

Early generated B1 B cells with restricted BCRs become chronic lymphocytic leukemia with continued c-Myc and low Bmf expression

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In mice, generation of autoreactive CD5⁺ B cells occurs as a consequence of BCR signaling induced by (self)-ligand exposure from fetal/neonatal B-1 B cell development. A fraction of these cells self-renew and persist as a minor B1 B cell subset throughout life. Here, we show that transfer of early generated B1 B cells from E μ -TCL1 transgenic mice resulted in chronic lymphocytic leukemia (CLL) with a biased repertoire, including stereotyped BCRs. Thus, B1 B cells bearing restricted BCRs can become CLL during aging. Increased anti-thymocyte/Thy-1 autoreactive (ATA) BCR cells in the B1 B cell subset by transgenic expression yielded spontaneous ATA B-CLL/lymphoma incidence, enhanced by TCL1 transgenesis. In contrast, ATA B-CLL did not develop from other B cell subsets, even when the identical ATA BCR was expressed on a Thy-1 low/null background. Thus, both a specific BCR and B1 B cell context were important for CLL progression. Neonatal B1 B cells and their CLL progeny in aged mice continued to express moderately up-regulated c-Myc and down-regulated proapoptotic Bmf, unlike most mature B cells in the adult. Thus, there is a genetic predisposition inherent in B-1 development generating restricted BCRs and self-renewal capacity, with both features contributing to potential for progression to CLL.

INTRODUCTION

In humans, B cell chronic lymphocytic leukemia (CLL) with CD5⁺ phenotype is a common form of adult leukemia with an incidence that increases with advancing age. A critical role of the BCR in development of CLL has been recognized by the presence of recurrent (stereotyped) BCRs, often with similar or identical Ig heavy chain third complementarity determining regions (HCDR3; Chiorazzi and Ferrarini, 2003; Stamatoopoulos et al., 2007). BCR signaling is able to induce expression of CD5 (Wortis et al., 1995). About half of CLL patients express an unmutated IgVH, which is often a marker of cases with a poorer prognosis than cases with a mutated IgVH (Hamblin et al., 1999), and unmutated CLL BCRs have been shown to be autoreactive and polyreactive (Hervé et al., 2005). These findings led to a proposal of multistep leukemogenesis: first, the generation of autoantigen-experienced B cells; second, their persistence and proliferation resulting from cross-reactivity with pathogens; and third, events leading to transformation and progression to CLL without BCR muta-

tion, as in cases with a more aggressive course (Chiorazzi and Ferrarini, 2011). However, it has long been debated how such autoreactive B cells with restricted BCRs are generated. Furthermore, recent data demonstrated that BCRs in CLLs often exhibit the capacity for autonomous signaling in the absence of an extracellular ligand, a feature not found in BCRs associated with other types of B cell lymphomas (Dühren-von Minden et al., 2012). This prompted the additional question of whether a stereotyped BCR plays a major role in B cell maintenance and/or transformation, independent of B cell context, once it is expressed.

In normal mice, generation of autoreactive mature CD5⁺ B cells, termed B1a, occurs as a positive outcome of fetal/neonatal B-1 B cell development from Lin28b⁺Let-7⁻ B-lineage precursors. In contrast, Lin28b⁻Let-7⁺ B lineage precursors become predominant in adult B-2 B cell development, and mature CD5⁺ B cell generation declined (Hardy and Hayakawa, 2001; Yuan et al., 2012; Zhou et al., 2015). Because some B-1-derived B cells self-renew and are maintained throughout life as a minor B cell subset (Hayakawa et al., 1986) termed B1 B cells (also called B-1 B cells), this prompted the question of whether early generated CD5⁺ B cells can become CLL in aged mice. In most WT mouse strains, development of CLL is rare. However, aggressive

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Abbreviations used: ATA, anti-thymocyte/Thy-1 autoreactive; ATA μ Tg, V_H3609 μ transgene; ATA μ KTg, V_H3609 μ /V_K21 κ transgene; AGcA, anti-intestinal goblet cell autoreactive; CLL, chronic lymphocytic leukemia; FO B, follicular B cell; GC, germinal center; HCDR3, heavy chain third complementarity determining region; HSC, hematopoietic stem cell; MZ B, marginal zone B cell; pB1a, peritoneal B1a; PBL, peripheral blood lymphocyte; PerC, peritoneal cavity; sB1a, splenic B1a; SLC, surrogate light chain; TCL1, T cell leukemia 1.

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CLLs in humans have higher levels of the T cell leukemia 1 (TCL1) oncogene, and transgenic expression of human TCL1 targeted to mouse B lineage cells ($E\mu$ -hTCL1 Tg) leads to a high incidence of $CD5^+$ CLLs during aging with biased utilization of unmutated BCRs (Bichi et al., 2002; Yan et al., 2006). One stereotyped BCR in mouse $TCL1^+$ CLL has an anti-nonmuscle myosin IIA autoreactivity, a feature also common to some human CLLs. Generation of mouse models with this autoreactive BCR by Ig transgenesis provided evidence that this particular BCR is restricted to the outcome of B-1 B cell development. Early generated B1 B cells with this BCR can develop CLL with aging, even without the TCL1 Tg, confirming that progression to CLL can occur from B-1-derived B1 B cells (Hayakawa et al., 2016). This Ig transgenic mouse model also demonstrated the importance of BCR structure, as not all early generated $CD5^+$ B1 B cells with a similar BCR could become CLL; there was a requirement for particular CDR3s in the V/D/J and V/J junctions (Hayakawa et al., 2016).

Here, we show that B1 B cells also generate CLLs with other stereotyped BCRs commonly found in mouse CLL, and that progression to CLL by B1 B cells is not only a result of their ability to express specific BCRs. The proto-oncogene *c-Myc* (*Myc*), deregulated in most human cancers, is one of the critical transcription factors regulating normal cell proliferation, growth, and also apoptosis in development and cell maintenance (Pelengaris et al., 2002; Nilsson and Cleveland, 2003; Delgado and León, 2010). *Myc* is broadly expressed during embryogenesis, regulating hematopoiesis. In the adult, *Myc* expression is maintained by normal dividing cells at a relatively consistent moderate level (Pelengaris et al., 2002; Delgado and León, 2010). In cellular processes, *Myc* also plays a role in apoptosis, in that it targets the Bcl-2 network, suppressing the prosurvival Bcl-2 and Bcl-X_L proteins (Nilsson and Cleveland, 2003). Bcl-2 family proteins are fundamental regulators of apoptosis, thus, the balance of anti- and proapoptotic proteins within the Bcl-2 family is important in determining cell survival.

Bim is a proapoptotic Bcl-2 family BH3-only protein expressed in a variety of cell types that is well known for playing various roles, including controlling the generation of autoreactive B and T cells during lymphocyte development (Bouillet et al., 1999, 2002; Enders et al., 2003). Bmf is also a BH3-only protein that was originally found as a regulator of anoikis in epithelial cells, similar to Bim (Puthalakath et al., 2001). Bmf is also expressed by hematopoietic cells, including B lymphocytes, where it controls the generation of autoreactive B cells (Labi et al., 2008). Both genes coding for Bim and Bmf proteins are located on chromosome 2 in mice, and the proteins share the ability to bind to cytoskeletal components through dynein light chain (DLC), sequestering Bim to the DLC1-microtubule and Bmf to the DLC2-actin-bound myosin V (Piñon et al., 2008). In response to certain cellular stress stimuli, Bim and/or Bmf are released from the cytoskeleton, localize to mitochondria, and neutralize prosurvival Bcl-2

family members to elicit apoptosis (Labi et al., 2008; Piñon et al., 2008; Grespi et al., 2010). Thus, reduction of Bim and/or Bmf can reduce apoptosis, promoting survival (Piñon et al., 2008; Labi et al., 2014). Although Bim continues to be expressed by immature through mature stage B cells, including by B1 B cells, we show that in B-1-derived B1 B cells, *Myc* is up-regulated and Bmf is down-regulated, in contrast to the majority of mature B cells generated from B-2 development. Thus, together with the ability to generate restricted autoreactive BCRs, the balance between proliferation and cell death in B1 B cells may confer a predisposition for CLL progression.

RESULTS

T cell-independent CLL generation in $E\mu$ -hTCL1 Tg mice

One potential feature in the evolution of CLL was T cell dependence, and germinal center (GC) generation through T cell CD40L-CD40 signaling was considered one possible mechanism (Zenz et al., 2010). To test this possibility, $E\mu$ -hTCL1 (TC) transgenic mice were crossed with $CD40^{-/-}$ mice. As shown in Fig. 1 A, PBL analysis of TC^+ $CD40^{+/-}$ and $CD40^{-/-}$ littermates at 6–7 mo of age, and CLL incidence comparison in Fig. 1 B, there was no strong reduction of $CD5^+B220^{lo}$ CLL incidence on a CD40-deficient background. Moreover, development of CLL also occurred in TC^+ mice lacking mature T cells as a result of $CD3\gamma\delta$ deficiency (Fig. 1 A). Thus, the high incidence of CLL generation in TC^+ mice is T cell independent, CD40 signal independent, and does not require GC formation.

Early generated B-1-derived B1 B cells progress to CLL in aged mice

Induction of CD5 expression in B cells, yielding B1a cells, can occur as a consequence of BCR signaling, without requiring either CD40 signaling or T cell help (Wortis et al., 1995). To assess whether B1a cells in TC^+ mice become CLL, we performed adoptive transfer experiments by cotransferring peritoneal B1a cells (pB1a), splenic B1a cells (sB1a), or splenic non-B1a B cells from TC^+ mice, together with BM cells from TC^- mice that differ in IgM allotype from the TC^+ mice. As shown in Fig. 2 A, 2-mo-old TC^+ mice had a small number of B1a cells ($CD5^+B220^{lo}$) in both the peritoneal cavity (PerC) and spleen that readily developed into CLL. In contrast, transferred cells derived from the dominant population of $CD5^-$ B cells ($CD5^-B220^{hi}$) did not develop tumors. For comparison, transfer of BM hematopoietic stem cells (HSCs) purified from the same TC^+ mice used for the B1a transfer resulted in generation of a normal $CD5^-$ B cell population, but most recipients did not develop CLL within 12 mo after transfer (Fig. 2 A, top). Only 2/11 cases developed CLL after transfer of BM HSCs from 2–3-mo-old TC^+ mice. Because the increase in B1a cells in PBL often began at 4–6 mo of age in TC^+ mice, some started to develop CLL by 6–8 mo, and most had developed CLL at 12 mo (Fig. 1 B), it appears that a loss of preexisting B1a cells by BM transfer alone resulted in a reduced incidence of CLL.

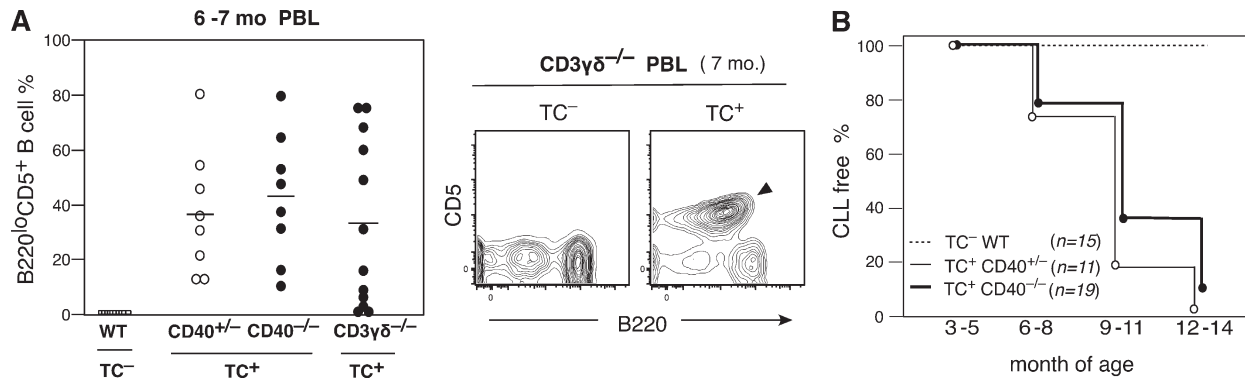


Figure 1. **T cell-independent CLL in E μ -TCL1 Tg mice.** (A) Increase in CD5⁺B220^{lo} B cell frequency in PBL of both CD40^{+/-} and CD40^{-/-} TC^{+/-} littermates (C.B17 background) and CD3 γ δ ^{-/-} TC^{+/-} mice (C.B17 and C57BL/6 mixed background), compared with WT (C.B17), at 6–7 mo. Mean is indicated by a bar. (right) B220^{lo}CD5⁺ B CLL in a 7-mo-old TC^{+/-}CD3 γ δ ^{-/-} mouse (marked) compared with a TC^{-/-} littermate. (B) Incidence of CLL over time. No strong reduction of CLL incidence occurred in CD40^{-/-} background.

To further assess the capacity of early generated B1 B cells to become CLL, B1a cells in spleen/PerC, or a mixture of B1a and B1b (CD5⁻ B220^{lo} B) cells in PerC, were purified from young (≤ 2 mo of age) TC⁺ mice, as B1 B; these cells were then cotransferred with TC⁻ BM cells, and CLL development was monitored by analyses of PBL (Fig. 2 B). As shown in Fig. 2 (C–E) and summarized in Fig. 2 F, CLL generation occurred, including from day 10 neonatal B1a. In day 10 neonates, the majority of mature AA4 (CD93⁻ CD5⁺B220^{lo} B1a cells were products of fetal/neonatal B-1 development (Hayakawa et al., 1985; Hardy and Hayakawa, 2001). Transfer of as few as 5×10^5 TC⁺ splenic IgM⁺ B1a cells from a day 10 neonatal TC⁺ littermate pool resulted in CD5⁺ CLL in aged recipients by 16 mo after transfer (Fig. 2 C). These B1a cell-derived neoplasms in aged mice became proliferative with a slight increase in cell size, as shown by scatter and retention of hTCL1 expression (Fig. 2 C). In most cases of CLL, in addition to predominance in PBL (Fig. 2, A and C), leukemias exhibited histological features of small lymphocytic lymphoma in spleens with parallels to human CLL (Morse et al., 2002; Fig. 2 D). Splenomegaly was common, presenting as aggressive CLL (Fig. 2 E). Transfer of B1 B cells from young TC⁺ mice consistently resulted in the development of CLL (Fig. 2 F). In addition, recipients of peritoneal B1 B cells (pB1) transferred without BM cells rapidly progressed to CLL as early as 2 mo (Fig. 2 F, right). Thus, early generated B1 B cells, predominantly with a B1a phenotype and progeny of B-1 development, can develop into CLL in aged mice.

CLLs of B1 B cell origin have stereotyped BCRs

Ig sequence analyses of 155 CLL samples from TC⁺ mice on a C.B17 background revealed biased V_H usage without mutation from the germline (Tables S2 and S3, HCDR3 list). Among these, stereotyped BCRs with identical V_H and V_K usage comprised >50% of cases (Table 1, five BCR set). The V_H gene nomenclature used here is based on Table S1, listed together with VBASE2 (Retter et al., 2005) names in

parenthesis (100% identity). These stereotyped BCRs were found in CLLs generated in the absence of CD40 signaling, confirming their T cell and GC independence. Furthermore, CLLs generated from cell transfers of B1 B cells of ≤ 2 -mo-old mice also exhibited stereotyped BCRs (Table 1 and Table S2). The finding that in set 5, the V_H11/V_K9 BCR was not detected was likely a result of the limited number of transfer CLL cases analyzed (total 20), as B cells with this BCR, with anti-bromelain-treated mouse RBC (BrMRBC)/phosphatidylcholine autoreactivity, are known to derive from early generated B1 B cells (Hardy et al., 1989; Wasserman et al., 1998). Set 3 BCR, with identical CDR3 and J segments, is an anti-nonmuscle myosin IIA autoantibody, previously confirmed to be restricted to B1 B cell, as B-1 B, by generation of BCR transgenic/knock-in mouse models (Hayakawa et al., 2016). Thus, B1 B cells develop into CLL with biased unmutated BCRs, including stereotyped BCRs.

Increased ATA B cell generation by B1 B cells yields a high incidence of MBL/CLL

Without the TCL1 transgene, CLL is a rare neoplasm in most WT mouse strains. However, increased frequency of certain germline (unmutated) autoreactive BCRs by transgenesis in B1 B cells leads to an increased incidence of CLL without the TCL1 Tg, on a C.B17 background. Set 3 V_HQ52/V_K9 BCR transgenesis was described previously (Hayakawa et al., 2016). V_H3609/V_K21 ATA (anti-thymocyte/Thy-1 autoreactivity) BCR transgenesis also led to a high incidence of CLL/lymphoma. In a V_H3609/D/J_H2- μ ^a Ig μ transgenic mouse line, ATA μ Tg, mature CD5⁺ ATA B cells, as B1a, are generated by B-1 development, dependent on the presence of a self-Thy-1 glycoform predominantly expressed by immature thymocytes in WT mice (Thy-1^{WT}; Gui et al., 1999; Hayakawa et al., 1999), as a positive selection. This is also true for V_H3609/D/J knock-in mice (unpublished data). This selection is independent of CD40 signaling (Fig. 3 A). In the ATA μ Tg mouse line, the frequency of ATA B cells in total

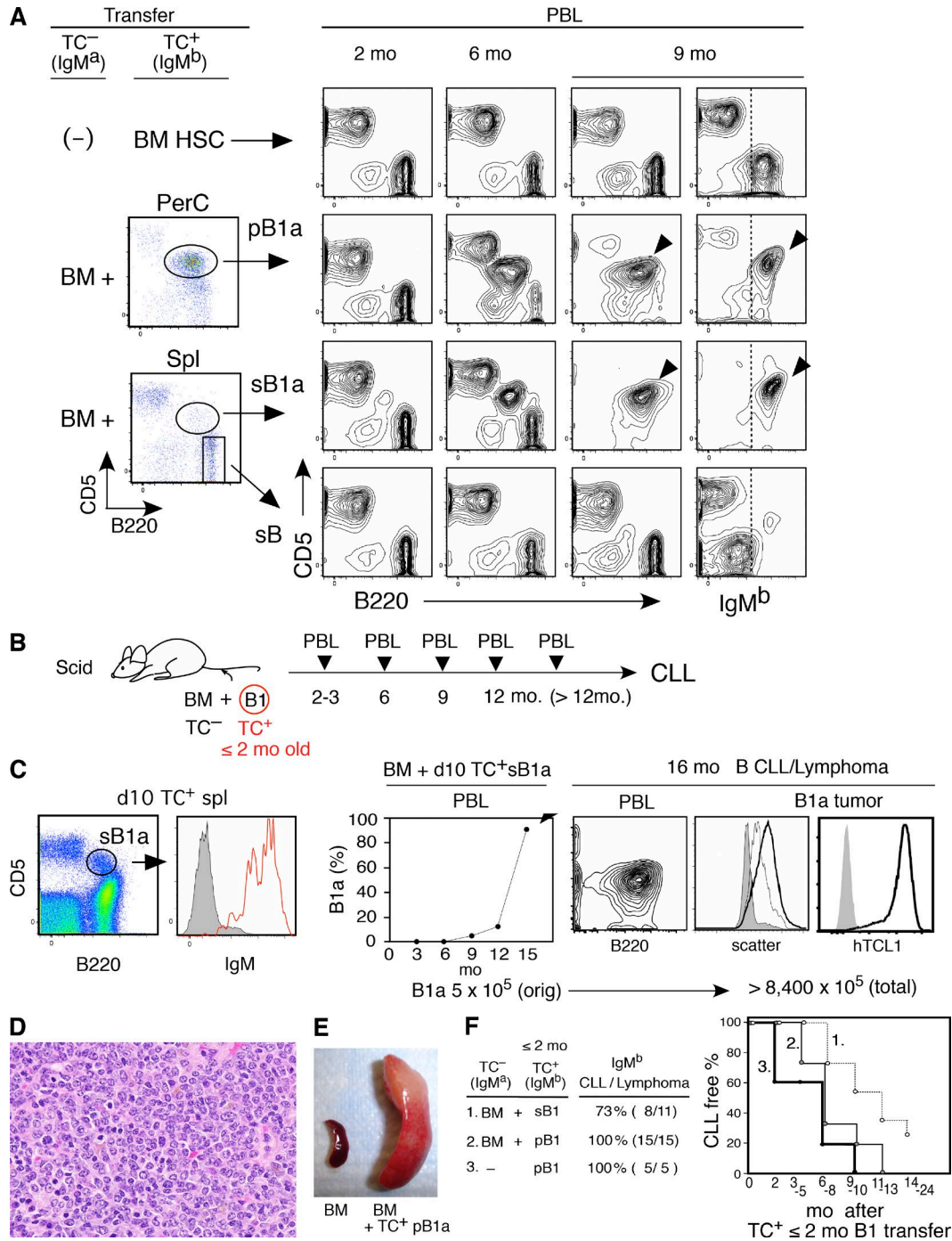


Figure 2. CLL development from early generated B-1-derived B cells. (A) Comparison of CLL incidence in C.B17.scid recipients transferred with BM HSC, B1a, and non-B1a B cells purified from 2 mo TC⁺ C.B17 mice. pB1a and sB1a (each 5 × 10⁵/recipient), and non-B1a spleen B cells (sB; 3 × 10⁶/recipient) were cotransferred with TC⁻ BALB/c BM. 2 mo after transfer, PBL showed similar numbers of B and T cells (top, using TC⁺ HSC alone were all IgM^b, others were predominantly IgM^a). Increase in B220^{lo}CD5⁺ B cells of IgM^b B1a and CLL development are marked. PerC, peritoneal cavity. Representative of six transfer cases using 2–3-mo-old mice. TC⁺ mice together with TC⁻ mice. (B) Cotransfer of purified B1 B cells from ≤2-mo-old TC⁺ C.B17 mice with TC⁻ BALB/c BM into C.B17.scid mice, monitoring PBL after transfer. (C) Transfer of B1a cells from 10-d-old TC^{-/-} mice and development of CLL. 10⁶ purified sB1a B cells (5 × 10⁵ TC⁺) from 12 10-d-old (TC^{+/-} × CB17)F₁ mice were transferred together with 5 × 10⁶ TC⁻ BM cells into CB.17. mice. (left) Surface IgM expression of sB1a cells. (right) Frequency of B1a cells in PBL of recipient after adoptive transfer (middle). Characteristic mouse CLL CD5⁺B220⁺ phenotype and lymphomatous spleen B cells (thick black line) compared with original transferred B1a cells (thin black line and CD5⁺B220⁺ B cells (gray). Expression of hTCL1 Tg in this CLL is visualized by cytoplasmic staining (gray, second step control). Total leukemia B cell number indicated as a sum of IgM^b B cells in PBL (2 × 10⁶), spleen (4.6

Table 1. Stereotyped BCRs in TCL1⁺ CLL in mice

CLL BCR set	Identical V _H /V _K BCR		TC ⁺	TC ⁺ CD40 ^{-/-}	≤2 mo B1 B cell origin
	IgH	IgL			
	V _H gene ^a	V _K gene ^b	n = 155	n = 18	n = 20
1	V _H 12-1 (V261)	V _K 4-91 (kf4)	40 (25.8 %)	4 (22.2%)	4
2	J558-64 (V332)	V _K 12-89 (fl12)	19 (12.3 %)	3 (16.6%)	2
3	Q52-1 (V222)	V _K 9-96 (ce9)	14 (9.0 %)	1 (5.6%)	2
4	J558-67 (V328)	V _K 1-117 (cr1)	10 (6.5 %)	1 (5.6%)	3
5	V _H 11-2 (V235)	V _K 9-128 (br9)	4 (2.6 %)	1 (5.6%)	nd

Presence of CLL stereotyped BCRs in TCL1⁺(TC⁺) CLL samples expressing identical V_H and V_K combination, including CD40 deficiency, and CLL developed by IgM⁺ B1 B cell transfer from TC⁺ neonatal/young mice (≤2 mo old). n, total sample number. nd, not detected.

^aV_H gene (see Table S1 and Vbase2 for parentheses).

^bV_K gene (Thiebe et al., 1999).

B cells often increased in PBL with age (detected by ATA idiotype antibody), with some becoming a monoclonal B cell lymphocytosis (MBL; >70% of total B cells; Fig. 3 B). A portion of these mice developed splenomegaly (unpublished data). Although most ATAμTg mice did not become leukemic without the TCL1 Tg, in the ATA Igμ/Igκ double-transgenic mouse line with dominance of ATA B cells among total B cells, total ATA B cell numbers increased in PBL, resulting in leukemia and splenomegaly as ATA B-CLL/lymphomas under the self-Thy-1 presence (TC⁻ Thy-1⁺ATAμκTg mice; 46%; 39/84; Fig. 3 C, left and middle). The presence of the TCL1 Tg further promoted the incidence of ATA B-CLL with splenomegaly (TC⁺ Thy-1⁺ATAμκTg mice; 77%; 46/60; Fig. 3 C, left, middle, and right). Thus, an increased frequency of B1 B cells expressing this ATA BCR by μ or κ transgenesis promoted the development of MBL/CLL.

Role of BCR together with B1 B cell origin, not BCR alone, in CLL generation

Using this strong TC⁺ ATAμκTg mouse model where ATA B cells increase, we next examined whether expression of the ATA BCR plays a major role in CLL development regardless of B cell context. As previously shown, and summarized in Fig. 4 (left; B-2 development), when ATAμκTg mice on a Thy-1 knockout background (Thy-1⁻) were crossed with *Lck*-Thy-1 transgenic mice expressing levels of Thy-1 different from the WT level, different B subsets were preferentially generated that all expressed the ATA BCR. Thy-1^{hi} (*Lck*-Thy-130), Thy-1^{lo} (*Lck*-Thy-10), and Thy-1⁻ (Thy-1⁻ background alone), respectively, generate arrested B cells (arr B), together with B1 B cells of B-1 origin, marginal zone B cells (MZ B), and follicular B cells (FO B; Fig. 4, top; Wen et al., 2005a). These ATAμκTg mice, including mice on a WT background (Thy-1^{WT}), were each crossed with TCL1 Tg mice. At

2 mo of age, the majority of ATA B cells in spleen of Thy-1^{hi} and Thy-1^{WT} mice are arrested at the T2 stage as an outcome of B-2 development, in contrast to the dominant population of mature ATA B1 B cells in PerC that derive from B-1. On a Thy-1^{lo} background, B cells are predominantly MZ B cells with a very few, nearly undetectable frequencies of B1a cells in both spleen and PerC. In Thy-1⁻ mice, ATA B cells become FO B cells in spleen, and the B1a cells present in PerC have BCRs from endogenous Ig rearrangements (IgM^b; Fig. 4, top right). These data from 2-mo-old adult mice were identical to those previously found with TC⁻ mice (Hayakawa et al., 2003; Wen et al., 2005a). Cells in which the V_H3609 IgH is associated with a different IgL, V_K19-17, become predominantly MZ B cells (CD21^{hi}CD24^{med}CD23^{lo/-}CD1d^{hi}CD5⁻), as well as FO B cells, with a different autoreactivity from ATA: an anti-intestinal goblet cell autoreactive (AGcA) BCR that primarily reacts with mucin 2 glycoprotein (Ichikawa et al., 2015; Fig. 4, bottom). These autoreactive AGcAμκTg mice were also crossed with TCL1 Tg mice as an example of BCRs with a different light chain, resulting in a B cell population dominated by MZ B (and FO B) cells, similar to the case with Thy-1^{lo} mice. All these B cell subsets expressed hTCL1 protein in the cytoplasm at similar levels (Fig. 4, right).

The CLL/lymphoma incidence data are summarized in Fig. 5 A. In mice with both Thy-1^{hi} and Thy-1^{WT} backgrounds, mature ATA B1 B cells are present and develop a high incidence of ATA B-CLL/lymphomas (Fig. 5 A, Gr. 1 and 2). In contrast, in mice with both Thy-1^{lo} and Thy-1⁻ backgrounds, CLL BCRs derived predominantly from B cells with endogenous rearrangements with a CD5⁺ B1a phenotype (Fig. 5 A, Gr. 3 and 4). A representative Thy-1⁻ case is shown in Fig. 5 B. To assess whether a lack of competitive B cells expressing endogenous BCRs leads to ATA B CLL generation, ATAμκTg Thy-1⁻ mice were further crossed with

× 10⁶), and peritoneal cavity (1.8 × 10⁶), as compared with the size of the initial TC⁺ inoculum. Representative of 15 cases with CLL/lymphoma generation by ≤2-mo-old mice. TC⁺ B1 B cell transfer. (D) H&E staining of spleen section with CLL/lymphoma of day 10 B1a cell origin. (E) Splenomegaly 8 mo after TC⁺ pB1a transferred together with TC⁻ BM cells, compared with a recipient of TC⁻ BM alone. (F) Summary of CLL/lymphoma incidence by purified B1 B cell transfer from ≤2-mo-old TC⁺ mice.

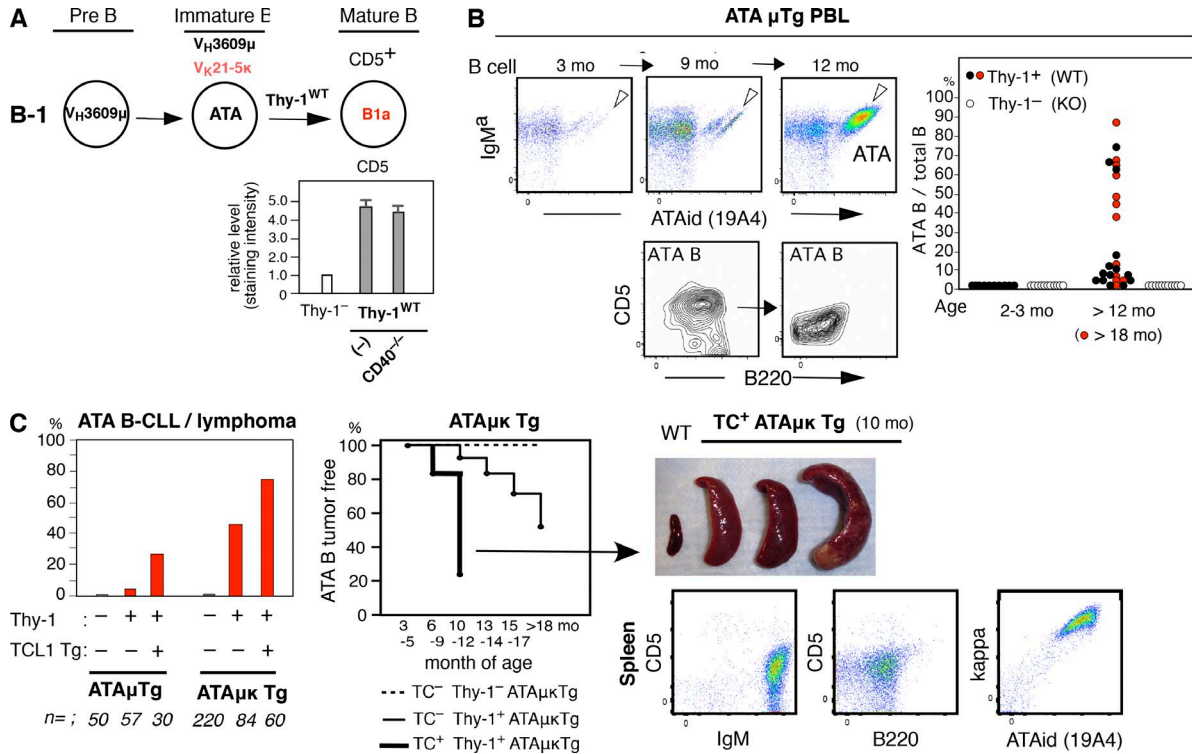


Figure 3. Increased incidence of MBL/CLL/lymphoma in B1 B cells with ATA BCR. (A) Self-Thy-1 antigen-dependent CD5 induction by ATA B cells through B-1 development does not involve CD40 signal. Comparative CD5 level by mature (AA4⁻) ATA B cells (ATAid⁺) in PerC in 2-mo-old ATA μ Tg mice in Thy-1^{-/-} versus WT (Thy-1^{+/+}) and CD40^{-/-} (*n* = 5; mean \pm SE). (B) Increased ATA B cell frequency in PBL B cells in aging ATA μ Tg mice. (left) ATA B increased case (marked) as MBL at 12 mo, with further B220 reduction. CD5 reduction also occurred in around half the cases of increased ATA B cells. (C) ATA B-CLL/lymphoma incidence in ATA μ Tg and ATA μ Tg mice with or without TCL1 Tg (left). Incidence over time by ATA μ Tg mice (middle). (right) Splenomegaly by TC⁺ATA μ Tg mice (>90% cases) together with CLL (top), and representative total spleen cell data analysis (bottom).

J_H knockout mice to maintain abundant ATA FO B cells into old animals. CLL B cells generated under this endogenous IgH⁻ condition were predominantly IgL edited B cells (Fig. 5, A, Gr. 5, and C), and still not ATA B cells. Thus, surface expression of the ATA BCR alone did not result in a heightened incidence of CLL. In addition to the absence of CLL development from ATA BCR-expressing MZ B and FO B subsets, AGcA BCR⁺ MZ B and FO B cells also did not develop into CLL (Fig. 5 A, Gr. 6).

Stereotyped BCRs (Table 1) were detected among endogenous B1a cell CLLs (Fig. 5 A and not depicted). Furthermore, sequencing of edited IgL in Gr. 5 and 6 revealed that the majority of such BCRs are associated with a typical B1a repertoire with V_H3609 IgH (Fig. 5 D). Among V_H3609 IgH-expressing B1a in ATA μ Tg mice, V_k21-5 (V_k21C) IgL⁺ cells normally predominate as ATA B cells, followed by V_k32 (gr32) IgL (Fig. 5 D, left). In ATA μ Tg mice under Thy-1⁻ J_H⁻ condition (Gr. 5), and in AGcA μ Tg mice on a WT self-Thy-1 background (Gr.6), edited IgLs were similar to the IgLs found in the B1a repertoire, including the ATA B CLLs found in AGcA μ Tg mice (Fig. 5 D, right). In young adult TC⁺AGcA μ Tg mice, 2 mo of age, the frequency of ATA B cells was very low; nevertheless, they were detected as B1a

(unpublished data). As summarized in Table 2, these results reveal the importance of B1 B cell context, and not just BCR alone, in promoting progression to CLL.

B1 B cells are the major source for ATA B-CLL

In adult ATA μ Tg mice, arrested B cells predominate in the splenic ATA B cell pool on Thy-1^{hi} and Thy-1^{WT} backgrounds. Thus, we asked whether these arrested B cells from B-2 development also became CLL. ATA B cells at the MBL/CLL stage often showed loss of CD5, in TC⁻ mice as well, as in several TC⁺ CLL cases (Fig. 6 A, bottom, shows a TC⁺ littermate case). TLR signaling for cell proliferation is one possible reason for reduced expression of CD5, as CD5 reduction occurs in mature ATA B1 B cells stimulated with TLR in vitro for 3 d (Fig. 6 B, top). However, IL-4 exposure together with BCR and CD40 signals also results in reduced CD5 expression by arrested ATA B cells (Fig. 6 B, bottom), similar to what was found in humans (Gagro et al., 2000). Because a fraction of mature T cells in spleen express the ATA B-reactive Thy-1 glycoform initially expressed by immature thymocytes (Hayakawa et al., 1990), interaction between ATA B and T cells may take place. Furthermore, T cell CD40L-CD40 signaling can augment survival of autoantigen-binding

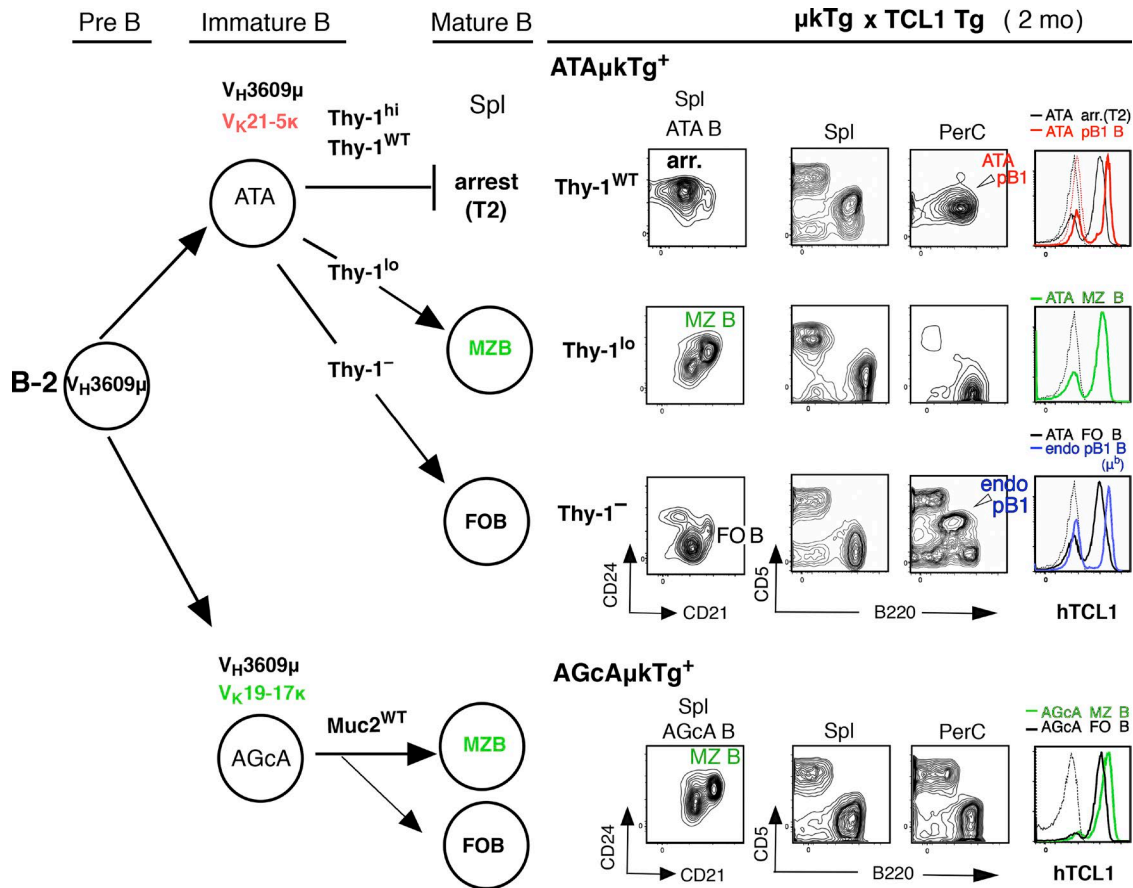


Figure 4. **hTCL1 expression by all B cell subsets.** (left) Summary of generation of different B cell subsets in spleen through B-2 development from BM; ATA B cell subset generation in ATA μ κTg mice by different levels of self-Thy-1 antigen exposure, and AGcA B cell subsets (dominantly autoreactive to mucin 2) in AGcA μ κTg mice, expressing different light chain from ATA BCR. (right) 2-mo-old TC⁺ ATA μ κTg (with different Thy-1 levels) and AGcA μ κTg mice. CD21/CD24 staining of ATA B or AGcA B cells in spleen (both constituting the dominant B cells among total B cells) to show arrested, MZ B, or FO B dominance, and B220/CD5 staining of total (lymphocyte) spleen and PerC. AA4 (CD93) and CD23 staining was also included to confirm each subset. Marked cell subsets for cytoplasmic hTCL1 staining analysis. CD21^{hi}CD24^{med}; MZ B, CD21^{med}CD24^{lo}; FO B. Thin dotted lines in hTCL1 staining are the data from TC⁻ μ κTg⁺ littermates as controls. Representative data of two to three samples each.

tolerant B cells (Lesley et al., 2006). These results suggested the possibility that CD40 signaling from interacting T cells may promote survival of arrested ATA B cells. However, CD40 deficiency in ATA μ κTg mice did not decrease ATA B-CLL incidence. ATA B cells increased in PBL of CD40^{-/-} mice (50%) similar to CD40^{+/+} mice (46%; Fig. 3 C), and CLL incidence was unaffected (Fig. 6 C). Thus, ATA B cell generation and progression to CLL was independent of CD40 signaling (Fig. 6 C).

To further assess this conclusion, we performed cotransfer experiments similar to those shown in Fig. 2, using 2-mo-old TC⁺ ATA μ κTg (IgM^a) mice, either BM, AA4⁺ (arrested) B cells in spleen, or peritoneal B1 B cells, together with C.B17 (IgM^b) BM (Fig. 6 D). Transfer of ATA pB1 cells of peritoneal origin showed consistent generation of CLL (5/5), often associated with loss of CD5 (Fig. 6 D right). In contrast, 3/5 cases of arrested ATA B transfer showed lack of CLL development, even after injection of threefold higher cell numbers

than pB1 from the same mice (Fig. 6 D, middle). Though 2/5 cases of arrested B transfer showed cell number increases at >12 mo after transfer, this result was not readily interpreted, as it was possible the CLL originated from a small contamination of mature ATA B1 B cells in spleen that increased in aged mice by self-renewal. As shown in Fig. 3 B, continued ATA B increase in circulating PBL occurs from young adult to older age in the same mouse, rather than as an acute increase in older mice, and CLL progression does not require CD40 signaling. These results prompted us to conclude that the major source for ATA B-CLL is B1 B cells and not arrested/tolerant ATA B cells.

Increased c-Myc and low Bmf in B1 B cells continues through the CLL/lymphoma stage

In human CLL, migration, survival, and proliferation of CLLs are regulated by the surrounding tissue microenvironment (Herishanu et al., 2011). In mice, after generation at the fetal/

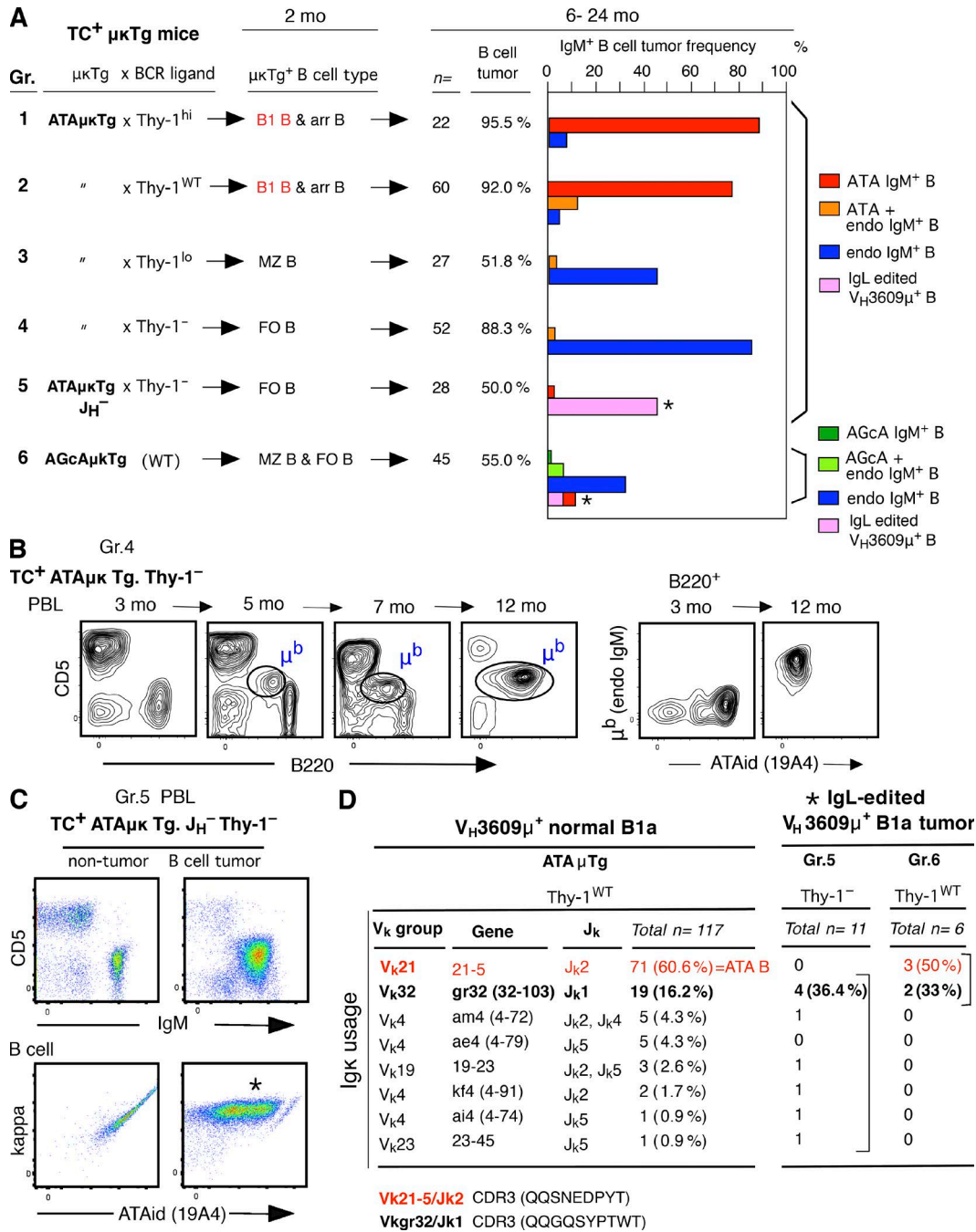


Figure 5. **CLL/lymphoma incidence predominantly arises from B1 B cells.** (A) Summary of IgM⁺ B cell tumor (CLL/lymphoma) incidence and frequency of tumor IgM with either original $\mu\kappa$ Tg BCR, endogenous IgM (IgM^b), or IgM^b coexpressed with original $\mu\kappa$ Tg BCR. In ATA $\mu\kappa$ Tg.J_H⁻Thy-1⁻, also in AGcA $\mu\kappa$ Tg mice, IgL edited B cell tumor occurred together with original V_H3609 μ IgH expression (*). (B) Representative case of endogenous IgM (IgM^b) increase as B220⁺CD5⁺ B cells in PBL of TC⁺ATA $\mu\kappa$ Tg.Thy-1⁻ mice (Gr. 4). As shown on right, original B220⁺CD5⁻ cells at 3 mo in PBL were circulating FO B cells with IgM⁺ ATA BCR, which did not develop B cell tumor. (C) TC⁺ATA $\mu\kappa$ Tg.J_H⁻Thy-1⁻ mouse PBL at the same 14 mo, without or with B cell tumor development. The edited IgL by this tumor B cells (*) was V_k19-23, together with V_H3609 μ IgH. (D) Edited IgL in V_H3609 μ ⁺ B tumors resemble normal mouse V_H3609 μ ⁺ B1a Igk. (left) Igk single-cell sequence data by V_H3609 μ ⁺ B cells (V_H3609id)*kappa⁺ in 2–3-mo-old ATA μ Tg mouse pB1a. Summary of data from four mice (107 shown of the total 117 κ listed). (right) Igk list of IgL-edited V_H3609 μ ⁺ B1a (CD5⁺) tumors. 9/11 of Gr5 and 5/6 of Gr 6 edited Igk were similar to normal mouse V_H3609 μ ⁺ B1a, including identical CDR3 use by V_k21/J_k2 and V_k32/J_k1, the most frequently expressed by V_H3609 μ ⁺ B1a (CD3 amino acids are shown).

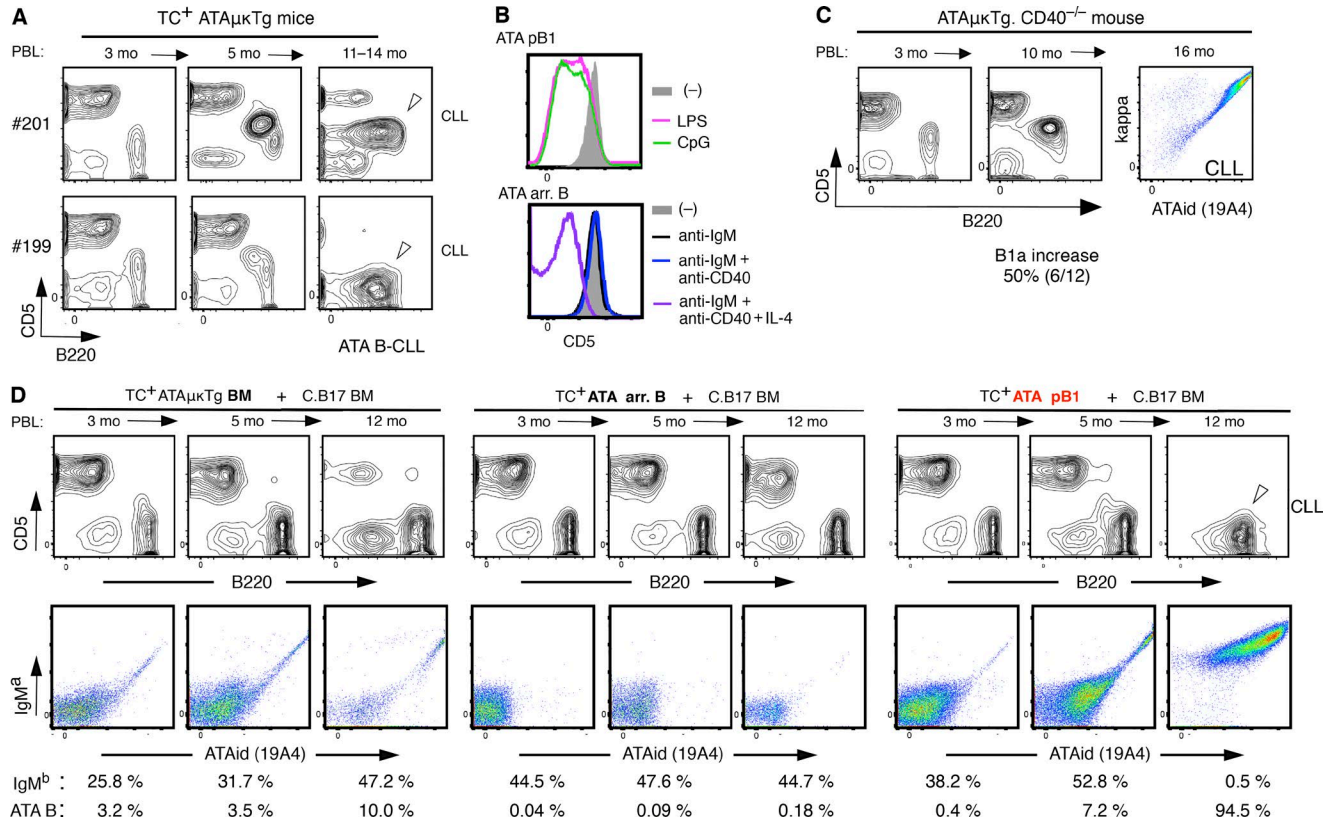


Figure 6. ATA B-CLL generation from mature B1 B cells than arrested B cells. (A) CD5 down-regulation occurrence at CLL stage in TC⁺ATAμκTg mice. CD5⁺ and CD5⁻ CLL (marked) found in two littermates, both with splenomegaly. CD5 down-regulation also occurred in TC⁻ ATA B cells as shown in Fig. 3 B. (B) 3 d after stimulation of ATA B cells in peritoneal cavity (pB1) and arrested B cells in spleen of ATAμκTg mice. Cell proliferation by LPS and CpG with CD5 down-regulation. Increased viability and proliferation by anti-IgM + anti-CD40 + IL-4 stimulation by arrested ATA B cells. Similar outcome by two experiments. (C) Data from one representative mouse with spontaneous CLL generation in ATAμκTg.CD40^{-/-} mice (6/12 cases). (D) Cotransfer of HSC enriched BM, arrested ATA B in spleen (arr. B), or ATA B in PerC (pB1) from TC⁺ATAμκTg (IgM^β) mice, together with C.B17 mouse BM. Percentage of C.B17-derived B cells (IgM^β) and ATA B in PBL are shown. ATA B-CLL generated by pB1 transfer is marked. Representative of five recipients each.

neonatal stage, B1 B cells migrate and circulate, and a fraction continues to self-renew, maintaining the B cell subset throughout life. As previously shown, early generated B1 B cells express the CXCR5 chemokine receptor, migrating to

CXCL13-expressing compartments to interact in the peritoneal cavity with CXCR13⁺ macrophages (Ansel et al., 2002), in the spleen to interact with follicular DCs (FDCs; Wen et al., 2005b). Stromal cell interactions may also be im-

Table 2. B1 B cell as a dominant origin of ATA μκTg⁺ B CLL/lymphoma

TC ⁺ μκTg mice	μκTg BCR ligand	μκTg ⁺ mature B cell	CLL/lymphoma in old aged μκTg B cell	CLL/lymphoma in old aged non-μκTg B cell
V _H 3609μ/V _L 21-5κ μκTg	WT	B1 B	++	+/-
V _H 3609μ/V _L 21-5κ μκTg	Thy-1 ^{hi}	B1 B	++	+/-
V _H 3609μ/V _L 21-5κ μκTg	Thy-1 ^{lo}	MZ B > FO B	-	+ B1a (endo IgM)
V _H 3609μ/V _L 21-5κ μκTg	Thy-1 ⁻	FO B	-	++ B1a (endo IgM)
V _H 3609μ/V _L 21-5κ μκTg.J _H ⁻	Thy-1 ⁻	FO B	-	+ B1a (IgL edited)
V _H 3609μ/V _L 19-17κ μκTg	WT	MZ B and FO B	-	+ B1a (endo IgM and IgL edited, including ATA B)

V_H3609μ/V_L21-5κ is ATA BCR, and V_H3609μ/V_L19-17κ is AGcA BCR, both with the same IgH expression. On a WT background in TCL1⁺ μκTg mice, ATA B cells become mature B1 B cells and AGcA B cells become mature MZ B (and FO B) cells. CLL/lymphoma developed predominantly from ATA B1 B cells, not AGcA B cells. ATA B cells expressed by MZ B or FO B cells under Thy-1^{lo} or Thy-1⁻ conditions in ATA μκTg mice did not become tumors in contrast with endogenous IgM-expressing CD5⁺ B cells (B1a). IgL editing was permissive for the generation of B1a cells with the ability to become CLL/lymphoma, including generation of V_L21-5κ ATA B-CLL in AGcA μκTg mice.

portant at the leukemic stage (Fig. S1). B1 B cells are cyclin D2 dependent (Solvason et al., 2000). *Ccnd2*, the gene encoding cyclin D2, together with *Cdk4*, are direct targets of *c-Myc* (*Myc*), thereby regulating the cell cycle (Pelengaris et al., 2002) with one distinctive feature of B1 B cells being moderately up-regulated *Myc* (Fig. 7 B). Various stimuli rapidly induce *Myc*, including BCR signaling; B1 B cells, with modestly increased cell size compared with FO B cells (Hayakawa et al., 1983), constitutively maintain up-regulated *Myc*, relative to FO B cells. All TC⁺ B CLL/lymphomas, including TC⁻ spontaneous ATA B CLL/lymphomas, express *Myc* mRNA at levels similar to or higher than early generated nonneoplastic B1 B cells (Fig. 7 B). *Myc* up-regulation in B1 B cells was also confirmed by elevated protein levels (Fig. 7, E and F). For B1 B cells at the preneoplastic stage, up-regulated *Myc* mRNA declines when the cells are cultured in medium with fetal calf serum alone in vitro (Fig. 7 C). This indicates the importance of continuous interaction by B1 B cells with the microenvironment.

In playing a role in homeostasis, *Myc* not only regulates the cell cycle, but apoptosis as well, targeting the Bcl-2 network (Nilsson and Cleveland, 2003). Thus, to understand the function of up-regulated *Myc* in B1 B cell self-renewal and sensitivity to CLL progression, Bcl-2 family genes were compared, initially by microarray analysis using nontumor pB1a and sB1a cells, in comparison to splenic FO B cells. The results showed no significant differences in expression of the majority of Bcl-2 network genes (antiapoptotic Bcl-2, Bcl-X_L, A1; proapoptotic Bax, Bak1, and Bok; and BH3-only proapoptotic Bad, Bid, Bim, Noxa, and Puma), except for slightly increased expression of antiapoptotic Mcl-1 in pB1a, and the consistently lowest level of BH3-only proapoptotic Bmf in both spleen and peritoneal cavity B1a cells. This led us to further analyze Bcl-2, Mcl-1, Bim, and Bmf, comparing mRNA levels between adult FO B cells and B1a cells during their initial generation in neonates (day 10–12), in adults (2 mo), and at the CLL/lymphoma stage, including tumor samples generated by B1 B cell transfer (Fig. 7 D, left). For ATA B cells, arrested B cells from adult mice were also included for comparison with B1 B cells (Fig. 7 D, right). As shown in Fig. 7 D, the major difference was up-regulated *Myc* together with down-regulated Bmf, consistently found in normal B1a cells, ATA B1 B cells, and in CLL/lymphoma samples, including the TC⁻ATA B cell/lymphomas. Bcl-2 levels were not up-regulated in normal B1a cells and CLL/lymphomas. Although Mcl-1 was slightly higher in pB1a- and also in B1 B-transferred cases, this was not consistently found in CLL/lymphomas. Arrested B cells showed a clear difference in Bmf mRNA levels from B1 B cells. BCR signaling is known to induce *Myc* and Bim (Craxton et al., 2005; Delgado and León, 2010), and expression of Bim and Bmf controls autoreactive B cell generation. Self-antigen-exposed-arrested ATA B cells, predominantly at the T2 stage, showed higher *Myc* and Bim mRNA, and Bmf mRNA was also slightly higher than for mature FO B cells in wild type mice. In contrast, Bmf mRNA was low in B1 B cells from the same ATA μ KTg mice.

As shown in Fig. 7 E, transcript levels of *Myc*, Mcl-1, and Bcl-2 were confirmed at the protein levels. For the BH3-only proteins, Bim and Bmf, transcriptional regulation, generation of multiple isoforms, and post-translational modifications occur (Piñon et al., 2008). Alternate splicing for Bim generates three Bim isoforms, and BimEL and BimL are widely expressed where BimEL plays a major role, including in B cells (Craxton et al., 2005). Normal pB1a cells in adult showed similar (or slightly lower) Bim protein level compared with FO B cells, confirming the mRNA data. However, some B cells at the tumor stage showed sharply reduced levels of Bim protein that were not reflected at the mRNA level. Thus, reduction of Bim occurred in B1 B cells in a proportion of CLLs due to post-transcriptional modification. For Bmf, two major isoforms, Bmf_{CUG} and Bmf_s, are generated in mice by an alternative start site detected by monoclonal anti-Bmf antibody (Grespi et al., 2010), and both isoforms are present in FO B cells (Labi et al., 2008). Bmf_s at 21 kD is the originally described Bmf protein (Puthalakath et al., 2001), and normal pB1a and sB1a, and all CLL/lymphomas showed low levels of Bmf_s. Bmf_{CUG} was also low in the majority of samples, although one tumor case showed no reduction (Fig. 7 E, and 2/12 cases in total). By using a polyclonal anti-Bmf antibody which detects mouse Bmf_s, we confirmed low Bmf in both B1 B cells and at the tumor stage in conjunction with up-regulated *Myc* (Fig. 7 F).

In addition to these *Myc* and Bmf data obtained with mature B1a cells in secondary sites (spleen and peritoneum), the newly generated immature (AA4⁺ IgM⁺ IgD⁻) B cells in the primary site of B-1 cell development, day 1 liver, already showed higher *Myc* and lower Bmf mRNA, relative to immature B cells in adult BM (Fig. 7 G). These immature B cells in primary sites are the immediate products from the pre-B cell stage to the stage controlling autoreactive B cell development. Thus, genetic predisposition difference between early fetal B-1 and adult B-2 development is continuing. As an outcome, fetal/neonatal B-1 development generated

CD5 up-regulated autoreactive B1 B cells with restricted BCRs, and with further up-regulated *Myc* and down-regulated Bmf cells that can self-renew and progress to CLL.

DISCUSSION

Fetal/neonatal B-1 B cell development in mice generates B1 B cells, and part of them renew throughout life. Here, we show that progression to CLL without BCR mutation can occur from B1 B cells, including cells initially generated during neonatal life. Self-renewal of B1 B cells is T cell independent, with continued expression of moderately up-regulated *c-Myc* and low Bmf, with the potential for CLL development in aged mice, being dependent on expression of certain BCRs expressed by B1 B cells. The importance of B1 B cells able to express particular BCRs for CLL/lymphoma development is shown by comparison between TC⁺ ATA μ KTg and AG-cA μ KTg mice. ATA B1 B cells arise from B-1 development associated with a high incidence of CLL/lymphoma. When

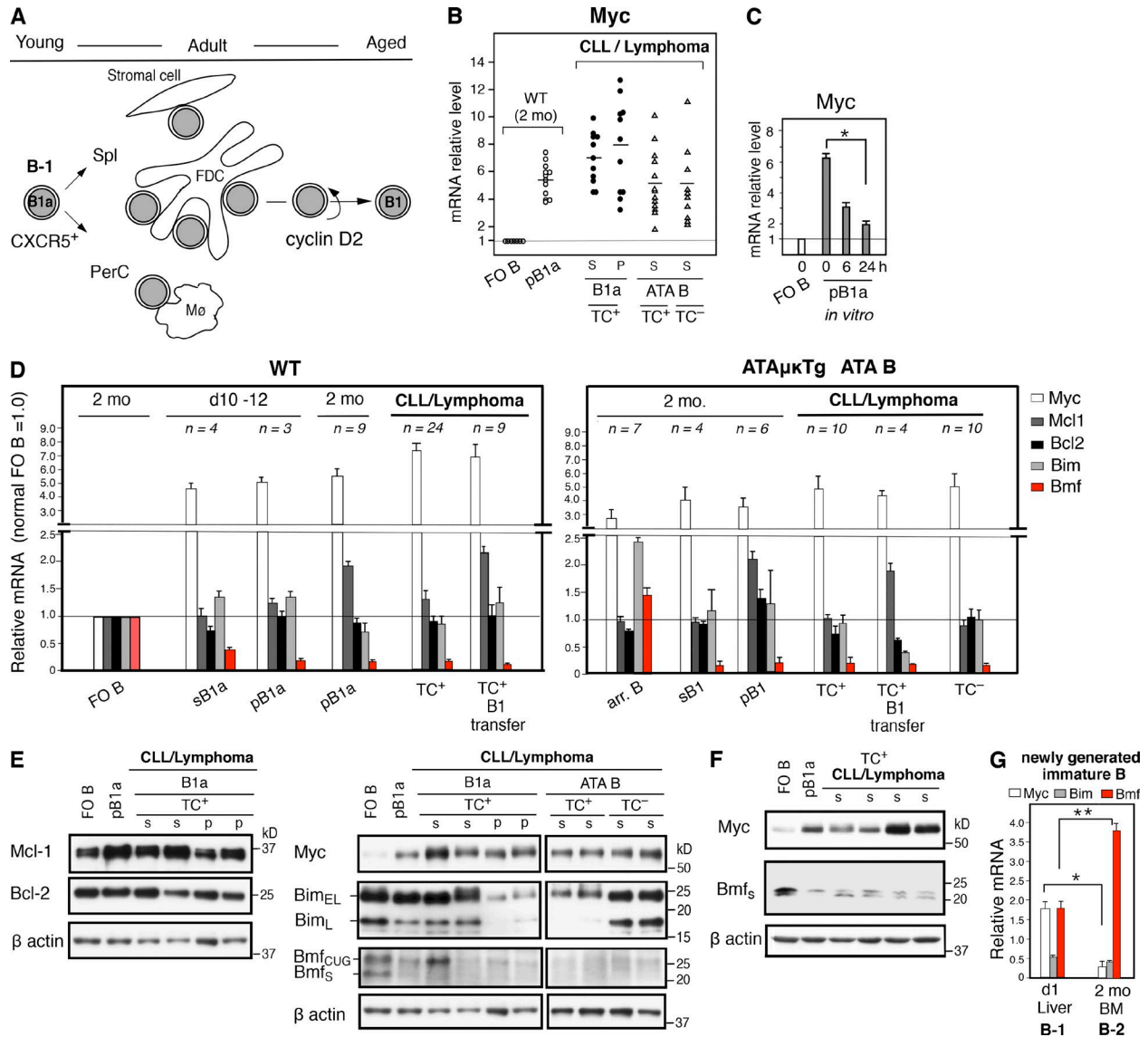


Figure 7. Continued up-regulated c-Myc and down-regulated Bmf by B1 B cells to become CLL. (A) CXCR5 up-regulated B1a cell migration, interaction, and renewal/maintenance from young to aged, with cyclin D2 dependence. (B) Myc mRNA levels by B1 B cells at normal and tumor stage, including TC⁻ ATA B cell tumors, relative to FO B cells. Mean percentages indicated in lines. S, spleen; p, peritoneal cavity. (C) Reduction of Myc mRNA by normal mouse (C.B17) B1a during 24-h culture. After plating in a 96-well U-plate for culture (with 10% FCS), the immediately harvested samples before 37°C incubation was defined as 0 h. FO B Myc mRNA levels were similar before and after culture (as 1.0). $n = 4$; mean \pm SE; *, $P = 0.0007$. (D) Myc, Mcl-1, Bcl-2, Bim, and Bmf mRNA levels by B1 B cells at TC⁻ nontumor and TC⁺ (and TC⁻) tumor stage relative to 2-mo-old FO B cells. (left) WT (C.B17) mice. (right) ATA B cells in ATA μ κTg mice, including arrested ATA B cells in spleen. Values are mean \pm SE. (E) Western blot of samples of FO B and pB1a cells in 2-mo-old WT mice together with TC⁺ CLL/lymphomas stained for Myc, Mcl-1, Bcl-2, Bim, Bmf, and β -actin. Another set of WT/ATA B cell tumors was assembled to compare Myc, Bim, Bmf, and β -actin. WT FO B and pB1a sample data were similar to WT data on left (not depicted). Representative data of two to three experiments. (F) Western blot using rabbit anti-Bmf, in comparison with Myc. (G) Myc, Bim, and Bmf mRNA levels in immature B cells (CD19⁺B220⁺AA4⁺IgM⁺IgD⁻) in day 1 liver and 2 mo BM. 2-mo-old spleen FO B cells in 1.0. C.B17 mouse. $n = 4$; mean \pm SE; *, $P = 0.016$; **, $P = 0.001$.

an Ig light chain is different from that of ATA BCR, such as AGcA BCR, expression of this BCR results primarily in development of MZ B cells, together with FO B cells, from B-2 development; these AGcA BCR-expressing B cells did not become CLL. Thus, the BCR is critical for development of CLL. Furthermore, we found that when we direct ATA BCR

expressing cells into the MZ or FO B cell pools from B-2 development by regulating the level of Thy-1 autoantigen, CLL with ATA BCR did not occur. Thus, these experiments permit the conclusion that possible autonomous signaling ability by biased BCRs is not the only factor leading to CLL progression. Instead, two factors promote CLL generation by

B1 B cells, the capability to express specific BCRs, and BCR expression in the context of B1 B cells.

In adult B-2 B cell development, which is responsible for generating the majority of mature B cells, expression of the proapoptotic protein Bmf is up-regulated at the pre-B cell stage in the BM and expression continues at the immature and transitional stages, thereby controlling generation of autoreactive B cells, together with Bim (Labi et al., 2008). After maturation, most B cells maintain expression of both Bmf and Bim. Reduction in Bmf and/or Bim levels promotes survival. Loss of Bmf (Bmf^{-/-}) in mice has an effect on B cells; immature and circulating B cells increase, there is a competitive survival advantage for mature B cells, and Bmf^{-/-} mice develop age-dependent, B cell-restricted lymphadenopathy (Labi et al., 2008). In contrast to such B-2 development generating mature B cells with normal levels of Bmf expression, B1 B cells generated by early B-1 development have the lowest levels of Bmf and continue to maintain low levels throughout life. Furthermore, we show here that, after initial mature B1a cell generation from B-1 development, which is known to be dependent on Myc together with N-Myc (Habib et al., 2007), B1 B cells continue to express moderately up-regulated Myc in vivo together with low expression of Bmf. In B-2 development, Myc plays a role at the early pre-BCR stage during association of IgH and surrogate light chain (SLC), inducing proliferation. However, Myc down-regulation occurs at the IgM⁻ pre-B cell stage (Yasuda et al., 2008) together with Bmf induction (Labi et al., 2008) before the initiation of IgM⁺ immature B cell development. Thus, distinctive B-lineage progression is occurring, generating B1a cells with restricted BCRs from B-1 development.

When Myc is overexpressed by the E μ -Myc transgene in mouse B-lineage cells, it promotes age-related development of pre-B and B cell tumors that, together with altered expression of the Bcl-2 family members, strongly accelerates B cell lymphomagenesis (Strasser et al., 1990; Egle et al., 2004). One such Bcl-2 members is Bmf. Bmf deficiency alone does not result in B cell tumors, but when combined with the E μ -Myc transgene, the deficiency results in increased B lymphomagenesis, including leukemia accompanied by splenomegaly (Frenzel et al., 2010). Down-regulated expression of Bmf and moderately heightened expression of Myc are normal characteristics of B1 B cells with the capacity to become CLL. Development of CLL is not random among B1 B cells, but rather depends on restricted expression of specific BCRs. As in human CLLs, mouse CLLs show biased BCR usage, including stereotyped (recurrent) BCRs. Stereotypic BCRs were expressed by >50% of TC⁺ CLL cases in mice we analyzed, and cell transfer experiments revealed that early generated B1 B cells developed CLLs expressing these BCRs. In addition to the stereotyped IgV_H/V_L BCRs, several IgV_H with similar HCDR3 sizes were also commonly found in TC⁺ CLL as biased V_H groups, and some were also confirmed to be of B1 B cell origin by adoptive transfer (Table S3). These BCRs were not selected solely by nonphysiolog-

ical overexpression of hTCL1 nor restricted to the C.B17 mouse background. Mouse strains that develop CD5⁺ B cell leukemia/lymphoma with aging on particular genetic backgrounds (Pennell et al., 1988), or mice with chromosomal deletions that included miR15a/16-1 controlling cell proliferation (Klein et al., 2010), both showed V_Hs shared with TC⁺ CLL V_H, including stereotyped BCRs (Table S2). A further example is provided by E μ -Bcl-1 (cyclin D1) transgenic mice on a C57BL/6 background. When Bcl-1 is overexpressed, CD5⁺ B cells increase during aging (Fig. S2). These B cells in aged mice show biased V_H usage, including all stereotyped BCR sets listed in Fig. S2 as potential B lymphoma precursors. Thus, CD5⁺ CLL/lymphoma in aged mice, promoted by genetic background or by transgenesis/gene knockout, show common recurrent unmutated BCRs, where B1 B cells with restricted BCRs are likely to be involved.

Among the five stereotyped BCRs listed, heavy chains of three BCRs (sets 3–5) generally do not associate strongly with SLC: V_HQ52-1 (Ox-1); all (Hayakawa et al., 2016), V_HJ558-67; 9/12 (unpublished data), V_H11; all (Wasserman et al., 1998), indicating a B-1 origin, as intense SLC association is not necessary in B-1 development for progression to the immature B cell stage (Wasserman et al., 1998). Set 1 V_H12 is known to be a B1a-associated BCR. As found in set 3 BCR, this V_H12 BCR also requires a restricted CDR3, and all V_H12⁺ CLLs expressed the glycine (G)-containing HCDR3 (Table S2). Such restricted V_H12-expressing B cells were already detected in day 1 newborn liver (Clarke and McCray, 1993). Set 2 V_H J558-64 (V332, 165.1) was also found in normal mouse B1a cells with identical CLL V_H/D/J sequences (unpublished data), and transfer of CD5⁺ B cells from aged mice into newborn also generated CD5⁺ B cell lymphoma with this V_H, without the TCL1 transgene (Förster et al., 1988). In aged E μ -Bcl-1 transgenic mice, B1a cells expressed all these stereotyped BCRs (Fig. S2). Thus, in addition to B1 B cell transfer experiments using \leq 2 mo mice, CLLs with these stereotyped BCRs clearly confirm continued presence of B1 B cells, from young to old, with the capacity to eventually progress to CLL/lymphoma. Although it remains unknown whether all CD5⁺ CLLs with unmutated biased V_H groups in mice are of B1 B origin, we show here that majority of TC⁺ CLLs we analyzed developed independent of CD40 signaling, not only CLLs with restricted stereotyped BCRs.

ATA BCR used in μ KTg mice with IgV_H3609/D/J IgH and V_k21-5/J IgL is not among the readily detected stereotyped BCRs listed in Table 1. However, the presence of B cells with anti-thymocyte/Thy-1 autoreactivity (ATA) is well known, including in 8-d-old BALB/c mice (Underwood et al., 1993). Though such B cells are not abundant normally, increased ATA CD5⁺ B cells and antibody production occurs in certain mouse strain backgrounds such as NZB and SM/J mice (Shirai and Mellors, 1971; Hayakawa et al., 1984, 1990). When we increased the frequency of cells expressing this ATA BCR by μ Ig transgenesis on a C.B17 background (BALB/c with congenic C57BL/Ka IgM allele), predominant expres-

sion of ATA BCR by B1 B cells occurred, including at the neonatal B1a stage (Hayakawa et al., 1999), and ATA $\mu\kappa$ Tg exhibited a higher incidence of CLL/lymphoma. This was also illustrated by set 3V_HQ52-1/V_k9 anti-nonmuscle myosin IIA BCR among stereotyped BCRs; this BCR is rare among B1 B cells, but fostering progression to CLL by Ig transgenesis (Hayakawa et al., 2016). Thus, expression of specific BCR, rather than frequency itself among the self-renewing B1 B cell pool, is key for progression to CLL by B1 B cells.

After the initial BCR selection to become mature B1a cells from B-1 development, the requirement for self-renewal and/or transformation may drive further BCR selection. Because unmutated germline BCRs have conformational flexibility (Wedemayer et al., 1997), polyreactivity can occur. Cross-reactivity differences are recognized among BCRs with similar autoreactivity, such as between V_H12 and V_H11 anti-phosphatidylcholine BCRs (Conger et al., 1991). Though present in both stereotyped BCR sets, the mice with V_H12 BCR exhibited a much higher incidence of CLL with splenomegaly. Thus, we suggest the importance of certain cross-reactivity, possibly combined with autonomous signaling, comprise roles that the restricted BCR contributes to CLL progression. Further, as for B1 B cell context, moderately up-regulated Myc and low Bmf by B1 B cells appear to play an important role. Continuous interaction occurs between B1 B cells and the surrounding tissue microenvironment, including FDCs and stromal cells. Tumor necrosis receptor family members BAFF and APRL provided by the microenvironment are known to promote B cell survival, and also increase the incidence of CLL in both mice and humans (Tsukada et al., 2002; Planelles et al., 2004; Enzler et al., 2009). Without BAFF, normal mature B cells are lost, whereas Bim and Bmf double deficiency promotes B cell survival in the absence of BAFF (Woess et al., 2015). It is likely that down-regulated Bim and Bmf, as found in a portion of mouse CLLs (Fig. 7 E), promotes survival. Thus, low Bmf and up-regulated Myc by B1 B cells combined with interactions with the microenvironment together with BCR signaling would promote survival, self-renewal, and/or increase in population numbers. Reduced expression and loss of CD5 by some B1 B cells, originally CD5⁺B1a, occurring during development of CLL suggests that TLR signaling is also likely to be involved in proliferation. Mouse B1 B cells are known to be the B cells capable of producing IL-10 (O'Garra et al., 1992), and this cytokine regulates the immune system. B1 B cells are a part of regulatory B cell population, and low-dose LPS TLR treatment *in vivo* rapidly induced IL-10 in the majority of TC⁺ mice (DiLillo et al., 2013). These suggest that an inflammatory signaling may also be involved to promote CLL/lymphoma development.

In human CLL, about half of patients express unmutated IgVH with more possibility for poor outcome than mutated IgVH. How mouse CLL relates to human CLL, particularly the CLL with unmutated BCR, is an ongoing issue that needs to be addressed. An issue related to the relevance

mouse CLL to human CLL is the occurrence of loss of a chromosomal region with synteny to human 13q14 including miR15a/16-1, an abnormality commonly found in human CLL (Hayakawa et al., 2016). This occurred dominantly in set 3 anti-nonmuscle myosin IIA BCR CLL, but also occurred in some CLLs with other stereotyped BCRs (such as set 1 and 2), particularly with cases showing infiltration of leukemic cells into subcutaneous tissues in mice. In addition to biased BCR usage in both human and mouse CLLs, overexpression of CXCR5 and cyclin D2 has been detected in human CLL (Delmer et al., 1995; Bürkle et al., 2007), resembling mouse B1 B cells. Although Myc was originally thought to be low in human CLL based on analysis of PBL, CLL present in the tissue environment, such as in lymph node, show detectable Myc, with an increased level in aggressive CLL (Herishanu et al., 2011). The role of Bmf in human B cells is not clear. However, three spliced variants of Bmf have been reported to be present in human B cells, including CLL, with two transcripts lacking the BH3 domain, different from mouse Bmf, and one of them with ability to counteract the proapoptotic function (Morales et al., 2004). Interestingly, Burkitt's lymphoma was originally described as a B cell neoplasm with a role for enhanced expression of Myc induced by chromosomal translocation, and these lymphoma cells also showed low levels of Bmf (Frenzel et al., 2010). This may indicate a prosurvival role for reduced Bmf in humans, as in mice.

Importantly, the differential Lin28b expression patterns between mouse B-1 and B-2 B-precursors are also found in human fetal and adult hematopoietic cells (Yuan et al., 2012). Thus, it will be interesting to know whether early generated CD5⁺ B cells in humans are also the outcome of restricted early generated B cell development with distinct control of the balance between cell proliferation and death by members of the Bcl-2 family. In mice, there is a clear distinction at the pro-pre-BCR stage; Lin28b⁺ Let-7⁻ in B-1 B cell development contrasts with Lin28b⁻ Let-7⁺ in adult B-2 B cell development (Zhou et al., 2015). Considering that the miR Let-7 down-regulates Myc (Sampson et al., 2007), low/negative Let-7 levels at the pro-pre-BCR stage in B-1 development may play a role for distinctive B-lineage progression, including pre-B and immature stages that allow generation of auto-reactive B cells associated with CD5 induction, different from B-2 development. Several other miR and protein differences are also recognized (Zhou et al., 2015). The differing Myc and Bmf levels in newly generated immature B cells, as shown here, is likely to result from such distinctive development. Understanding this early B-lineage process will be critical for testing this hypothesis as to how B cells can self-renew and be maintained throughout life, with an ability to become CLL.

MATERIALS AND METHODS

Mice

μ -hTCL1 Tg mice were backcrossed onto the C.B17 mouse background for more than six generations. Transgenic mouse lines, V_H3609 μ (ATA μ Tg), V_H3609/V_k21-5 $\mu\kappa$ (ATA $\mu\kappa$ Tg;

3369) and $V_H3609/V_k19-17 \mu\kappa$ (AGcA $\mu\kappa$ Tg; EP67), Thy-1 knockout, *lck*-Thy-1 transgenic lines, J_H knockout, all on C.B17 mouse background, have been previously described (Hayakawa et al., 1999, 2003; Wen et al., 2005a; Ichikawa et al., 2015). C.B17, BALB/c, CD40^{-/-}.C.B17, and C.B17.*scid*, were all bred and maintained in our laboratory animal facility. CD3 $\gamma\delta$ ^{-/-} mice were a gift from D. Kappes, originally provided by C. Terhorst (Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA; Wang et al., 1998). All animal experiments were conducted under a protocol (#00-10) approved by the FCCC Institutional Animal Care and Use Committee (IACUC).

Flow cytometry analysis, sorting, and reagents

Multicolor flow cytometry analysis, sorting, and monoclonal antibody reagents, including rat anti-mouse Ig idiotype antibodies, P9-19A4 (anti-ATA idiotype), together with anti- κ to detect ATA B cells, and P9-10C7 (anti- V_H3609 idiotype) together with P9-13H8 (anti- V_k21 and V_k19) to detect AGcA B cells. These have been described previously (Hayakawa et al., 2003; Ichikawa et al., 2015). Anti-hTCL1 antibody (27D6/20) originally provided by C. Croce (Bichi et al., 2002) was conjugated to PE (phycoerythrin) for cytoplasmic staining.

B cell leukemia/lymphoma analysis, diagnosis, and purification

PBL analysis was performed every 2–3 mo for each mouse. A predominance of B1a (CD19⁺B220^{lo}CD5⁺) in TC⁺ Tg mice and/or ATA B (ATAid⁺ IgM^b) in TC⁺ and TC⁻ ATA $\mu\kappa$ Tg mice in PBL (>70% of B cells), with significant increase of total PBL number (>10-fold) was defined as CLL stage. The majority of CLL samples expanded by >100-fold increased B cells in PBL compared with normal B cells, often with splenomegaly. These CLL samples were used for tissue analysis and B cell purification. For Ig sequencing, gene expression analysis, and Western blotting, leukemia/lymphoma B cells (all IgM⁺) in TC⁺ mice and TC⁺ B cell-transferred mice were purified as CD19⁺B220^{lo}CD5^{+/lo} by flow cytometry sorting. For ATA CLL (TC⁺ and TC⁻), B220^{lo}CD5^{+/lo} ATAid (19A4)⁺ IgM^bB cells were purified. Histological diagnoses were made using formalin-fixed, paraffin-embedded sections that were stained with H&E using established criteria (Morse et al., 2002).

Ig sequencing

Sequencing Ig μ and Ig κ variable regions of tumor B cell BCRs by μ or κ PCR with consensus V primers was performed using standard procedures in most cases. When sequences were not recovered, specifically for V_k9-96 (ce9) and V_k1-117 (cr1) κ , 5' RACE cloning was done using the SMARTer RACE cDNA amplification kit (Takara Bio Inc.). For single-cell sequencing, individual cells were deposited using a FACSariaII (BD), directly onto either AmpliGrid AG480F slides (Beckman Coulter), or onto 96-well plates prepared with SuperScript III RT kit (Life Technologies) as previously described (Ichikawa et al., 2015; Hayakawa et al.,

2016). The efficiency of obtaining sequences was 70–90%. κ -chain single-cell sequencing of $V_H3609\mu^+$ peritoneal CD5⁺ B cells in ATA μ Tg mice was done by staining with P9-10C7 (anti- V_H3609 id) together with anti- κ as previously described (Ichikawa et al., 2015); the V_k usage list is a summary of data from three mice.

Mouse V gene nomenclature

The V_H gene family name is based on Johnston et al. (2006). V_H gene nomenclature was made based on BALB/mouse IgMV $_H$ gene analysis, with >99% match with genes in Vbase2 for the majority (Table S1); listed CLL BCR data are a 100% match. Table S1 shows total V_H gene nomenclature list together with the matches from Vbase2, IMGT, and GenBank. Because C.B17 mouse is a congenic mouse line with IgH locus of C57BL/Ka, on a BALB/c mouse background, V_H gene sequencing data showed V_H allele representing C57BL. V_k gene nomenclature is based on Thiebe et al. (1999).

B1 B cell and bone marrow cell cotransfer

≤2 mo B1 B cell transfer. For days 10–13 TC⁺ B1 B cell transfer, CD5⁺ B220^{lo} B1a B cells or total B cells (B220⁺ κ^+ including B1a) were purified from mixed spleens of (TC^{+/-} x CB17) F₁ littermates (30–50% of littermates were TC Tg⁺). For 2–4 wk, B1 B transfer, B1a B cells from spleen or peritoneal cavity (or total B cells in peritoneal cavity) were used. From 5–8 wk mice, TC^{+/-} B1a B cells from spleen and peritoneal cavity were sorted. These purified B1 B cells, either 1–10 × 10⁵ cells from 10 d to 4 wk or 5–15 × 10³ cells from 5–8 wk donors were injected intravenously into each recipient, transferred alone or with total bone marrow cells (5 × 10⁶) from 2-mo-old TC⁻ BALB/c mice into C.B17 *scid* recipients (lightly irradiated, 3 Gy, 1 d prior).

2 mo B1 B cell transfer. BM HSC-enriched fraction, B1a, and non-B1a B cells (B220^{hi}CD5⁻ in spleen) were purified from 2 mo TC⁺ C.B17 mouse (pool of two mice). pB1a and sB1a (each 5 × 10⁵/recipient), and non-B1a spleen B cells (sB; 3 × 10⁶/recipient) were cotransferred with TC⁻ BALB/c BM (5 × 10⁶/recipient) to C.B17 *scid* recipients. For TC⁺ bone marrow stem cell transfer alone, stem cell/progenitor enriched fractions (CD19⁻ IgM⁻ CD5⁻ and low side scatter) were sorted from 2–3-mo-old TC⁺ mice, and transferred (5 × 10⁵ cells per *scid* recipient). Cellular origins of B cells in recipients were distinguished by anti-IgM allotype staining.

ATA B and bone marrow cell cotransfer

2–4-mo-old TC⁺ ATA $\mu\kappa$ Tg (IgM^a Tg) mice were used for purification, and then cotransferred with 2–3 mo C.B17 mouse (IgM^b) BM into C.B17 *scid* mice by intravenous injection. Per recipient: bone marrow stem cell/progenitor enriched fraction (CD19⁻ IgM⁻ CD5⁻; 10⁶), arrested ATA B cells (B220⁺AA4⁺ IgM^b; 1.5 × 10⁶), or peritoneal ATA B cells (6B2⁺AA4⁺ IgM^b; 5 × 10⁵), together with total C.B17 mouse bone marrow cells (5 × 10⁶). PBL was analyzed by

staining with (CD19, B220, CD5, ATAid, and IgM^a) and (CD19, B220, ATAid, IgM^a, and IgM^b).

ATA B cell stimulation in vitro

Cell sorter purified ATA B1a cells (B220⁺CD5⁺IgM^{b-}) in peritoneal cavity (pB1) and arrested cells (B220⁺AA4⁺IgM^{b-}) in spleen in ATA μ KTg mice were cultured in a 96-well U-plate at 2×10^5 cells in 150 μ l medium (with 10% FCS)/well (in duplicate), with or without stimulation. Stimulating reagents: LPS (Sigma-Aldrich) at 20 μ g/ml, CpG (InvivoGen) at 3 μ g/ml, goat F(ab)₂ anti-IgM (The Jackson Laboratory) at 10 μ g/ml, anti-CD40 (eBioscience) at 5 μ g/ml, and recombinant murine IL-4 (PeproTech) at 10 ng/ml.

Quantitative RT-PCR assay

Gene expression was quantitated by real-time PCR, using TaqMan assays from Applied Biosystems, an ABI 7500 real-time thermal cycler, and ABI software (Life Technologies). Relative gene expression levels were normalized using β -actin values as a standard.

Western blotting

FO B cells (B220⁺CD21^{med}CD23⁺AA4⁻) and pB1a (CD19⁺B220^{lo}CD5⁺) from 2-mo-old C.B17 mice and CLL/lymphoma (CD19⁺B220^{lo}CD5^{+/lo} in TC+WT or B220^{lo}CD5^{+/lo} ATAid⁺ in TC⁻ or TC⁺ ATA μ KTg mice) were purified by cell sorting (2×10^6 cells/tube), and cell lysates were subjected to SDS-PAGE. Anti-c-Myc, anti-Mcl-1, and HRP goat anti-rabbit IgG antibodies were all obtained from Cell Signaling Technologies. Anti-Bcl-2 (BioLegend) with HRP rat anti-mouse IgG1 (Southern Biotech) was also used. Rat anti-Bim 3C5 and rat anti-Bmf 17A9 were both purchased from Enzo Life Sciences, together with HRP-goat anti-rat (Cell Signaling Technology). Rabbit anti-Bmf (Abcam; ab9655) was used together with HRP goat anti-rabbit IgG (Cell Signaling Technology). Anti-b actin was obtained from Bethyl Labs.

Online supplemental material

Fig. S1 shows TC⁺ CLL-stromal cell interaction. Fig. S2 shows BCR usage by B1a, increased in aged E μ -Bcl-1 transgenic mice. Table S1 lists mouse V_H gene nomenclature. Table S2 lists BCR-stereotyped sets with HCDR3 and LCDR3 used by TC⁺ CLLs (total 155 samples). Table S3 lists BCR-stereotyped sets with other V_H/CDR3 used by TC⁺ CLLs (total 155 samples). Tables S1–S3 are available as Excel files.

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