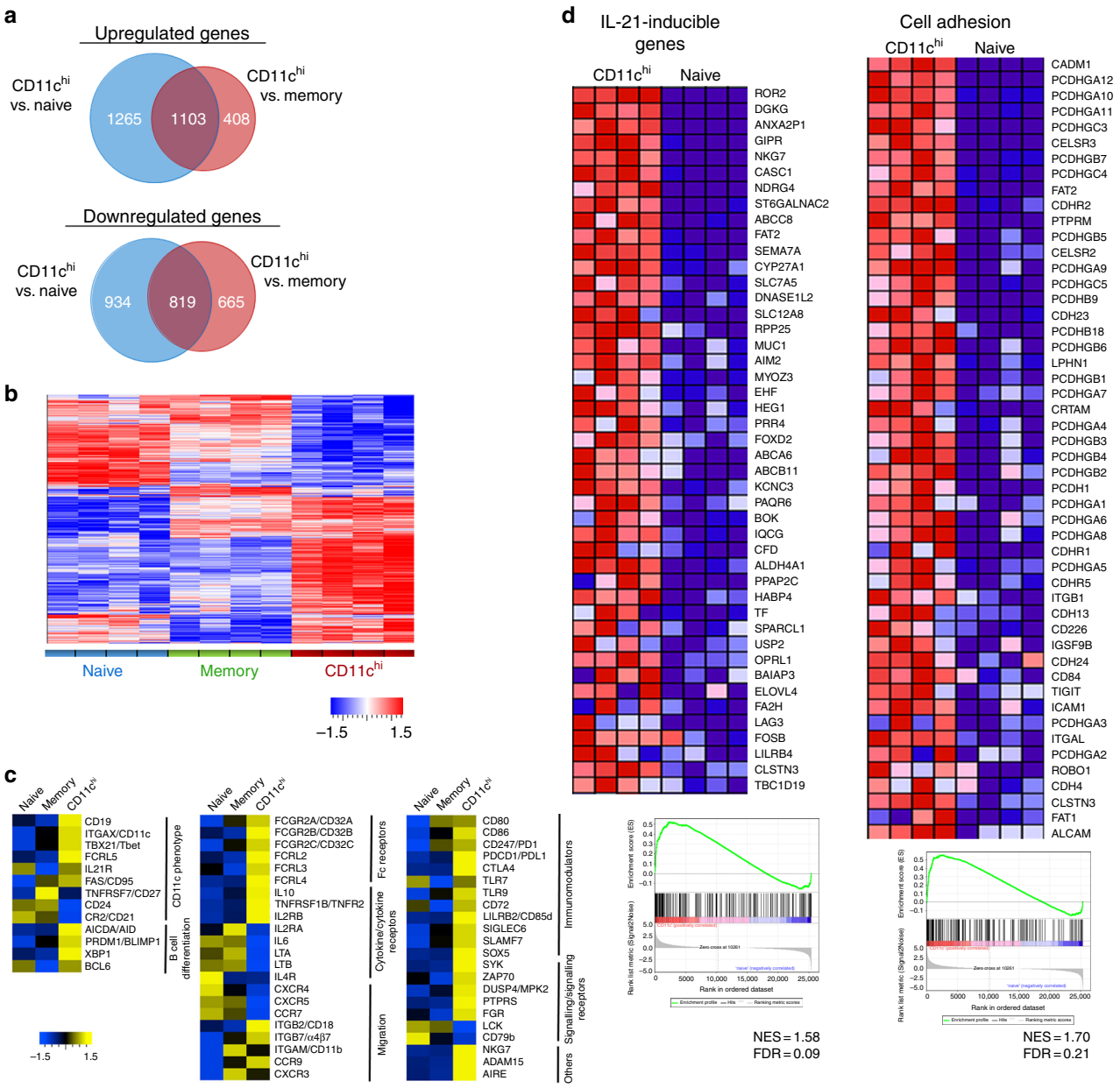


**Fig. 5** Unique transcriptome profile of CD11c$^{hi}$ B cells in SLE. **a** Venn diagram showing number of genes with significant upregulation or downregulation comparing CD11c$^{hi}$ cells with naive or memory B cells. Sorting strategy shown in Supplementary Fig. 11b. Significance is defined as Fold change (FC) >2 and False discovery rate (FDR) <0.05. **b** Heatmap showing expression pattern of genes with significant change (FC >2, FDR <0.05) in at least one of the group comparisons (CD11c$^{hi}$ vs. naive or CD11c$^{hi}$ vs. memory B cells), and passed cut-off of median counts per million (CPM) >50 in at least one of the three cell populations. Log2 transformed CPM values were used for plotting. Genes were clustered using hierarchical clustering. Red indicates higher expression, and blue indicates lower expression. Colour bar indicates *Z* score. **c** Heatmap showing expression pattern of representative genes with relevant functions. Median log2 transformed CPM values for each cell population were used for plotting. Yellow indicates higher expression, and blue indicates lower expression. Colour bar indicates *Z* score. **d** Gene set enrichment analysis (GSEA) showing pathways enriched in CD11c$^{hi}$ B cells compared to naive B cells. Heatmap shows the expression of the core genes that contribute to pathway enrichment (red, high expression; blue, low expression). NES: normalised enrichment score, FDR: false discovery rate

produced IgG upon co-culture with activated T cells (Supplementary Fig. 8a, b, Supplementary Table 5). Previously, we reported that plasma cell differentiation of total human B cells by activated T cells require de novo production of IL-21[34]. Consistent with our previous data, plasma cell differentiation of both memory and CD11c$^{hi}$ B cells was largely inhibited upon neutralisation of either IL-21 or CD40L (Supplementary Fig. 8c).

**IL-21 regulates expansion of CD11c$^{hi}$ B cells**. Next, we investigated whether IL-21 was involved in the differentiation of these cells, as unlike other memory B cell populations, CD11c$^{hi}$ B cells expressed high density of IL-21R. To this end, CD11c$^{-}$ naive B cells were isolated from the peripheral blood of SLE patients and stimulated with a combination of activators. Remarkably, IL-21 in combination with anti-IgM and anti-CD40 resulted in upregulation of CD11c expression in the majority of naive B cells

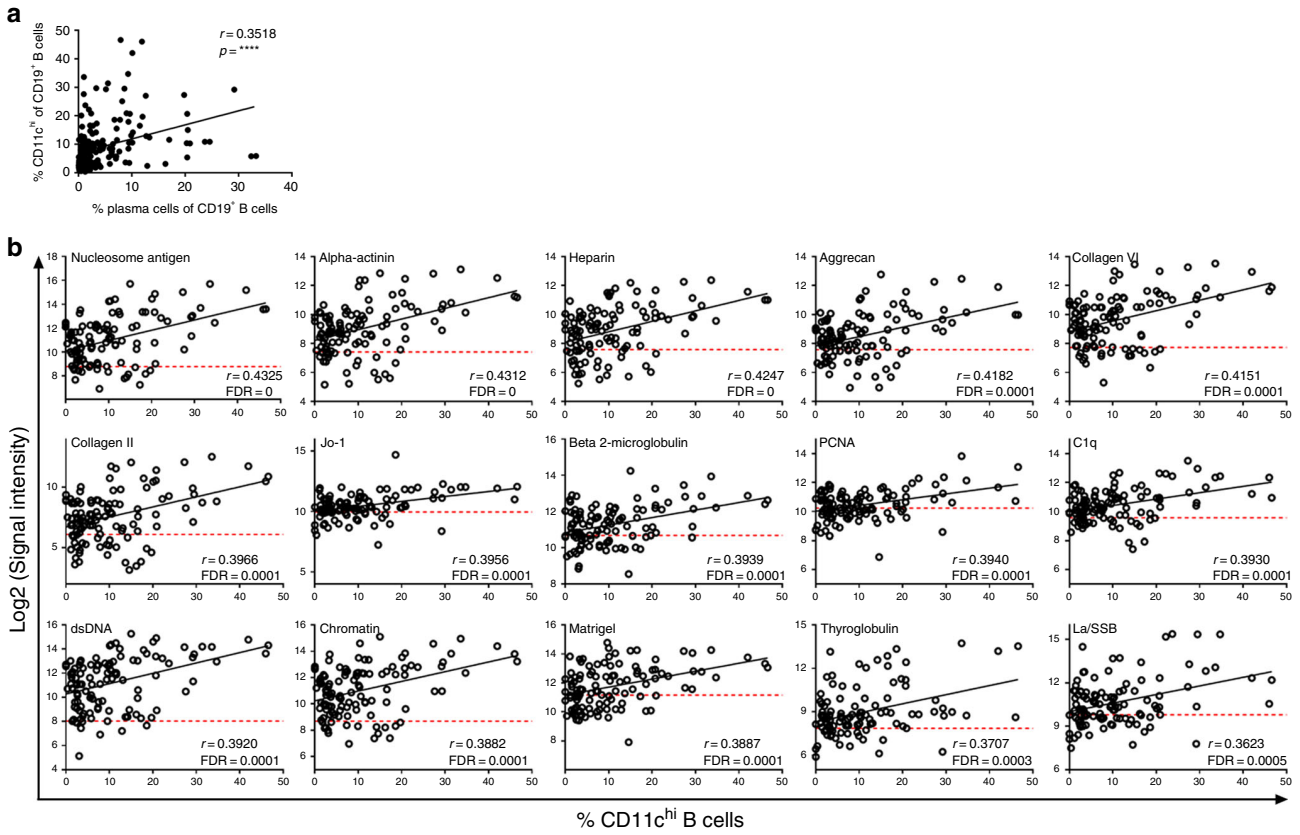


**Fig. 6** Increase of CD11c$^{hi}$ B cells significantly correlates to plasma cells and a distinct set of IgG autoantibodies in SLE. **a** %CD11c$^{hi}$ B cells (of CD19$^{+}$ cells) was correlated to %CD38$^{hi}$CD27$^{hi}$ plasma cells (of CD19$^{+}$ cells) in SLE blood. (*r*: Pearson correlation coefficient, ****$p < 0.0001$, $n = 189$). **b** Serum autoantibodies were screened for reactivity to 95 self-antigens. Scatter plots show the top 15 correlated autoantibodies (*r*: Pearson correlation coefficient, FDR: false discovery rate, $n = 114$ total SLE patients with 71 unique donors). Red dashed line indicates median log2 (signal intensity) of the autoantibodies of healthy donors ($n = 49$)

(Fig. 8a, Supplementary Table 6). While anti-IgM with anti-CD40 in the absence of IL-21 increased CD11c levels in approximately a third of the naive B cells, IL-21 was required for maximum expression (Fig. 8a, b).

We next asked if IL-21 co-stimulation resulted in upregulation of only CD11c, or if IL-21 was driving the differentiation of these cells. Thus, we examined expression of other phenotypic markers. Strikingly, this stimulation cocktail resulted in the differentiation of cells that closely resembled that of CD11c$^{hi}$ B cells present in the blood of SLE patients, namely, low CD27 expression with high densities of T-bet, FcRL5 and IL-21R (Fig. 8a, c). The ability of IL-21 to increase CD11c expression was not unique to SLE naive B cells, as naive B cells from healthy donors also increased CD11c expression after IL-21 co-stimulation (Supplementary Fig. 9a, b). Stimulation through TLR9 did not result in a similar profile but did allow for further B cell expansion.

**CD11c$^{hi}$ B cells in blood do not associate with age**. A population of CD11c$^{+}$CD27$^{+}$ B cells has been described to accumulate in the blood of aged women with RA[11]. Analysis of the circulating CD11c$^{hi}$ B cells from healthy donors and this lupus cohort revealed that there was no apparent correlation between the frequencies of CD11c$^{hi}$ B cells to the age of female healthy donors or SLE patients (Supplementary Fig. 10a, b). In fact, the female lupus patients with the highest percent of CD11c$^{hi}$ B cells were present in the younger individuals that tended to decrease with age (Supplementary Fig. 10b). Furthermore, these B cells were also noted in men with SLE where a negative correlation was noted between CD11c$^{hi}$ B cells and age (Supplementary Fig. 10b). Consistent with our studies, B cells of similar phenotype to CD11c$^{hi}$ B cells were also found not to be age associated in healthy donors[57] or patients with multiple sclerosis[13].

## Discussion

Here we describe a population of CD11c$^{hi}$ B cells that are highly expanded in lupus and present in target tissues. Our data suggest that CD11c and other integrin and chemokine receptors are guiding these cells to sites of inflammation where they may contribute to immunopathology upon encountering activated T cells and differentiate locally into plasma cells with autoreactive specificities. Further, we show that CD11c$^{hi}$ B cells are associated with LN and malar rash but not with other SLE subtypes. Clinically, the pathophysiology that drives the various SLE sub-manifestations is not well understood. However, both active LN and malar rash are associated with high dsDNA autoantibodies[58]. Moreover, active LN is also associated with hypocomplementemia. Importantly, we show that CD11c$^{hi}$ B cells significantly correlated with both anti-dsDNA levels, as well as with low complement present in the serum. We also show that CD11c$^{hi}$ B cells were poised to differentiate into plasma cells and appear to produce the majority of autoantibodies (including specificities capable of binding nuclear antigens) when compared to plasma cells differentiated from other B cell subsets from the same individual. Our data suggest that CD11c$^{hi}$ B cells may contribute to immunopathology of kidney and skin through local production of autoantibodies with pathogenic potential.

Of note, plasma cells differentiated from CD11c$^{hi}$ B cells also produced significantly higher levels of autoantibody to the RNA-associated autoantigens Sm and SmD, compared to plasma cells differentiated from either naive or memory B cells. Others have described similar findings with B cells that share phenotypic similarities of CD11c$^{hi}$ B cells including CD19$^{hi}$ B cells from SLE donors which can be driven to plasma cells that produce anti-Sm autoantibodies[8]. Autoantibodies present in sera of SLE, including

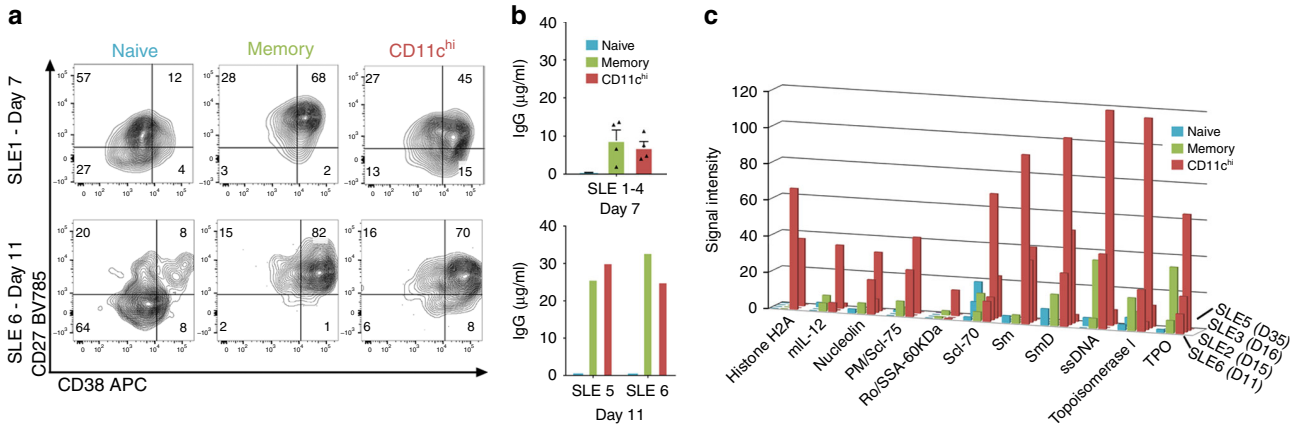


**Fig. 7** CD11c$^{hi}$ B cells are poised to differentiate into plasma cells and are the major producers of autoantibody. **a**–**c** B cells from SLE patients were sorted into CD19$^{+}$CD11c$^{-}$CD27$^{-}$ naive (blue), CD11c$^{-}$CD27$^{+}$ memory (green), or CD11c$^{hi}$ (red) B cells and added to anti-CD3-activated T cells. Sorting strategy shown in Supplementary Fig. 11c. For several experiments, an early and late time point was evaluated from separate culture wells. Each SLE number represent an independent donor examined at one or more time points. **a** After 7 or 11 days of culture, CD19$^{+}$CD3$^{-}$ B cells were analysed for CD27$^{+}$CD38$^{hi}$ plasma cell phenotype. **b** IgG in the supernatant was determined by ELISA (for day 7, data are represented as mean ± SEM of four independent experiments, or day 11, two independent experiments). **c** Autoantibodies in the supernatant were screened for reactivity to 95 self-antigens on culture day indicated. Bar plot shows significantly increased autoantibodies ($p < 0.05$) in CD11c$^{hi}$ vs. naive B cells as described in Methods

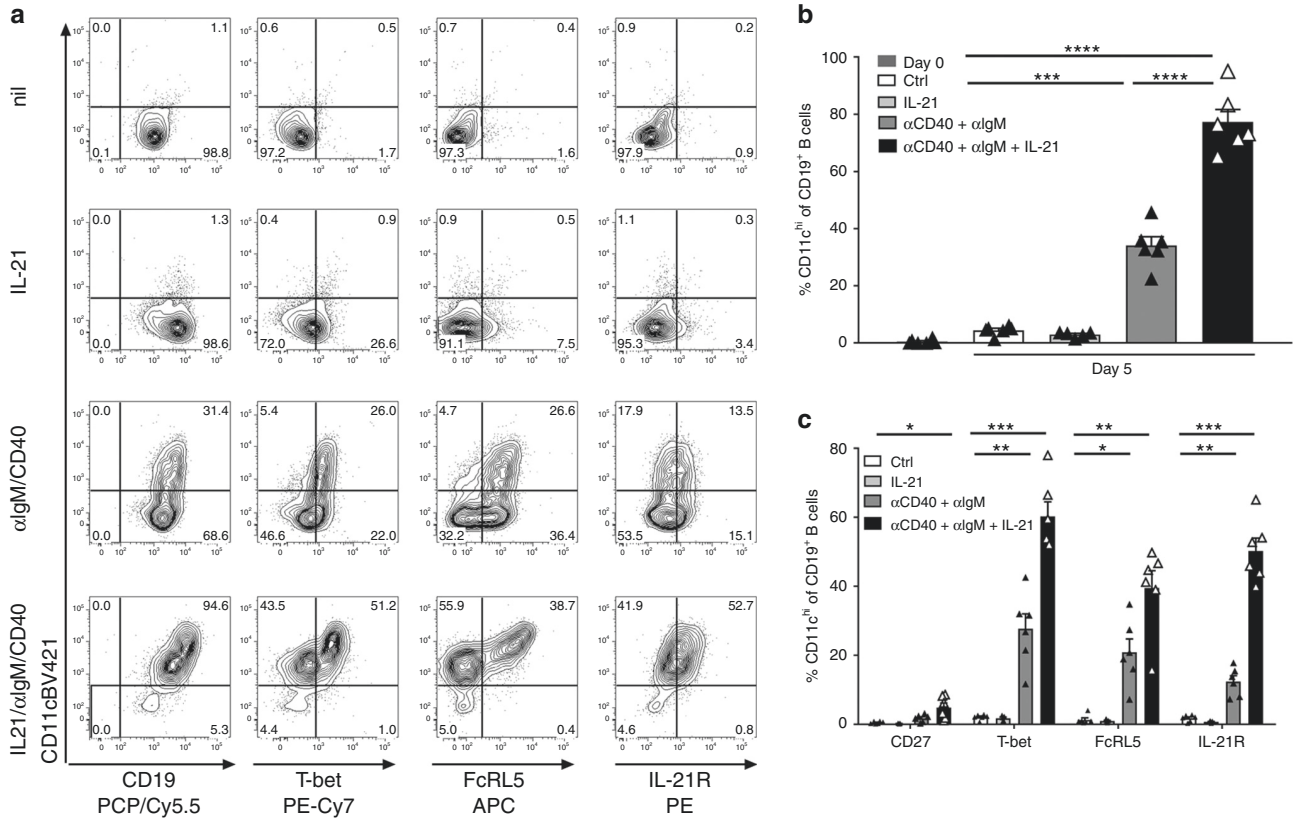


**Fig. 8** IL-21 co-stimulates the CD11c$^{hi}$ B cell phenotype. CD11c$^{-}$ naive B cells were sorted from the blood of 6 unique SLE patients and activated with a combination of stimulators as indicated. **a**–**c** Cell surface phenotype was evaluated after 5 days of culture of CD19$^{+}$ B cells excluding IgD$^{-}$CD38$^{hi}$ plasma cells. **b** Enumeration (mean ± SEM) of the frequencies of CD11c$^{hi}$ cells of CD19$^{+}$ B cells either post sort (day 0, $n = 6$) or after 5 days of culture. **c** Enumeration (mean ± SEM) of the frequencies of CD11c$^{hi}$CD19$^{+}$ B cells with the indicated phenotype. **b**, **c** $n = 6$ independent experiments of unique SLE donors, expect for IL-21 stimulation only, where $n = 5$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ as determine by unpaired $t$ test with Welch's correction

anti-Sm, significantly associate with the frequency of CD19$^{hi}$ or CD27$^{-}$IgD$^{-}$ 'double-negative' B cells[5,8]. The concept that CD11c$^{hi}$ T-bet$^{+}$ B cells in human autoimmune disease are skewed to autoreactive specificities is supported by mouse studies. In mice, CD11c$^{+}$ ABCs stimulated with TLR7 agonist produce anti-Sm autoantibodies where in vivo depletion of these CD11c$^{+}$ ABC, results in substantial reduction of anti-Sm autoantibodies after immunisation with TLR7 agonist[11]. Furthermore, in murine models of lupus, loss of T-bet expression greatly reduces B cell-related disease manifestations[22], including autoantibody titres, while T cell manifestations are spared[19], suggesting T-bet expression in B cells is playing a critical role in the regulation of autoreactive responses.

CD11c$^{hi}$ B cells express a unique cell surface and transcriptome phenotype not noted on other B cell subsets. While intermediate densities of CD11c are found on B cells that appear to contain classical CD27$^{+}$ memory B cells, T-bet expression was limited only to those B cells that express very high densities of CD11c, comparable to that noted on dendritic cells. In mice, B cell-expressed T-bet has been shown to be required to drive class switch to IgG2a[19] and involved with B cell survival[20]. In humans, B cell-expressed T-bet has been described in autoimmunity (this report and refs. [10,59]), or after parasitic infection[46], suggesting that this cell population is expanded after chronic B cell activation. As CD11c$^{hi}$ B cells associate with disease severity scores in SLE, this may suggest that increases of these cells in the periphery may also reflect tissue mobilisation by ongoing activation. This is supported by the presence of CD20$^{+}$CD11c$^{+}$ B cells in nephritic kidney, as well as the pattern of expression of chemokine receptors where CD11c$^{hi}$ B cells downregulate lymphoid tissue–chemokine receptors, and upregulate chemokine receptors associated with recruitment into inflamed tissues[52]. These data have clinical implications for patients with LN where we revealed a significant correlation of CD11c$^{hi}$ B cells in these patients. Further studies focusing specifically on this subset will help better define the pathophysiology of LN. CD11c$^{hi}$ B cells may have important implications for identifying SLE patients at risk of developing LN and could prove clinically useful as a predictive biomarker, which is largely not available for LN.

Another unique marker of these cells is FcRL5 where high expression of this antigen was limited to CD11c$^{hi}$ B cells. FcRL is a family of proteins that share homology with the Fc gamma receptor I, where FcRL5 binds IgG of all subclasses, with strongest binding by IgG1 and IgG2[60]. FcRL5 signalling can result in both inhibition as well as stimulation, when B cells are co-stimulated through either the BCR or TLR9, respectively[61,62]. Such a dual response is possible due to the association of FcRL5 with both activating (ITAM) and inhibitory (ITIM) motif elements[63]. Other FcR, or FcR-like receptors that express ITAM and ITIM motifs were also upregulated in these CD11c$^{hi}$ B cells. This includes high expression of CD32B (ITIM), unusual expression of CD32A (ITAM) that is not expressed on other B cells subsets, and CD32C, whose extracellular domain encompasses the CD32B antigen, with the intracellular region containing the CD32A ITAM motif[64], and LILRB2/CD85d, an Ig-like receptor that contains an ITIM domain. This suggest that these FcR or FcR-like receptors compete for binding of Ig immune-complexes present in SLE, that could result in either a positive or negative signal dependent on complex interactions of these molecules.

IL-21, a master regulator of B cell responsiveness, has wide reaching actions in determining how B cells respond to their environment, capable of inducing B cell activation, CSR, plasma cell differentiation or death, depending on the context of co-stimulation[29]. Normally IL-21R is down regulated when a B cell differentiates from a naive to a memory B cell[49]. These CD11c$^{hi}$ B cells, that appear antigen experienced, uncommonly express high densities of IL-21R, and mRNA associated with IL-21R signalling. In cultures of human naive B cells, IL-21 co-stimulation drove the differentiation of CD11c$^{+}$T-bet$^{+}$FcRL5$^{+}$IL-21R$^{+}$ B cells, consistent with other studies that show IL-21 induced T-bet expression in B cells after TLR stimulation[16,28]. Moreover, IL-21 was involved in the differentiation of these cells to plasma cells, similar to what we have previously reported for total human B cells[34]. Importantly, IL-21, and IL-21-producing T cells are increased in SLE[41,42] and correlate to specific B and T cell subsets[41,43]. Taken together, these data suggest that among IL-21's many roles in activation and differentiation of B cells, over expression of this cytokine in SLE may contribute to autoimmune pathology through influencing the expansion of CD11c$^{+}$ T-bet$^{+}$ B cells with autoreactive specificities.

Other unusual phenotypic components of these memory B cells are lower expression of CD27 and CD40 (compared to memory B cells), which suggests that these B cells may not have productive interactions with T cells that express the ligands for these molecules. However, despite low expression of these antigens, the CD11c$^{hi}$ B cells readily differentiated into plasma cells capable of secreting autoreactive IgG in B cell/T cell co-cultures. Thus, these cells do not appear anergic, as has been reported of B cells with similar phenotype described in RA or Sjögren's Syndrome[10,12], but rather responded maximally to activated T cells. Others have reported that a single stimulus was not sufficient to induce activation of memory B cells with a similar phenotype to CD11c$^{hi}$ B cells, but rather require several activation signals to induce proliferation and differentiation[57], which would be delivered by activated T cells in our system.

In human B cells, CD27 is a differentiation antigen induced after activation of naive B cells that continues to upregulate as memory B cells differentiate into plasma cells. Interaction of CD27 with T cell-expressed CD70 regulates B cell activation, plasma cell differentiation and Ig production[2,3,65]. However, CD70 expressed on activated T cells can also downregulate CD27 expression on B cells[65]. In this regard, T cells isolated from lupus patients over-express CD70, which correlates with disease activity[66]. Taken together, these studies suggest that the CD11c$^{hi}$ memory B cells described here may have recently encountered CD70 expressed on activated T cells, resulting in downregulation of CD27.

In summary, we show that a population of CD11c$^{hi}$ T-bet$^{+}$ B cells is significantly expanded in SLE that may have the capacity to differentiate into autoreactive plasma cells upon encounter with activated T cells. Given their unique phenotype, these cells have the potential to be targeted by biologics, which may prove efficacious in autoimmune diseases where autoantibodies are believed to have a pathogenic role.

## Methods

**Cells from healthy donors**. Human whole blood was collected after informed written consent from healthy volunteers recruited by the MedImmune Blood Donor program. Healthy control donors consisted of healthy MedImmune or AstraZeneca employees, who were anonymously enroled in the MedImmune Research Specimen Collection Program. Donors with HIV infection, hepatitis B or C virus, Human T-lymphotropic virus, or syphilis were excluded. Informed written consent for blood draws was obtained from the donor. Peripheral blood mononuclear cells (PBMCs) were isolated from CPT tubes (BD Biosciences) after centrifugation. Naive B cells were negatively selected using MACS cell separation kit #130-091-150 (Miltenyi Biotec), which routinely yielded greater than 85% purity. The degree of purity and initial cell phenotype were determined by flow cytometry for all experiments on day 0.

**B cells from autoimmune donors**. Lupus and RA blood samples were obtained from the Warren G. Magnuson Clinical Center Blood Bank (Bethesda, MD) as approved by the National Institute of Arthritis and Musculoskeletal and Skin Diseases/National Institute of Health and isolated as described above. The demographics and clinical characteristics of these donors are shown in Supplementary

Table 1 (SLE) and Supplementary Table 2 (RA). Naive B cells from SLE were sorted as $CD19^+CD11c^-CD27^-$ and post sort purity was greater than 90%.

**B cells from spleen and tonsil**. Tonsil (four males, 4, 18, 23 and 37 years old and three females, 19, 22 and 30 years old) and spleen (female, 64 years old, male, 80 and 82 years old) tissue was acquired from the National Disease Research Interchange (Philadelphia, PA). Tissues were cut and mashed gently to remove all lymphocytes. Tonsil cells were lysed with ammonium chloride and washed with RPMI 10% FBS. Splenocytes were isolated by ficoll separation and washed with RPMI 10% FBS. B cells from tonsil and spleen were visualised by flow cytometry as described below.

**Culture conditions of purified SLE B cells**. Sort purified SLE naive peripheral blood B cells were cultured at a density of $0.5–1 \times 10^5$ B cells per well and healthy donor naive peripheral blood B cells were cultured at a density of $1.5 \times 10^5$ B cells per well in 96-well round-bottom plates in a final volume of 200 µl complete medium. Culture medium for all experiments was RPMI 1640 (Invitrogen) supplemented with 10% FCS, penicillin-streptomycin (100 units/ml penicillin, 100 mg/ml streptomycin), 2-mercaptoethanol (55 mM), L-glutamine (2 mM), and HEPES (5 mM). At initiation of culture, B cells were stimulated with a combination of IL-21 (40 ng/ml, MedImmune, LLC), anti-CD40 (0.1 µg/ml, goat IgG, R&D Systems), and anti-IgM $F(ab')_2$ (5.0 µg/ml, Jackson ImmunoResearch Laboratories). The concentration of antibodies used in these experiments was determined based on maximal inhibition achieved in extensive titration studies. B cells were cultured for 5 days prior to examination. The healthy donor naive B cells were also stimulated with CpG-B (1 µg/ml, Invivogen). In order to calculate the total number of $CD11c^{hi}$ B cells present after 5 days of culture, the total number of events within the $CD11c^{hi}$ gate that was collected in 70 µl of FACS buffer on the BD LSR II flow cytometer (BD Biosciences) was back calculated to the total volume of 100 µl.

**Flow cytometry**. Freshly isolated lymphocytes of blood, spleen, tonsil or cultured B cells were stained in round-bottom plates for 30 min at 4 °C in a total volume of 100 µl. All fluorochrome-conjugated mouse anti-human mAbs and quantity used for flow cytometry are shown in Supplementary Table 7. Mouse anti-human PE-Cy7-anti-IgG (clone G18-145, BD Biosciences) and APC-anti-IgA (clone IS11-8E10, Miltenyi Biotec) were used for IgG and IgA surface staining. For intracellular T-bet staining, cells were surface stained, fixed using eBio fix/perm kit (eBioscience#00-5523), washed and stained in 1 × eBio fix/perm buffer. Cells were analysed on a BD LSR II flow cytometer (BD Biosciences) using FACSDiva software. Mouse IgG1 kappa Isotype Control, PE-Cyanine7 (eBioscience#25-4714-42) was used as isotype control for T-bet staining. All conditions were collected for the same amount of time, thus the number of events displayed is reflective of the relative cell number.

**Autoantigen arrays**. For autoantibodies measure in sera: Healthy donor and lupus patient sera were screened for reactivity to a panel of 95 autoantigens (UT-Southwestern (UTSW) Microarray Core Facility, Dallas, TX) as described[67]. Array signal intensities were normalised and log2 transformation was performed to ensure normal distribution. Pearson correlation test was performed with percentage of $CD11c^{hi}$ cells and values of each autoantigen using R. FDR was calculated using the Benjamini–Hochberg procedure. Autoantibodies with significant correlation were defined as FDR ≤0.05. Heatmap was plotted using R.

For autoantibodies measure in cell supernatant: B cells were sorted from total of 13 SLE patients (for a total of six independent experiments; SLE 1-6) into $CD19^+CD11c^-CD27^-$ "naive" B cells, $CD19^+CD11c^-CD27^+$ "memory" B cells, or "$CD19^+CD11c^{hi}$" B cells and cultured as described below (B cell/T cell co-cultures). Some samples were pooled from multiple donors to achieve sufficient cell numbers for culture: sample SLE1, 2, and 5 were pooled from three donors; SLE3 and 4 were from individual donor; SLE6 was pooled from two donors. Supernatant was collected between 7 and 35 days of culture (day 7 for SLE1 and 4, day 11 for SLE 6, day 15 for SLE2, day 16 for SLE 3, day 35 for SLE5 (for SLE5, naive and CD11c only, no memory condition) and screened for reactivity to a panel of 95 autoantigens, using the UTSW platform as described above. Supernatant from sample SLE 5 and 6 were diluted in order to have sufficient volume for autoantigen arrays. For SLE 6, supernatant from all conditions was diluted by 50%; and for SLE 5, supernatant from all conditions was diluted by 25%. Signal intensities were normalised and log2 transformed, and group comparisons were performed using the R Limma package based on linear model and modified *T* test. Significance was defined as *p*-value <0.05.

**Cell sorting and determination of RTL**. B cells were enriched from PBMC from either individual healthy donors or two pooled healthy donors (to increase cell yield) with RosetteSep human B cell enrichment cocktail (StemCell Technologies#15064). The enriched B cells were stained as described above with 5 µl anti-CD19 BUV395, 1 µl anti-CD38 APC, 5 µl anti-CD11c BV421 (All from BD Biosciences) and 10 µl anti-CD27 BV785 (BioLegend) per $1 \times 10^6$ cells. Using a FACS Aria Fusion (BD Biosciences), $CD19^+CD11c^-$ B cells were sorted as naive ($CD27^-CD38^+$), memory ($CD27^+CD38^-/CD38^+$), or plasma cells ($CD27^{hi}CD38^{hi}$). $CD19^+CD11c^+$ B cells were sorted as $CD27^-CD38^-$ B cells. Post sort purity was greater than 90%; B cells from five independent donors were examined. In one of the sorts insufficient cells were obtained from $CD11c^+$ B cells and plasma cells to extract data. The RTL of sorted B cell populations were determined using Telomere PNA Kit/FITC for flow cytometry (Dako #K5327), per manufacturer's instructions.

**Sorting and culture of B cell/T cell co-cultures**. Peripheral blood B cells were enriched with RosetteSep human B cell enrichment cocktail (StemCell Technologies#15064). The enriched B cells were stained as described above. Using a FACS Aria Fusion (BD Biosciences), B cells were sorted as: $CD19^+CD11c^-CD27^-$ B cells naive B cells, $CD19^+CD11c^-CD27^+$ B cells memory B cells or $CD19^+CD11c^{hi}$ B cells. Some experiments used pooled sorted B cells from multiple donors to achieve sufficient cell numbers for culture. T cell/sorted B cells were cultured as previously described[34]. Briefly, $CD4^+$ T cells were mitomycin-C treated (30 µg/ml, Sigma Aldrich) for 30 min at 37 °C then washed and rested in complete media at 37 °C for an additional 30 min. $1.0 \times 10^5$ mitomycin-C treated $CD4^+$ T cells were cultured with $0.2–0.5 \times 10^5$ purified B cells (per 96 well) and co-cultured in a final volume of 200 µl. T cells were stimulated with T cell Activation/Expansion kit (Miltenyi Biotec#130-091-441) in a 2:1 T cell to bead ratio. Culture medium for these experiments was RPMI 1640 (Invitrogen) supplemented with 10% FCS, penicillin–streptomycin (100 units/ml penicillin, 100 µg/ml streptomycin), 2-mercaptoethanol (55 µM), L-glutamine (2 mM), and HEPES (5 mM). After 7 or 11 days of culture, cells were stained by flow cytometry as described above for plasma cell phenotype and IgG in the supernatant was determined as described below. Several experiments were set up in duplicate to allow for analysis of secreted autoantibodies at later culture time points.

**Immunoglobulin production**. Secreted Ig was quantified by ELISA after stimulation of B cells for the indicated number of days. Ninety six-well flat-bottom plates were coated overnight at 4 °C with either 5 µg/ml of goat anti-human IgM or goat anti-human IgG diluted in PBS. Plates were washed and blocked with 0.2% BSA in PBS. Supernatants were diluted and incubated in plates overnight. Bound Ig was detected with goat anti-human IgG-alkaline phosphatase (0.2 µg/ml, Bethyl Laboratories) diluted in blocking buffer. Plates were developed with SigmaFast p-Nitrophenyl phosphate Tablets (Sigma Aldrich), and specific absorbance was measured at 405 nm using a SpectraMax microplate reader (Molecular Devices).

**ELISpot assay for detection of IgG-secreting plasma cells**. ELISpot was performed according to manufactures instructions (Human IgG ELISpotBASIC HRP, Mabtech). Briefly, PVDF ELISpot plates (MAIPS4510, Millipore) were Ethanol treated and coated with capture antibody (MT91/145, Mabtech) at a concentration of 15 µg/ml in PBS and incubated overnight at 4 °C. Total PBMCs or sorted $CD11c^{hi}$ B cells ($CD19^+CD11c^{hi}$) or plasma cells ($CD19^+CD27^{++}CD38^{++}$) from SLE donors ($n = 4$) were added to wells at indicated concentrations in complete media and incubated for 16 h at 37 °C. The plates were then washed and incubated with detection mAb (MT78/145, Mabtech) at a concentration of 1 µg/ml for 2 h at RT, followed by streptavidin-horseradish peroxidase conjugate for 1 h at RT and developed with precipitating TMB substrate (Mabtech) for 15 min. The images were captured using an Immunospot reader (Cellular Technology Limited).

**RNAseq and data analysis**. Peripheral blood B cells from SLE patients ($n = 4$ independent samples) were isolated using human B cell enrichment kit (StemCell Technologies, catalogue #19054) and stained as described above. Using a FACS Aria Fusion (BD Biosciences), B cells from SLE patients were sorted as: $CD19^+CD11c^-CD27^-IgD^+$ naive B cells, $CD19^+CD11c^-CD27^+IgD^-$ memory B cells, $CD19^+CD11c^{hi}IgD^+$ B cells or $CD19^+CD11c^{hi}IgD^-$ B cells. B cells from healthy donor ($n = 4$ independent samples) or RA patients ($n = 4$ independent samples) were sorted as: $CD19^+CD11c^-CD38^{inte}IgD^+$ B naive B cells ($n = 3$ used for analysis), $CD19^+CD11c^-CD38^{inte}IgD^-$ B cells memory B cells, $CD19^+CD11c^-CD38^-IgD^-$ memory B cells or $CD19^+CD11c^{hi}CD38^-IgD^-$ B cells. The purity of the sorted populations was routinely >90%.

RNA was isolated using PicoPure RNA isolation kit (Thermo Fisher Scientific, catalogue#: KIT0204). RNAseq was performed at MedImmune Deep Sequencing and Microarray Core. RNA sequencing data (RNASeq) data was generated using the Illumina standard library preparation and sequencing protocols as described[68]. In brief, mRNA-seq libraries were generated using the TruSeq RNA Sample Preparation kit (Illumina, catalogue #RS-122-2001) and sequenced on the HiSeq 2000 platform according to the manufacturer's recommendations. Paired end FASTQ files of 90 mer sequence reads were generated. For RNASeq data, the average read count per mate was 50 million. Quality of the RNAseq data, such as the overall sequencing score, over-represented reads, kmer presence, was evaluated using the FastQC package[68].

For sorted SLE B cell subsets, sequencing reads were aligned to human reference genome hg19 using Hisat2 (v2.0.2)[69]. Default parameters were used. Raw counts were generated using HTseq[70]. Data was normalised and CPM values for each gene were generated using the Deseq2 package. Group comparisons were performed in Deseq2 using generalised linear model assuming negative binomial distributions[71]. False discovery rate (FDR) was generated with Benjamini and Hochberg correction. Genes with significant expression change was defined as FC ≥2 and FDR ≤0.05. For sorted healthy donor or RA B cell subsets, STAR 2.5.2a was used to map reads to

human genome (HG19). The count data was normalised using rlog implemented in Deseq2. Pooled *t* test was used for group comparison. Significant change was defined as FC >2 and *p*-value <0.05. Pathway analysis was performed using GSEA[72]. Pathway enrichment FDR was generated from *p*-values with Benjamini and Hochberg correction. Other graphing and statistics were performed using R.

**Immuno-histochemical staining of kidney sections**. Formalin-fixed paraffin embedded blocks of Kidney needle biopsies form LN patients were purchased from either Conversant Bio or Tissue solutions ($n = 11$, 2 Class II, 3 Class III, 6 class IV). Five-micrometre sections were cut at MedImmune Pathology department and Florescent staining performed on fully automated Ventena autostainer. Slides were loaded onto Ventena, and then went through rehydration and $H_20_2$ block then antigen retrieval using Cell Conditioning 1. The slides were stained with mouse anti-CD20 clone L26 (Ventena), followed by HRP labelled secondary mouse Omnimap (Ventena) then detected by FITC (Ventena). Slides went through heat denaturation to neutralise any unbound HRP and stripped of the primary Ab using Tris EDTA at 120 degrees for 28 min. The slides then through a 2nd incubation with Rabbit anti-CD11c clone EP134Y (Abcam) followed by HRP labelled Rabbit HQ secondary (Ventena), then detected with Rhodamine (Ventena). Nuclei were stained with DAPI (Molecular probes) and slides mounted using prolong media (Life technologies). Images acquisition using Leica SP5 inverted confocal scope using a 40× oil objective. To quantify infiltration of $CD20^+CD11c^+$ B cells in kidney biopsies, between 1–6 200X section per biopsy was examined. Sections were considered to contain B cell infiltrates if more than 10 $DAPI^+CD20^+$ cells were noted, where the 'B cell-positive' sections contained between 13–118 B cells (mean of 48 B cells per section). The sections that were considered 'B cell-negative', contained between 0 and 8 B cells. These sections were then enumerated for the number of cells positive for DAPI, CD20 and CD11c. If more than one section per biopsy was examined, the mean number of $CD11c^+CD11c^+$ cells/section was used for quantification.

**Statistical analysis**. Group comparison was performed using unpaired *t*-test and Welch's correction was applied as equal variance was not necessarily satisfied (Figs. 3c, 4g, 4h, 8b, 8c, Supplementary Figs. 2b, 3a, 3b, 4d, 10a). Mann–Whitney *U*-test was applied when the values were not normally distributed (Fig. 1b–d, Supplementary Fig. 1b). Correlation was performed using Pearson correlation test (Supplementary Figs. 1c, 4e, 10b). Significance was noted by *p* value: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$, $****p < 0.0001$. The above tests were performed using GraphPad Prism (GraphPad software). Statistical analysis for autoantigen arrays and RNAseq was described above in "Autoantigen arrays" and "RNAseq and data analysis".

**Study approval**. For healthy donors of MedImmune employees, all protocols and informed consent forms were approved by Chesapeake Institutional Review Board (Protocol 2010-001 version 4.0). For lupus and RA donors, the studies were approved by the Institutional Review Board of the National Institute of Arthritis and Musculoskeletal and Skin Diseases (protocol 94-AR-0066, and 00-AR-0222, respectively).

**Data availability**. All relevant data are available from the corresponding author on request. The RNAseq data have been deposited in Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE110999.

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## Author contributions

S.W. and V.K. conducted and analysed the majority of experiments. J.W. analysed and interpreted the autoantibody array data and RNAseq data and performed statistical tests for the majority of the experiments. J.L.K. performed the majority of the RA studies as well as supported and interpreted several experiments. B.N. and P.G. performed, supported and interpreted experiments. S.R. orchestrated clinical lupus sample handling, flow cytometry, acquisition and laboratory data under the guidance of M.S. Autoimmunity Molecular Medicine Team ran flow cytometry experiments for over 300 lupus and healthy donor samples under the guidance of M.S. R.G.M., Z.M., S.H., and R.S. provided the clinical and lab data, including disease activity indices, analysis and interpretation of results. N.H., C.M. and H.L. performed the RNA seq under the guidance of K.S. K.Z. and R.H. performed and analysed the IHC studies. M.P.C. supported experiments assessing T-bet expression and contributed to the writing of the manuscript. R.E. under the guidance of R.K. conceived, designed, and supervised the project. R.E. wrote the manuscript with input from all authors.

## Additional information

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**Competing interests:** S.W., J.W., V.K., B.N., S.R., K.Z., R.H., C.M., H.L., N.H., Autoimmunity Molecular Medicine Team, K.S. and R.K. are full-time employees and shareholders of MedImmune/AstraZeneca. R.E. and J.L.K. were employees at MedImmune and owners of AstraZeneca stock. They are currently employees at and shareholder at Viela Bio. The remaining authors declare no competing interests.

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